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Jeremy C. Wright
Diane J. Burgess *Editors*

Long Acting Injections and Implants

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Long Acting Injections and Implants

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This book is dedicated to friends and family. Especially both editors dedicate the book to their respective parents (George Gartly Burgess and Violet Isobel Burgess; William C. Wright and Jenny J. Wright).

Preface

The evolving complexity of human therapeutics requires the development of novel drug delivery systems. This book encapsulates in a single volume the concepts essential for understanding the science and technology associated with the research and development of long acting injections and implants. It provides a comprehensive overview of the scientific and regulatory challenges associated with these delivery systems.

Critical contributions to this area are the array of formulations that make up the spectrum of long acting injections and implant dosage forms. These include microspheres, liposomes, in situ forming depots, suspensions, implants, lipophilic solutions, and osmotic implants. Such formulations have the potential to maintain therapeutic drug concentrations for durations from days to months, can be engineered to maintain characteristics such as zero-order or pulsatile drug release, and, in some cases (e.g., liposomes) can provide targeted drug delivery to the site of action. These attributes lead to increased patient compliance and convenience, reduced fluctuations in plasma profiles, and reduced plasma concentrations making it possible to administer higher drug concentrations to the site of action while reducing the overall dose. Thus, unwanted side effects can be minimized or reduced. In addition, long acting injections and implants can provide a means for the delivery of drugs that are subject to degradation in the harsh environment of the gastrointestinal tract, that undergo extensive first pass metabolism, or that exhibit poor bioavailability when such molecules are orally administered.

Long Acting Injections and Implants begins with chapters that provide basic concepts explained in a simple, clear, and concise manner. In subsequent chapters additional material, expansions on the basic scientific concepts underpinning research and development of such dosage forms, and examples of technological developments in this area are comprehensively reviewed and discussed. The introductory chapter provides a brief description of the types of systems and major areas of current application and research. The *Historical Overview* chapter provides a chronological overview of the historical developments associated with long acting injections and implants providing sufficient background to enable the reader to appreciate the historical development of the area and to use that knowledge as a foundation for the

development of the next generation of products. The *Host Response* chapter introduces the reader to the body's response to biomaterials/foreign bodies and the influence of environmental conditions on the design and development of long acting injections and implants. The *Anatomy and Physiology* chapter describes the biological features of the site of administration that are relevant to the development of long acting injections and implants. The following two chapters provide comprehensive information on drug candidates, clinical objectives, and disease states. The next series of chapters of the book focus on aspects related to the research and development of specific injection and implant dosage form types. In addition to the systems mentioned above, chapters are also provided on micro- and nanoemulsions, PEGylation of nanocarriers, self-assembling lipid formulations, microfabricated technologies, drug eluting stents, delivery of peptides and proteins, and delivery of vaccines. These overviews are followed by chapters that describe and discuss special considerations unique to the injection route including sterilization and in vitro release testing (and in vivo/in vitro correlation). In the final chapter of the book, an overview of the regulatory considerations associated with the registration of long acting injections and implants is provided.

Long Acting Injections and Implants has been written with the objective of both enlightening someone just starting in the field (e.g., a new scientist or experienced scientist switching fields) and while at the same time providing the in-depth knowledge that is beneficial for a skilled worker in the field. It is hoped that the reader will find this volume useful and intriguing for both the variety of scientific system types that provide long acting therapy and for the wide range of scientific and technical topics that are involved in the research, development, and registration of long acting injections and implants that provide state of the art therapy to patients.

Cupertino, CA — Jeremy C. Wright
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Chapter 1
An Introduction to Long Acting Injections and Implants

Diane J. Burgess and Jeremy C. Wright

Abstract This chapter provides an overview of the area of long acting injections and implants, summarizing the contents of the book. As appropriate, the reader is directed to the relevant chapters.

The therapeutic utility of many drugs can be enhanced by prolonged administration. Constant, extended drug levels at the site of action can improve efficacy and reduce side effects. A number of modalities can achieve sustained drug levels including: repeated oral administration, repeated topical (transdermal) administration, repeated pulmonary administration, repeated parenteral administration, delivery via infusion pumps, and long acting injections and implants. Long acting injections and implants offer a number of advantages (some of which are shared with other long acting administration modalities). First is extended duration of action. Repeated bolus administration of a drug (either oral or parenteral) can result in the classic "peak and valley" pattern of drug administration (see Fig. 1.1) wherein plasma C_{max} can exceed the toxic threshold (leading to undesirable side effects) and plasma C_{min} can fall below the minimum threshold for efficacy. Alternatively, prolonged, long acting drug delivery can result in steady levels that are maintained above the minimum threshold for therapeutic efficacy but below the toxic threshold, resulting in prolonged drug action (i.e., better pharmacodynamics) without unwanted side effects. For some therapies, side effects may be a matter of inconvenience to the patient, but for other therapies (e.g., cancer chemotherapy) the impact and potential benefits can potentially be much greater.

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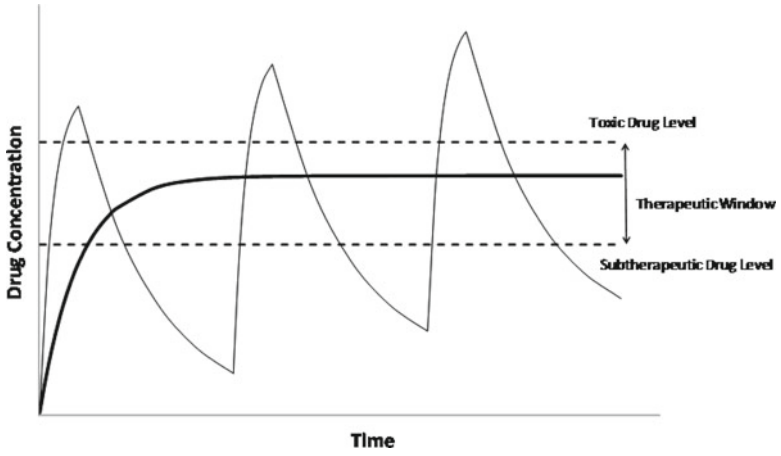

Fig. 1.1 Conceptual depiction of plasma drug concentrations after repeated administration from a conventional dosage form (*thin line*) and from a controlled release system (*bold line*)

Less frequent administration via long acting injections and implants can be significantly more convenient for the patient and the health care provider by reducing the number of times a drug must be administered. This reduced frequency of administration can result in savings for the health care system and can also enhance patient compliance with the therapeutic regimen (less skipped doses, etc.) thereby increasing the probability of the desired therapeutic outcome. Additionally, patient compliance can be enhanced when side effects are minimized by long acting injections and implants.

Parenteral administration may be the most logical or only choice for drugs with low oral bioavailability or extensive first pass metabolism. Long acting injections and implants may be applied to either systemic delivery of a drug (for example, systemic delivery of an antimetastatic chemotherapeutic agent) or applied to local delivery (for example, an analgesic system applied directly to a surgical incision or a chemotherapeutic system applied directly to a brain tumor site).

Sustained parenteral drug delivery (including long acting injections and implants) began to emerge as a subarea of pharmaceutics in the mid-twentieth century. Advances in pharmacokinetics and pharmacodynamics highlighted the need for sustained drug levels in plasma or in the target tissues in order to achieve the desired therapeutic response. The development of the field of long acting injections and implants was propelled by advances in pharmaceutical chemistry (including biotechnology) and advances in materials science, especially polymer technology. (See Chap. 2 for more information on the development of the field).

A wide variety of different types of long acting injections and implants have been designed and tested. New systems seem only to be limited by the extensive creativity of the innovators in this field and advancing scientific knowledge. Nevertheless, design of long acting systems requires knowledge of the physicochemical properties of

the system under development, the physicochemical properties of the drug being delivered, and the pharmacokinetic and pharmacodynamic properties of the drug, including toxicities associated with elevated drug levels. Drugs and diseases that have been treated or investigated for application of long acting injections and implants are discussed in Chaps. 5 and 6. Additionally, the body will respond to the administration of long acting injections and implants. The understanding of the foreign body response has progressed greatly, and the foreign body response must be considered in the development of long acting injections and implants. As is well known to those working in the field, even the administration of systems composed of materials considered to have good biocompatibility will engender some sort of foreign body response (see Chap. 3). Additionally, if the system is intended for producing sustained systemic levels of the drug, then the biological characteristics of the injection site that affect drug transport and uptake must be considered (see Chap. 4).

Some of the first implantable sustained release preparations involved compressed tablets of steroids, wherein the slow dissolution of the steroid from the tablet provided sustained release of the active agent. A significant improvement occurred due to advances in polymer technology, specifically the development of polymeric materials that possessed significant biocompatibility. It was recognized that a polymeric rate-controlling membrane (RCM) could be placed between the body and a drug reservoir, and this would lead to controlled release of the drug (RCM/reservoir drug delivery system). This led to the development of RCM drug delivery systems for ocular applications, intrauterine inserts, and, finally, to contraceptive implants such as the Norplant® system consisting of an outer cylindrical membrane of polydimethylsiloxane and the steroid levonorgestrel in the interior of the cylinder. An additional contraceptive implant utilizing similar design concepts is the Implanon®, which utilizes poly(ethylene-*co*-vinyl acetate) as the RCM for the delivery of etonogestrel. Implants utilizing a hydrogel RCM (Hydron® Implant) were also developed and applied to the delivery of the LHRH agonist, histrelin acetate. (See Chap. 2 for additional discussion of nondegradable implants).

Another subclass of RCM systems are the osmotic implant systems. In these systems, the RCM controls the rate of water diffusion into the system, with the delivery of an equal volume of the drug solution or suspension from the system reservoir. These systems yield steady, zero-order delivery of drugs. Use of a catheter with these systems can enable site-specific delivery of a therapeutic agent. The ALZET® pump was developed and commercialized for animal research applications and has been extensively applied in a wide range of investigational areas. Osmotic systems for human applications are based on a design with an outer cylinder of titanium (DUROS® implant) and a piston that separates the osmotic layer from the drug reservoir. DUROS implants have provided delivery of agents from 1 month to over 1 year for treatment of pain, cancer, diabetes, and hepatitis (see Chap. 17).

Lipophilic (oily) solutions and suspensions are one of the original dosage forms in this field. They contain an oil, such as sesame oil or castor oil, and the drug as either a solution or suspension. These depots can be quite elegant in design and

simple to manufacture. These systems have been applied to the delivery of a number of drugs (e.g., steroids, antipsychotics), with durations of action as long as 6 weeks. [See Chap. 7 on oily (lipophilic) solutions and suspensions].

Biodegradable polymers are inherently attractive for drug delivery applications because of two potential major attributes: first, if the polymer erodes only at the surface, then it would seem possible to engineer systems yielding nearly constant release. Second, the system can be expected to completely erode, thereby eliminating the need for a procedure to remove an implanted or injected system at the end of the delivery lifetime.

Biodegradable copolymers of lactic and glycolic acid (PLGA) have been extensively investigated for the delivery of a large number of drugs, including small molecules, peptides, proteins, vaccines, and other biomolecules. The degradation rates of these polymers can be modified by varying the ratio of lactic to glycolic acid in the copolymer.

The development of microspheres based on PLGA has been one of the major successful applications of long acting injections and implants. Microspheres can range in size from 1 to 999 μm. In general, microspheres are suspended in an aqueous vehicle prior to administration through a small gauge needle. The microspheres may have either a continuous polymeric matrix wherein the drugs are homogeneously dispersed or a shell-like wall surrounding the drug reservoir/core. The terms microspheres and microcapsules are often used synonymously. Research and development of PLGA-based microspheres has led to the regulatory approval and commercialization of a number of products including the Lupron Depot® (leuprolide), the Trelstar® depot (triptorelin), the Nutropin® depot (somatropin), and Risperal® Consta® (risperidone) for applications ranging from prophylactic treatment of prostate cancer to growth hormone deficiency to antipsychotic therapy (see Chap. 10).

Additionally, biodegradable implants composed of PLGA were investigated for a number of applications. The systems can be implanted via trocars or, if the implant size is small enough, these systems can be delivered via standard-sized needles. The Zoladex® implant (PLGA matrix system delivering the LHRH analog goserelin) was developed for the treatment of prostate cancer, breast cancer, and endometriosis and has been commercialized worldwide.

Other biodegradable polymers have also been investigated and utilized for the development of commercial products. Polyanhydride polymers are utilized for the Gliadel® wafer for the delivery of carmustine (BCNU) for the treatment of brain tumors (glioblastomas). The polyorthoester family of polymers has been widely investigated and regulatory approval has been sought for a product for the delivery of granisetron. (See Chap. 2 for additional discussion of biodegradable polymers).

In situ forming systems have been developed by a number of investigators. These systems can be injected through standard syringe needles but then gel or form viscous depots once injected. Drug release through the depot is thus sustained for periods of time from days to months. Major examples of these dosage forms include the Atrigel® system (based on PLGA copolymers), the SABER™ system (based on sucrose acetate isobutyrate) and the ReGel® system [based on polyethylene glycol (PEG)–PLGA copolymers]. These systems have been applied to cancer therapy, analgesia, and other areas (see Chap. 9).

Liposomes constitute another system for injectable drug delivery. Liposomes are self-closed structures composed of one or more curved lipid bilayers (lamella) that entrap part of the solvent into their interiors and are usually spherical in shape. Hydrophilic groups of the lipids are on the outside and inner surfaces of the lamella, hydrophobic groups are in the interior of the lamella. Liposomes can be utilized for drug delivery either by loading the drug into the interior of the liposome or into the lamella. Additionally, PEG can be covalently attached to the lipid molecules that constitute the lamella (termed a "Stealth" liposome) and this modification extends the circulating half-life. The most notable example of long-circulating liposomes is Doxil®, a PEGylated liposome product containing doxorubicin. Liposome formulations of doxorubicin exhibit reduced toxicity and a higher efficiency as a result of passive tumor targeting via the "Enhanced Permeation and Retention (EPR)" effect. The EPR was discovered by Maeda and coworkers who discovered that a nanoscale construct accumulated or was trapped within tumor tissue. They concluded that the vasculature in the tumors was "leaky" and the lymph drainage system was not yet functioning efficiently. (See Chaps. 2 and 11 for additional information).

An additional application of lipids involves lipid-based liquid crystalline (LC) phases that can be formed by molecular self-assembly. These systems consist of specific liquid mixtures of naturally occurring polar lipids and small amounts of solvents (FluidCrystal® Injection depot). The systems can be injected subcutaneously or intramuscularly. Upon contact with the aqueous fluids present in the tissue, the lipids self-assemble into reversed LC phases, thereby effectively entrapping dissolved or dispersed drugs. Drugs can be released over periods of days to months. The system is applicable to the delivery of small molecule drug substances, peptides, and proteins (see Chap. 16).

Liposome and microspheres are dispersed in a liquid carrier prior to injection. There are a number of other dispersed long acting injection systems, including aqueous suspensions, emulsions, nanosuspensions, and surface-modified nanocarriers.

Sustained release formulations of sterile aqueous suspensions (particle size of 0.1 μm or greater) are generally intended for injection by the subcutaneous, intramuscular, or intra-articular routes. Compared to aqueous solution formulations, aqueous suspension formulations can be relatively fast releasing or exhibit sustained release with readministration intervals from hours to months, depending on the formulation and the properties of the active ingredient. A number of formulation parameters must be assessed to prepare a formulation that exhibits acceptable functionality in the hands of the user. Additionally, an understanding of the rate limiting step in the absorption of the active into the systemic circulation (or target site for local delivery) is required to optimize the formulation and the delivery of the active. Examples of active ingredients formulated as aqueous suspensions include steroids and antibiotics (see Chap. 8).

Micro- and nanoemulsions may be utilized to overcome formulation challenges such as solubilization of poorly aqueous-soluble drugs and/or protection of drugs susceptible to hydrolysis. Emulsions may be oil-in-water (O/W) or water-in-oil (W/O) or multiple emulsions (O/W/O and W/O/W). Emulsions are thermodynamically unstable; however, emulsion stability can be substantially improved using suitable emulsifiers and viscosity-enhancing agents. The method of preparation can

affect the droplet size distribution and, hence, the stability. Drug release rates from emulsion systems are determined by both the carrier and the drug characteristics. A number of emulsion systems have been commercialized (see Chap. 12).

Nanosuspensions represent another possible approach to the problem of drug candidates that are poorly soluble in water and exhibit poor bioavailability. Nanosuspensions are generally considered as consisting of particles with mean diameters below 1,000 nm. Nanoparticles may be produced via techniques that are applicable to most drug candidates and amenable to scale-up. Nanoparticulate formulations have been approved for antipsychotic therapy (Invega Sustenna®) and for the treatment of breast cancer (Abraxane®, a nanoparticle formulation of paclitaxel with albumin which provides for targeted delivery of the chemotherapeutic to the tumor through albumin mediated uptake) (see Chap. 13).

Since nanoparticulate pharmaceutical carriers are often rapidly cleared from the body, can be unstable at physiological conditions, and may be taken up by the mononuclear phagocytic system, surface modification is often used to extend the carrier lifetime. A number of synthetic polymers have been investigated as surface modifiers, with PEG being highly favored because of a number of attractive properties. Additionally, a targeting ligand can be attached to a PEGylated nanocarrier, especially if the receptor is overexpressed in the target tissue, as occurs for certain cancer cells (see Chap. 14).

Initially long acting implants and injections were developed for small molecule drugs. With the development of techniques for producing commercially significant quantities of peptides and proteins, investigations were launched into the design of systems for peptides and proteins, oftentimes exploiting and adapting existing technologies. These efforts resulted in the development of a number of techniques or systems for the delivery of peptides and proteins as long acting injections and implants. Included in these techniques/systems are synthesis of drug substance analogs with improved pharmacokinetic characteristics, the addition of high molecular weight entities (such as PEG) to the drug substance to modify release characteristics and the ADME profile and incorporation of the peptide or protein in a long acting drug delivery system. Examples of long acting drug delivery systems include PLGA microspheres and PLGA implants (especially for LHRH analogs), osmotic implants for peptide and protein delivery, in situ gelling systems, and self-assembling lipid systems. (See the chapters on the individual technologies and Chap. 20 for additional information).

As noted above, the covalent linking of polyethylene glycol ("PEGylation") to protein and other molecules has been a major area of research and development over the last several decades. Examples of commercial products resulting from these efforts include PEG-interferons (Pegasys®, PEG-Intron®) and PEG-GCSF (Neulasta®) among others. PEGylation results in an extended circulation half-life, although usually with some loss of activity and binding affinity. Overall, there is an improvement in product performance. Additionally, PEGylation has the potential to reduce immunogenicity. The attached PEG moieties can be either linear or branched and may attach to multiple sites on the protein. Thus, it is possible for PEGylation to yield mixtures of conjugate isomers; current development favors PEGylated molecules with minimized heterogenicity (see Chaps. 15 and 20).

Another area of biomolecule delivery that has been investigated is the sustained release of stable vaccine antigens. Vaccines are considered to be one of the most safe and effective medical interventions currently available. Micro- and nanoparticles prepared from PLGA represent a promising delivery system for vaccine antigens and have been shown to improve immunogenic responses in mammals relative to administration of soluble antigen. These systems have the potential to eliminate the need for booster vaccinations. Moreover, PLGA microparticles can trap and retain the vaccine antigens in local lymph nodes and protect them from proteolytic degradation, ensuring longer stimulation by the antigen. Development of PLGA protein antigen vaccine systems requires careful optimization of the microencapsulation method and selection of PLGA type and excipients. Because of cost considerations, the first clinical use of this dosage form for vaccines may be for treatment of diseases such as cancer instead of prophylactic immunization in healthy patient populations (see Chap. 21).

A major advance for "implantable" systems has been the development of drug-eluting stents (DES). Stents are tiny metal wire structures intended to keep arteries open following balloon angioplasty. While bare metal stenting produced improvements in restenosis rates compared to balloon angioplasty alone, a fairly high restenosis rate was still observed. DES that slowly release a potent antirestenotic drug were introduced to the market in 2002 and have resulted in very significant decreases in restenosis rates compared to bare metal stents. The first two commercially available DES were the CYPHER® Stent (a sirolimus-eluting stent marketed by Cordis Corporation) and the TAXUS® Stent (a paclitaxel-eluting stent by Boston Scientific). These DES consist of a metal stent that has been coated with the antirestenotic drug dispersed in polymer matrix. DES have been very successful and widely adopted. In addition to the CYPHER and TAXUS stents, DES have been introduced by other manufacturers. The ultimate stent design might be a fully bioresorbable DES and this concept is under investigation (see Chap. 19).

DES are considered combination products (drug + device) by regulatory agencies, a product classification that overlaps in some aspects with drug delivery systems and long acting implants. Examples of other combination products that have been investigated include antibiotic-impregnated bone cement matrices and implanted insulin pumps containing a glucose sensor that enables feedback control. While combination products are not discussed in extensive detail in this book, combination products are expected to expand as an area of investigation and product development, borrowing concepts and technologies from long acting injections and implants.

Another intriguing area for the development of long acting injections and implants involves utilization of the microfabrication technologies originally developed for the semiconductor industry. These technologies can be exploited in a number of ways. Implantable, addressable arrays with microreservoirs of drug can be implanted and drug release triggered electronically. Alternatively, the microfabrication technologies can be utilized to produce particles of uniform shape and size (and perhaps uniform surface properties) that can be exploited for various drug delivery applications (see Chap. 18).

There are a number of other important considerations required for development of a long acting system, obtaining approval from the appropriate regulatory authorities and for distribution into widespread commercial application so that patient therapy can be improved. Some of these considerations are unique to injections and implants (as compared to topical and oral delivery).

First, systems must be sterile. Sterilization may be achieved through terminal methods, such as irradiation or steam autoclave, or systems may be produced via an aseptic process. Because of its lower sterility assurance level, aseptic processing is less favored by regulatory agencies. Some innovative paradigms for achieving sterilization are under development, which may aid the commercial development of long acting injections and implants. In addition to sterility, systems must also meet requirements for endotoxin levels. These and other considerations are described in more detail in Chap. 22.

Second, a major advantage of long acting implants and injections is their ability to maintain a relatively constant drug concentration at the site of interest. In vitro drug release testing is of substantial utility for the development of new long acting implant and injection systems provided that there is correspondence between the in vivo performance of the systems and the in vitro test results. Establishment of an in vivo/in vitro correlation for drug release can thus help streamline system development and is also of substantial utility for regulatory approval and quality control of products via specifications on in vitro drug release. There are various in vitro release testing methods, which can be chosen based on availability, dosage form specifications, and drug properties. These methods are categorized into three groups: sample and separate, flow through, and dialysis. Although there are currently no standard methods for in vitro release testing of controlled release parenterals, standard dissolution apparatus specified by the US Pharmacopeia have been adapted in some cases and the development of compendial monographs is possible (see Chap. 23).

Finally, new parenteral products must be shown to be safe and effective via appropriately controlled clinical studies, resulting in the submission of a New Drug Application in the United States or other appropriate submission in other regions and countries. For these regulatory applications, specifications need to be developed to ensure consistent product performance across batches and throughout the shelf life of that product. This in turn necessitates an appreciation of the physiological variables and critical quality attributes that influence product performance. Some of these critical attributes may differ among the various types of long acting injection and implant systems described in this book. The assessment of critical quality attributes and manufacturing processes provides the basis for establishing appropriate quality standards for new drug products. Chapter 24 provides an overview of the questions and background information that regulators may consider when reviewing long acting injection and implant products.

As described above, the field of long acting injections and implants encompasses a wide range of system types and enables the delivery of drugs from days to years. A number of very innovative and commercially successful products have been developed, which have provided improved therapy through more precise control of drug levels and better patient compliance with the therapy regimen. It is expected

that additional innovative products will be developed and gain regulatory approval, many of them based on the science and technology described in this book. While it is difficult to precisely predict the future, it seems reasonable to assume that, in addition to products based on more mature technologies, nanoscale systems, targeted systems, and more combination products will enter the market place. Better test methods for drug release will be developed and the activity in this field will result in increasing regulatory guidance and scrutiny.

Chapter 2
Historical Overview of Long Acting Injections and Implants

Jeremy C. Wright and Allan S. Hoffman

Abstract Long acting injections and implants emerged as a sub-area of pharmaceutics in the twentieth century, with companies dedicated to the field being established in the 1960s and 1970s. The field contains a wide range of system types. This chapter summarizes the historical development of the field, including rate-controlled membrane concepts, biodegradable polymer concepts, surface-releasing systems, liposomes, targeted/nanoscale systems, and microelectronic systems.

2.1 Introduction

Sustained parenteral drug delivery began to emerge as a clearly defined sub-area of pharmaceutics in the middle of the twentieth century. The development of the field has been significantly influenced by advances in pharmacokinetics and pharmacodynamics, which served to highlight the need for controlled, extended drug delivery and sustained drug plasma/tissue levels in achieving desired therapeutic responses.

In the 1960s and 1970s, companies dedicated to controlled delivery were established (e.g., Alza, Elan). The field of long acting injections and implants consists of trends and technological developments that converge, diverge and sometimes reconverge, somewhat reconfigured. Two major trends have been the development of pharmaceutical chemistry (including biotechnology) and advanced materials science, especially polymer technology.

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The field has encompassed dissolution-controlled systems, liposomes and micelles, oleaginous depots, membrane rate-controlled implants, micro- and nanoparticles, extended circulation conjugates and in situ forming systems. Some of these systems "borrow" concepts or materials from another class, with the objective of providing long acting therapy. Systems in this field include those that provide zero-order (constant rate) delivery of drugs and sustained-release systems that provide long acting therapy, though not necessarily at a constant rate. Long acting injections and implants can provide systemic, local, or targeted therapy. Systems can also be viewed as macroscale, microscale, or nanoscale [1]. This chapter provides an overview of the historical development of this field with an emphasis on systems that have achieved clinical or commercial success; individual following chapters provide more details on key system types.

2.2 Early History

By the 1930s, it was recognized that implanted pellets containing hydrophobic compounds could provide sustained release of drugs [2]. Examples of these pellet systems included pellets containing estradiol for the treatment of prostate cancer and pellets containing testosterone for the treatment of testosterone deficiency [3].

Additionally, it was recognized that depot formulations of drugs or esters with very low water solubility could also provide extended delivery. These depots often utilized oleaginous vehicles. Examples include procaine penicillin G in an aqueous vehicle and fluphenazine decanoate in a sesame oil vehicle as an antipsychotic preparation ([3–5]; oil-based solutions and suspensions are discussed further in the chapter on oily (lipophilic) solutions and suspensions).

In the early 1960s, T. Higuchi presented the now classic "Higuchi model" [6]. While originally for release of drug dispersed in an ointment, the model was subsequently applied to the release of drugs from a variety of matrix systems. The Higuchi model (2.1) indicates that extended drug release will be observed from solid drug dispersed in a matrix, but will vary with (time)$^{1/2}$:

$$M_t / M_\infty = 2\left\{DC_s(2C_0 - C_s)t\right\}^{1/2} / C_0 l \tag{2.1}$$

In this equation, C_0 is the total concentration, D is the diffusivity and C_s is the solubility of the drug in the matrix. The surface area and thickness of the depot are denoted by A and l, respectively. Equation (2.1) describes release from a rectangular slab, so that $M_\infty = Al\,C_0/2$. It should be noted that the above model is for a drug delivery system where the rate of diffusion of the drug through the system matrix is the rate-controlling phenomena. The form of the model given above assumes that there is rapid transport of the drug through any diffusional boundary layer at the surface of the system.

2.3 Rate-Controlling Membrane Concepts

In the mid-1960s, while circulating rabbit blood inside a Silastic® (silicone rubber) arterio-venous shunt, Folkman discovered that if the tubing was exposed to anesthetic gases on the outside, the rabbits would fall asleep [7]. He proposed that short, sealed segments of such tubing containing a drug could be implanted and form the basis of a constant rate drug delivery system [8].

Further work in the 1960s and 1970s led to the establishment of the rate-controlling membrane (RCM)/reservoir drug delivery system (DDS) concept as yielding a constant delivery rate and producing a zero-order, flat pharmacokinetic profile. The first commercial RCM product was the Ocusert® that was developed and commercialized in the early 1970s by ALZA Corporation for the treatment of glaucoma. It was an elliptical-shaped planar system that was inserted into the cul-de-sac of the eye and delivered pilocarpine at a controlled rate for 1 week. The product utilized poly(ethylene-co-vinyl acetate) (polyEVA) as the RCM, thereby introducing this versatile material for controlled-release applications. This product was followed by the Progestasert® (also ALZA Corporation), a T-shaped device that was inserted into the uterus and released progesterone for a 1-year period for contraception. The RCM of this system was also polyEVA, further demonstrating the utility of this polymer [9]. Subsequently, the Population Council developed a contraceptive subcutaneous implant system comprised of six silicone rubber tubes (crosslinked polydimethylsiloxane) containing the steroid levo-Norgestrel. The system was trade named the Norplant® (Fig. 2.1) and has a 5-year delivery duration. It was introduced

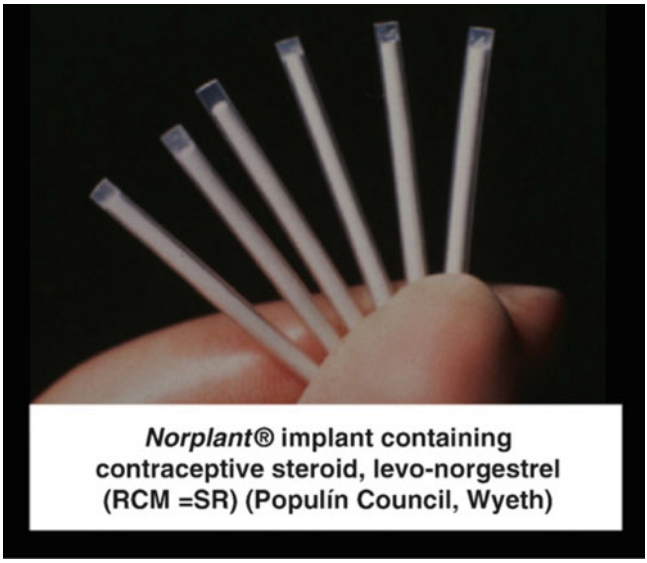

Fig. 2.1 Photograph of the Norplant® System (reprinted from [1] with permission from Elsevier)

in certain countries in 1983, but it was not until 1990, that Norplant was approved in the United States. Later in the United States, Norplant became associated with removal problems due to operator inexperience leading to poor insertion technique and thereby resulting in explantation difficulties. Norplant® was withdrawn from the U.S. market in 2002, but is still available in other countries. Organon has recently developed a similar system (Implanon®), using polyEVA as the RCM for the delivery of etonogestrel for up to 3 years. Implanon was approved by the FDA in 2006. Additionally, following the ALZA work with polyEVA, the polymer was investigated for protein delivery but was not commercialized for this application [10].

Additionally, implants utilizing a hydrogel RCM were investigated leading to the development of the Hydron® Implant, a nondegradable reservoir implant capable of long-term (1 year or longer) delivery. This technology has been utilized for the delivery of the LHRH agonist, histrelin acetate for the treatment of precocious puberty (Supprelin® LA) and for prostate cancer (Vantas®) (http://www.endo.com, accessed July 2010) (see Sect. 2.4 for other delivery systems for LHRH analogues).

The osmotic pump is a variant of the rate-controlled membrane system. Building on earlier work [11] and beginning in the 1970s and continuing into the 1990s, Theeuwes and coworkers at Alza developed a family of osmotic pump systems. This work led first to the development of the ALZET® pump that provides zero-order delivery when implanted into research animals [12] and subsequently to the DUROS® osmotic implant system for human therapy ([13]; also see the chapter on systems based on osmosis). Both of these systems are zero-order, diffusion-controlled systems with RCMs, but the difference is that in the osmotic systems the RCM controls a constant rate of water diffusion into the system, forcing an equal volume of the drug solution or suspension out of the system reservoir through the delivery orifice. The RCM in the ALZET pump is based on cellulose esters (e.g., cellulose acetate), while the RCM in the DUROS system is polyurethane.

2.4 Biodegradable Polymer Concepts

Biodegradable polymers are inherently attractive for drug delivery applications because of two potential major attributes: first, if the polymer erodes only at the surface, then it would seem possible to engineer systems yielding sustained or constant release. Second, for parenteral applications, the system can be expected to completely erode, thereby eliminating the need for a procedure to remove the system at the end of the delivery lifetime.

Investigations of biodegradable polymers of poly(hydroxy acids) for drug delivery applications began in the 1960s and 1970s and the polymers continue to be utilized today. These polymers were developed for sutures in the 1960s and 1970s. Schmitt and Polestina at Davis & Geck (Cyanamid Co.) synthesized poly(glycolic acid) (PGA) for use as a degradable suture [14]. Ethicon added lactic acid to the composition, licensed the PGA technology from Davis and Geck, and introduced the degradable poly(lactic-co-glycolic acid) (PLGA) suture (Vicryl®).

In the late 1960s, Boswell and Scribner at Dupont developed microparticle and pellet depot delivery systems by adding drugs to PLA [15]. In parallel, in the 1970s, researchers at Southern Research Institute and the University of Alabama at Birmingham (UAB) (e.g., Cowser, Lewis and Beck) were investigating and clinically testing steroid-loaded PLGA microparticles for contraceptive drug delivery [Tice T (2008) personal communication]. Additionally, Gilding and Reed carried out important early studies on the properties and in vitro degradation of PLGA copolymers [16, 17].

While much of the early development of PLGA systems focused on small molecule drugs, a number of research groups and pharmaceutical companies began investigating the delivery of leutinizing hormone-releasing hormone (LHRH) analogues. In the late 1970s, under a Southern Research Institute project sponsored by Syntex, a Southern/Syntex team developed and patented long acting (1-month) PLGA microparticles for the delivery of LHRH [18]. In Europe in 1986, Debiopharm introduced a PLGA microparticle system for the delivery of triptorelin [D-Trp-6 LHRH; Decapeptyl®] as a treatment for prostate cancer. It was the first injectable, degradable microparticle depot drug delivery system to obtain regulatory approval and is still on the market today [Tice T (2008) personal communication]. Takeda Pharmaceutical Company (Takeda-Abbott Pharmaceutical Co. (TAP) in the United States) also developed a PLGA microsphere product for the delivery of a LHRH analogue (leuprolide, Lupron® Depot), licensing the Syntex patent [18] in addition to filing other patents. The Lupron Depot was introduced in the United States in 1989.

Additionally, implants composed of PLGA were investigated for a number of applications, including delivery of LHRH analogues. The Zoladex® implant (PLGA matrix system delivering the LHRH analogue goserelin) was developed for the treatment of prostate cancer, breast cancer and endometriosis, and was commercialized world-wide [20, 21].

Through the 1970s and 1980s, emulsion/solvent evaporation techniques or phase separation techniques were utilized to prepare microparticles for drug delivery applications. Gombotz and coworkers at Enzytech developed the Prolease® process in the early 1990s for fabricating PLGA microparticles. This process utilizes an ultrasonic sprayer and a liquid nitrogen/ethanol bath [22]. This process has been applied to the development of the Neutropin Depot (for delivery of the protein recombinant growth hormone) and Risperdal® Consta® (for delivery of risperidone, an antipsychotic drug).

In the 1970s, Heller and Choi at ALZA synthesized the first in a series of degradable polyorthoester (POE) polymers. Heller and coworkers continued to develop the POE family [23]. Recently (2009), a POE product delivering granisetron was submitted to the U.S. FDA for approval.

In the late 1980s, Dunn and coworkers at Southern Research Institute developed injectable, degradable drug depot systems of PLA or PLGA. These systems are in situ forming “implants” generated by subcutaneous or IM injections of drug/polymer/solvent formulations, with subsequent phase separation and solvent loss [24]. (This drug depot was extended into the clinic by Atrix, Inc. and has resulted in several

approved products, including the Eligard® system for the delivery of the LHRH analogue leuprolide; refer to the chapter on in situ forming systems).

Langer and coworkers developed a family of polyanhydride biodegradable polymers. The Gliadel® wafer utilizes this technology for the delivery of carmustine (BCNU) for the treatment of brain tumors (glioblastomas) [25]. Brem pioneered the clinical application of these systems and the product was approved by the FDA in 1996.

Thermally responsive, aqueous solutions of di-block and tri-block copolymers of PLGA–PEG were investigated by Kim and Byun at the University of Utah as degradable depot systems. These systems are trade named "Re-Gel®" (MacroMed, Inc.) and have been applied to the delivery of anticancer products and other drugs ([26]; refer to the chapter on in situ forming systems).

Another biodegradable polyester class based on copolymers of poly(ethylene glycol terephthalate) (PEG-T) and poly(butylene terephthalate) (PBT) was investigated for drug delivery applications by Feijen and coworkers at Twente University beginning in the 1990s [27]. A microparticle depot formulation of this copolymer and alpha interferon, trade named Locteron®, is currently under clinical investigation for the treatment of hepatitis [28].

2.5 Surface-Releasing Systems

Release of the anticoagulant heparin from polymer surfaces was an early, successful drug delivery system. Introduced into the clinic in the early 1960s, the concept was based on an ionic complex of heparin, an anionic polysaccharide, with a cationic surfactant that was hydrophobically imbedded in the surface of the polymer being "heparinized." The heparin was gradually released by exchange with the ions present in blood, thereby inhibiting coagulation of blood at the surface of the polymer until the heparin concentration decreased to low levels, usually after several days (refer to Plate and Valuev [29] for a review of heparinized surfaces).

A more recent application of drug-loaded surface coatings are the drug-eluting stents (DES) for the treatment of occluded or partially occluded arteries [30]. First approved in 2002, these stents have been one of the most successful recent drug delivery systems and have been widely adopted. The DES with the earliest regulatory approvals in the United States are the Cypher® stent (rapamycin, Johnson & Johnson) and the Taxus® stent (paclitaxel, Boston Scientific). These stents release smooth muscle cell (SMC) antagonists that retard SMC proliferation, the main cause of vessel stenosis. These DES are designed as matrix delivery systems; drug particles are blended with the polymer, which in the Cypher® stent is a polyblend of poly(butyl methacrylate) and polyEVA, while the polymer utilized in the Taxus® stent is a novel tri-block copolymer of polystyrene–polyisobutylene–polystyrene (SIBS). (Refer to the chapter on drug eluting stents).

DES are considered drug–device combination products, a product classification that overlaps with drug delivery systems and long acting implants.

Additional combination products that have been investigated include, among others, antibiotic impregnated bone cement matrices and implanted insulin pumps containing a glucose sensor that enables feedback control [31].

2.6 Nanotherapeutics, Conjugates, Liposomes, Micelles, and Targeting

Three key technologies: Three key scientific discoveries led to three key technologies that have been major factors in stimulating the growth in research and translational activity in nanotherapeutics. The first was the development of "PEGylation" technology in the late 1970s and early 1980s. The term "PEGylation" refers to polyethylene glycol-conjugated drugs or carriers. The second key development, "active targeting," was based on the concept of the "magic bullet," originally described by Ehrlich in the early 1900s. It became possible upon the discovery of monoclonal antibodies in the mid-1970s. (The use of a polyclonal antibody to target a drug was described in 1958 [32], while the use of monoclonal antibodies did not happen until after their discovery in 1975.) The famous peptide ligand, RGD [33], was also first described around the same time (in 1980). Active targeting was accomplished by attaching such targeting moieties directly to the drug or to its nanocarrier. The third key development was the discovery of the "enhanced permeation and retention effect" (EPR) in the early 1980s, wherein nanocarriers are entrapped within tumors. It is interesting that all three key technologies originated in the 1970s–1980s.

2.6.1 PEGylation

PEGylation was developed to enhance both the circulation time and the stability (against enzyme attack or immunogenic recognition) of recombinant protein drugs [34]. Enzon, a pioneering PEGylation company, was founded in the beginning of the 1980s. The first products were PEGylated enzymes such as asparaginase and glutaminase, which metabolize asparagine and glutamine, essential nutrients for leukemic cancer cells. These products were followed by PEGylated interferons and PEGylated G-CSF (refer to the chapters on protein and nanocarrier PEGylation for additional information).

2.6.2 Enhanced Permeation and Retention Effect

The "Enhanced Permeation and Retention" effect, or EPR, was discovered by Maeda and coworkers in 1984. The team was conducting animal studies with a novel polymer–drug conjugate, styrene-maleic anhydride (SMA) conjugated to

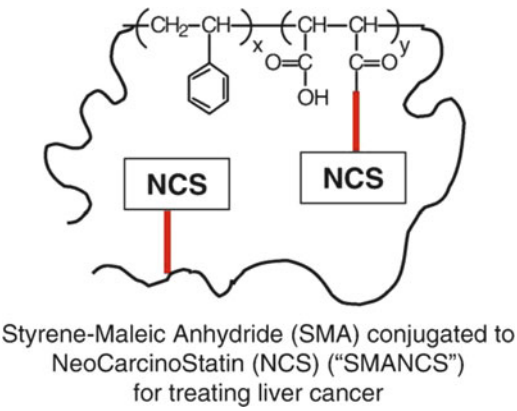

Fig. 2.2 Diagram of "SMANCS" (polymer–drug conjugate of poly(styrene-comaleic anhydride) (SMA) and NeoCarcinoStatin (NCS)) (reprinted from [1] with permission from Elsevier)

neocarcinostatin (NCS), an anticancer peptide drug (Fig. 2.2). They bound a blue dye to albumin and discovered that it accumulated in the tumor tissue of an experimental animal. Maeda concluded that the rapidly forming vasculature in such solid tumors was "leaky" and the lymph drainage system was not yet functioning efficiently, causing the entrapment of the nanoscale albumin–dye complex within the tumor tissue. They also injected SMANCS and saw the same effect [35].

2.6.3 Liposomes and Micelles

Liposomes were first described by Bangham in the 1960s. Their potential for drug delivery led to research in this area, wherein hydrophilic drugs could be loaded in the aqueous core of the liposome, or hydrophobic drugs could be loaded in the lipid bilayer shell [36]. Woodle and Martin at Liposome Technologies Inc. (LTI) developed a PEGylated liposome-doxorubicin product, with PEG being grafted to the lipid components of the liposome's lipid bilayer. The product, termed a "Stealth®" liposome and trade-named Doxil®, exhibited enhanced circulation times and accumulation in tumor tissues. It was approved by the FDA in 1995 for the treatment of AIDS-related Kaposi's sarcoma (Fig. 2.3) ([37]; also refer to the chapter on liposomes).

Another nanocarrier, polymeric micelles, were developed for drug delivery in 1990. Kataoka, Okano and Yokoyama synthesized A–B block copolymers of a block of a partially butylated aspartic acid that was conjugated to a PEG block. These copolymers spontaneously formed PEGylated polymeric micelles above a very low critical micelle concentration (CMC). The micelles had a PEG corona with a hydrophobic butyl aspartate core. Small hydrophobic drugs could be loaded into the core, either by physically loading or by conjugating the drug to the amino acid's remaining pendant acid groups. Additionally, the terminal OH groups of the PEGs

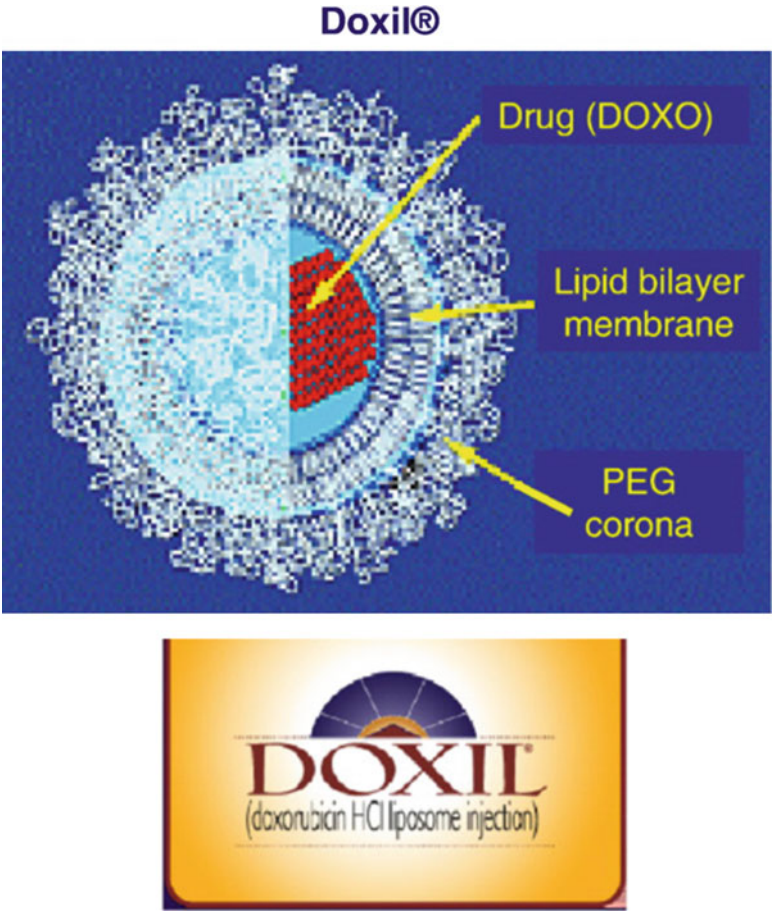

Fig. 2.3 Schematic of the PEGylated lipsome Doxil®, which contains the anticancer drug doxorubicin (reprinted from [1] with permission from Elsevier)

could be coupled to cell-specific ligands for targeted delivery (Fig. 2.4) [19, 38, 39]. Independent of Kataoka et al.'s work, drug-loaded PEGylated micelles based on the Pluronic® family of PEO-PPO-PEO tri-block copolymers were investigated by Kabanov and coworkers [40]. A number of different PEGylated micelles are now under clinical investigation for the delivery of small molecule drugs.

2.6.4 Polymer–Drug Conjugates, Targeting and Nanotherapeutics

The concept of polymer–drug conjugates or "nanotherapeutics" independently arose at several research centers in the mid to late 1970s. In the mid-1970s, PEGylated protein drugs were being synthesized and tested by Davis and

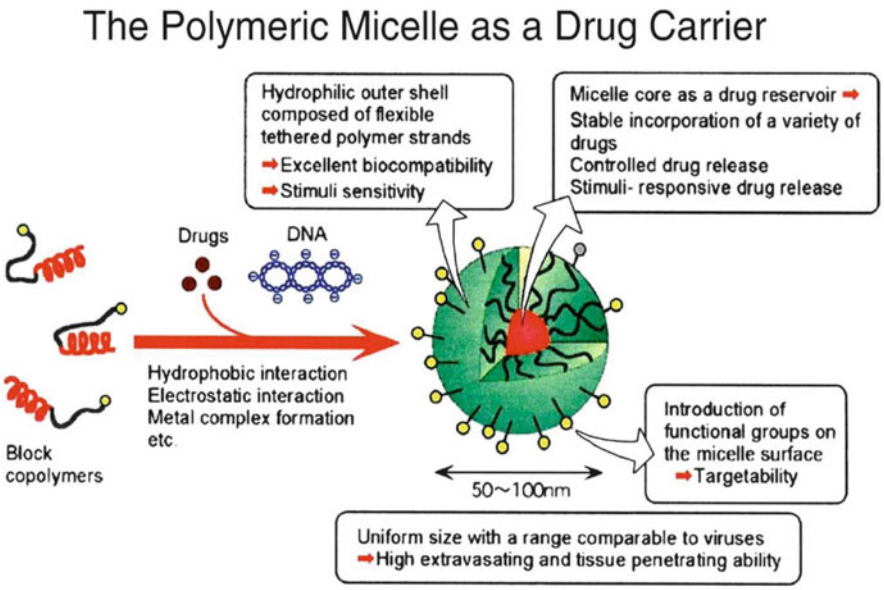

Fig. 2.4 Diagram of the PEGylated polymeric micelle drug carrier (reprinted from [1] with permission from Elsevier)

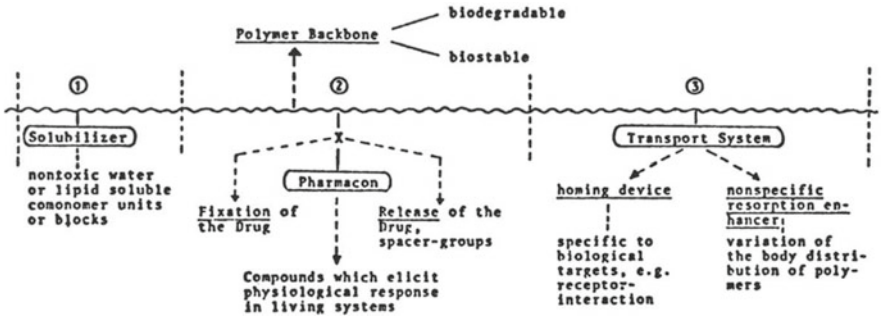

Fig. 2.5 Schematic from Ringsdorf [41] illustrating proposal for the concept of the "complete" polymer–drug construct, including targeting (reprinted from [1] with permission from Elsevier)

Aubuchowski in the United States. In 1975 in Mainz, Germany, Ringsdorf independently outlined the concept of a targeted, polymer–drug conjugate (Fig. 2.5) [41]. Independent of those scientists, and also in the mid-1970s, Kopecek in Prague described the conjugation of drugs to their new polymer carrier, poly(hydroxypropyl methacrylamide) (PHPMA). A drug was conjugated to PHPMA by pendant, degradable peptide linkages. Later, in collaboration with Duncan, the tetrapeptide linkages were designed to be degradable by a lysosomal enzyme, cathepsin B [42–44]. The polymer synthesis and characterization were conducted in Prague while the conjugate's

drug action was tested in the UK in collaboration with Duncan and Lloyd (Interestingly, Kopecek was introduced to Duncan and Lloyd by Ringsdorf). Rihova in Prague found PHPMA to be nonimmunogenic, and Cassidy, a UK clinician, led the clinical investigations, which included doxorubicin and other small molecule anticancer drugs. Active targeting for liver cancer therapy was accomplished with ligands such as galactose, a membrane receptor ligand for hepatocytes. This international success story has significantly influenced the field of nanoscale polymeric therapeutics and several reviews have been published [45, 46].

Albumin-drug carriers have also been developed. These drug nanocarriers are, in some senses, similar to PEGylated drug nanocarriers. Abraxane® (Abraxis Bioscience) is a nanoparticle of albumin and paclitaxel (http://www.drugs.com/pro/abraxane.html, accessed 28 Aug 2010) that has received regulatory approval for the treatment of breast cancer.

2.7 Microelectronic/Microfabricated Technologies

In the period from 1960 to the present, microelectronic technology has experienced a huge growth in capacity and almost unimaginable reductions in size and cost. For drug delivery, the technology was initially applied via improved control and capabilities for pump systems. In the 1990s, Langer, Cima, Santini and coworkers applied microelectronic chip technology to implantable drug delivery via a chip containing addressable, drug-filled reservoirs covered with a film that could be disintegrated via electric current [47]. This type of system can provide a variety of drug delivery patterns. Sensing elements may also be included in the microelectronic system, thereby enabling feedback controlled drug delivery. Since the initial publications, the concept of microelectronic/microfabricated drug delivery systems has been investigated and extended by a number of investigators. (Refer to Chap. 18).

2.8 Conclusion

This chapter has attempted to provide an overview of the major developments in long-lasting injections and implants over the last 50 years or so. Polymer technology, pharmaceutical chemistry and biotechnology have enabled significant advances in the field, with products as diverse as nonerodible implants, erodible implants, microspheres, liposomes, targeted nanoparticles, and microelectronic implants. There have been significant benefits to the patient and to the practitioner in terms of efficacy, side effects, convenience, duration of therapy, and compliance with the treatment regimen. These historical systems can generally be characterized as macro- or microscale, but, as indicated above, work at the nanoscale has progressed into clinical applications.

Disclaimer

In a summary survey such as this chapter, it is impossible to describe every system developed since the mid-twentieth century and it is impossible to know of and include all such examples and to properly credit all the key people who helped to bring the various technologies and devices to the clinic. The authors apologize in advance for all omissions.

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Chapter 3
Host Response to Long Acting Injections and Implants

James M. Anderson

Abstract Perspectives on the in vivo biocompatibility of drug delivery systems to be considered in the design, development, and evaluation of drug delivery systems are presented. Temporal events occurring in response to the implanted/injected drug delivery system are presented, including early events following injection/implantation, acute inflammation, chronic inflammation, granulation tissue formation, foreign body reaction, and fibrosis/fibrous encapsulation. Both nonbiodegradable and biodegradable/resorbable systems are discussed. Important in the identification of biocompatibility of any drug delivery system is the intensity and/or time duration of the various components of the inflammatory reaction, wound healing responses, and foreign body responses. Finally, a section on immunotoxicity (acquired immunity) has been included due to current events in development of controlled sustained release systems focused on delivery of biologically reactive agents that may potentially initiate an adaptive immune response.

3.1 Introduction

In the research and development of implantable or injectable long acting drug delivery systems, in vivo biocompatibility studies play an important role in determining the safety and efficacy of these devices. The evaluation of the biocompatibility of implantable or injectable drug delivery systems requires an understanding of the inflammatory and healing responses of implantable materials. For delivery systems, this includes an appreciation of the inflammatory and healing responses of degradable/resorbable systems as well as nondegradable systems. In vivo biocompatibility

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studies commonly utilize subcutaneous implantation and examples of the author's efforts and the efforts of others in the literature will be utilized to illustrate important issues pertinent to the biocompatibility of implantable delivery systems. As the desired goal of delivery systems is to deliver or release a drug or therapeutic agent either locally or systemically in vivo, studies that concomitantly monitor the delivery of therapeutic agents in conjunction with the biocompatibility of the system are important. From this perspective, examples utilizing the cage implant system will be presented. The cage implant system permits the simultaneous in vivo analysis of the release of therapeutic agents and components of the inflammatory and healing responses that dictate the overall biocompatibility of the system. It is not our intent to provide a complete literature review of biocompatibility studies that have been carried out on degradable/resorbable and nonbiodegradable materials utilized for implantable delivery systems but, rather, to present significant perspectives on the in vivo biocompatibility of drug delivery systems that should be considered in the design, development, and evaluation of drug delivery systems.

3.2 Overview

Inflammation, wound healing, and foreign body responses are generally considered as parts of the tissue or cellular host responses to injury. Table 3.1 lists the sequence of these events following injury. From the drug delivery system perspective, placement in the in vivo environment involves injection, insertion, or surgical implantation, all of which injure the tissues or organs involved [1–6]. The implantation procedure initiates a response to injury and the mechanisms are activated to produce healing. The degree to which the homeostatic mechanisms are perturbed and the extent of pathophysiologic responses and their resolutions are measures of host reactions to the biomaterial or drug delivery system. Host reactions are tissue dependent, organ dependent, and species dependent.

The sequence of events following implantation of a drug delivery system or biomaterial is illustrated in Fig. 3.1 and Table 3.1. The size, shape, and chemical and physical properties of the biomaterial and the physical dimensions and properties of the material, prosthesis or device may be responsible for variations in the intensity and time duration of the inflammatory and wound healing processes. Thus, intensity and/or time duration of the inflammatory reaction may characterize the biocompatibility of a drug delivery system or biomaterial [1, 3, 5, 7, 8].

3.3 Early Events Following Injection/Implantation

Inflammation is generally defined as the reaction of vascularized living tissue to local injury. Inflammation serves to contain, neutralize, dilute, or wall off the injurious agent or process [9]. In addition, it sets into motion a series of events that may heal

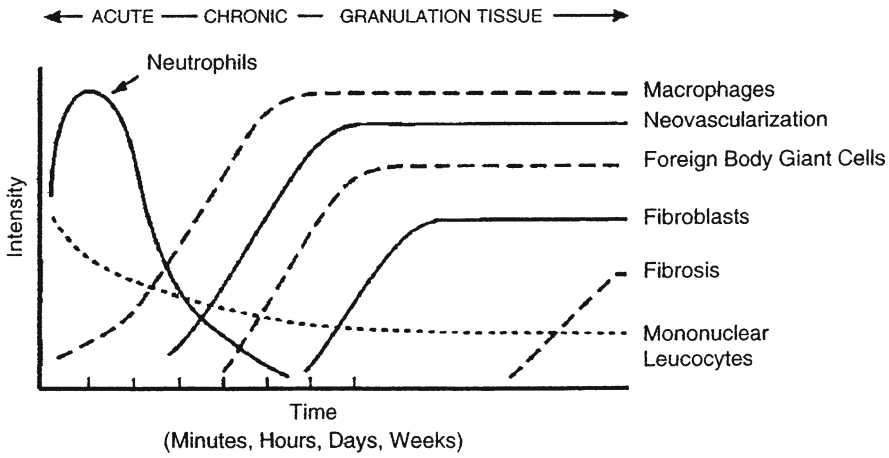

Fig. 3.1 The temporal variation in the acute inflammatory response, chronic inflammatory response, granulation tissue development, and foreign body reaction to implanted biomaterials. The intensity and time variables are dependent upon the extent of injury created in the implantation and the size, shape, topography, and chemical and physical properties of the biomaterial

Table 3.1 Sequence of host reactions

Injury
Blood–material interactions
Provisional matrix formation
Acute inflammation
Granulation tissue
Foreign body reaction
Fibrosis/fibrous capsule development

and reconstitute the injection/implant site through replacement of the injured tissue by regeneration of native parenchymal cells, formation of fibroblastic scar tissue, or a combination of these two processes.

Immediately following injury, there are changes in vascular flow, caliber, and permeability. Fluid, proteins, and blood cells escape from the vascular system into the injured tissue in a process called exudation. Following changes in the vascular system, which also include changes induced in blood and its components, cellular events occur and characterize the inflammatory response. The effect of the injury, drug delivery system, or biomaterial in situ on plasma or cells can produce chemical factors that mediate many of the vascular and cellular responses of inflammation (Table 3.2).

Blood–material interactions and the inflammatory response are intimately linked, and in fact, early responses to injury involve mainly blood and vasculature. Regardless of the tissue or organ into which a biomaterial is implanted, the initial inflammatory response is activated by injury to vascularized connective tissue. Since blood and its components are involved in the initial inflammatory responses, blood clot formation and/or thrombosis also occur. Blood coagulation and

Table 3.2 Important chemical mediators of inflammation derived from plasma, cells, or injured tissue

Mediators	Examples
Vasoactive agents	Histamine, serotonin, adenosine, endothelial derived relaxing factor (EDRF), prostacyclin, endothelin, thromboxane A_2
Plasma proteases	
Kinin system	Bradykinin, kallikrein
Complement system	C3a, C5a, C3b, C5b–C9
Coagulation/fibrinolytic system	Fibrin degradation products, activated Hageman factor (FXIIA), tissue plasminogen activator (tPA)
Leukotrienes	Leukotriene B_4 (LTB_4), hydroxyeicosatetranoic acid (HETE)
Lysosomal proteases	Collagenase, elastase
Oxygen-derived free radicals	H_2O_2, superoxide anion, nitric oxide
Platelet-activating factors	Cell membrane lipids
Cytokines	Interleukin 1 (IL-1), tumor necrosis factor (TNF)
Growth factors	Platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF-α or TGF-β), epithelial growth factor (EGF)

thrombosis are generally considered humoral responses and may be influenced by other homeostatic mechanisms such as the extrinsic and intrinsic coagulation systems, the complement system, the fibrinolytic system, the kinin-generating system, and platelets. Thrombus or blood clot formation on the surface of a biomaterial is related to the well-known Vroman effect, in which a hierarchical and dynamic series of collision, absorption, and exchange processes, determined by protein mobility and concentration, regulate early time-dependent changes in blood protein adsorption. From a wound-healing perspective, blood protein deposition on the implant surface is described as provisional matrix formation. Blood interactions with biomaterials are generally considered under the category of hematocompatibility.

Injury to vascularized tissue in the injection/implantation procedure leads to immediate development of the provisional matrix at the implant site. This provisional matrix consists of fibrin, produced by activation of the coagulation and thrombosis systems, and inflammatory products released by the complement system, activated platelets, inflammatory cells, and endothelial cells. These events occur early, within minutes to hours following implantation of a medical device. Components within or released from the provisional matrix, i.e., fibrin network (thrombosis or clot), initiate the resolution, reorganization, and repair processes such as inflammatory cell and fibroblast recruitment. The provisional matrix appears to provide both structural and biochemical components to the process of wound healing. The complex three-dimensional structure of the fibrin network with attached adhesive proteins provides a substrate for cell adhesion and migration. The presence of mitogens, chemoattractants, cytokines, and growth factors within the provisional matrix provides for a rich milieu of activating and inhibiting substances for various cellular proliferative and synthetic processes. The provisional matrix may be viewed as a naturally derived, biodegradable, sustained release system in which mitogens, chemoattractants,

cytokines, and growth factors are released to control subsequent wound-healing processes. In spite of the increase in our knowledge of the provisional matrix and its capabilities, our knowledge of the control of the formation of the provisional matrix and its effect on subsequent wound healing events is poor. In part, this lack of knowledge is due to the fact that much of our knowledge regarding the provisional matrix has been derived from in vitro studies, and there is a paucity of in vivo studies that provide for a more complex perspective. Little is known regarding the provisional matrix, which forms at drug delivery system, biomaterial, or medical device interfaces in vivo, although attractive hypotheses have been presented regarding the presumed ability of materials and protein-adsorbed materials to modulate cellular interactions through their interactions with adhesive molecules and cells.

The predominant cell type present in the inflammatory response varies with the age of the inflammatory injury (Fig. 3.1). In general, neutrophils predominate during the first several days following injury and then are replaced by monocytes as the predominant cell type. Three factors account for this change in cell type: neutrophils are short lived and disintegrate and disappear after 24–48 h; neutrophil emigration from the vasculature to the tissues is of short duration; and chemotactic factors for neutrophil migration are activated early in the inflammatory response. Following emigration from the vasculature, monocytes differentiate into macrophages and these cells are very long-lived (up to months). Monocyte emigration may continue for days to weeks, depending on the injury and implanted biomaterial, and chemotactic factors for monocytes are activated over longer periods of time. In short-term (24 h) implants in humans, administration of both H1 and H2 histamine receptor antagonists greatly reduced the recruitment of macrophages/monocytes and neutrophils on polyethylene terephthalate surfaces [10]. These studies also demonstrated that plasma-coated implants accumulated significantly more phagocytes than did serum-coated implants.

While injury initiates the inflammatory response, the chemicals released from plasma, cells, or injured tissues mediate the inflammatory response. Important classes of chemical mediators of inflammation are presented in Table 3.2. Several points must be noted in order to understand the inflammatory response and how it relates to biomaterials. First, although chemical mediators are classified on a structural or functional basis, different mediator systems interact and provide a system of checks and balances regarding their respective activities and functions. Second, chemical mediators are quickly inactivated or destroyed, suggesting that their action is predominantly local (i.e., at the implant site). Third, generally the lysosomal proteases and the oxygen-derived free radicals produce the most significant damage or injury. These chemical mediators are also important in the degradation of biomaterials [11].

3.4 Acute Inflammation

Acute inflammation is of relatively short duration, lasting for minutes to hours to days, depending on the extent of injury. Its main characteristics are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes (predominantly

neutrophils). Neutrophils [polymorphonuclear leukocytes (PMNs)] and other motile white cells emigrate or move from the blood vessels to the perivascular tissues and the injury (implant) site. Leukocyte emigration is assisted by "adhesion molecules" present on leukocyte and endothelial surfaces. The surface expression of these adhesion molecules can be induced, enhanced, or altered by inflammatory agents and chemical mediators. White cell emigration is controlled, in part, by chemotaxis, which is the unidirectional migration of cells along a chemical gradient. A wide variety of exogenous and endogenous substances have been identified as chemotactic agents. Specific receptors for chemotactic agents on the cell membranes of leukocytes are important in the emigration or movement of leukocytes. These and other receptors also play a role in the transmigration of white cells across the endothelial lining of vessels and activation of leukocytes. Following localization of leukocytes at the injury (implant) site, phagocytosis and the release of enzymes occur following activation of neutrophils and macrophages. The major role of the neutrophil in acute inflammation is to phagocytose microorganisms and foreign materials. Phagocytosis is seen as a three-step process in which the injurious agent undergoes recognition and neutrophil attachment, engulfment, and killing or degradation. In regard to biomaterials, engulfment and degradation may or may not occur, depending on the size, form, and properties of the biomaterial or drug delivery system.

Although biomaterials and injected microsphere drug delivery systems are not generally phagocytosed by neutrophils or macrophages because of the disparity in size (i.e., the surface of the biomaterial is greater than the size of the cell), certain events in phagocytosis may occur. The process of recognition and attachment is expedited when the injurious agent is coated by naturally occurring serum factors called "opsonins." The two major opsonins are immunoglobulin G (IgG) and the complement-activated fragment, C3b. Both of these plasma-derived proteins are known to adsorb to biomaterials, and neutrophils and macrophages have corresponding cell-membrane receptors for these opsonization proteins. These receptors may also play a role in the activation of the attached neutrophil or macrophage. Other blood proteins such as fibrinogen, fibronectin, and vitronectin may also facilitate cell adhesion to biomaterial surfaces. Owing to the disparity in size between the biomaterial surface and the attached cell, frustrated phagocytosis may occur. This process does not involve engulfment of the biomaterial but does cause the extracellular release of leukocyte products in an attempt to degrade the biomaterial.

Neutrophils adherent to complement-coated and immunoglobulin-coated nonphagocytosable surfaces may release enzymes by direct extrusion or exocytosis from the cell [12]. The amount of enzyme released during this process depends on the size of the polymer particle, with larger particles inducing greater amounts of enzyme release. This suggests that the specific mode of cell activation in the inflammatory response in tissue depends upon the size of the implant and that a material or drug delivery system in a phagocytosable form (i.e., powder, particulate, or nanomaterial) may provoke a different degree of inflammatory response than the same material in a nonphagocytosable form (i.e., film). In general, materials greater than

5 μm are not phagocytosed, while materials less than 5 μm, i.e., nanomaterials, can be phagocytosed by inflammatory cells.

Acute inflammation normally resolves quickly, usually in less than 1 week, depending on the extent of injury at the implant site. However, the presence of acute inflammation (i.e., PMNs) at the tissue/implant interface at time periods beyond 1 week (i.e., weeks, months, or years) suggests the presence of an infection.

3.5 Chronic Inflammation

Chronic inflammation is less uniform histologically than acute inflammation. In general, chronic inflammation is characterized by the presence of macrophages, monocytes, and lymphocytes, with the proliferation of blood vessels and connective tissue. Many factors can modify the course and histologic appearance of chronic inflammation.

Persistent inflammatory stimuli lead to chronic inflammation. While the chemical and physical properties of the biomaterial in themselves may lead to chronic inflammation, motion in the implant site by the biomaterial or infection may also produce chronic inflammation. The chronic inflammatory response to biomaterials is usually of short duration and is confined to the implant site. The presence of mononuclear cells, including lymphocytes and plasma cells, is considered chronic inflammation, whereas the foreign-body reaction with the development of granulation tissue is considered the normal wound-healing response to implanted biomaterials (i.e., the normal foreign-body reaction). Chronic inflammation with the presence of collections of lymphocytes and monocytes at extended implant times (weeks, months, years) also may suggest the presence of a long-standing infection. The prolonged presence of acute and/or chronic inflammation also may be due to toxic leachables from a biomaterial [7, 13].

The following example illustrates this point. In vivo implantation studies were carried out subcutaneously in rats and rabbits with naltrexone-sustained release preparations that included placebo (polymer only) beads and naltrexone-containing beads [14]. Histopathological tissue reactions utilizing standard procedures and light microscopic evaluation to the respective preparations were determined at days 3, 7, 14, 21, and 28. The only significant histological finding in both rats and rabbits at any time period was the persistent chronic inflammation that occurred focally around the naltrexone-containing beads. The focal inflammatory cell density in both rats and rabbits was higher for the naltrexone beads than for the placebo beads at days 14, 21, and 28, respectively. This difference in inflammatory response between naltrexone beads and placebo beads increased with increasing time of implantation. Considering the resolution of the inflammatory response for the placebo beads with implantation time in both rats and rabbits, it is suggested that the naltrexone drug itself is identified as the causative agent of the focal chronic inflammation present surrounding the naltrexone beads in the implant sites.

The important lesson from this case study is the necessary use of appropriate control materials. If no negative control, i.e., placebo polymer-only material, had been used, the polymer in the naltrexone-containing beads also would have been considered as a causative agent of the extended chronic inflammatory response. Similar chronic inflammatory responses have been identified with drugs, polymer plasticizers and other additives, fabrication and manufacturing aids, and sterilization residuals. Each case presents its own unique factors in a risk assessment process necessary for determining safety (biocompatibility) and benefit versus risk in clinical application.

Lymphocytes and plasma cells are involved principally in immune reactions and are key mediators of antibody production and delayed hypersensitivity responses. Although they may be present in nonimmunologic injuries and inflammation, their roles in such circumstances are largely unknown [15, 16]. Little is known regarding humoral immune responses and cell-mediated immunity to synthetic biomaterials. The role of macrophages must be considered in the possible development of immune responses to synthetic biomaterials. Macrophages and dendritic cells process and present the antigen to immunocompetent cells and thus are key mediators in the development of immune reactions.

Nanoparticle and microparticle delivery systems that are directly injected into the blood may be taken up and cleared by specific mononuclear phagocytes found in organs of the reticuloendothelial system (RES). In particular, Kupffer cells in the liver, fixed and free macrophages and dendritic cells in the spleen and lymph nodes, and pleural and peritoneal macrophages in the serous cavities can remove or phagocytose blood-borne particulate delivery systems. This effectively removes them from their intended site of action and may reduce the efficacy of such systems.

Monocytes and macrophages belong to the mononuclear phagocytic system (MPS), also known as the RES. These systems consist of cells in the bone marrow, peripheral blood, and specialized tissues. Table 3.3 lists the tissues that contain cells belonging to the MPS or RES. The specialized cells in these tissues may be responsible for systemic effects in organs or tissues secondary to the release of components or products from implants through various tissue–material interactions (e.g., degradation products) or the presence of implants (e.g., microcapsule or nanoparticle drug-delivery systems).

The macrophage is probably the most important cell in chronic inflammation because of the great number of biologically active products it can produce. Important classes of products produced and secreted by macrophages include neutral proteases, chemotactic factors, arachidonic acid metabolites, reactive oxygen metabolites, complement components, coagulation factors, growth-promoting factors, cytokines, and acid.

Phagolysosomes in macrophages can have acidity as low as pH of 3.5 and direct microelectrode studies of this acid environment have determined pH levels as low as 3.5. Moreover, only several hours are necessary to achieve these acid levels following adhesion of macrophages [17–21]. As indicated earlier, frustrated phagocytosis may occur in macrophages adherent to surfaces of drug delivery systems

Table 3.3 The mononuclear phagocytic system

Tissues	Cells
Implant sites	Inflammatory macrophages, foreign-body giant cells
Liver	Kupffer cells
Lung	Alveolar macrophages
Connective tissue	Histiocytes
Bone marrow	Macrophages
Spleen and lymph nodes	Fixed and free macrophages
Serous cavities	Pleural and peritoneal macrophages
Nervous system	Microglial cells
Bone	Osteoclasts
Skin	Langerhans' cells, dendritic cells
Lymphoid tissue	Dendritic cells

and biomaterials. This frustrated phagocytosis can result in the release of acid from the macrophage phagolysosomes and thus the pH that may be present in the privileged zone between the membrane of the macrophage and the surface of the material or drug delivery system may be as low as 3.5. This marked decrease in pH, i.e., marked increase in acidity, has implications for in vitro studies of drug release from delivery systems. In vitro drug release studies from delivery systems are commonly carried out at a pH of 7.0 but this in vitro condition does not replicate that which is possible in vivo. Biodegradable and bioerodible polymer delivery systems may undergo acid-catalyzed chain cleavage in the biodegradation process and thus it is appropriate to carry out in vitro studies at pH values of 3.0, 5.0, and 7.0 to determine the potential effect of the increased acidity in the privileged zone between the adherent macrophage and drug delivery surface.

Growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), TGF-α/epidermal growth factor (EGF), and interleukin-1 (IL-1) or tumor necrosis factor (TNF-α) are important to the growth of fibroblasts and blood vessels and the regeneration of epithelial cells. Growth factors released by activated cells can stimulate production of a wide variety of cells; initiate cell migration, differentiation, and tissue remodeling; and may be involved in various stages of wound healing.

3.6 Granulation Tissue

Within 1 day following implantation of a biomaterial (i.e., injury), the healing response is initiated by the action of monocytes and macrophages. Fibroblasts and vascular endothelial cells in the implant site proliferate and begin to form granulation tissue, which is the specialized type of tissue that is the hallmark of healing inflammation. Granulation tissue derives its name from the pink, soft granular

appearance on the surface of healing wounds, and its characteristic histologic features include the proliferation of new small blood vessels and fibroblasts. Depending on the extent of injury, granulation tissue may be seen as early as 3–5 days following injection/implantation of a drug delivery system or biomaterial.

The new small blood vessels are formed by budding or sprouting of preexisting vessels in a process known as neovascularization or angiogenesis [22, 23]. This process involves proliferation, maturation, and organization of endothelial cells into capillary vessels. Fibroblasts also proliferate in developing granulation tissue and are active in synthesizing collagen and proteoglycans. In the early stages of granulation tissue development, proteoglycans predominate but later collagen, especially type III collagen, predominates and forms the fibrous capsule. Some fibroblasts in developing granulation tissue may have the features of smooth muscle cells, i.e., actin microfilaments. These cells are called myofibroblasts and are considered to be responsible for the wound contraction seen during the development of granulation tissue. Macrophages are almost always present in granulation tissue. Other cells may also be present if chemotactic stimuli are generated.

The wound-healing response is generally dependent on the extent or degree of injury or defect created by the implantation procedure [24–28]. Wound healing by primary union or first intention is the healing of clean, surgical incisions in which the wound edges have been approximated by surgical sutures. Healing under these conditions occurs without significant bacterial contamination and with a minimal loss of tissue. Wound healing by secondary union or second intention occurs when there is a large tissue defect that must be filled or there is extensive loss of cells and tissue. In wound healing by secondary intention, regeneration of parenchymal cells cannot completely reconstitute the original architecture and much larger amounts of granulation tissue are formed that result in larger areas of fibrosis or scar formation. Under these conditions, different regions of tissue may show different stages of the wound-healing process simultaneously.

Granulation tissue is distinctly different from granulomas, which are small collections of modified macrophages called epithelioid cells. Langhans' or foreign-body-type giant cells may surround nonphagocytosable particulate materials in granulomas. Foreign-body giant cells (FBGCs) are formed by the fusion of monocytes and macrophages in an attempt to phagocytose the material.

3.7 Foreign Body Reaction

The foreign-body reaction to biomaterials is composed of FBGCs and the components of granulation tissue (e.g., macrophages, fibroblasts, and capillaries in varying amounts) depending upon the form and topography of the implanted material [3]. Relatively flat and smooth surfaces such as those found on breast prostheses have a foreign-body reaction that is composed of a layer of macrophages one to two cells in thickness. Relatively rough surfaces such as those found on the outer surfaces of expanded polytetrafluoroethylene (ePTFE) or Dacron vascular prostheses have a foreign-body reaction composed of macrophages and FBGCs at the surface. Fabric

materials generally have a surface response composed of macrophages and FBGCs, with varying degrees of granulation tissue subjacent to the surface response. A similar response with varying degrees of granulation tissue is seen with injected aggregates of microcapsules. Granulation tissue may form in the interstitial space or volume between the microcapsules and vasculature and, if present, may facilitate systemic circulation of drugs released from microcapsules or microspheres.

As previously discussed, the form and topography of the surface of the biomaterial determine the composition of the foreign-body reaction. With biocompatible materials, the composition of the foreign-body reaction in the implant site may be controlled by the surface properties of the biomaterial, the form of the implant, and the relationship between the surface area of the biomaterial and the volume of the implant. For example, high-surface-to-volume implants such as fabrics, porous materials, particulate, or microspheres will have higher ratios of macrophages and FBGCs in the injection/implant site than smooth-surface implants, which will have fibrosis as a significant component of the implant site.

The foreign-body reaction consisting mainly of macrophages and/or FBGCs may persist at the tissue–implant interface for the lifetime of the implant (Fig. 3.1). Generally, fibrosis (i.e., fibrous encapsulation) surrounds the biomaterial or implant with its interfacial foreign-body reaction, isolating the implant and foreign-body reaction from the local tissue environment. Early in the inflammatory and wound-healing response, the macrophages are activated upon adherence to the material surface [29].

Although it is generally considered that the chemical and physical properties of the biomaterial are responsible for macrophage activation, the subsequent events regarding the activity of macrophages at the surface are not clear. Tissue macrophages, derived from circulating blood monocytes, may coalesce to form multinucleated FBGCs. It is not uncommon to see very large FBGCs containing large numbers of nuclei on the surface of biomaterials. While these FBGCs may persist for the lifetime of the implant, it is not known if they remain activated, releasing their lysosomal constituents, or become quiescent [30].

Figure 3.2 demonstrates the progression from circulating blood monocyte to tissue macrophage to FBGC development that is most commonly observed. Indicated in the figure are important biological responses that are considered to play an important role in FBGC development. Material surface chemistry may control adherent macrophage apoptosis (i.e., programmed cell death) that renders potentially harmful macrophages nonfunctional, while the surrounding environment of the implant remains unaffected. The level of adherent macrophage apoptosis appears to be inversely related to the surface's ability to promote diffusion of macrophages into FBGCs, suggesting a mechanism for macrophages to escape apoptosis.

Figure 3.3 demonstrates the sequence of events involved in inflammation and wound healing when medical devices are implanted. In general, the PMN predominant acute inflammatory response and the lymphocyte/monocyte predominant chronic inflammatory response resolve quickly (i.e., within 2 weeks) depending on the type and location of the implant. Studies using IL-4 or IL-13, respectively, demonstrate the role for Th2 helper lymphocytes and/or mast cells in the development

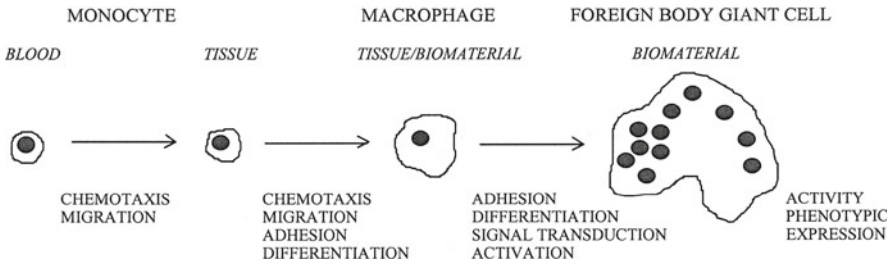

Fig. 3.2 In vivo transition from blood-borne monocyte to biomaterial adherent monocyte/macrophage to FBGC at the tissue–biomaterial interface. Little is known regarding the indicated biological responses, which are considered to play important roles in the transition to FBGC development

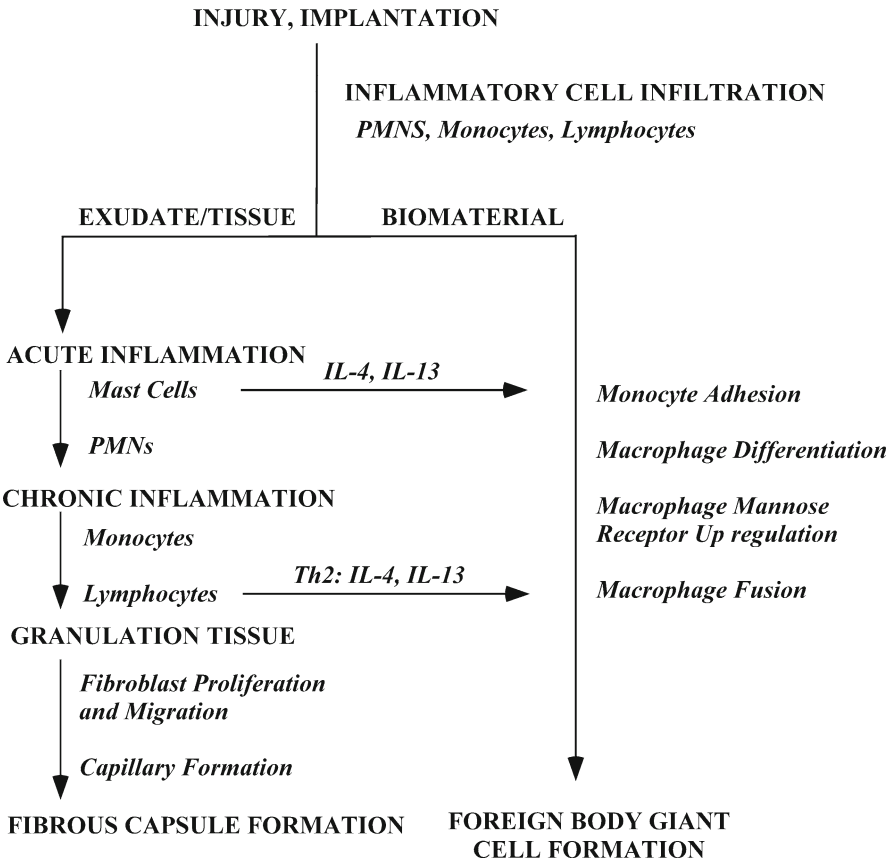

Fig. 3.3 Sequence of events involved in inflammatory and wound-healing responses leading to FBGC formation

of the foreign body reaction at the tissue/material interface [31, 32]. Integrin receptors of IL-4-induced FBGC are characterized by the early constitutive expression of αVβ1 and the later induced expression of α5β1 and αXβ2, which indicate potential interactions with adsorbed complement C3, fibrin(ogen), fibronectin, factor X, and vitronectin [31, 33–39]. Interactions through indirect (paracrine) cytokine and chemokine signaling have shown a significant effect in enhancing adherent macrophage/FBGC activation at early times, whereas interactions via direct (juxtacrine) cell–cell mechanisms dominate at later times [40, 41]. Th2 helper lymphocytes have been described as "anti-inflammatory" based on their cytokine profile, of which IL-4 is a significant component.

3.8 Fibrosis/Fibrous Encapsulation

The end-stage healing response to biomaterials is generally fibrosis or fibrous encapsulation. However, there may be exceptions to this general statement (e.g., porous materials inoculated with parenchymal cells or porous materials implanted into bone). As previously stated, the tissue response to implants is in part dependent upon the extent of injury or defect created in the implantation procedure and the amount of provisional matrix.

Repair of implant sites can involve two distinct processes: regeneration, which is the replacement of injured tissue by parenchymal cells of the same type, or replacement by connective tissue that constitutes the fibrous capsule. These processes are generally controlled by either (1) the proliferative capacity of the cells in the tissue or organ receiving the implant and the extent of injury as it relates to the destruction, or (2) persistence of the tissue framework of the implant site.

The regenerative capacity of cells allows them to be classified into three groups: labile, stable (or expanding), and permanent (or static) cells. Labile cells continue to proliferate throughout life; stable cells retain this capacity but do not normally replicate; and permanent cells cannot reproduce themselves after birth. Perfect repair with restitution of normal structure can theoretically occur only in tissues consisting of stable and labile cells, whereas all injuries to tissues composed of permanent cells may give rise to fibrosis and fibrous capsule formation with very little restitution of the normal tissue or organ structure. Tissues composed of permanent cells (e.g., nerve cells and cardiac muscle cells) most commonly undergo an organization of the inflammatory exudate, leading to fibrosis. Tissues of stable cells (e.g., parenchymal cells of the liver, kidney, and pancreas); mesenchymal cells (e.g., fibroblasts, smooth muscle cells, osteoblasts, and chondroblasts); and vascular endothelial and labile cells (e.g., epithelial cells and lymphoid and hematopoietic cells) may also follow this pathway to fibrosis or may undergo resolution of the inflammatory exudate, leading to restitution of the normal tissue structure.

The condition of the underlying framework or supporting stroma of the parenchymal cells following an injury plays an important role in the restoration of normal tissue structure. Retention of the framework with injury may lead to restitution of

the normal tissue structure, whereas destruction of the framework most commonly leads to fibrosis. It is important to consider the species-dependent nature of the regenerative capacity of cells. For example, cells from the same organ or tissue but from different species may exhibit different regenerative capacities and/or connective tissue repair.

Following injury, cells may undergo adaptations of growth and differentiation. Important cellular adaptations are atrophy (decrease in cell size or function), hypertrophy (increase in cell size), hyperplasia (increase in cell number), and metaplasia (change in cell type). Other adaptations include a change by cells from producing one family of proteins to another (phenotypic change), or marked overproduction of protein. This may be the case in cells producing various types of collagens and extracellular matrix proteins in chronic inflammation and fibrosis. Causes of atrophy may include decreased workload (e.g., stress-shielding by implants), and diminished blood supply and inadequate nutrition (e.g., fibrous capsules surrounding implants).

Local and systemic factors may play a role in the wound-healing response to biomaterials or implants. Local factors include the site (tissue or organ) of implantation, the adequacy of blood supply, and the potential for infection. Systemic factors may include nutrition, hematologic derangements, glucocortical steroids, and preexisting diseases such as atherosclerosis, diabetes, and infection.

3.9 Immunotoxicity (Acquired Immunity)

Current efforts in the development of controlled or sustained release systems are now focused on the delivery of biologically reactive agents such as growth factors, cytokines, and other biologics that may have the potential to initiate an adaptive immune response. Of special concern with these types of systems is the development of immunotoxicity or acquired immunity [42]. The following is a brief overview of mechanisms by which acquired immunity may develop and possibly result in immunotoxicity.

The inflammatory (innate) and immune (adaptive) responses have common components. It is possible to have inflammatory responses only with no adaptive immune response. In this situation, both humoral and cellular components that are shared by both types of responses may only participate in the inflammatory response. Table 3.4 indicates the common components to the inflammatory (innate) and immune (adaptive) responses. Macrophages and dendritic cells are known as professional antigen-presenting cells (APCs) responsible for the initiation of the adaptive immune response.

The acquired or adaptive immune system acts to protect the host from foreign agents or materials and is usually initiated through specific recognition mechanisms and the ability of humoral and cellular components to recognize the foreign agent or material as being “nonself” [43, 44]. Generally, the adaptive immune system may be considered as having two components: humoral or cellular. Humoral components include antibodies, complement components, cytokines, chemokines, growth factors,

Table 3.4 Common components in the inflammatory (innate) and immune (adaptive) responses

Components
Complement cascade components
Immunoglobulins
Cellular components
Macrophages
Natural killer (NK) cells
Dendritic cells
Cells with dual phagocytic and antigen presenting capabilities

and other soluble mediators. These components are synthesized by cells of the immune response and, in turn, function to regulate the activity of these same cells and provide for communication between different cells in the cellular component of the adaptive immune response. Cells of the immune system arise from stem cells in the bone marrow (B lymphocytes) or the thymus (T lymphocytes) and differ from each other in morphology, function, and the expression of cell surface antigens. They share the common features of maintaining cell surface receptors that assist in the recognition and/or elimination of foreign materials. Regarding controlled or sustained release systems, the adaptive immune response may recognize the biological components, modifications of the biological components, or degradation products of the biological components, commonly known as antigens, and initiate immune response through humoral or cellular mechanisms.

Components of the humoral immune system play important roles in the inflammatory responses to foreign materials. Antibodies and complement components C3b and C3bi adhere to foreign materials, act as opsonins and facilitate phagocytosis of the foreign materials by neutrophils and macrophages that have cell surface receptors for C3b. Complement component C5a is a chemotactic agent for neutrophils, monocytes and other inflammatory cells and facilitate the immigration of these cells to the implant site. The complement system is composed of classic and alternative pathways that eventuate in a common pathway to produce the membrane attack complex (MAC), which is capable of lysing microbial agents. The complement system, i.e., complement cascade, is closely controlled by protein inhibitors in the host cell membrane that may prevent damage to host cells. This inhibitory mechanism may not function when nonhost cells are used in tissue-engineered devices.

T (thymus-derived) lymphocytes are significant cells in the cell-mediated adaptive immune response, and their cell-adhesion molecules play a significant role in lymphocyte migration, activation, and effector function. The specific interaction of cell membrane adhesion molecules, sometimes also called ligands or antigens, with APCs produce specific types of lymphocytes with specific functions. Table 3.5 indicates cell types and function in the adaptive immune response. Obviously, the functions of these cells are more numerous than that indicated in Table 3.5 but the major function of these cells is provided to indicate similarities and differences in the interaction and responsiveness of these cells. Effector T cells (Table 3.6) are produced when their antigen-specific receptors and either the CD4 or the CD8

Table 3.5 Cell types and function in the adaptive immune system

Cell type	Function
Macrophages (APC)	Process and present antigen to immunocompetent T cells
	Phagocytosis
	Activated by cytokines, i.e., IFN-γ, from other immune cells
T cells	Interact with antigen-presenting cells (APCs) and are activated through two required cell membrane interactions
	Facilitate target cell apoptosis
	Participate in transplant rejection (type IV hypersensitivity)
B cells	Form plasma cells that secrete immunoglobulins (IgG, IgA, and IgE)
	Participate in antigen–antibody complex mediated tissue damage (type III hypersensitivity)
Dendritic cells (APC)	Process and present antigen to immunocompetent T cells
	Utilize Fc receptors for IgG to trap antigen–antibody complexes
Natural killer (NK) cells (non-T, non-B lymphocytes)	Innate ability to lyse tumor, virus infected, and other cells without previous sensitization
	Mediates T and B cell function by secretion of IFN-γ

Table 3.6 Effector T lymphocytes in adaptive immunity

Th1 helper cells	CD4+
	Proinflammatory
	Activation of macrophages
	Produces IL-2, interferon-γ (IFN-γ), IL-3, tumor necrosis factor-α, GM-CSF, macrophage chemotactic factor (MCF), migration inhibitor factor (MIF)
	Induce IgG2a
Th2 helper cells	CD4+
	Anti-inflammatory
	Activation of B cells to make antibodies
	Produces IL-4, IL-5, IL-6, IL-10, IL-3, GM-CSF, and IL-13
	Induce IgG1
Cytotoxic T cells (CTL)	CD8+
	Induce apoptosis of target cells
	Produce IFN-γ, TNF-β, and TNF-α
	Release cytotoxic proteins

coreceptors bind to peptide–MHC (major histocompatibility complex) complexes. A second, costimulatory signal is also required and this is provided by the interaction of the CD28 receptor on the T cell and the B7.1 and B7.2 glycoproteins of the immunoglobulin superfamily present on APCs. B lymphocytes bind soluble antigens through their cell-surface immunoglobulin and thus can function as professional APCs by internalizing the soluble antigens and presenting peptide fragments of these antigens as MHC:peptide complexes. Once activated, T cells can synthesize the T cell growth factor interleukin-2 and its receptor. Thus, activated T cells secrete and respond to interleukin-2 to promote T cell growth in an autocrine fashion.

Table 3.7 Selected cytokines and their effects

Cytokine	Effect
IL-1, TNF-α, INF-γ, IL-6	Mediate natural immunity
IL-1, TNF-α, IL-6	Initiate nonspecific inflammatory responses
IL-2, IL-4, IL-5, IL-12, IL-15 and TGF-β	Regulate lymphocyte growth, activation and differentiation
IL-2 and IL-4	Promote lymphocyte growth and differentiation
IL-10 and TGF-β	Downregulate immune responses
IL-1, INF-γ, TNF-α and MIF	Activate inflammatory cells
IL-8	Produced by activated macrophages and endothelial cells
	Chemoattractant for neutrophils
MCP-1, MIP-α and RANTES	Chemoattractant for monocytes and lymphocytes
GM-CSF and G-CSF	Stimulate hematopoiesis
IL-4 and IL-13	Promote macrophage fusion and foreign-body giant cell formation

Cytokines are the messenger molecules of the immune system. Most cytokines have a wide spectrum of effects, reacting with many different cell types, and some are produced by several different cell types. Table 3.7 presents common categories of cytokines and lists some of their general properties. It should be noted that while cytokines can be subdivided into functional groups, many cytokines such as IL-1, TNF-α, and IFN-γ are pleotropic in their effects and regulate, mediate, and activate numerous responses by numerous cells.

Immunotoxicity is any adverse effect on the function or structure of the immune system or other systems as a result of an immune system dysfunction [42, 43]. Adverse or immunotoxic effects occur when humoral or cellular immunity needed by the host to defend itself against infections or neoplastic disease (immunosuppression) or unnecessary tissue damage (chronic inflammation, hypersensitivity or autoimmunity) is compromised. Potential immunological effects and responses that may be associated with one or more of these effects are presented in Table 3.8. Hypersensitivity responses are classified on the basis of the immunologic mechanism that mediates the response. There are four types: type I (anaphylactic), type II (cytotoxic), type III (immune complex), and type IV cell-mediated (delayed) hypersensitivity. Hypersensitivity is considered to be increased reactivity to an antigen to which a human or animal has been previously exposed, with an adverse rather than a protective effect. Hypersensitivity is a synonym for allergy. Type I (anaphylactic) reactions and type IV (cell-mediated delayed hypersensitivity) reactions are the most common. Types II and III reactions are relatively rare and are less likely to occur with medical devices and biomaterials; however, with sustained release systems containing potential antigens, i.e., proteins, types II and III reactions must be considered in biological response evaluations.

Type I (anaphylactic) hypersensitivity reactions are mediated by IgE antibodies which are cytotropic and affect the immediate release of basoactive amines and other mediators from basophils and mast cells followed by recruitment of other

Table 3.8 Potential immunological effects and responses

Effects
Hypersensitivity
Type I – anaphylactic
Type II – cytotoxic
Type III – immune complex
Type IV – cell-mediated (delayed)
Chronic inflammation
Immunosuppression
Immunostimulation
Autoimmunity
Responses
Histopathological changes
Humoral responses
Host resistance
Clinical symptoms
Cellular responses
T cells
Natural killer cells
Macrophages
Granulocytes

inflammatory cells. Type IV cell-mediated (delayed) hypersensitivity responses involve sensitized T lymphocytes that release cytokines and other mediators that lead to cellular and tissue injury. Type IV hypersensitivity (cell-mediated) reactions are initiated by specifically sensitized T lymphocytes. This reaction includes the classic delayed-type hypersensitivity reaction initiated by CD4+ T cells and direct cell cytotoxicity mediated by CD8+ T cells. The less common type II (cytotoxic) hypersensitivity involves the formation and binding IgG and/or IgM to antigens on target cell surfaces that facilitate phagocytosis of the target cell or lysis of the target cell by activated complement components. Type II hypersensitivity (cytotoxic) is mediated by antibodies directed toward antigens present on the surface of cells or other tissue components. Three different antibody-dependent mechanisms may be involved in this type of reaction: complement-dependent reactions, antibody-dependent cell-mediated cytotoxicity, or antibody-mediated cellular dysfunction. Type III immune complex hypersensitivity is present when circulating antigen–antibody complexes activate complement whose components are chemotactic for neutrophils that release enzymes and other toxic moieties and mediators leading to cellular and tissue injury.

Immunologic reactions that occur with organ transplant rejection also offer insight into potential immune responses. Mechanisms involved in organ transplant rejection include T cell-mediated reactions by direct and indirect pathways and antibody-mediated reactions. Immune responses may be avoided or diminished by using autologous or isogeneic cells in cell/polymer scaffold constructs. The use of allogeneic or xenogenic cells incorporated into the device require prevention of immune rejection by immune suppression of the host, induction of tolerance in the

host, or immunomodulation of the tissue-engineered construct. The development of tissue-engineered constructs by immunoisolation using polymer membranes and the use of nonhost cells have been compromised by immune responses. In this concept, a polymer membrane is used to encapsulate nonhost cells or tissues thus separating them from the host immune system. However, antigens shed by encapsulated cells were released from the device and initiated immune responses [45].

Although exceptionally minimal and superficial in its presentation, the previously discussed humoral and cell-mediated immune responses demonstrate the possibility that any known tissue-engineered construct may undergo immunologic tissue injury. To date, our understanding of immune mechanisms and their interactions with controlled release system tissue-engineered constructs is markedly limited. One of the obvious problems is that preliminary studies are generally carried out with nonhuman tissues and immune reactions result when tissue-engineered constructs from one species are used in testing the device in another species. Ideally, tissue-engineered constructs would be prepared from cells and tissues of a given species and subsequently tested in that species. While this approach does not guarantee that immune responses will not be present, the probability of immune responses in this type of situation is markedly decreased.

The following examples provide perspective to these issues. They further demonstrate the detailed and in-depth approach that must be taken to appropriately and adequately evaluate tissue-engineered constructs or devices and their potential adverse responses.

The inflammatory response considered to be immunotoxic is persistent chronic inflammation. With biomaterials, controlled release systems and tissue-engineered devices, potential antigens capable of stimulating the immune response may be present and these agents may facilitate a chronic inflammatory response that is of extended duration (weeks, months). Regarding immunotoxicity, it is this persistent chronic inflammation that is of concern as immune granuloma formation and other serious immunological reactions such as autoimmune disease may occur. Thus, in biological response evaluation, it is important to discriminate between the short-lived chronic inflammation that is a component of the normal inflammatory and healing responses versus long-term, persistent chronic inflammation that may indicate an adverse immunological response.

Immunosuppression may occur when antibody and T cell responses (adaptive immune response) are inhibited. Potentially significant consequences of this type of response are frequent and serious infections resulting from reduced host defense.

Immunostimulation may occur when unintended or inappropriate antigen-specific or nonspecific activation of the immune system is present. From a biomaterials and controlled release system perspective, antibody and/or cellular immune responses to a foreign protein may lead to unintended immunogenicity. Enhancement of the immune response to an antigen by a biomaterial with which it is mixed ex vivo or in situ may lead to adjuvancy, which is a form of immunostimulation. This effect must be considered when biodegradable controlled release systems are designed and developed for use as vaccines.

Table 3.9 Representative tests for the evaluation of immune responses

Functional assays
Skin testing
Immunoassays (e.g., ELISA)
Lymphocyte proliferation
Plaque-forming cells
Local lymph node assay
Mixed lymphocyte reaction
Tumor cytotoxicity
Antigen presentation
Phagocytosis
Degranulation
Resistance to bacteria, viruses, and tumors
Phenotyping
Cell surface markers
MHC markers
Soluble mediators
Antibodies
Complement
Immune complexes
Cytokine patterns (T-cell subsets)
Cytokines (IL-1, IL-1ra, TNFα, IL-6, TGF-β, IL-4, IL-13)
Chemokines
Basoactive amines
Signs of illness
Allergy
Skin rash
Urticaria
Edema
Lymphadenopathy

Autoimmunity is the immune response to the body's own constituents, which are considered in this response to be autoantigens. An autoimmune response, indicated by the presence of autoantibodies or T lymphocytes that are reactive with host tissue or cellular antigens may, but not necessarily, result in autoimmune disease with chronic, debilitating and sometimes life-threatening tissue and organ injury.

Representative tests for the evaluation of immune responses are given in Table 3.9. Table 3.9 is not all-inclusive and other tests may be applicable. The examples presented in Table 3.9 are only representative of the large number of tests that are currently available. Table 3.9 is informative but incomplete as in the future, direct and indirect markers of immune response may be validated and their predictive value documented thus providing new tests for immunotoxicity. Direct measures of immune system activity by functional assays are the most important types of test for immunotoxicity. Functional assays are generally more important than tests for soluble mediators, which are more important than phenotyping. Signs of illness may be important in vivo experiments but symptoms may also have a significant role in studies of immune function in clinical trials and postmarket studies.

As with any type of test for biological response evaluation, immunotoxicity tests should be valid and have been shown to provide accurate, reproducible results that are indicative of the effect being studied and are useful in a statistical analysis. This implies that appropriate control groups are also included in the study design.

Immunogenicity involving a specific immune response to a biomaterial is an important consideration as it may lead to serious adverse effects. For example, a foreign, nonhuman, protein may induce IgE antibodies that cause an anaphylactic (type I) hypersensitivity reaction. An example of this type of response is latex protein found in latex gloves. Low molecular weight compounds such as chemical accelerators used in the manufacture of latex gloves may also induce a T cell-mediated (type IV) reaction resulting in contact dermatitis. Tests for type I, e.g., antigen-specific IgE, and type IV, e.g., guinea pig maximization tests, hypersensitivity should be considered for materials with the potential to cause these allergic reactions. In addition to hypersensitivity reactions, a device may elicit autoimmune responses, i.e., antibodies or T cells that react with the body's own constituents. An autoimmune response may lead to the pathological consequences of an autoimmune disease. For example, a foreign protein may induce IgG or IgM antibodies that cross-react with a human protein and cause tissue damage by activating the complement system. In a similar fashion, a biomaterial or controlled release system which has a gel or oil constituent may act as an adjuvant leading to the induction of an autoimmune response. Even if an autoimmune response (autoantibodies and/or autoreactive T lymphocytes) is suggested in preclinical testing, it is difficult to obtain convincing evidence that a biomaterial or controlled release system causes autoimmune disease in animals. Therefore, routine testing for induction of autoimmune disease in animal models is not recommended.

Babensee and coworkers have tested the hypothesis that the biomaterial component of a medical device, by promoting an inflammatory response, can recruit APCs (e.g., macrophages and dendritic cells) and induce their activation, thus acting as an adjuvant in the immune response to foreign antigens originating from the histological component of the device [46–52, 67]. Utilizing polystyrene and polylactic-glycolic acid microparticles and polylatic-glycolic scaffolds together with their model antigen, ovalbumin, in a mouse model for 18 weeks, Babensee et al. demonstrated that a persistent humoral immune response that was Th2 helper T cell dependent, as determined by the IgG1, was present. These findings indicated that activation of CD4+ T cells and the proliferation and isotype switching of B-cells had occurred. A Th1 immune response, characterized by the presence of IgG2a was not identified. Moreover, the humoral immune responses for all three types of microparticles were similar indicating that the production of antigen-specific antibodies was not material chemistry dependent in this model. Babensee suggests that the presence of the biomaterial functions as an adjuvant for initiation and promotion of the immune response and augments the phagocytosis of the antigen with expression of MHC class II and costimulatory molecules on APCs with the presentation of antigen to CD4+ T cells.

The use of appropriate animal models is an important consideration in the safety evaluation of controlled release systems that may contain potential immunoreactive

materials. A recently published study involving the in vivo evaluation of recombinant human growth hormone (rhGH) in poly(lactic-*co*-glycolic acid) (PLGA) microspheres demonstrates the appropriate use of various animal models to evaluate biological responses and the potential for immunotoxicity. Utilizing biodegradable PLGA microspheres containing rhGH Cleland et al. used rhesus monkeys, transgenic mice expression rhGH, and normal control (Balb/C) mice in their in vivo studies [53]. Rhesus monkeys were utilized for serum assays in the pharmacokinetic study of rhGH release as well as tissue responses to the injected microcapsule formulation. Placebo injection sites were also utilized and a comparison of the injection sites from rhGH PLGA microspheres and placebo PLGA microspheres demonstrated a normal inflammatory and wound healing response with a normal focal foreign body reaction. To further examine the tissue response, transgenic mice were utilized to assess the immunogenicity of the rhGH PLGA formulation. Transgenic mice expressing a heterologous protein have been previously used for assessing the immunogenicity of sequence or structural mutant proteins. With the transgenic animals, no detectable antibody response to rhGH was found. In contrast, the Balb/C control mice had a rapid onset of high titer antibody response to the rhGH PLGA formulation. This study points out the appropriate utilization of animal models to not only evaluate biological responses but also one type of immunotoxicity (immunogenicity) of controlled release systems.

3.10 Subcutaneous Implantation Studies

In developing drug delivery systems for implantation, one must evaluate the biocompatibility of the system and its components [8, 54, 55]. In general, these studies are based on histological observations and morphologic evaluations and are subjective in their approach. The biocompatibility of biomaterials or drug delivery systems with tissue is usually described in terms of the acute and chronic inflammatory response, foreign body reaction, and fibrous capsule formation seen at various time intervals after implantation of the respective material or system (Fig. 3.1).

Histologic evaluation of the tissue adjacent to the implanted material or system as a function of implant time has been the most commonly used method of evaluating the biocompatibility of the material or system. Gourlay et al. have described a method for grading acute and subacute tissue reactions following the implantation of powders in rats [56]. Weighting factors for the various components of the tissue response, such as muscle cell damage, thickness of tissue reaction, and cell density, are used to provide an overall biocompatibility score. Autian et al. have examined the biocompatibility of many implant materials using a primary acute-toxicity screening program [57, 58].

We also have used the subcutaneous implantation method for the biocompatibility evaluation of drug delivery systems as well as materials utilized in drug delivery systems [14, 59–61, 63]. Presented here are several examples of our studies to evaluate the biocompatibility of drug delivery systems and materials utilizing the

subcutaneous implantation method. Appropriate utilization of this method and appropriate interpretation of the findings using the subcutaneous implantation method require a perspective and knowledge of inflammatory and wound-healing responses to injury. An overview of these responses has been presented in the previous section.

As discussed previously, the form of a biomaterial or drug delivery system may dictate the biological responses when the material or system is implanted or injected. The degradability or nondegradability of a material or system in a given form also may dictate the inflammatory and wound-healing responses. These topics will be discussed in the following examples.

Biodegradable and nonbiodegradable microspheres, microcapsules, and other forms of particulate offer great therapeutic potential for the control of systemic or localized disease, or the control of physiological responses. These forms of delivery systems offer the distinct advantage of being injectable. Size also may play a role in the inflammatory and wound-healing responses to particulate types of materials and delivery systems. Particles ranging in size from 10 μm or greater usually are not phagocytosed or engulfed by macrophages and FBGCs in the inflammatory and healing responses. Thus, the main biological interaction is a surface or interfacial interaction. Particles smaller than 10 μm in dimension, i.e., nanoparticles, may be phagocytosed or engulfed by macrophages or FBGCs and thus may be subjected directly to the cytoplasmic contents of these cells that may include high acidity, i.e., low pH, degradative enzymes, and oxygen radicals. It is possible that the phagocytosis of nanoparticles may alter the phenotypic expression of macrophages and/or FBGCs that could result in the release of other potent inflammatory or wound-healing mediators that could alter the tissue response [62]. These mediators include the aforementioned acidity, degradative enzymes, oxygen radicals, as well as cytokines and growth factors produced and released by these cells.

We have carried out the in vivo biocompatibility testing of Medisorb 65/35 D,L-lactide-glycolide copolymer microspheres to characterize the tissue response of these biodegradable implants [61]. These microspheres ranged in diameter from 25 to 150 μm. Microspheres in an aqueous dextran vehicle were sterilely injected subcutaneously into the back of Sprague–Dawley rats such that the implantation site was below the paniculus carnosa muscle. Animals were sacrificed at 15, 30, 45, 60, 75, 90, 120, and 150 days following injection. Gross and microscopic analyses of all injection sites were performed. At the different time periods, injection sites were removed, fixed in buffered formaldehyde solution, and serially sectioned. The tissue sections with injection sites were embedded in paraffin and glass slides with 5 μm tissue sections were prepared using standard histological procedures. Slides of each implant site were stained by three methods: haematoxylin and eosin for the cellularity and number of cells; Masson's trichrome and picrosirius red for fibrous capsule formation and collagen deposition.

Histopathological evaluation was performed by two methods utilizing light microscopy. The first method was the evaluation of the tissue response of the injection site using the following factors: degeneration or necrotic changes of the tissue, inflammation, FBGC formation, and collagen deposition in the fibrous capsule

formed surrounding the injection sites. For the collagen deposition analysis, polarized microscopy was used with the picrosirius red stain. The second method was the evaluation of the types of cells surrounding the microspheres to characterize the tissue/implant interface. In this analysis, the types of cells at the interface were determined.

The characteristic normal foreign body reaction to the injected microspheres was observed at 30 days implantation. Acute and chronic inflammatory responses following injection have resolved by the 30-day time period. Two wound healing responses were identified. The first is the fibrous encapsulation of the total injection site, i.e., the aggregate of microcapsules, and the second response is the normal foreign body reaction to the individual microcapsules within the injection site. The injection site has undergone fibrous encapsulation as the resolution and progression of the inflammatory and wound-healing responses to this biocompatible material have occurred at a much faster rate than the biodegradation/bioresorption of the microspheres.

The implant site showed voids that are indicative of the microspheres surrounded by elements of a normal foreign body reaction to the microspheres. Macrophages and FBGCs are identified at the surfaces of the microspheres. The interstitial spaces between the microcapsules contain macrophages and fibroblasts, i.e., elements of the granulation tissue response. The fibroblasts produced collagen, i.e., fibrosis. The space available between individual microcapsules determines the extent or degree of the granulation tissue response. In close-packed systems where minimal space is present, only the foreign body reaction with the presence of macrophages and FBGCs will be observed. As the space between microspheres increases, the granulation tissue response increases and may become predominant as the macrophage and FBGCs in the foreign body reaction are found only on surfaces of biocompatible microspheres.

With time, the size of the implant/injection site as well as the individual microcapsules decreased and a clearer definition between the macrophages and FBGCs at the surface of the microcapsules and the interstitial fibroblasts and fibrosis was observed. Over this time period, no acute or chronic inflammation was seen indicating that the biodegradation of the Medisorb 65/35-D,L-lactide-glycolide microspheres did not lead to adverse inflammatory or wound-healing responses. With time, biodegradation of the microcapsules leads to smaller microcapsules, eventually reaching particle sizes that can be phagocytosed. At this point, an expected increase in macrophage density and decrease in FBGC number and density is observed. At 120 days, a few individual microspheres were identified with a normal foreign body reaction at their interface and surrounded by fibrous (collagen) tissue. At 150 days, a small fibrotic scar, less than 20% the size of the initial injection site, remained with no foreign body reaction.

This example illustrates the classic inflammatory and wound-healing responses to microcapsules injected in subcutaneous soft tissue. It is noteworthy that both the size and shape of the injection site as well as the size and shape of the microcapsules control the wound healing responses [61]. Both responses must be considered when the subcutaneous implant injection method is used for biocompatibility testing.

As indicated earlier, both the size and shape of implanted delivery systems may modify the inflammatory and healing responses providing for a variation in the biological response. This is true for systems that lose their integrity either through the release of drugs or chemical/physical changes that may occur in the polymer matrix over the implantation interval.

We have investigated the biocompatibility and acute histopathological responses to a series of biodegradable polypeptide polymers that show promise for implantable drug delivery systems [59, 60]. These polymers, poly[(*tert*-butyloxycarbonyl-methyl) glutamates], POMEGs, were obtained by partial esterification of polyglutamic acid with *tert*-butyl bromoacetate. Three different polymers were synthesized from the same initial batch of polyglutamic acid with nominal degrees of esterification of 25, 50, and 80%. Rods, 1 mm in diameter and 10 mm in length, were extruded, sterilized, and subcutaneously implanted in rats. Experimental details and results of this study have been published [59]. As expected, increasing the ester content of these polymers increased their in vivo stability and their hydrophobic character.

The tissue response to the POMEG 25% showed two zones of differing inflammatory response. The first zone, which surrounded the entire implant site, was composed of resolving chronic inflammation with low cell density. This is the most common response to polymer implants of this shape and size at this short time interval, i.e., 3 days. The second inflammatory zone is the acute inflammatory response present within the interstitial spaces of the implant that have been created by swelling and fragmentation of the polymer. Following implantation, the POMEG 25% polymer had undergone swelling and fragmentation, creating particles and new surfaces to which the inflammatory response has responded. This inflammatory response consists of acute inflammatory cells, that is, polymorphonuclear leukocytes. The acute inflammatory response present within the interstitial spaces created by the formation of particulate shows polymorphonuclear leukocytes present within these spaces between particles.

When the ester content of these polymers was increased to 50%, the swelling and fragmentation phenomenon decreased and only a localized response was identified at the implant surfaces at short time intervals. At this short time interval, two zones of inflammatory response were identified. The inflammatory zone at the implant surface shows swelling and minimal fragmentation of the polymeric implant at its surface with the presence of chronic inflammatory cells, monocytes and lymphocytes, within the interstitial spaces created by this phenomenon. The outer zone of response, which interfaces with the skeletal muscle, is composed of a lower density of chronic inflammatory cells and the inflammatory response in this zone is resolving. Macrophages are identified at the interface with skeletal muscle. These cells will initiate the formation of granulation tissue. It should be noted that the interior of this implant at 3 days has not undergone swelling or fragmentation. However, longer time period implantations indicated that this swelling and fragmentation phenomenon was progressive and both swelling and fragmentation increased with implant time.

A further increase in esterification of the POMEG polymers to 80%, POMEG 80%, produced a hydrophobic implant that did not undergo swelling and fragmentation out to 1 year implantation. End-stage healing had occurred with the development

of a fibrous capsule and similar histological response with this polymer was identified at 3, 6, 9, and 12 months implantation [63].

The classic wound-healing response to hydrophobic polymers or polymers that do not undergo biodegradation or bioresorption is characterized by a single layer of monocytes and FBGCs that are present at the polymer surface. This classic foreign body reaction, indicating biocompatibility, is surrounded by a thin fibrous capsule that is composed predominantly of collagen with a few fibroblasts present within the capsule. End-stage fibrous capsule formation does not usually contain the vascular capillaries that are easily recognized in the developing granulation tissue, which leads to fibrous capsule formation [64]. In general, in end-stage fibrous capsule formation, the vascular capillaries disappear and the fibrous capsule may be seen to shrink in regard to its thickness. This observation of shrinkage is actually a condensation or compaction of the collagenous fibers in the fibrous capsule. This condensation or compaction leads to an increase in density of the collagen fibers. This classic end-stage fibrous capsule formation with the normal foreign body reaction at the implant interface is seen with polymers such as polyethylene, poly(ethylene-vinyl acetate), and other nondegradable polymers [8, 64].

3.11 Cage Implant System

To place biocompatibility on an objective and quantitative basis, we have investigated the use of the cage implant system to determine the dynamic nature of cell function occurring at the implant site [13, 54, 63, 65, 66]. The cage system containing the biomedical polymer or the drug–polymer system allows the evaluation of inflammatory exudates that surround the material. The cage implant system provides a simple means by which the inflammatory exudate that bathes the biomedical polymer within the cage can be monitored serially without sacrificing the animal. After the withdrawal of a small fraction of the inflammatory exudate that surrounds the biomedical polymer or drug–polymer delivery system, total white cell concentrations, polymorphonuclear leukocyte concentrations, mononuclear leukocyte concentrations, extracellular alkaline phosphatase activity, acid phosphatase activity, and other components of the inflammatory exudate can be determined quantitatively. Thus, sensitive, reliable, reproducible, and quantifiable assays can be used to determine the temporal variations and cell function in the inflammatory exudate that interacts with the implanted biomedical polymer or drug–polymer delivery system. In addition, the cage system also allows the determination of the release characteristics of drug delivery systems with time by appropriate quantitation of the drug present in the inflammatory exudate at various time intervals. The cage system provides correlative, comparative, and quantitative information on the release characteristics and cellular response and activation, that is, biocompatibility of delivery systems.

We present here two examples of how this cage system can be used to determine the biocompatibility of drug–polymer systems. Details regarding the use of the cage implant system have been presented in the literature [13, 65, 66].

The first system to be discussed is a gentamicin–silicone rubber monolithic delivery system that had been developed for insertion in prosthetic heart valves for the treatment of prosthetic valve endocarditis [54]. Subcutaneous implant studies of this system suggested that there was an increased inflammatory response with the gentamicin–silicone rubber system compared to comparable implants of silicone rubber alone. To quantitatively investigate this observation, we utilized the cage implant system. The gentamicin–silicone rubber system displayed an increased inflammatory response at days 4 and 7 following implantation when compared to the silicone rubber specimens alone. The inflammatory response to the silicone rubber specimens was comparable to the empty cage controls, and the exudate cellular and enzyme concentrations for the silicone rubber implants were not statistically significantly different from the control values. The total white cell concentration in the exudates at days 4 and 7 for the gentamicin–silicon rubber system was statistically significantly higher than the control values and the silicone rubber values. Differential cell counts at day 4 showed that polymorphonuclear leukocytes were responsible for the increase in white cell concentrations at day 4 and mononuclear leukocytes were responsible for the increased white cell concentrations at day 7. It is noteworthy that the acute inflammatory response resolved and by days 14 and 21, the inflammatory response of the gentamicin–silicone rubber system was comparable and not statistically significantly different than the silicone rubber material. Extracellular acid phosphatase and alkaline phosphatase activity were measured in the exudates and the gentamicin/silicone rubber system showed an increase in alkaline phosphatase activity at day 4 when compared to the silicone rubber but no increase was noted at day 7 for this enzyme and no differences at days 4, 7, and 14 were noted in the extracellular acid phosphatase activity for the gentamicin–silicone rubber system compared to the silicone rubber.

These results indicate that the gentamicin, released from the silicone rubber system, was responsible for the increased acute inflammatory response. Gentamicin released from the silicone rubber into the exudate was monitored and the release characteristics were pseudo first order with an initial burst effect. The day 4 values were 40.7 ± 20.8 μg ml^{-1} but at day 7, this had decreased to 8.7 ± 1.5 μg ml^{-1} and the day 14 and 21 values were below 5 μg ml^{-1}. The conclusion that the released gentamicin caused the increased inflammatory response is not surprising; gentamicin is a drug that has well-documented clinical nephrotoxic effects. Utilization of the cage implant system and comparison of the drug–polymer to the polymer allowed us to appropriately identify the released drug as the agent eliciting the increased local inflammatory response.

The cage implant technique also was used to identify the effect of hydrocortisone acetate loaded poly(D,L-lactide) film on the inflammatory response [66]. Results from these studies showed that the hydrocortisone acetate-D,L-polylactic acid system dramatically inhibited all aspects of inflammation in the cage system over the 21-day time period of the experiment. White cell concentrations in the hydrocortisone acetate-D,L-polylactic acid exudates were markedly lower than the white cell concentrations of both the empty cages and the D,L-polylactic acid controls throughout the implantation period. The hydrocortisone acetate inhibited the

accumulation of all types of leukocytes, i.e., in the inflammatory exudates within the cage, including polymorphonuclear leukocytes, macrophages, and lymphocytes. Examination of the tissue response to the cages was carried out 21 days after implantation and it was obvious that the hydrocortisone acetate-D,L-polylactic acid implants markedly inhibited fibrous capsule formation surrounding the cage implants. This indicated that the final stage of the healing response, fibrosis, or scar formation, was repressed. The quantitative information derived from exudate analysis from the cages at the various time intervals of the experiment. These studies in a quantitative fashion indicate that hydrocortisone acetate in a biodegradable delivery system can inhibit the acute inflammatory response, chronic inflammatory response, granulation tissue development, and fibrous capsule formation. Thus, hydrocortisone acetate represses all components of the inflammatory and wound-healing responses. Utilization of the cage system and statistical evaluation of the quantitative results from these in vivo studies permitted these conclusions to be drawn regarding the broad activity of hydrocortisone acetate in the in vivo environment.

These two examples demonstrate the usefulness of the cage implant system in providing quantitative information regarding the in vivo inflammatory and wound-healing responses of drug delivery systems. Moreover, the cage implant technique permits the statistical comparison between drug carrying materials and the materials themselves, as well as the release profiles of drugs from drug delivery systems.

3.12 Conclusion

Host response evaluation of implantable or injectable long acting drug delivery systems is critical to determining the safety and biocompatibility of such systems. This chapter presents an overview of the inflammatory, wound-healing, and foreign body responses generally considered as parts of the tissue or cellular host response to the injection or implantation of long acting drug delivery systems. To provide a foundation and perspective for future efforts in the drug delivery arena, a section on immunotoxicity (acquired immunity) has been included. This section provides an overview of the common responses expected for immunotoxicity as it relates to the bioactive agent released from the respective delivery system. Given the current and future development of tissue-engineered constructs that utilize controlled drug or bioactive agent delivery, immunotoxicity evaluation may play a significant role in determining the safety of these systems. A section on the subcutaneous implantation of drug delivery systems with serial analyses of the temporal sequence of events of the inflammatory and wound-healing responses has been included. This method, when coupled with pharmacokinetic studies, can provide an overall appreciation of the biocompatibility and drug-releasing characteristics of a system or device. Finally, a section on the utilization of the in vivo cage implant system has been included and this system permits a more detailed and quantitative analysis of the events that occur in the inflammatory and wound-healing responses to an implanted drug delivery system. The cage implant system permits sensitive, reliable, reproducible, and quantifiable assays that can be used to determine the temporal variations in

inflammatory cell concentration and function. An understanding of the mechanisms and methods presented in this chapter can permit the early identification of factors that may compromise or obviate the biocompatibility of given drug delivery systems. Use of these types of studies is important in the research and development of drug delivery systems. Early identification of problems permits new design criteria to be introduced into the research and development process for drug delivery systems.

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Chapter 4
Anatomy and Physiology of the Injection Site: Implications for Extended Release Parenteral Systems

Arlene McDowell and Natalie J. Medlicott

Abstract Understanding how the biological environment contributes to drug release following administration is increasingly becoming a focus for drug delivery research. Achieving therapeutic levels of a bioactive relies on appropriate drug release following parenteral administration that must be complimentary to subsequent drug absorption, distribution, metabolism and elimination. The biological characteristics of the injection site can have an influence on the drug absorption process. In this chapter the intravenous, intramuscular and subcutaneous routes for parenteral administration of extended release products will be discussed.

4.1 Introduction

There are many extended and controlled release injectable systems used to deliver drugs in human and veterinary medicine [1–5]. These systems are prepared from a variety of biocompatible materials and aim to release drug for an extended period following injection or implantation. Drug release is governed by the design of the dosage form, although the biological environment often influences drug release [6–9]. Understanding how the biological environment contributes to drug release following administration is increasingly becoming a focus for drug delivery research.

Extended release parenteral delivery systems range from relatively simple aqueous suspensions that prolong drug release due to slow dissolution at the injection site to more sophisticated in situ gelling implants and polymeric biodegradable microparticulate systems [4, 10, 11]. For example, long acting intramuscular aqueous suspensions of penicillin have been available since the 1950s [12] and oily

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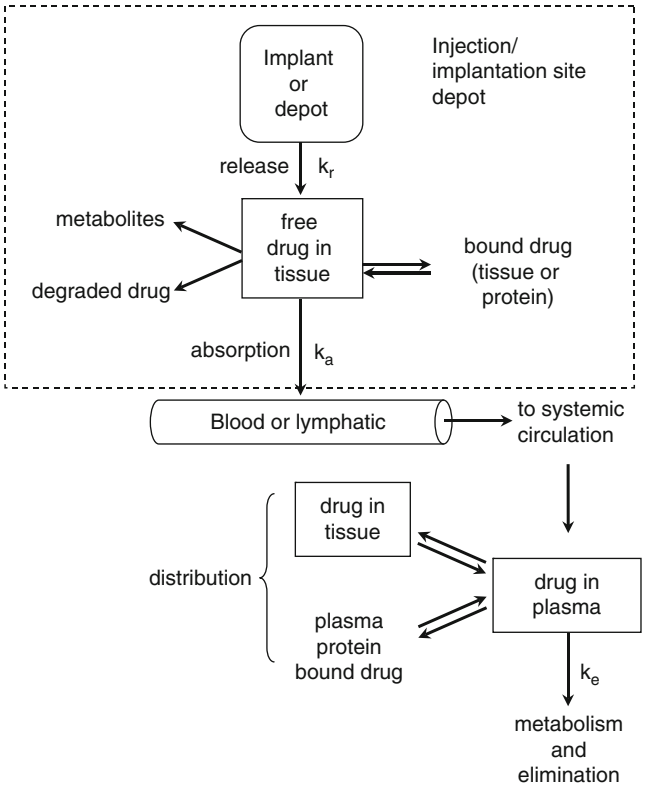

Fig. 4.1 Schematic showing pharmacokinetics for a drug administered as an extended release intramuscular or subcutaneous system

formulations of neuroleptic drugs since the 1970s [13]. More recently, sustained release microparticulate polymeric formulations of leuprolide have been developed for prostate cancer [1, 14, 15]. For all implanted extended release delivery systems, balancing the in vivo drug release from the delivery system with drug absorption, distribution, metabolism and elimination processes is key to achieving target drug levels in the body (Fig. 4.1). Hence, careful consideration of drug pharmacokinetics and knowledge of target plasma and tissue drug concentrations can guide the development of extended and controlled release parenteral drug delivery systems. One useful way to categorize parenteral delivery systems has been suggested by Washington et al. for intramuscular injections [16]. According to this, injectable delivery systems can be divided into those in which the pharmacokinetics are predominately controlled by the implant (or device) and those controlled by the process of absorption into blood or lymphatic capillaries at the implant site (i.e. perfusion limited pharmacokinetics), as shown in Fig. 4.2. If drug release from the device is slow and drug absorption from the tissue is fast, then a situation results where the appearance of drug in the blood is closely controlled by the release characteristics of the extended release device (Type I). At the other extreme, an aqueous

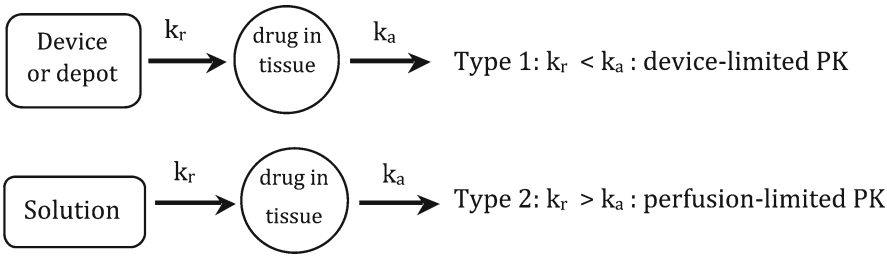

Fig. 4.2 Pharmacokinetic (PK) classification of intramuscular implantable delivery systems. Modified from Washington et al. [16]

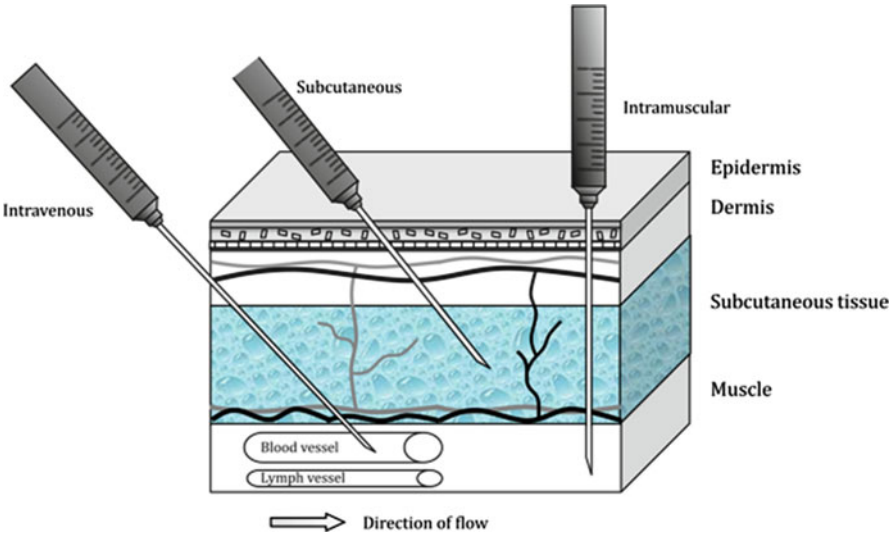

Fig. 4.3 Potential sites for injection of extended release parenteral dosage forms

solution of a water-soluble drug provides its dose immediately following injection and in this case, the absorption processes (k_a) may limit the appearance of drug in the blood (Type II). Many extended release parenteral delivery systems fall between these two extremes so it is important to consider the biological processes at injection sites that contribute to drug absorption. A further example of extended release systems are those designed to deliver drugs locally at the implantation site. For these local delivery systems, a balance between drug release and drug absorption that maintains a constant local drug concentration may be the goal. The previous chapter has shown that while implantable extended release systems must be biocompatible, they cannot be considered biologically inert because the patient will react to the presence of the implant.

The aims of this Chapter are to review the anatomy and physiology of injection sites (intravenous, intramuscular and subcutaneous – Fig. 4.3) and to summarize the biological variables that affect drug release and absorption at these sites.

4.2 Intravenous Route

The intravenous route of administration for injectable products provides direct access into the blood stream and so therapeutic agents delivered by this route are available immediately in the systemic circulation. The plasma concentration is determined by the initial dose injected, the drug distribution and rates of metabolism and elimination according to Eqs. (4.1) and (4.2) for first-order elimination kinetics.

$$C_0 = \frac{\text{Dose}}{V}, \tag{4.1}$$

$$C_t = C_0 \times e^{-kt}, \tag{4.2}$$

where: C_0 = initial plasma concentration, V = volume of distribution, C_t = plasma concentration at time t and k = first-order elimination rate constant.

Controlled drug release can be achieved using the intravenous route when particulate systems such as liposomes or polymeric nanoparticles are used [2, 17, 18]. The prolonged effect of these dosage forms is primarily attributed to the time taken for the bioactive to be released from the circulating particles. Properties of nanoparticles such as: method of preparation, method of drug association (e.g., encapsulated or surface-adsorbed) and the type of polymer used can be manipulated to alter the drug release profile and the in vivo fate of the drug. For example, nanoparticles that have the drug dispersed uniformly throughout a matrix generally release drug in a first-order process controlled by diffusion and polymer degradation [19]. Nanoparticulate systems administered by the intravenous route can also be used to facilitate drug targeting to specific tissues and cell types. Active targeting can be achieved through surface modifications, such as conjugation of ligands, e.g., biotin to poly(D,L-lactide-co-glycolide) nanoparticles to deliver paclitaxel to tumors [20].

Restrictions are placed on the size of particulates that can be injected into the circulatory system so that controlled release systems designed for intravenous use are typically within colloidal range with diameters less than 500 nm [21]. With increasing research into particulate delivery systems, understanding of the tissue distribution of colloidal particles following administration into the blood has advanced. Moghimi et al. [22], in a review of long-circulating and target-specific nanoparticles, reported that passive tissue distribution patterns of intravenously administered particles depended on size and deformability of the particles as well as their surface chemistry. Slack et al. [23] showed polystyrene-divinylbenzene microparticles with diameters 7.4 and 11.6 μm were deposited mainly in the lungs, while particles with diameter 3.4 μm deposited in the liver and spleen following intravenous administration. Smaller nanoparticles (less than 150 nm) appear to be distributed more widely than larger colloids with particles seen in the liver, spleen, bone marrow, bone, heart, kidney and stomach [21, 24, 25]. At tumor sites and sites of tissue inflammation, the spaces between endothelial cells lining blood capillaries are enlarged so that correctly sized nanoparticles can escape the vasculature into

underlying tissue in these sites [25–27]. This mechanism is exploited in the targeting of cytotoxic drugs to tumors [28, 29] or anti-inflammatory drugs to sites of tissue inflammation [27]. Tumors at different sites of the body, however, appear to have different levels of microvasculature porosity, which may affect the accumulation of particulate delivery systems [25]. Pore sizes ranging from 200 nm to 1.2 μm were reported with experimental subcutaneous tumors, but lower pore size (less than 500 nm) was reported in brain tumors. The microvascular pore size also varied depending on the tumor cell line [25]. At even smaller sizes, particles may be eliminated from the body by filtration through the kidney. Choi et al. suggested that particles less than 5 nm diameter were freely filtered in the kidneys, while those with diameter greater than 5 nm were retained in the body [30]. This suggests that there is also a lower particle size limit (5 nm) as well as the higher one (500 nm) for particulate systems administered via the intravenous route.

4.3 Intramuscular Route

The intramuscular site is reached by injection through the hypodermis into the underlying skeletal muscle. The structure of musculature is such that vasculature extends into the muscle and each muscle fiber is surrounded by a number of capillaries lying parallel to each fiber with transverse vessels between muscle fibers [31]. Thus, muscle tissue is typically highly perfused with blood for the delivery of oxygen and nutrients to muscle cells and for the removal of waste material and so can be utilized for the systemic delivery of therapeutics. Extracellular fluid in skeletal muscle is reported to have a pH of 7.1 at rest but decreases with exercise to 6.8 due to lactate accumulation [32].[1] This slightly acidic pH may influence release and absorption properties of weakly acidic or basic drugs. Lymphatic vessels are also present within the connective tissue that surrounds the muscle fibers and bundles; however, the lymph system is more extensive in the subcutaneous site compared to the intramuscular site [33]. Drug characteristics that promote absorption into the lymphatic system are discussed later.

The most common muscles into which injections are made in humans are the gluteus maximus, vastus lateralis and deltoid. Blood flow in these muscles is reported to be fastest in the deltoid, and slowest in the gluteus maximus giving rise to potential differences in drug absorption rates at different sites of administration [34]. Differences in muscle perfusion can be expected to have the greatest effect on drug absorption when uptake into injection site capillaries is the rate-limiting step in drug absorption. For example, more rapid absorption of diazepam from injections into the deltoid muscle compared with the vastus lateralis has been reported [35]. Additionally, when muscle perfusion is the rate-limiting step for drug absorption, activities that increase local blood flow such as exercise, and local muscle massage may be expected to increase the rate of drug absorption following intramuscular injection [36]. Increased absorption has been reported for both intramuscular administration of

[1] pH can also be lowered by the foreign body reaction to implants (see Chap. 3).

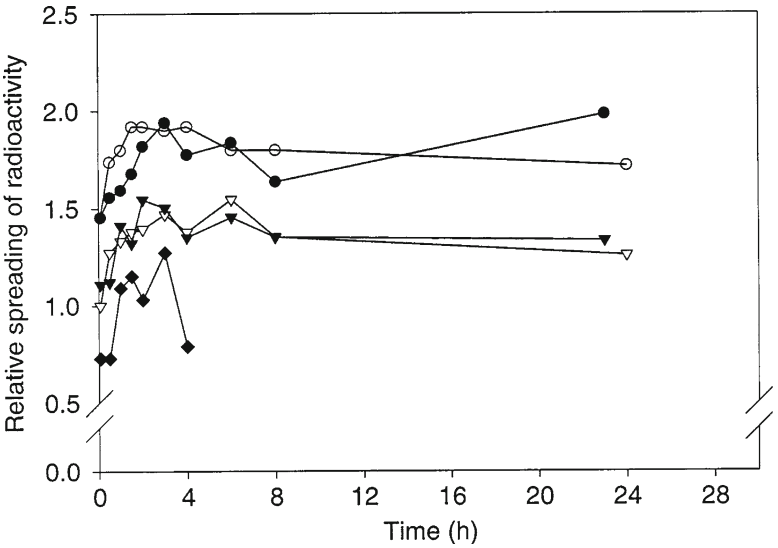

Fig. 4.4 Graph of the spread following intramuscular injection of hydrophilic radioactive markers into thigh muscle of rabbit. Spreading is represented as relative to aprotinin encapsulated in an emulsion with 60% w/w aqueous phase. (*Open circle*) 30% w/w aqueous phase emulsion containing aprotinin; *open inverted triangle* 60% w/w aqueous phase emulsion containing aprotinin; (*Filled circle*)30% w/w aqueous phase emulsion containing radioactive pertechnetate; (*filled inverted triangle*) 60% w/w aqueous phase emulsion containing radioactive pertechnetate; (*filled diamond*) solution of aprotinin in PBS administered intramuscularly. Figure with permission from Bjerregaard et al. [39]

penicillin and diazepam with exercise [37]. However, when drug release from the depot is slow, then the effects of increased muscle perfusion may not be great. Soni et al. showed no significant effects of injection site, massage or muscle activity on plasma levels from a depot injection of fluphenazine decanoate [38]. Muscle activity may also have an effect on the surface area available for drug release for low to intermediate viscosity systems as exercise could affect the spreadability of the extended release depot within the muscle tissue. For example, absorption of the protein aprotinin [39] was found to be greater from formulations with a lower viscosity that spread more extensively within the muscle compared to the formulations with higher viscosity, 30% and 60% w/w emulsions, respectively (Fig. 4.4).

The amount of fat associated with the muscle can also modulate absorption from intramuscular injections and has been given as an explanation for the slower rate of drug absorption following injection into the gluteus maximus in females compared to males [40]. Cockshott et al. have shown that injections intended for intramuscular administration into the gluteal muscle may not reach the muscle and may indeed be made most of the time into the fat surrounding the muscle [41]. Their study of 63 men and 60 women indicated that at any given weight, the skin to muscle distance in the gluteal region was approximately 2.5 cm greater in women than in men. If a standard 3.5 cm length needle was used, then under 5% of women would receive

the intramuscular injection at the desired depth of at least 0.5 cm into the muscle [41]. Hence, the reported slower absorption following intramuscular injection into the gluteus maximus site may be due to injection into fat overlying the muscle rather than muscle itself.

4.4 Subcutaneous Route

The subcutaneous tissue or hypodermis is situated directly below the dermis layer of the skin and exterior to the muscle layer [16]. The characteristic feature of this site is the storage of dietary adipose within loose connective tissue in the interstitial space [16]. The interstitial space comprises a collagen network embedded in a gel of glycosaminoglycans, salts and proteins [42] and has a pH of 7.3 [43]. The accessibility of the subcutaneous site and relative ease of injection with short, fine needles contribute to its popularity for self-administration of medications such as insulin. Implants, particulates or in situ gelling systems can sit comfortably within the connective tissue of the subcutaneous site and release drug that is then absorbed into surrounding blood or lymph capillaries.

Extended release injections containing contraceptive hormones have been in use since the late 1960s, initially with intramuscular Depo Provera®, a long acting aqueous suspension of medroxyprogesterone acetate administered once every three months [44] and more recently a lower dose subcutaneous form, depo-subQ Provera [45]. The duration of effective contraceptive action was further extended with the introduction of levonorgestrel containing polydimethylsiloxane implants. The first of these were the Norplant® implants of the 1980s, which gave slow release over 5 years following subcutaneous administration. Six rod shaped implants were needed for treatment with Norplant, and re-design of this product allowed the number of implants to be reduced to two [46]. More recently a single rod implant for subcutaneous administration has been developed incorporating etonogestrel in ethylene vinyl acetate (Implanon™) to provide contraceptive effects over a period of three years [47].

Blood perfusion in the subcutaneous tissue is recognized to be lower than in the intramuscular site, which translates into comparatively slower absorption, lower maximum plasma concentrations and longer times to maximum plasma concentration as illustrated for the antibiotic cefotaxime in sheep (Fig. 4.5). A further delay in the appearance of drug within the systemic circulation results if drug is absorbed into subcutaneous lymphatic capillaries because time is required for the drug to transverse the lymphatic system and enter the blood circulation. The lymphatic system has unidirectional flow and maintains interstitial pressure through the collection of fluid and proteins from the interstitial fluid through a series of draining lymph nodes and is eventually returned to systemic circulation [49]. Lymph capillaries are more permeable than blood capillaries because they are lined with a single layer of endothelial cells that have an incomplete basal layer and lack coherent tight junctions between adjacent endothelial cells [42]. The composition of protein in the lymph is similar to that in blood plasma and lymph flow rate is 100–500 times

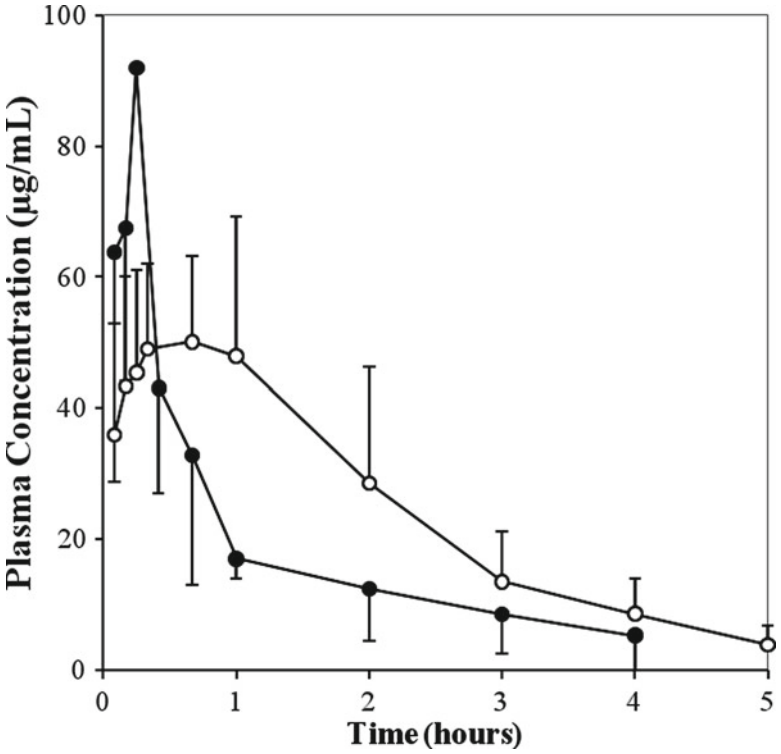

Fig. 4.5 Comparative plasma concentrations following injection of an aqueous solution cefotaxime (50 mg/kg) via the (*filled circle*) intramuscular and (*open circle*) subcutaneous routes in sheep. Data with permission from Guerrini et al. [48]

slower than the flow in blood vessels [49]. The importance of the lymphatic system for absorption of drugs is increasingly recognized for large molecular weight protein drugs that do not partition well into blood capillaries [50]. Small drug molecules (< 1 kDa) are predominantly absorbed into blood capillaries as their small size means they can partition relatively easily across the capillary endothelium and their diffusion through the interstitial fluid is not restricted [51, 52]. For larger peptide and protein compounds, absorption into blood vessels is limited primarily by their poor permeability across the capillary endothelial cell wall due to their large size. Consequently, these remain in the extracellular fluid until taken up by the lymphatic system. Porter and Charman have shown that the fraction of a dose absorbed into the lymphatic system is directly proportional to molecular weight [50]. Compounds with a molecular weight greater than 30 kD have been shown to be predominantly absorbed from into the lymphatic capillaries [52]. Colloidal particles may also be absorbed into the lymphatic system, with an optimal size for uptake reported to be 10–100 nm [49] and larger particles taking longer to be absorbed [53]. Whilst absorption into the lymphatic vessels appears not to be selective, the rate of

diffusion of the solute through the extracellular matrix of the interstitial fluid is determined by size, charge and hydrophilicity of the drug and so will influence lymphatic uptake [49, 50].

The spread of formulations within the subcutaneous tissue is another potentially important variable affecting in vivo drug release as it will have an effect on the available surface area across which drug can escape from the delivery system. The influence of depot spreadability on drug absorption can be demonstrated by the effects of co-administration of formulations with the enzyme hyaluronidase. This enzyme degrades hyaluronic acid within the subcutaneous interstitial matrix and basement membrane, reducing the resistance to flow of injected material through the subcutaneous tissue [54]. Bookbinder et al. [55] described the effects on subcutaneous injection spread on co-administration of a recombinant form of hyaluronidase with trypan blue dye. The area of spread of the trypan blue dye was dose dependant over 0.05–5.0 units hyaluronidase per injection and because the enzyme acts only on the hyaluronic acid and not the collagen network, a significant structural matrix still existed. This enzyme also allowed the spread of small particulates (less than 200 nm) within the subcutaneous tissue [55].

4.5 Effects of the Tissue Response on Extended Release Parenteral Systems

For many extended release parenteral systems, extensive in vitro characterization is carried out and understanding of the effects of formulation and processing variables on the release characteristics are determined. In vitro release methods to study the release of drugs from extended release parenteral delivery systems make only small attempts to simulate the in vivo environment and methods used may be categorized as: sample and separate, continuous flow and dialysis-based methods as recently reviewed by Larsen et al. [51]. These methods mimic the poorly stirred, limited fluid conditions that are expected in intramuscular and subcutaneous sites and little is done to account for the effect of the tissue inflammatory reaction on release characteristics.

For intravenous nanoparticulate systems, protein adsorption to the particulate surface and uptake by the reticuloendothelial system reduce the circulation time within the blood. Much recent work has shown that these effects can be reduced by surface pegylation so that residence time of nanoparticulate systems in the circulation are now significantly extended [56]. This strategy has had wide application in the formulation of particulate-targeted delivery systems. Ideally, the drug remains within the particulate system while it is circulating in the blood, but is released following deposition in the target tissue (e.g. tumor). In vitro evaluation of particle size and retention of the drug within the nanoparticle are important studies to characterize these delivery systems prior to in vivo use. However, it is the rate of in vivo particle opsonization, uptake by reticuloendothelial system cells and interactions with microvasculature that will ultimately determine the in vivo fate and effectiveness of intravenously injected nanoparticles.

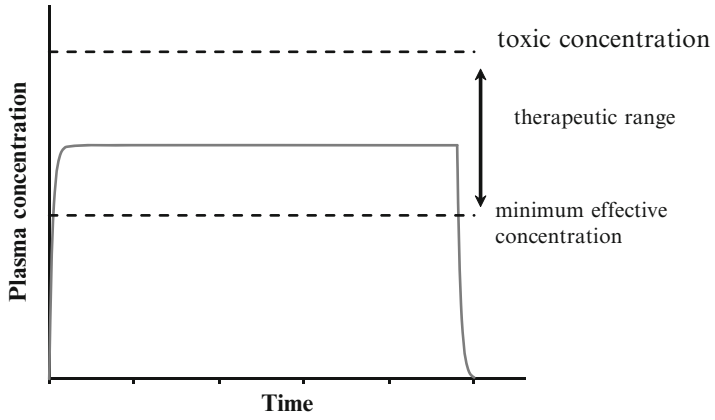

Fig. 4.6 Plasma versus time profile for an ideal controlled release delivery system

Intramuscular and subcutaneous extended release systems are expected to stay at the administration site and release drug slowly over time to achieve constant plasma drug concentrations as shown in Fig. 4.6. For some systems, the biological environment has a large influence on the in vivo release profile and understanding how drug release occurs in vivo may allow optimization of in vivo release profiles to achieve target plasma drug concentrations. The biological environment can trigger physical change in the injected materials such as the solution to semi-solid transformation occurring for in situ gelling systems ([57–60]; see also Chap. 9). For others the opposite occurs and a solid material may be implanted, which hydrates to form a semi-solid depot at the injection site [61, 62]. In situ gelling implant materials transition from liquid to solid in response to environmental changes such as temperature changes, e.g., poloxamer gels [63, 64], or loss of organic solvent into surrounding tissue, e.g., polylactide-co-glycolide organic solutions [65–67]. The tissue reaction at the implantation site can additionally influence the in vivo performance and may be involved in drug release. Kempe et al. described the in vivo solidification process for PLGA implants from *n*-methyl-2-pyrrolidone solutions as a two-stage process involving firstly surface solidification to form a shell over about 30 min, followed by a slower process of complete solidification over 24 h [67]. In the first stage of solidification they reported about 75% of the polymer precipitated. As well as altering the physical form of the implanted material, the biological environment may affect drug release and implant degradation and erosion rates. An early example was reported by Olanoff et al. for a tetracycline containing methacrylate tri-laminate film system [68, 69]. These films released tetracycline with a zero-order profile in both in vitro studies [69] and following subcutaneous implantation in rats [68]. The tissue response 1–2 weeks after implantation was described as mild inflammatory with tissue oedema, loose granulation tissue and the beginning of fibrous capsule formation. The fibrous capsule developed further over the following 4–9 weeks

with dense collagen fibrils and little cellular infiltration [68]. In vivo release rates of 123 and 158 μg/day for two film formulations were shown to be approximately equal to the tetracycline elimination rates (110 ± 32 and 153 ± 42 μg/day, respectively). This resulted in reasonably steady plasma concentrations of tetracycline (0.6 – 1.0 μg/mL) over 3–14 days post-implantation for the films with release rate of 158 μg/day. Knowing the in vivo release rate (i.e. the drug input rate) allowed development of a pharmacokinetic model for controlled release tetracycline [70]. Further to this, Anderson et al. went on to describe the diffusional barrier effects of the fibrous capsule to in vivo gentamicin release from silicone implants [7] and then naltrexone release from polylactide-co-glycolide beads and microparticles [71]. They showed a well-developed fibrous capsule surrounded silicone implants at 4 weeks. This capsule comprised three zones; one immediately surrounding the implant (approx. 2 μm thickness), then the fibrous capsule (10 – 15 μm thickness) that was surrounded by an outer zone. The outer zone contained blood capillaries but the inner two zones were avascular and therefore these inner two layers may represent a diffusional barrier [7]. Around polylactide-co-glycolide beads (bead diameter ≈ 1.5 mm) a similar tissue response and fibrous layer formation was observed. In contrast, the tissue reaction to microspheres was an accumulation of inflammatory cells (monocytes, lymphocytes polymorphonuclear neutrophils and foreign body giant cells) over 3 days. The presence of inflammatory cells decreased by day 7 but giant cell numbers increased. Interestingly, the fibrous capsule appeared to take around 2–4 weeks to fully develop, which means for systems which are designed to give extended release (i.e. greater than 2–4 weeks) this additional barrier may assist extension of the duration of release. For the polylactide-co-glycolide bead example in Yamaguchi and Anderson's study, steady plasma levels of naltrexone were achieved from days 3 to 28 [71]. For a cholesterol-based implant, another group has shown that the fibrous capsule may contribute to uptake of lipid material and hence influence release of drug [6]. They reported the fibrous capsules surrounding norethisterone–cholesterol implants and interactions of cells within the fibrous capsule with the lipid material. Foam cells were observed in the inner layer of the surrounding capsule containing lipid and their involvement in an absorption mechanism was suggested. Again, the outer layers of the fibrous capsule contained blood and lymphatic capillaries [6]. These studies confirm the importance of the inflammatory response and fibrous capsule not only as a potential diffusional barrier to drug release from extended release implants but as a more complex biological interface which can potentially enhance or delay drug release from implanted systems.

For particulate systems, particle size influences residence time at the injection site as phagocytic cells will take up particles of appropriate size, shape and surface characteristics. Generally particles with diameters less than 0.5 μm are reported to undergo phagocytosis; however, particle shape and surface characteristics will also influence this process [72, 73]. The surface properties also appear to influence the tissue response and Daugerty et al. have described differences in cellular infiltration between PLGA microparticles depending on their surface hydrophilicity [8]. Microparticles prepared using poly-D,L-lactic acid with hydrophobic end groups, formed clumps within subcutaneous tissue in which granulation tissue appeared

to be excluded. In comparison, when poly-D,L-lactic acid with hydrophilic end groups was used and the particle surface was more hydrophilic, cellular infiltration was seen between individual microparticles. This suggests tissue structure surrounding particulates is dependant of particle surface hydrophobicity and effects on in vivo drug release may be important especially as granulation tissue is rich in blood capillaries.

4.6 Concluding Remarks

Extended release delivery systems are increasingly investigated for administration by the parenteral route. For these systems the biological environment in which they are placed can be shown to affect their in vivo drug release characteristics. Understanding specific effects of the tissue response on drug release and delivery system degradation may allow better design of extended release parenteral delivery systems which achieve target in vivo release profiles which translate into optimal plasma concentration versus time profiles for the therapeutic goal.

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Chapter 5
Drugs for Long Acting Injections and Implants

Jie Shen and Diane J. Burgess

Abstract Long acting injections and implants have been developed for controlled drug delivery to improve therapeutic effects, decrease dosing frequency, and also avoid potential drug toxicity. Many pharmaceutical agents, such as biomacromolecules (*e.g.* peptides, proteins and gene therapeutics), drugs with poor bioavailability, and drugs for local delivery are good candidates for developing long acting injections and implants. However, not all drugs are suitable for these formulations (for example, drugs with high dose or narrow therapeutic index).

5.1 Introduction

Drug formulations should deliver the API (active pharmaceutical agent) to the site of action at the correct therapeutic concentration for as long as the treatment is desired. The therapeutic and the physicochemical properties of a drug determine the preferred route of administration (oral or parenteral) as well as the most appropriate delivery systems.

Due to convenience and patient acceptability, oral drug delivery is the most attractive and preferred route for administering medicines. However, some drugs cannot be tolerated via the oral route due to the acid environment and the variety of proteolytic enzymes (proteases) in the gastrointestinal tract (GIT). These conditions tend to degrade or metabolize drugs such as proteins and peptides, resulting in low bioavailability [1]. Moreover, patients who are unable to tolerate oral ingestion (*e.g.* patients with aspiration problems or GI disorders) require nonoral administration (*e.g.* parenteral administration) to receive medications and nutrition. Parenteral administration (*e.g.* intravenous, intramuscular, and subcutaneous) is also useful

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for: *i)* drugs with poor oral bioavailability; *ii)* for treatment of patients with physical or mental states that would make other routes (*i.e.* oral or inhalation) difficult and *iii)* site specific drug delivery. Local delivery can be achieved via direct injection or implantation into target organs or via delivery systems that allow passive or active targeting. This allows high doses of drugs to reach specific organs or tissue sites (*e.g.* eyes, brain cancer, joints, central nervous systems, and heart), enhancing therapeutic effects while avoiding systemic side effects. Considering the biocompatibility problems associated with multiple injections and the potential systemic side effects resulting from high peak drug concentrations, long acting injections and implants are often preferred [2–4].

Several drug classes lend themselves to parenteral administration, including anti-inflammatory medicines (fentonyl, dexamethasone), antipsychotic medicines (risperidone), anti-cancer drugs (paclitaxel, cytokines), medicines for the treatment of coronary artery disease (simvastatin), protein and peptide drugs as well as gene therapeutics. Whether a drug is suitable for long acting parenteral formulation depends on its physicochemical properties as well as its pharmacokinetic and pharmacodynamic profiles. This chapter briefly outlines examples of drugs that are suitable candidates for long acting injections and implants. Table 5.1 lists examples of long acting injections and implants that are currently on the market.

5.2 Drug Candidates for Long Acting Parenteral Formulation Based on Drug Characteristics

Many different kinds of drugs have been successfully formulated into long acting injections and implants. This section focuses on the physicochemical properties of drugs that make them good candidates for long acting parenteral formulation.

5.2.1 Proteins and Peptides

With the advances in pharmaceutical biotechnology, therapeutic proteins and peptides represent a rapidly growing portion of marketed drugs. Proteins and peptides are ideal drugs for the treatment of numerous diseases as a result of their high selectivity and their ability to provide effective and potent action at low doses [5]. However, the use of proteins and peptides as therapeutic agents is hampered by their physicochemical properties and biological instability. In addition, most therapeutic peptides and proteins are hydrophilic with MW (molecular weight) >700 Da [6–8], which results in low permeation and absorption. Moreover, proteins and peptides are susceptible to rapid degradation by enzymes (especially by digestive enzymes in GIT), which is largely prohibitive of oral delivery [9, 10]. Even when

Table 5.1 Examples of long acting injections and implants that are currently on the market

Drugs	Administration route	Duration	Application	References
Carmustine	Intracranial implantation	2–3 weeks	Glioblastoma	GLIADEL® Wafer
Dexamethasone	Intravitreal implantation	1–3 months	Macular edema	OZURDEX™
Etonogestrel	Subdermal implantation	3 years	Birth control	IMPLANON®
Fluocinolone acetonide	Intravitreal implantation	30 months	Uveitis	RETISERT®
Ganciclovir	Intravitreal implantation	5–8 months	AIDS-related Cytomegalovirus (CMV) Retinitis	Vitrasert®
Goserelin acetate	Subcutaneous implantation	1 or 3 months	Prostate cancer, breast cancer or endometriosis (non-cancerous condition)	ZOLADEX®
Histrelin acetate	Subcutaneous implantation	1 year	Central precocious puberty	SUPPRELIN® LA
Lanreotide	Subcutaneous injection	4 weeks	Acromegaly	Somatuline® Depot
Leuprolide acetate	Subcutaneous or intramuscular implantation	12 months	Prostate cancer	Viadur™
	Intramuscular injection	1–3 months	Central precocious puberty	Lupron Depot®
Morphine sulfate	Epidural injection	48 h	Postoperative pain relief	DepoDur®
Naltrexone	Intramuscular injection	1 month	Alcohol dependence	Vivitrol®
Octreotide acetate	Intramuscular (intragluteal) injection	4 weeks	Metastatic carcinoid tumors and Vasoactive Intestinal Peptide (VIP) secreting adenomas	Sandostatin LAR® Depot
Risperidone	Intramuscular injection	2 weeks	Schizophrenia and Bipolar I Disorder	RISPERDAL® CONSTA®
Somatropin	Subcutaneous injection	2–4 weeks	Growth hormone deficiency	Nutropin Depot®
Testosterone	Subcutaneous implantation	3–6 months	Deficiency or absence of endogenous testosterone	Testopel™ Pellets
Triptorelin pamoate	Intramuscular injection	1–3 months	Prostate cancer	Trelstar® Depot

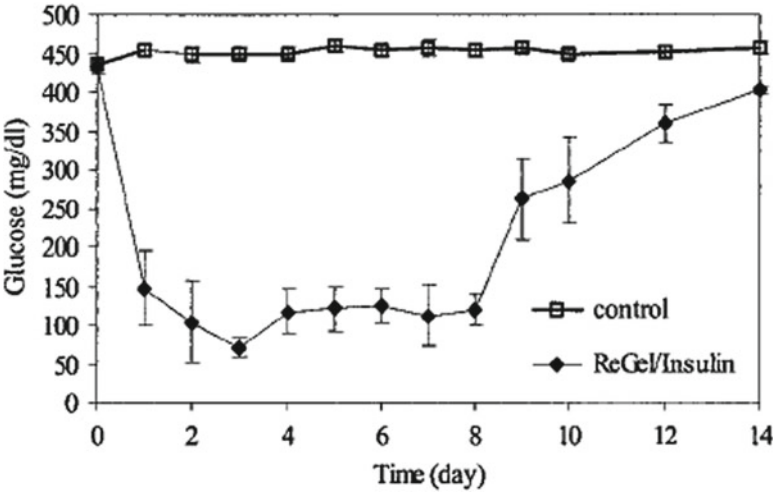

Fig. 5.1 Blood glucose levels in Zucker diabetic fatty rats. The graph represents the average ± SD, and each group was composed of five rats (reprinted, by permission, from Choi and Kim [19])

administered parenterally, proteins and peptides have short half-lives as a result of enzymatic degradation and therefore, repeated doses are required to maintain therapeutic levels.

As a consequence of the above limitations of protein and peptide therapeutics, long acting injections or implants have been developed for these products. Examples include in situ injectable depot-forming systems, microspheres, biodegradable and nonbiodegradable polymeric implants, implantable pump systems, and drug-eluting stents [3, 11–14]. The reader is referred to the following chapters in this book on in situ forming gels; microsphere technologies; osmotic implants; biodegradable technologies; drug eluting stents; delivery of peptides and proteins via injections and implants. One of the difficulties in developing injectable or implantable controlled-release delivery systems for proteins and peptides is their instability during processing and storage [15]. Proteins and peptides are sensitive to conditions, such as low and high pH, extremes of temperature, shear stress as well as surface interactions [16–18]. Despite these processing challenges, several products are available in the market (Table 5.1).

In addition to the above products, much research is ongoing into the development of new long acting parenteral products. For example, ReGel™, which is a thermally reversible and biodegradable hydrogel platform for parenteral delivery is being considered for a long acting insulin formulation. This insulin formulation has been shown to maintain blood glucose levels in the euglycemic range in type II diabetic animals for a 2-week period (see Fig. 5.1) [19].

Vascular endothelial growth factor (VEGF) is a potential treatment option for peripheral and myocardial vascular diseases as well as neovascularization in the areas of tissue trauma. However, current VEGF delivery is limited by its short in vivo half-life (30 min) and its overall dose is limited by off-target site toxicity issues [20]. To overcome these problems, VEGF entrapped in PLGA microspheres

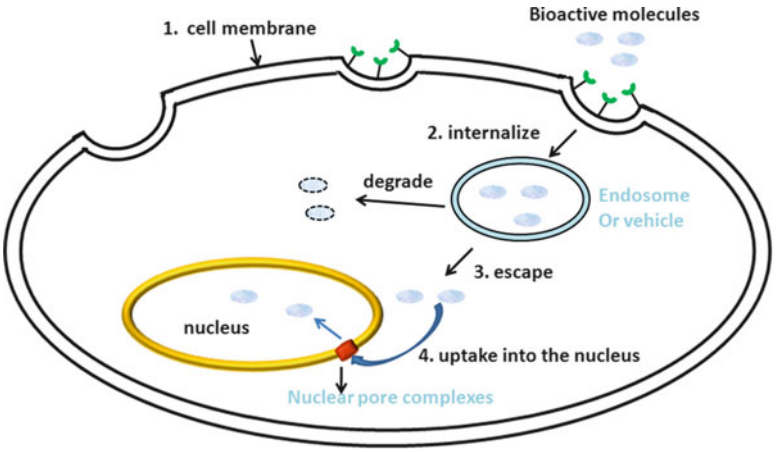

Fig. 5.2 Schematic diagram of cellular internalization of gene therapeutics. 1. cellular recognition: absorptive, receptor-mediated; 2. internalization: receptor-mediated endocytosis, phagocytosis, pinocytosis; 3. passage through the cytoplasm following endosomal escape (therapeutic DNA is subject to possible degradation by cytosolic nucleases); 4. uptake into the nucleus through the nuclear pore complexes and mediated by the cellular nuclear import machinery

has been administered (subcutaneously, intravitreally and subretinally) for continuous localized delivery over periods up to 1 month and has successfully resulted in local neovascularization [21, 22].

5.2.2 Gene Therapeutics

Gene therapy holds great promise for the treatment of a number of human diseases with increased efficiency and specificity, and accordingly low toxicity. The process of gene expression includes the following steps: a gene is transcribed to mRNA in the nucleus and translated into a peptide in the ribosome; the peptide goes through folding, glycosylation, and/or multimeric formation, resulting in the production of a functional protein. Accordingly, gene therapy requires the transgene expression of encoded genes; thus, the administered gene should reach the nucleus of the target cell [23]. Before entering the nucleus, the DNA therapeutic must overcome a series of extracellular and intracellular barriers, which can limit its efficiency. The extracellular barriers include transport to the desired cell populations, potential degradation in the plasma. Intracellular barriers include cellular internalization, endosomal escape, vector unpacking, potential degradation in the cytoplasma, and transport into the nucleus [24, 25] (see Fig. 5.2).

To overcome the extracellular and intracellular barriers mentioned above and provide a therapeutic effect without unwanted cytotoxicity or immune responses, a safe and efficient nonviral gene delivery system is required. Long acting gene delivery systems can protect DNA from nuclease degradation and provide prolonged therapeutic levels of encoded proteins.

The most widely investigated gene therapeutic sustained release systems include cationic liposomes, cationic polymers and nano/micro spheres. These systems have been administered i.v./i.m. to be taken up by the target tissues and provide sustained release and gene expression [26]. A biodegradable polycationic polyphosphoester (PPE-EA) was used to condense plasmid DNA via its strong negative charge. PPE-EA condensed DNA can protect DNA from nuclease and serum degradation. Moreover, PPE-EA/DNA complexes generated increased systemic levels of interferon-α2b in mouse serum via intramuscular injection when compared to naked DNA injection [27]. DNA has also been incorporated into hyaluronan (HA) microspheres, resulting in sustained release for approximately 2 months in vitro. In vivo transfection showed that HA-DNA microspheres could transfect in skeletal muscle for 3 weeks after a single injection and target specific cellular receptors to distinguish E- and P-selectin expressing cells [28]. Biodegradable and biocompatible PLGA polymer has also been used as a gene therapeutic carrier. Encapsulated plasmid DNA (pDNA) in submicron size PLGA particles (pDNA-NP) exhibited higher transfection effects than those obtained in the naked pDNA group at 28 days after a single intramuscular injection. The long-term transfection effect suggests that more pDNA-NP were taken up by the cell and PLGA could protect pDNA against biological degradation and sustained release pDNA [29].

Furthermore, DNA entrapped in hydrogels or scaffolds have been injected or implanted in tissues/cavities as a sustained gene delivery platform for tissue engineering [24, 30]. Due to their stabilizing and opsonization-inhibiting properties, lipoplex- and polyplex-loaded collagen sponges have been developed that can mediate sustained gene delivery and promote gene transfection following subcutaneous implantation [31].

Gene silencing via small interfering RNAs (siRNA) delivery offers potential for treating a wide variety of diseases or disorders through sequence-specific silencing of expression of specific proteins [32, 33]. To mediate gene-silencing activity, intact double-stranded siRNAs need be introduced into the cellular cytoplasm [34]. The effects of siRNA are transient even though they are highly suppressive and therefore repeated administration is necessary to achieve a persistent effect [35–37]. Mesoporous silicon particles loaded with neutral nanoliposomes containing siRNA achieved sustained antitumor gene silencing without observable concurrent toxicity for at least 3 weeks via a single i.v. administration [38]. Injectable, macroscopic, biodegradable hydrogel systems have been developed to permit long-term gene silencing effects at specific sites in a minimally invasive manner [39].

5.2.3 Drugs of Poor Oral Bioavailability

Oral delivery is the most convenient and widely accepted route of drug administration. Achieving good oral bioavailability is often the biggest hurdle for potential new medicines. Oral bioavailability is the term usually applied to the proportion of drug reaching the systemic circulation in vivo after oral administration (p.o.), compared

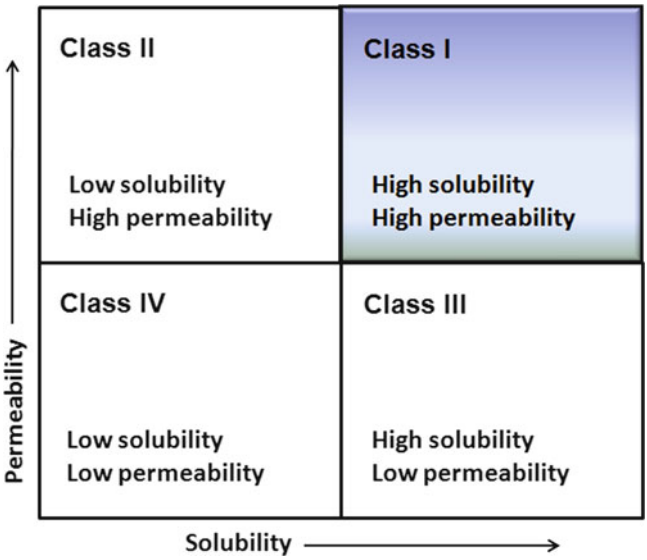

Fig. 5.3 Biopharmaceutics classification system of drugs. The oral absorption of BCS Class II drugs can be markedly enhanced by optimal formulation design, whereas the best solution to improve the oral bioavailability of BCS Class IV drugs is to go back to the lead optimization phase of drug discovery and modify their structures for the appropriate physicochemical properties to enhance permeability

to the same dose administered intravenously (i.v.). This fraction (F) is normally presented as a percentage and is calculated from the relative areas of the plasma concentration/time curves (AUCs) for the drug given by the two routes and normalized for dose, *i.e.*:

$$F\% = \left(\text{AUC p.o.} / \text{AUC i.v.}\right) \times 100\%. \tag{5.1}$$

Factors that may limit oral bioavailability include poor absorption, first-pass metabolism and other means of rapid clearance of drug before it reaches the systemic circulation. The absorption of drugs following oral administration is affected by many factors such as physicochemical properties (*e.g.* solubility, diffusivity, stability, and p*K*a) and physiological barriers (*e.g.* transit time through the different GI segments and absorption mechanisms) [40]. Based on the biopharmaceutics classification system (BCS), drug substances are classified into four categories according to their solubility and permeability properties, as shown in Fig. 5.3. Only drugs with high solubility and high permeability, which are categorized as BCS class I, can achieve high oral bioavailability [41].

More than 40% of compounds identified through combinatorial screening programs are poorly soluble in water. These molecules are difficult to formulate using conventional approaches and are associated with innumerable formulation-related performance issues. Controlled injectable or implantable drug delivery systems are

a frequently used alternative delivery approach for several poorly soluble drugs, such as chemotherapeutic agents and anesthesia agents [42].

Many anticancer drugs with potent bioactivity are highly lipophilic with extremely limited water solubility. Hence, formulation of these drugs is a challenging issue in cancer therapy [43]. Injectable or implantable formulations allow these drugs to bypass oral absorption barriers and gain direct entry into the general circulation or targeting site. Paclitaxel (PTX), an effective drug for the treatment of several solid tumor malignancies, is hampered clinically by its extremely poor water solubility (<1 μg/mL) and therefore ineffective peroral delivery [44]. Polyester-based micelles and nanoparticles for parenteral delivery of taxanes have been successfully used to improve PTX delivery [45]. Paclimer® is a biodegradable polymer microsphere formulation containing 10% (w/w) paclitaxel. A phase I trial of Paclimer® showed paclitaxel continued to be released for at least 8 weeks after intraperitoneal (i.p.) administration [46]. Camptothecin, an inhibitor of the DNA-replicating enzyme topoisomerase I with very low aqueous solubility (2 μg/mL), has been formulated as an in situ forming hydrogel to enhance the safety and efficacy of this chemotherapeutic agent [47, 48]. Poor oral bioavailability limits the use of curcumin and other dietary polyphenols in the prevention and treatment of cancer. In a phase I clinical trial, oral consumption of 3.6 g of curcumin daily resulted in low plasma concentrations (11.1 nmol/L) [49]. Minimally invasive strategies that can provide effective and sustained tissue concentrations of curcumin will help translate the preclinical efficacy of curcumin to the clinic. A single dose of curcumin PLGA microparticles resulted in sustained systemic availability of curcumin and inhibited the growth of MDA-MB-231 xenografts [50].

Lipid nanocarriers, such as liposomes, nanoemulsions, and nanoparticles have also been demonstrated as potential parenteral delivery systems for poorly soluble therapeutics. For example, the oral bioavailability of the potent antimalarial agent – artemether (ARM) is low due to its poor solubility. The current oily injection of ARM although overcoming its solubility problems has some disadvantages, such as pain on injection and slow and erratic absorption on intramuscular administration [51]. The ability of nanostructured lipid carrier (NLC) systems for sustained delivery has been applied to ARM [52]. The NLC formulation offered significant improvement in the antimalarial activity and duration of action of ARM [53].

5.3 Drug Candidates for Long Acting Parenteral Formulation Based on Therapeutic Activity

In this chapter, drug candidates that lend themselves to long acting parenteral formulation based on their therapeutic activity are discussed. The reader is referred to the next chapter in this book on disease and clinical applications for specific clinical case studies on implants and injections used for treatment of contraception, musculoskeletal diseases as well as osteoporosis and bone fractures.

5.3.1 Drugs for Systemic Delivery

The term systemic delivery refers to the delivery of a drug to the site of action via the blood circulation. Long acting parenteral formulations can maintain stable, pharmacologically relevant plasma levels, and avoid fluctuation in plasma concentrations that may occur with repeat dose administration of immediate release formulations. Examples of drug candidates that are delivered parenterally through the systemic circulation include pain relieving drugs, therapeutics for alcohol or opioid dependence, antipsychotic agents, and hormones.

5.3.1.1 Intense Pain Therapeutics

Pain is an unpleasant sensory experience commonly produced by damage to body tissues. Intense pain is a most common and feared symptom associated with cancer, surgery and labor as well as other conditions and diseases [54–56]. There are two types of pain relieving drugs – analgesics and anesthetics. Analgesia is the relief of pain without total loss of feeling or muscle movement. Anesthesia is blockage of all feeling, including pain. For the treatment of intense pain, the use of analgesics and anesthetics in combination has been suggested [57].

Systemic analgesics are often given via injection into a muscle or vein. However, currently available treatments of intense pain have relatively limited duration of activity due to the short plasma half-lives of these drugs. In addition, some of these drugs may cause severe toxicity due to their low LD50 values [58]. To achieve continuous relief of intense pain, long acting injectable or implantable controlled release systems have been developed to prolong therapeutic effects, and to decrease toxicity and side effects (*e.g.* bleeding, loss of alertness).

For example, implantable polymer-based biodegradable drug delivery systems are being intensively investigated by various research groups. PLGA rods containing a combination of analgesics (morphine, codeine and hydromorphone) and anesthetics (bupivacaine, BP) showed improved alleviation of pain compared to PLGA rods containing only one API [59]. Moreover, a new DepoFoam technology has been developed, which consists of microscopic, spherical particles with hundreds of nonconcentric aqueous chambers that can encapsulate drug. A single epidural injection of DepoDur™ loaded with morphine sulfate was shown to manage postoperative pain up to 48 h after cesarean surgery [60].

5.3.1.2 Drugs Used for Treatment of Alcohol Dependence

Pharmacotherapy is commonly used to assist individuals with alcohol dependence by easing both the craving for the substance and the withdrawal symptoms. Disulfiram (antabuse), naltrexone, and acamprosate are among the most commonly used drugs for treatment of alcohol dependence [61].

The efficacy of oral delivery of these therapeutics is limited by poor patient compliance and fluctuations in plasma concentrations following daily drug administration [62]. To overcome these limitations, attempts have been made to develop injectable or implantable extended-release formulations. For example, Vivitrex® (naltrexone injectable suspension) was designed to provide therapeutic levels of naltrexone over 1 month via intramuscular injection. Intramuscular repeat dose administration of Vivitrex® in alcohol-dependent patients showed that sustained injectable naltrexone was generally safe and well tolerated, and resulted in reduction in heavy drinking among treatment-seeking alcohol-dependent patients during 6 months of therapy [63, 64]. Intramuscular injection of naltrexone-loaded PLGA microspheres (Medisorb®) achieved stable, pharmacologically relevant plasma levels of naltrexone for at least 28 days [62]. Nonerodible rods that provide 6 months delivery of nalmefene (a pure opioid antagonist structurally similar to naltrexone) have been successfully developed to achieve prolonged therapeutic activity [65].

5.3.1.3 Contraceptives

The pharmacological approach to birth control is still mainly achieved by oral or transdermal delivery of contraceptive steroids. However, daily ingestion and subsequent daily variations in blood concentrations associated with oral administration can cause unwanted side effects [66]. Transdermal delivery systems can result in insufficient or excessive mean blood concentrations due to variation in an individual's skin permeability as well as poor patient compliance [67].

Long acting injections and implants have attracted considerable attention as effective delivery systems for contraceptive steroids to ensure a longer period of optimum blood concentrations and avoid systemic side effects [68–72]. A biodegradable levonorgestrel-releasing implant made of poly (ε-caprolactone)/Pluronic F68 has been shown to prevent pregnancy in rats for 2 years without toxicity [73]. Levonorgestrel and ethinyl estradiol loaded PLGA microspheres maintained constant steroid levels for 15 weeks following intramuscular injection [67].

5.3.2 Drugs for Local Delivery

Direct injection or implantation can deliver high doses of drugs into specific organs and tissue sites (*e.g.* brain cancer, joints), and thus, enhance therapeutic effects and avoid systemic side effects.

5.3.2.1 Drugs for Biosensors and Other Implantable Devices

Technological advances in biomaterials, tissue engineering, biotechnology, and polymer science have enabled the development of long acting parenteral systems including drug-eluting stents, biosensors, scaffolds for tissue engineering and other

implantable devices for local delivery [24, 74–76]. Although commonly used polymers, such as collagen, chitosan, hyaluronan, PLGA, PEG and PVA, are considered to be relatively biocompatible, the biocompatibility of the implantable devices still remains a critical issue in limiting device longevity and functionality, particularly in the case of biosensors [77–79].

Implantable devices have been shown to typically trigger a cascade of immunogenic reactions in response to the tissue trauma generated during implantation as well as the long-term presence of foreign material in the body tissue. This foreign body response (FBR) includes an initial acute inflammatory phase and a subsequent chronic inflammatory phase [80, 81]. The reader is referred to the chapter in this book on host response to long acting injections and implants. To overcome FBR, biocompatible materials have been used to coat implantable devices to mask the underlying surface, and steroidal/nonsteroidal anti-inflammatory drugs have been used to minimize and/or control the FBR at the implant site [82, 83]. Oral administration of these drugs may not achieve adequate local concentrations, and their long-term systemic use can cause major side effects (*e.g.* withdrawal symptoms, electrolyte imbalance, peptic ulcer, and osteoporosis). Therefore, sustained local delivery of anti-inflammatory drugs is desired.

Microsphere- and nanoparticle-based drug delivery systems, microparticles embedded in hydrogel matrices and implants containing drug-filled reservoirs have been investigated to deliver anti-inflammatory drugs such as dexamethasone to the implant site to prevent inflammation and fibrous encapsulation [84, 85]. It has been shown that these systems are effective in controlling FBR. However, as soon as drug release is complete, the inflammatory process begins, indicating that a constant release of drug at the local site is required throughout the functional lifetime of the device [86, 87].

In the case of biosensors, it is also important to ensure adequate blood supply at the local site. For this, controlled release of angiogenic factors has been investigated [22]. Localized concurrent elution of VEGF and dexamethasone has been shown to overcome the anti-angiogenic effects of dexamethasone [22].

5.3.2.2 Anti-cancer Therapeutics

Many anti-cancer drugs are cell-cycle specific, requiring adequate blood levels over prolonged time periods to achieve maximum cell-kill. Cancer therapies are typically delivered systemically in order to reach all tissues within the body, eliminating potential metastases. However, there are limitations associated with achieving optimal dose levels at the primary tumor site (this is particularly true in the brain). Medications must be injected directly at the site of action, since the systemic levels necessary to reach therapeutic levels in these target zones would result in systemic toxicity. However, many chemotherapeutic drugs have very rapid plasma clearance, leading to short tumor exposure times. Therefore, sustained as well as localized delivery is desirable to achieve elevated drug concentrations at the tumor site, to help prevent postoperative metastasis as well as reduce systemic toxicity [88, 89].

For the treatment of brain tumors, the blood brain barrier presents a major obstacle [90–92]. The Gliadel® wafer was the first clinically approved chemotherapeutic implant for intracranial delivery. It consists of a carmustine (BCNU)-eluting implant fabricated by a polyanhydride copolymer. Clinical trials have shown that this product is safe and effective in the treatment of both recurrent and newly diagnosed malignant brain tumors [93, 94]. Microcapsule containing antineoplastic RNAse proteins (amphibinases) have also been used for intracranial delivery to decrease toxicity as well as increase local therapeutic activity [95]. Paclitaxel in ReGel® (OncoGel®) has been developed for sustained local delivery of paclitaxel over approximately 6 weeks following a single administration. The phase I study of OncoGel® showed that OncoGel® was well tolerated and remained at the local injection site [96, 97].

5.3.2.3 Drugs for Musculoskeletal Diseases

Due to residual infection and poor penetration of therapeutics into the musculoskeletal systems (*e.g.* bone bed, joint), localized concentrations of antibiotics are essential for the treatment of infectious musculoskeletal diseases (*e.g.* osteomyelitis, arthritis, and osteonecrosis) [98–100]. Long acting injections and implants have advantages over oral or transdermal administration due to the delivery and maintenance of high tissue concentrations of antibiotics to eradicate infection and prevent relapse [101–103].

Direct intra-articular injection of corticosteroid (betamethasone sodium phosphate) loaded PLGA nanospheres has been shown to prolong local anti-inflammatory action in the joints of arthritic rabbits without biological damage [104]. A biodegradable thermosensitive implant composed of poly(ethylene glycol) monomethyl ether (mPEG)–PLGA copolymer has also been developed to form an in situ gelling implant for treatment of bone infection [105].

5.3.3 Other Drugs

There are some other drugs that cannot be administrated orally, such as antipsychotic drugs that require immediate onset of action, or any medication to be given to a patient who is unable or unwilling to ingest anything orally.

For patients with schizophrenia, premature discontinuation of antipsychotic drug therapy is a common phenomenon. Many patients do not experience the full benefit of antipsychotic drug therapy, and suffer frequent relapses or exacerbations which require rehospitalization, often in the context of psychiatric emergency. Long acting depot antipsychotics can mitigate nonadherence, and possibly reduce the risk of relapse. For example, long acting risperidone microspheres have been successfully used for this purpose [106–109]. Similarly, a long acting suspension of olanzapine has been shown to be an effective long-term treatment of schizophrenia [110].

5.4 Vaccines

Vaccines are responsible for decreasing the mortality and morbidity of many diseases by exploiting the immune system's ability to "remember" prior encounters with a pathogen. The most effective mechanism for the elimination of infectious diseases is the use of vaccination. However, this often requires repeated administration. Vaccines administered using an immunization schedule require two to three injections spaced over several weeks or months, followed by booster shots [111, 112].

Injectable polymeric particles (usually microspheres) represent an exciting approach to reduce the dose frequency and optimize the immune response via selective targeting of antigen to antigen presenting cells. The reader is referred to the chapter in this book on injectable PLGA systems for delivery of vaccine antigens. PLGA microparticle-based vaccines have been shown to provide persistent levels of neutralizing antibodies as well as exhibit immunological memory [113]. Long acting PLGA microspheres loaded with recombinant hepatitis B surface antigen (HBsAg) resulted in a response similar to that of three injections of HBsAg alone [114]. Microparticles used for vaccine delivery can also improve the efficacy via adjuvant activity, since microparticles are able to simulate macrophages, monocytes, and dendritic cells [115, 116].

5.5 Conclusions

Many drugs cannot achieve ideal therapeutic effects, and even have severe side effects due to their physicochemical properties, bioavailability or lack thereof, as well as their pharmacokinetic and pharmacodynamic profiles. Long acting injections and implants can facilitate drug delivery achieving therapeutic concentrations at a particular action site for a specified period of time, and avoiding potential systemic drug toxicity. However, not all drugs are suitable for controlled-release technology. Drugs requiring high dose and/or those with a narrow therapeutic index are undesirable for long acting delivery.

The use of controlled release technology is also dependent on the disease state or therapeutic outcome desired. The treatment of diseases such as diabetes, cancers, cardiovascular, and musculoskeletal system diseases, can be significantly improved by long acting parenteral formulations. Long acting parenteral formulations are also useful for the purpose of contraception and vaccination.

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Chapter 6
Diseases and Clinical Applications that Can Benefit from Long Lasting Implants and Injections

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Abstract Development of long-term implants is challenging due to stringent requirements of biocompatibility, necessitating minimal or absent adverse affects during the period of active utilization and thereafter. Implants designed for load-bearing applications must integrate with host tissue to create a stable environment. For instance, improvements that allow for better hip implant integration with the surrounding bone will prevent implant loosening and greatly improve the quality of patient life. Use of long-term implants significantly reduces the number of operative procedures while integration with drug-delivery techniques has tremendous potential to improve patient outcomes. Drug-delivery techniques have been utilized for contraception in the form of dermal-patches, vaginal rings, and intrauterine devices; all of which can be applied and removed by the patient thus minimizing medical appointments. This chapter will discuss implants for treatment of diabetes to contraception, from fracture healing to chemotherapy that are commercially available, and new treatment strategies that are being explored.

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6.1 Introduction

The use of medical implants for the treatment of injury and diseases has increased steadily with implants being used in every organ of the human body. Ideally, medical implants must not have adverse effects and must integrate with the host tissue to perform accurately as intended for the desired application. For load-bearing applications, they must have biomechanical properties comparable to autologous tissues and for long-term drug delivery they must enable controlled release of the drug either continuously or on demand. Today, the development of successful implants involves interdisciplinary components which are critical to their approval and commercial success.

The major challenges during development of medical implants are our limited ability to simulate the complexities of the biological environment; the current lack of reliable computer modeling of the in vivo performance characteristics of implants; and difficulties in evaluating the synergistic contributions of materials, design features, and therapeutic drug regimens. The ability to predict the long-term in vivo performance of medical implants is of vital interest and the extrapolation of in vitro data to the in vivo environment remains largely unproven.

Implants intended for long-term use reduce the impact of invasiveness and integrate with the surrounding host tissue to form a more stable tissue environment. Such drug-delivery systems require less frequent calibration and the increased delivery durations positively improve patient treatment and outcomes. Biodegradable polymeric implants offer unique advantages in promoting integration and growth of the host tissue, and providing at the same time functionality to the individual. The degradation products are nontoxic and readily eliminated from the body. This chapter will discuss long-term use implants for a variety of clinical applications and focus in depth on musculoskeletal uses.

6.2 Diabetes

Degenerative diabetes is a persistent hyperglycemia condition arising from the destruction of pancreatic B cells. Insulin is produced by specialized cells located within the pancreas and secreted in response to levels of glucose, fatty acids, and amino acids. It is modulated by certain neurological signals, hormones, and pharmacological agents. Individuals with diabetes do not produce or have insufficient production of insulin which limits the nutrient uptake mainly of glucose and disturbs carbohydrate, fat, and protein metabolism processes [1–3]. Type 1 diabetes is an autoimmune disorder characterized by leukocyte invasion of islet tissue and this is followed by B cell death and thus lack of sufficient insulin. Type 2 diabetes is associated with genetic disposition and various environmental determinants such as obesity, bad nutrition and low exercise and thus is the most prevalent degenerative metabolic disease in adults. The manifestation of type 2 diabetes can be delayed or reversed by correcting factors such as dietary habits and by increased physical activity.

Glycemic control is maintained in diabetics by replacement of insulin. Insulin administered subcutaneously will be absorbed into the bloodstream. The injections should provide basal insulin requirements to satisfy a 24 h period covering food intake, minimize glucose fluctuations, and be able to provide for corrections if needed. Therapy can be in the form of multiple injections per day or in the form of an infusion via a device that is implanted subcutaneously, or worn externally with leads into the subcutaneous tissue [4]. Insulin administered by multiple injections daily will have varied time to action and will be present in the bloodstream for short durations characterized by rapid metabolism leading to decreasing dosage with time. Infusion pump devices require frequent monitoring of blood glucose and can deliver a bolus dose to match the basal needs of the individual and the dose can be altered based on the need to cover meals and glycemic fluctuations in the individual [5]. Current insulin pumps are worn by the individual externally and insulin is delivered via insertion of a catheter into the subcutaneous tissue of the abdominal wall. The invasiveness, requirement for sensor or probe change every 2–3 days and low convenience in daily life have given rise to alternatives such as fully implantable systems. Here, the glucose sensor covered with an angiogenic layer is exposed to interstitial fluid. Neovascularization at the sensing site improves oxygen supply and stabilizes the sensor–tissue environment. The sensor signal is transmitted in real-time to the fully implanted insulin pump located in the abdomen wall by subcutaneous leads and thus can deliver insulin on demand. The insulin delivery system is based on a closed-loop feedback system which is optimal for long-term use. In the future, cell-based therapies offer a more robust and less invasive therapy with potentially lower costs over the life of the individual [6]. For example, transplantation or regeneration of the pancreas can restore the body's natural endocrine function [7–9]. Islet cells from human cadaveric donors and animals are being investigated for transplantation or encapsulation. A key feature is the transplantation of an adequate number of islets producing insulin and the need of an immunosuppressive regime for maintenance avoiding graft rejection and improved implant survival.

6.3 Prostate Cancer

Prostate cancer is the sixth most common form of cancer and the second leading cause of cancer in men affecting one in six of all men in America. Prostate is a small gland in the male reproductive system found below the bladder and in front of the rectum. The prostate surrounds part of the urethra, the tube that empties urine from the bladder. Because of the prostate's location, the flow of urine can be slowed or stopped if the prostate grows too large. In 2009, more than 192,000 men were diagnosed with prostate cancer, and more than 27,000 men will die from the disease [10]. That places the occurrence of a new case every 2.7 min and results in a man dying from prostate cancer every 19 min. It is estimated that there are more than two million American men currently living with prostate cancer. This is a heterogeneous disease in terms of its causes and progressions are modulated by varied factors such as genetic, environmental, nutritional, and hormonal.

The majority of clinically approved cytotoxic cancer drugs possess a narrow therapeutic window due to high systemic toxicity and lack of tumor selectivity which limits their clinical success. The poor specificity of cytotoxic drugs in terms of bio-distribution as well as pharmacology at the cellular level can be improved by adopting drug delivery principles [11, 12]. The use of radiotherapy and tumor removal is used as the last salvage treatment for advanced disease stage to control and relieve symptoms, with the aim of prolonging and improving quality of life.

Current and future approaches for prostate cancer treatment involve development of drug delivery systems which combine prodrug and active targeting of the cancer cells. One approach is self-assembled micelles containing prodrugs and is functionalized with specific targeting moieties such as prostate-specific membrane antigen (PSMA) [13]. PSMA is a type 2 integral membrane glycoprotein abundantly expressed on the surface of prostate carcinoma and the neovasculature of most other solid tumors at all stages, and thus serves as an attractive target. PSMA functionalized prodrug micelles will improve the drug solubility, minimize cytotoxicity, and target prostate cancer epithelial cells [14]. Active drug will be generated at the site of action following peptide spacer cleavage/degradation in the tumor environment. This platform can produce numerous chemotherapeutic drug formulations for prostate cancer treatment. Such delivery systems can significantly improve the performance of chemotherapeutic drugs and contribute to prolonging a good quality of life.

Localized prostate cancer can be put in complete remission with practices such as radiotherapy and radical prostatectomy. Current approaches are focused on early detection and treatment by minimally invasive radiotherapy. Radiotherapy offers complete cure in selected individuals when a sufficiently high dosage is administered. Radiotherapy can be administered by an external X-ray beam which needs to pass through various superficial tissues and organs if the target tumor is interior. However, the X-ray beam will radiate the tissues it passes through to reach the site of tumorgenesis. In another approach, a radioactive source can be delivered/implanted at the location of the prostate tumor to deliver it locally within the tumor. In permanent transplantation, a low dose seed such as iodine 125 or palladium 103 having a dose rate <40 cGy/h is implanted permanently [15, 16]. High dose rate iridium 192 is implanted interstitially temporarily to deliver a high radiation dose over a short period to time and removed after the desired dosage is delivered [17, 18]. The success of the localized radiotherapy approach relies largely on early detection using noninvasive imaging technique and biochemical analysis of blood samples. In advanced cases of prostate cancer, surgical removal of the tumor will have to be performed.

6.4 Contraception

In the United States, unintended or unplanned pregnancies are estimated to be as high as 49% of all pregnancies [19]. The incidences of teenage pregnancies have been decreasing and are attributed to reduced sexual activity, but largely due to the

growing use of effective contraception strategies. The most common form of contraception till date has been oral contraceptives. Their success critically depends on the proper and daily ingestion of the pill which can be a barrier to effective use. Approximately 47% of oral contraceptive users miss at least one pill per 28-day pack and 22% miss two or more pills [20]. The yearly failure rates among users of oral contraceptives is approximately 8% and higher in adolescents as compared to adults [21]. Repeat pregnancies in the first 6 months were lowest in adolescents using contraceptive implants and DMPA as compared to oral contraceptives. The first generation of oral contraceptives delivered very high doses of estrogen and was found to raise the risk of stroke, blood clots, and heart attacks. Later generations, contained lower hormonal doses and were combined with other synthetic hormone drugs. This reduced the risk of blood clots and stroke, but other side effects such as weight gain and acne were reported in many women. Increasing obesity contributes to hypertension, diabetes, and cardiovascular problems. The increase in weight can possibly put at risk reproductive health due to diminished ovulation, decreased response to fertility treatment, and adverse pregnancy outcomes [22]. Currently available reversible nondaily options include hormonal transdermal patch, hormonal vaginal ring, progestin-only injections, subdermal implants, and intrauterine contraception device [23, 24]. Ortho Evra (Ortho-McNeil, Raritan, NJ) is a transdermal patch that releases both ethinyl estradiol and norelgestromin and works to inhibit ovulation and also affects the cervical mucus and endometrium which contribute to its efficacy [23]. The patch has some unique disadvantages such as problems of adhesion and adverse skin reactions. This necessitates patch replacement and in some cases pigment changes and local inflammation have been observed at the site of adhesion. Another option is contraceptive rings which are placed in the vagina for 3 weeks and then removed for a withdrawal bleed. A single ring can inhibit ovulation up to 5 weeks. The ring is easily inserted and removed by the majority of women and most did not report any discomfort during sexual activity. Intrauterine contraceptives, such as Mirena, are small implantable contraceptive devices that can easily be removed and replaced. Mirena works by thickening the mucus of the cervix, thinning the lining of the uterus, and by blocking sperm from reaching or fertilizing eggs. The hormones are released continuously from the implant for up to 5 years making it relatively easy to prevent unwanted pregnancies.

Newer contraceptive devices deliver progestin hormone which is available both as an intramuscular injection and a subdermal implant [25]. The hormonal drug works by inhibiting ovulation, thinning of the endometrial lining, and thickening of the cervical mucus. One progestin-only injection inhibits ovulation for 14 weeks, and is administered at 3-month intervals. Side-effects include irregular bleeding, weight-gain with long-term use and most significantly a delayed return to fertility with average time being 10 months after the last injection; however, fertility is not affected by long-term use of the progestin-only injection. The subdermal implant is a single rod providing a controlled release of the drug daily and effective contraception up to 3 years [26]. The implant is placed under the skin of the inner upper arm by trained healthcare provider under local anesthetic. It is removed easily and there is no delay in return to fertility as all subjects were ovulating within 6 weeks after

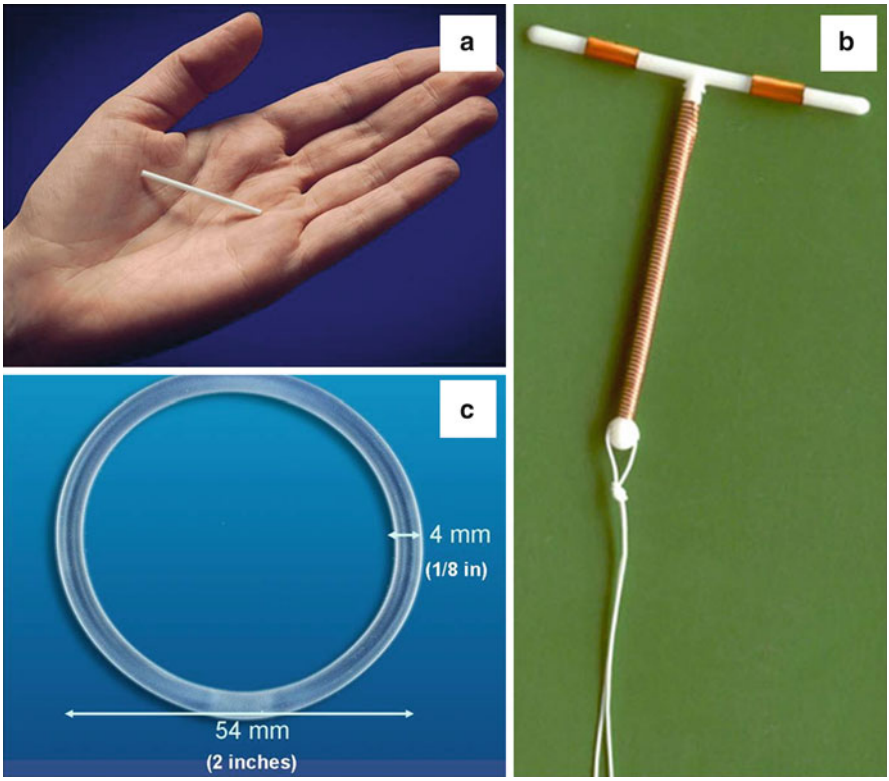

Fig. 6.1 Contraceptive implants. (**a**) Single rod implant (Implanon, Schering-Plough Corp.) made of ethylene vinyl acetate (EVA) and delivering progestin etonogestrel. (**b**) Copper intrauterine device (T 380A Paragard, Duramed Pharmaceuticals, Inc.) releases free copper and copper salts that impact the uterine lining and is approved for 10 years of use. (**c**) Contraceptive vaginal ring (NuvaRing, Schering-Plough Corp.) made of EVA is inserted by the patient themselves

removal. These newer long-term implantable contraceptives deliver the required dosages and minimize the inconvenience of daily use of the contraceptive. The chance of missing the dosage is significantly reduced and thus unexpected pregnancies. Figure 6.1 shows commercially available contraceptive implants intended for long-term use.

6.5 Musculoskeletal System

6.5.1 Tendon

Disorders of the tendon are commonly reported both in the workplace and in sport activities and severe injury leads to significant morbidity of the individual for long periods of time [27–30]. These injuries include both acute and chronic ruptures, and

also those sustained from overuse and aging exhibiting an inflammation and degeneration of the tendon. Tendinopathies are observed with disordered collagen arrangement and fiber separation accompanied by increase in proteoglycans ground substance. A conservative treatment approach is generally preferred with the goal of reducing pain and return of full mobility. Extensive rest of the affected area, stretching and strengthening exercises, application of ice and analgesics are all considered and it may take up to 6 months for the condition to improve. A clinical approach to treatment of primary and secondary tendon rupture is surgical repair. The tendon at this stage is characterized by defrayed tendon tissue unable to sustain daily tensile loads with a high risk of re-rupture. Surgical repair using grafts would enhance tissue healing by providing augmentation which will result in highly effective treatment leading to a stronger repaired tendon tissue. These implants are intended to integrate with the host tissue over the healing period and not be recovered.

Allografts and xenografts are commonly used in tendon reconstruction and repair procedures [31]. These commercially available products are largely sourced from human fascia, porcine small intestinal submucosa, and from equine, bovine and porcine epicardium. Biological scaffolds such as Restore (DePuy Orthopaedics, Warsaw, IN), GraftJacket (Wright Medical Technology, Arlington, TN), Zimmer Collagen (Zimmer, Warsaw, IN), TissueMend (Stryker Orthopaedics, Mahwah, NJ), CuffPatch (Arthrotek, Warsaw, IN), and Bio-Blanket (Kensey Nash, Exton, PA) are commonly used [32–35]. These biological tissues are processed through a series of steps involving removal of lipids or fat deposits, disruption of cellular and DNA materials, cross-linking and sterilization. They are predominately composed of type I collagen, and have surface chemistry and structural composition that mimics the native tendon tissue. The bioactive nature of these scaffolds endorses in vivo integration of the implant by promoting the attachment, migration, and proliferation of cells into the acellular biological graft. These scaffolds are limited in having low mechanical properties, nonspecific induction ability, undefined degradation rate, and variation in biocompatibility depending on the source which can cause inflammatory response and implant rejection.

Synthetic scaffolds allow for better control and optimized matching of the physio-chemical properties to suit the application needs. Synthetic scaffolds can be reproducibly made with custom properties such as mechanical strength, porosity, and degradation rate. Artelon Sportmesh (Biomet, Warsaw, IN), Gore-Tex patch (W.L. Gore & Associates, Flagstaff, AZ), and Poly-tape (Xiros, Leeds, UK) are popular commercially available synthetic scaffolds that have regulatory approval for human use. They are made of materials such as polyester, polypropylene, polyarylamide, dacron, carbon, silicone, and nylon. Artelon and Sportmesh are made of biodegradable polyurethane urea polymer and intended for reinforcement of soft tissues such as rotator cuff, Achilles, and patellar tendon. Lars ligament and polytape synthetic grafts are composed of polyesters such as ethylene terephthalate and developed as nonabsorbable grafts for ACL augmentation and reconstruction. These grafts are largely employed for augmentation of the repair and not regeneration of tendon tissue such as in case of a gap defect. Figure 6.2 shows commercially available tendon repair/augmentation device that are applied surgically.

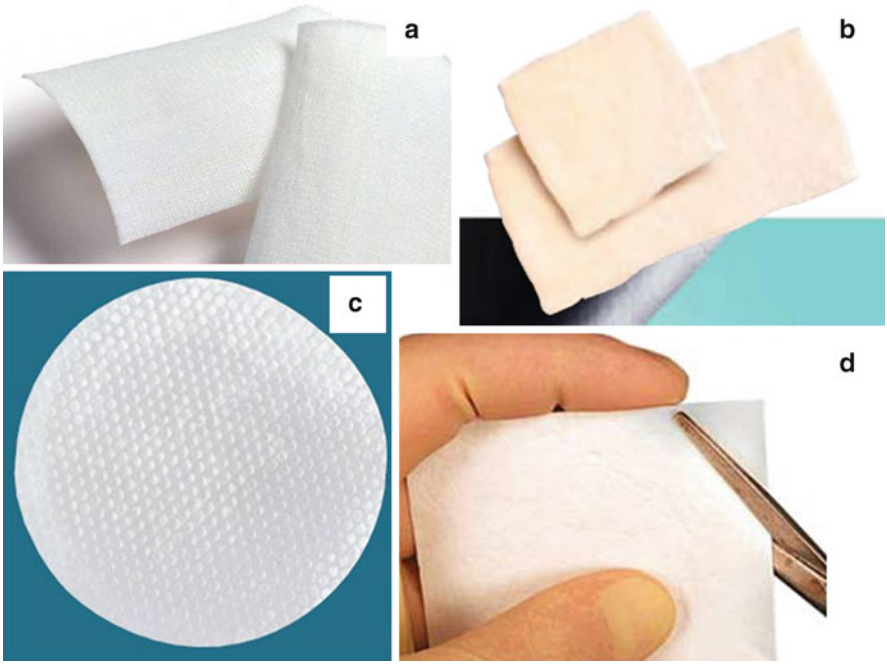

Fig. 6.2 Commercially available tendon scaffolds. (**a**) SportMesh Soft Tissue Replacement (Biomet Sports Medicine) is a degradable poly(urethaneurea)implant that is sutured over torn or degenerative tissue for reinforcement. (**b**) GraftJacket Regenerative Tissue Matrix (Wright Medical) is a human acellular tissue graft that has demonstrated excellent tensile strength and suture retention properties. (**c**) Restore Orthobiologic Soft Tissue Implant (DePuy Orthopaedics) is a restorable scaffold to reinforce soft tissue repair including reinforcement of the rotator cuff, patellar, Achilles, biceps, quadriceps, and other tendons. (**d**) TissueMend Soft Tissue Repair Matrix (Stryker Orthopaedics) is an acellular collagen membrane used to augment the repair and reinforcement of soft tissues. It serves as a scaffold for cellular in-growth that is gradually remodeled by the body's own tissues

Current laboratory research is investigating new polymers and methodology to fabricate grafts for tendon and ligament tissue regeneration [36–39]. Nanofiber-based scaffolds are being designed for rotator cuff and Achilles tendon tissue engineering [40]. These scaffolds are largely composed of polyesters such as PLGA and have fibers having nano and micro diameter. These fibers are fabricated by electrospinning technique resulting in a scaffold composed of nonwoven fibers. They are biocompatible and conducive to cell attachment, migration and proliferation and the dimension of the fibers have a profound effect on directing cellular response and matrix properties.

Regenerative capabilities of the novel tissue-engineered constructs are further enhanced by delivering multipotent stromal cell populations using the novel biological and synthetic grafts [41, 42]. Stromal cell populations can regenerate into various tissues such as bone, cartilage, and nerve under appropriate and timely

provided stimulus and environmental conditions. Stem cells have been described as being capable of differentiating into tendon phenotype in various animal models such as the horse, rabbit, mouse, and rhesus monkey [43]. Bone marrow stromal cell (bMSC) seeded knitted PLGA fiber scaffolds have been investigated for Achilles tendon repair in rabbit models. bMSC/PLGA-treated tendons showed more mature collagen fibers than in the PLGA alone treated groups. Additionally, a higher rate of tissue formation and remodeling was observed at the early time points in the bMSC/PLGA group. Current research brings together tissue-engineering strategies to match implant degradation rate to tissue regeneration and further exert considerable control over the regenerative/repair process leading to faster functional recovery of the individual.

6.5.2 Osteoporosis and Bone Fractures

Osteoporosis is bone disease characterized by reduced bone formation or increased bone resorption. The imbalance between the process of bone resorption and bone formation leads to low bone density in the individual. The compromised bone strength predisposes the patient to an increased risk of fracture. Elderly populations are at the highest risk of bone fracture and thus leading to significant morbidity and poor healing response to the injury. It is estimated that by 2012, 40% of women and 14% of men over the age of 50 years will sustain osteoporotic fractures and thus experience drastically reduced function and quality of life [44–46]. Osteoporosis is thus considered a public health problem with enormous financial burden to the individuals affected. The most common fractures associated with osteoporosis are that of hip, vertebrae, and distal radius [47]. Surgical repair is necessary and involves the use of long-lasting implants, stabilizing rods, and screws to stabilize the fracture site. More importantly, the implants need to integrate with the host. The healing capacity of bone is reduced with osteoporosis and is reflected in the increased failure rate of implant fixation largely due to implant pull-out and cut-through phenomena. Early implant failure is caused by weak structure of the cancellous bone and overloading of the fracture site. Late implant failure is related to impaired healing of the fracture site and excessive mechanical loading of the bone–implant interface. One popular mechanism is to augment implant fixation with bone graft substitutes which may enhance the stability at the bone–implant interface [47, 48]. An ideal bone-graft substitute must provide scaffolding for osteoconduction, growth factors for osetoinduction, and progenitor cells for osteogenesis [49, 50]. Autografts are the gold standard and contain osteoblasts, endosteal osteoprogenitor cells, and a structural matrix that will act as a scaffold which will readily integrate with the host. However, their supply is limited and there are increased issues concerning donor site morbidity. Allografts are the next best option; however, they carry risk of disease transmission. Various techniques are employed to minimize the risk of disease transmission such as freezing, enzymatic degradation, freeze drying, and sterilization procedures to destroy all possible cells and contaminants. The allograft bone

substitutes largely retain the osteoconductive potential and provide a scaffold template which serves to promote cell attachment, migration, and proliferation. Demineralized bone matrix (DBM) produced by acid extraction of allograft cortical bone is extensively used. DBM contains type I collagen which provides osteoconductive properties and osteoinductive growth factors such as bone morphogenetic protein (BMP), fibroblast growth factor (FGF), insulin growth factor (IGF), platelet-derived growth factor (PDGF), and transforming growth factor-beta (TGF-B).

Calcium phosphate cements (CPC) are composed of calcium phosphate and are capable of self-setting into a hard mass [51]. They have controlled setting times and have excellent moldability properties enabling it to fill void spaces and conform to irregular-shaped defects. The setting reaction starts with the dissolution of the salts in an aqueous system which then supplies the Ca and P ions which precipitate in the form of HA under isothermic conditions and physiological pH. In the operating room, calcium phosphate powder and fluid are mixed into an injectable paste either manually or with the use of a mixing machine. As it is injected, the cement integrates with the adjacent bone forming a solid structure that is mechanically very stable. After injection, the paste hardens to form a carbonated calcium-phosphatidyl apatite very similar to the mineral phase of bone. Fully cured calcium phosphate cement has compressive strength between that of cancellous and cortical bone, but tensile and shear strength much lower than cancellous bone. Studies have demonstrated that 95% of calcium phosphate is resorbed in 26–86 weeks [52]. Under loading, the augmented specimens were significantly stiffer and failed at higher loads. In a randomized clinical study where fractures of the femur neck was fixed with two cannulated screw alone or in combination with calcium phosphate cement, it was shown that stability of the fixation improved in the early postoperative phase (up to 6 weeks). The authors reported that the augmented fracture has a total displacement of mean 1.9 mm as compared to 5.5 mm in the control group. The mobilization of the patient was easier during the early postoperative phase; however, there was a higher overall rate of re-operation in the augmented group due to nonunion or avascular necrosis in the follow-up period of 24 months. Different studies have reported the progress of bone formation around calcium phosphate cements, and these materials have demonstrated both osteoconductive and osteoinductive properties. Histologically, at 2 weeks, spicules of living bone with normal bone marrow and osteocytes in lacunae can be seen in the cement. At 8 weeks, the cement is almost totally surrounded by mature bone. At this stage, no resorption of the cement is typically observed.

Calcium phosphate precipitates into HA at low temperatures which allows for the addition of BMPs as a lyophilized power to the cement or the aqueous component during mixing just prior to injection [51]. The low temperature of the cementing reaction minimizes the risk of heat damage to the protein. When BMPs are incorporated into the cement, the retention profile is prolonged when compared to surface release. The CPC is resorbed by osteoclasts and multi-nucleated giant cells during which the incorporated BMPs are released. Figure 6.3 depicts bone graft substitutes from varied sources and in different forms that are commercially available.

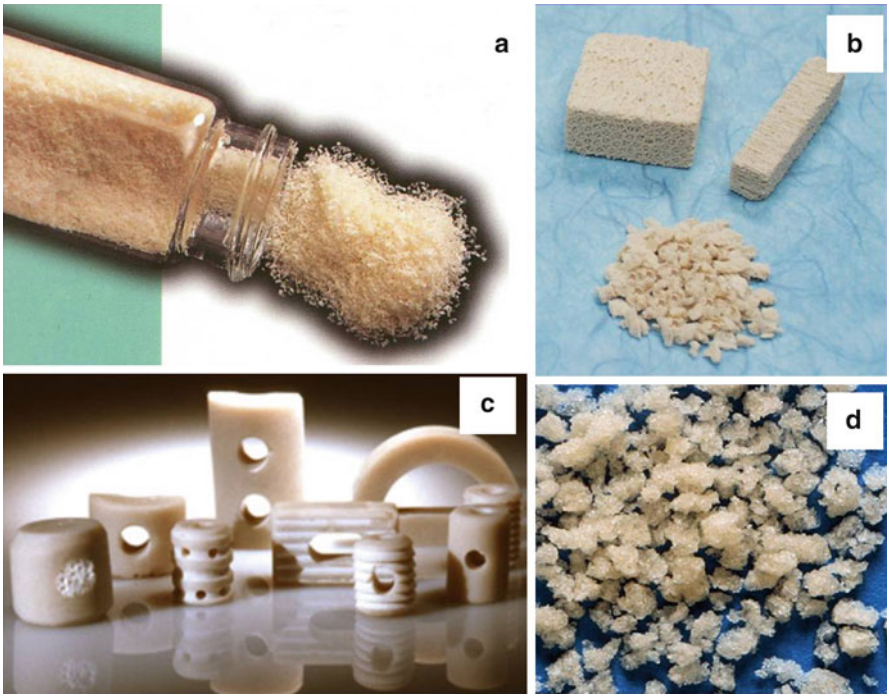

Fig. 6.3 Bone graft substitutes. (**a**) Allogro (AlloSource) is demineralized bone matrix. (**b**) ProOsteon (Interpore Cross International, Inc.) is produced from hydroxyapatite and available in either a particulate or a block form by chemically treating sea coral. Polymer-based bone graft substitutes include both. (**c**) Solid forms such as Cortoss and Rhakoss (Orthovita, Inc.). (**d**) Particulate forms such as Immix Extenders (Osteobiologics, Inc.) which are completely resorbed by the body

6.5.3 Nucleus Pulposa Replacement

In patients suffering from degenerative disc disease, the current disease management protocols are shifting toward preserving spinal mobility and minimizing tissue dissection. Replacement of the nucleus pulposus and retainment of the native annular fibrosus is proposed as an alternative to spinal fusion surgery. Following discectomy or in cases of progressive disc degeneration, there is substantial reduction in the disc height of the vertebral bodies resulting in altered biomechanics and restricting spinal mobility [53]. The NP loses its hydrated nature and loss of proteoglycans leads to slackening. The reduced disc height and volume of the vertebral body increases the load on the NP leading to increased cell necrosis and decrease in the synthesis of relevant matrix components. Increased static loading of the vertebral bodies can initiate disc degeneration as evidenced in animal models. The vertebral column is connected by a complex system of joints, intervertebral discs, ligaments, and muscles which function in unison to preserve segmental motion and provide global

stability of the spine. At the local level, the interaction between the semifluid NP and the rigid annulus fibrosus provides the biomechanical properties necessary for maintenance of stability. In patients who suffer from progressive disc degenerative disease, and have also undergone surgical discectomy, the delicate balance within the vertebral body is disturbed [54]. It begins with gradual dehydration of the NP which alters the tension applied to the fibers of the disc annulus under loading. The outer region of the annulus will bulge outward and the inner region will bulge toward the center of the disc under axial loading. This is thought to promote circumferential tears in the annulus which will further decrease its ability to resist shear forces. The fiber annulus that is normally under tension in healthy vertebral bodies will collapse altering the transfer of stress under loading between the individual discs. This local reduction in disc height will significantly alter the mechanical stability of the vertebral column and without timely treatment will promote further degeneration of the disc joint [55]. The decreased height of the disc will cause the facet joints to bear increasing loads and thus undergo hypertrophy and become a source of pain. The decreased stiffness and increased range of motion can manifest itself in abnormal muscle, ligament, and tendon loading leading to back pain.

The use of biomaterials for nucleus replacement provides to augment the nucleus pulposus preventing disc height loss and the associated biomechanical and biochemical changes that negatively impact the stability of the vertebral column [53, 56]. An injectable material will be ideal to restore the disc volume and can be delivered to the disc space using a minimally invasive technique. The injected material considered should restore the biomechanical functions of the annulus by placing the annular fibers in tension without risk of significant toxicity or rejection. The implant device must most importantly be able to withstand the repeated physical loading and ideally integrate with the host by tissue formation. There are two types of NP replacement devices, one example is intradiscal implants that are similar in property to the native NP tissue and the other is in situ curable polymers that harden after implantation. Additionally, injectable biomaterials will allow for incorporation and uniform dispersion of cells and/or therapeutic agents.

Hydrogels are three-dimensional expandable polymers that can absorb and release water depending on load, and have been extensively explored for nucleus replacement. Prosthetic Disc Nucleus (Raymedica, Minneapolis, Minnesota) is a hydrogel pellet encased in a polyethylene jacket. It can absorb 80% of its weight in water and it allows the device to swell restoring and maintaining the native disc height. The polyethylene jacket is inelastic and allows fluid to pass into the hydrophilic core allowing the device to expand. The jacket restrains the height gained so as to prevent fractures of the vertebral endplates. The device has performed well in mechanical endurance tests which have revealed its ability to maintain disc height, implant form, and viscoelasticity up to 50 million cycles with loads ranging from 200 to 800 N. The implant was biocompatible without any systemic toxicity and carcinogenicity.

An injectable in situ curing polymer commercially available is NuCore (Spine Wave Inc., Shelton, CT) which is composed of a copolymer of silk and elastin structural proteins suited for elasticity and toughness. The polymer is composed of synthetically designed repeat blocks of amino acid sequences fabricated using gene

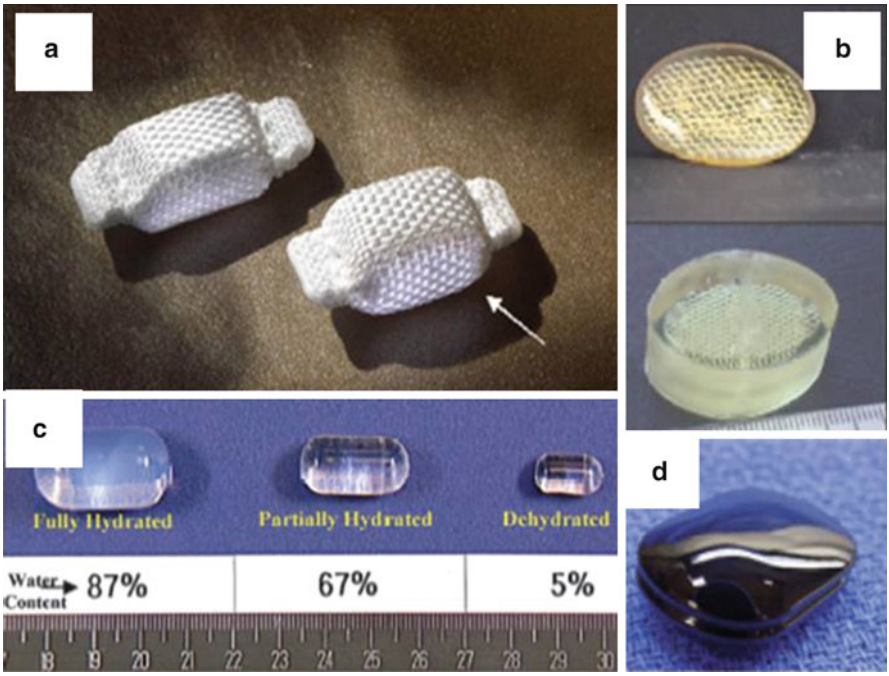

Fig. 6.4 Nucleus pulposus replacement device. (**a**) PDN-SOLO device in dehydrated and hydrated states (Raymedica, Inc.). (**b**) Aquerelle poly(vinyl alcohol) hydrogel being hydrated (Stryker Spine). (**c**) Neudisc hydrogel pre- and posthydration (Replication Medical, Inc.). (**d**) EBI regain a rigid nucleus disc replacement device (EBI)

template-directed synthesis. The copolymer consists of two silk block and eight elastin blocks per polymer repeat and synthesized using recombinant techniques in *Escherichia coli*. One of the elastin blocks is modified to enable chemical crosslinking. The purified protein is mixed with a crosslinking agent at the time of implantation. The polymer is injected through the annular defect and adheres to the surrounding tissue within the disc space as it cures. The material fills the void space and seals the annulotomy. Extensive evaluation shows the material mimics the mechanical properties of native NP and is nontoxic and biocompatible. Figure 6.4 shows various devices developed for nucleus replacement. Most are supplied in a dehydrated form and can be implanted using minimally invasive procedures.

6.5.4 Spine

When back pain becomes severe enough to disable a patient such that they can no longer function in the activities of daily living, a surgical option is considered. Spinal fusion is a surgical procedure used to correct the problems arising due to a

fractured (broken) vertebra; spinal deformities (spinal curves or slippages); pain from motion; spinal instability, and cervical disc herniations [57–59].

In fusion, one or more of the vertebrae of the spine are united together ("fused") so that motion no longer occurs between them. Bone grafts are placed around the spine during surgery [60, 61]. The body then heals the grafts over several months – similar to healing a fracture – which joins, or "welds," the vertebrae together. The spine may be approached and the graft placed from the back (posterior approach) or from the front (anterior approach), or by a combination of both. The ultimate goal of fusion is to obtain a solid union between two or more vertebrae. Fusion may or may not involve use of supplemental hardware (instrumentation) such as plates, screws, and cages [62]. Instrumentation is sometimes used to correct a deformity, but usually is just used as an internal splint to hold the vertebrae together while the bone grafts heal. The surgery also stops the progress of spinal deformity, such as scoliosis [63]. Scoliosis is an "S"-shaped curvature of the spine that sometimes occurs in children and adolescents. Fusion is indicated for very large curves or for smaller curves that are getting worse.

Another condition that is treated by fusion surgery is actual or potential instability. Instability refers to abnormal or excessive motion between two or more vertebrae. It is commonly believed that instability can either be a source of back or neck pain or cause potential irritation or damage to adjacent nerves. Although there is some disagreement on the precise definition of instability, many surgeons agree that definite instability of one or more segments of the spine is an indication for fusion.

Cervical disc herniations that require surgery usually involve not only the removal of the herniated disc (discectomy), but also fusion. With this procedure, the disc is removed through an incision in the front of the neck (anteriorly) and a small piece of bone is inserted in place of the disc. Although disc removal is commonly combined with fusion in the neck, this is not generally true in the lower back (lumbar spine).

6.5.5 Arthritis

Osteoarthritis, also known as degenerative arthritis, is caused by the breakdown and eventual loss of the cartilage present at the joints. Cartilage protein serves as a "cushion" between the bones of the joints. Among the over 100 different types of arthritis conditions, osteoarthritis is the most common, affecting over 20 million people in the United States [64]. Osteoarthritis commonly affects the hands, feet, spine, and large weight-bearing joints, such as the hips and knees. With aging, the water content of the cartilage increases, and the protein makeup of cartilage degenerates. Eventually, cartilage begins to degenerate by flaking or forming tiny crevasses. In advanced cases, there is a total loss of cartilage cushion between the bones of the joints. Repetitive use of the worn joints over the years can irritate and inflame the cartilage, causing joint pain and swelling. Loss of the cartilage cushion causes friction between the bones,

leading to pain and limitations of joint mobility. Inflammation of the cartilage can also stimulate new bone outgrowths (spurs, also referred to as osteophytes) to form around the joints. Osteoarthritis occasionally can develop in multiple members of the same family, implying a hereditary (genetic) basis for this condition.

During arthrocentesis, a sterile needle is used to remove joint fluid for analysis [65, 66]. Joint fluid analysis is useful in excluding gout, infection, and other causes of arthritis. Removal of joint fluid and injection of corticosteroids into the joints during arthrocentesis can help relieve pain, swelling, and inflammation. Arthroscopy is a surgical technique whereby a doctor inserts a viewing tube into the joint space. Abnormalities and damage to the cartilage and ligaments can be detected and sometimes repaired through the arthroscope. If successful, patients recover from the arthroscopic surgery much more quickly than from open joint surgery. X-rays of the affected joints can suggest osteoarthritis. The common X-ray findings of osteoarthritis include loss of joint cartilage, narrowing of the joint space between adjacent bones, and bone spur formation. Simple X-ray testing can be very helpful to exclude other causes of pain in a particular joint as well as assist in decision making as to when surgical intervention should be considered. Bony enlargement of the joints from spur formations is characteristic of osteoarthritis.

Treatment with oral cortisone is generally not used in treating osteoarthritis, but when injected directly into the inflamed joints, it can rapidly decrease pain and restore function. Since repetitive cortisone injections can be harmful to the tissues and bones, they are reserved for patients with more pronounced symptoms. For persisting pain of severe osteoarthritis of the knee that does not respond to weight reduction, exercise, or medications, a series of injections of hyaluronic acid (Synvisc, Genzyme Corporation, Cambridge, MA) into the joint can sometimes be helpful, especially if surgery is not being considered [67]. The products seem to work by temporarily restoring the thickness of the joint fluid, allowing better joint lubrication and impact capability, and perhaps by directly affecting pain receptors.

Surgery is generally reserved for those patients with osteoarthritis that is particularly severe and unresponsive to the conservative treatments. Arthroscopy, discussed above, can be helpful when cartilage tears are suspected. Osteotomy is a bone-removal procedure that can help realign some of the deformity in selected patients, usually those with knee disease. In some cases, severely degenerated joints are best treated by fusion (arthrodesis) or replacement with an artificial joint (arthroplasty) [68, 69]. Joint replacement has become the treatment of choice to restore function in severely arthritic joints with the most common involving joints of the knee and hip, followed by the shoulder. These can bring dramatic pain relief and improved function. Most total hip and knee replacements are for individuals having osteoarthritis and the majority of the patients hospitalized for such undergo joint replacement. For example, in an arthritic knee the damaged ends of the bones and cartilage are replaced with metal and plastic surfaces that are shaped to restore knee movement and function. In an arthritic hip, the damaged ball (the upper end of the femur) is replaced by a metal ball attached to a metal stem fitted into the femur and a plastic socket is implanted into the pelvis, replacing the damaged socket. Although hip and knee replacements are the most common joints replaced, this surgery can be

performed on other joints, including the ankle, foot, shoulder, elbow, and fingers. The materials used in a total joint replacement are designed to enable the joint to move just like a normal joint. The prosthesis is generally composed of two parts: a metal piece that fits closely into a matching sturdy plastic piece. Several metals are used, including stainless steel, alloys of cobalt and chrome, and titanium. The plastic materials are durable and wear-resistant (polyethylene). Plastic bone cement may be used to anchor the prosthesis into the bone. Joint replacements can also be implanted without cement when the prosthesis and the bone are designed to fit and lock together directly.

Surgical innovation has led to a technique for the repair of isolated splits of cartilage (fissures) of the knee [70]. In this procedure, a patient's own cartilage is actually grown in the laboratory, then inserted into the fissure area and sealed over with a "patch" of the patient's own bone covering the tissue. While this is not a procedure for the cartilage damage of osteoarthritis, it does open the door for future cartilage research. These and other developing areas hold promise for new approaches to an old problem.

6.6 Conclusion

This chapter characterizes long-lasting medical implants into two distinct categories. One includes all drug delivery devices which enable precisely controlled delivery of potent therapeutic molecules. These medical implants are either removed after very long duration or biodegrade into nontoxic products that are readily eliminated from the body. The design challenges involve precise dosage delivery, localized effect, stable tissue integration, and minimize low daily convenience to the individual.

The other category of long-term implants includes those having significant mechanical properties that can improve functionality of the injured or diseased individual. Most such implants are intended for the musculoskeletal applications such as hip and knee replacement, augmentation of torn tendons and ligaments, and plates and screws to stabilize fractures and spinal problems. Many are metal and polymer based with extremely low or no degradability. These are intended for lifelong use or till implant failure. Design challenges involve high biocompatibility of the medical device and strong integration with the host tissue and the ability to restore limited to full functionality of the individual.

Future products will be directed toward regeneration of tissues wherein the implant will serve as a template to provide early functionality and direct the cell growth and phenotype differentiation. This may be achieved by the material properties of the implant and also by delivery of molecular cues to direct the cellular response. Such implants will undergo controlled degradation matched by tissue growth rate and provide limited to full functionality. The tissue is expected to be fully regenerated by the host cells.

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Chapter 7
Oily (Lipophilic) Solutions and Suspensions

Susan W. Larsen, Mette A. Thing, and Claus Larsen

Abstract Formulation of drugs in the form of lipophilic solutions or suspensions may lead to a considerable prolongation of drug action following parenteral administration. The in vivo performance of such oil depot injectables are influenced by three major parameters, i.e., the rate of drug release from the oil vehicle, the degree of drug load, and the syringeability. Thus, the aim of this chapter is mainly to give a brief overview over various means to manipulate in vitro characteristics including (1) vehicle viscosity, (2) drug solubility in oil vehicles, and (3) drug partitioning between oil and aqueous buffer. Furthermore, the utility of in vitro release models in the design of lipophilic drug formulations and for quality control purposes is discussed.

7.1 Introduction

Usually pharmacologically active agents endowed with optimal structural configuration for eliciting the desired therapeutic response in the target area do not possess the best molecular forms and properties for either the incorporation into a suitable drug delivery system (DDS) or the delivery to and adequate retention at the site of ultimate action. This situation may exist for novel biologicals intended for parenteral administration. For such therapeutic macromolecules, the drug delivery methodology may impact efficacy as much as the nature of the drug molecule itself [1]. Likewise, the selection of advanced sustained-release injection principles for small-molecule drugs can affect the therapeutic value significantly [2]. Since the introduction

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Table 7.1 Oil depot preparations intended for intramuscular administration

Drug substance	Product (manufacturer)	Oil vehicle	Duration of action
Cyproterone acetate	Androcur®-Depot (Schering)	Castor oil with benzyl benzoate	2 weeks
Estradiol valerate	Prognyn®-Depot (Schering)	Castor oil with benzyl benzoate	2–6 weeks
Estradiol valerate	Delestrogen® (Monarch Pharmaceuticals)	Sesame oil with chlorbutanol	4 weeks
Fluphenazine decanoate	Flufenazin Dekanoat "Squibb" (Bristol-meyers Squibb)	Sesame oil with benzyl alcohol	2–4 weeks
Cis(Z)-fluphenthixol	Fluanxol® Depot (Lundbeck)	MCT	2–4 weeks
Haloperidol decanoate	Serenase® Dekanoat (Janssen-Cilag)	Sesame oil with benzyl alcohol	3–4 weeks
Haloperidol decanoate	Serenase® Dekanoat (Ortho-McNeil Pharmaceutical)	Sesame oil with benzyl alcohol	4 weeks
Nandrolone decanoate	Deca-Durabolin® (Organon)	Sesame oil with benzyl alcohol	3–4 weeks
Perphenazine decanoate	Trilafon® dekanoat (Schering-Plough)	Sesame oil with propyl parahydroxy benzoate	2–4 weeks
Testosterone cypionate	Depot-Teststerone® (Pharmacia and Upjohn)	Cottonseed oil with benzyl benzoate and benzyl alcohol	2–4 weeks
Testosterone enanthate	Delastryl® (BTG Pharmaceuticals)	Sesame oil with chlorbutanol	2–4 weeks
Testosterone enanthate	Testoviron®-Depot (Schering)	Castor oil with benzyl benzoate	3–4 weeks
Testosterone enanthate and testosterone propionate	Testoviron®-Depot (Schering)	Castor oil with benzyl benzoate	2 weeks
Zuclopehthixol acetate	Cisordinol-Acutard® (Lundbeck)	MCT	1–2 days
Zuclopehthixol decanoate	Cisordinol®-Depot (Lundbeck)	MCT	2 weeks

MCT medium-chain triglyceride (see Table 7.3)

of the early types of sustained-release injectables, mainly lipophilic (oily) solutions (Table 7.1) and aqueous suspensions, additional formulation principles (like microspheres and liposomes) have received considerable attention. A number of liposome and microsphere-based products have been approved for parenteral administration in areas such as cancer and schizophrenia [3–5]. Development of these types of DDSs might, however, be far from straightforward. Drawbacks to their use encounter risk of development of hypersensitivity reactions [6] and lack of physical stability [7] (liposomes) as well as rather unpredictable bulk degradation reactions leading to batch to batch fluctuations in the overall drug release profile and problematic manufacture (microspheres) [8, 9]. Key attributes of simple lipophilic solutions include uncomplicated manufacture (including terminal sterilization), favorable

long-term stability as well as the possibility to design depots with tailored delivery characteristics. Thus, for novel drug candidates requiring administration in the form of a parenteral depot, development of a lipophilic solution-based injectable should be considered a realistic option. The main focus of this chapter is on in vitro characterization of the latter formulation type. The in vivo fate of lipophilic solutions is only briefly commented on since more comprehensive treatises of in vivo behavior of oil-based injectables can be found in previous overviews [10, 11]. Furthermore, the utility of oil-based suspensions is briefly discussed.

7.2 Oil–Drug Solutions

Long acting oil solutions of steroid esters (for contraception and hormone replacement therapy [12]) as well as antipsychotics [13] have been used in the clinic for more than four decades. Despite the worldwide use of oil depot injectables in the management of schizophrenia, detailed information of this formulation type is sparse as regards both basic in vitro characterization and pharmacokinetic behavior. The limited literature related to the pharmacokinetics of depot antipsychotics can, at least in part, be ascribed to the lack of sufficiently sensitive bioanalytical procedures in the early 1960s [14]. Selected pharmacokinetic data are given in Table 7.2 and examples of pharmacokinetic profiles obtained in human after intramuscular injection of two oil depot products are depicted in Fig 7.1. In this section, attempts have been made to compile basic in vitro characteristics of lipophilic solutions with particular attention on means to manipulate (1) vehicle viscosity, (2) drug solubility in oil vehicles, and (3) drug partitioning between oil and aqueous buffer. These three basic physicochemical parameters might significantly impact overall depot performance by affecting depot syringeability/injectability, degree of drug load, and rate of drug release from the vehicle in vivo. Furthermore, the utility of in vitro drug release models in the formulation development phase as well as in quality control is discussed.

7.2.1 Structure and Basic Properties of Oil Vehicles

Vegetable oils have been used as vehicles in the majority of parenteral long acting lipophilic solutions (Table 7.3). Synthetic fatty acid esters such as isopropyl myristate and ethyl oleate constitute alternative vehicles. Vegetable oils contain various triglycerides in different proportions where castor oil, in particular, deviates from the other oils by the high content of a fatty acid (ricinoleic acid) with a hydroxy group. The fatty acid composition of vegetable oils influences vehicle density and viscosity (Table 7.3). In general, vegetable oils exhibit an acceptable chemical stability. It should be stressed, however, that triglycerides containing unsaturated fatty acids might be susceptible to autoxidation, a degradation pathway catalyzed by heat and light.

Table 7.2 Selected pharmacokinetic data of some depot antipsychotics obtained after intramuscular administration of different ester derivatives in oil solutions

Drug	Ester	Oil vehicle	t_{max} (days)	$t_{½}$ (days)	Model	Method of analysis	Dosage	References
Fluphenazine	Enanthate	Sesame oil	2	3.5–4	Patients	Determination of plasma radioactive fluphenazine after separation from radioactive metabolites by solvent extraction. ^{14}C-labeled fluphenazine decanoate was administered	Single dose	Curry et al. [15]
Fluphenazine	Decanoate	Sesame oil	0.3–1.5	7–10	Patients	Determination of plasma radioactive fluphenazine after separation from radioactive metabolites by solvent extraction. ^{14}C-labeled fluphenazine decanoate was administered	Single dose	Curry et al. [15]
Fluphenazine	Base	Sesame oil	0.1	0.1 and 4[a]	Male beagle dogs	Determination of total plasma radioactivity after administration of ^{14}C-labeled fluphenazine base	Single dose	Dreyfuss et al. [16]
Fluphenazine	Enanthate	Sesame oil	4	10	Male beagle dogs	Determination of total plasma radioactivity after administration of ^{14}C-labeled fluphenazine enanthate	Single dose	Dreyfuss et al. [17]

Fluphenazine	Decanoate	Sesame oil	11	34	Male beagle dogs	Determination of total plasma radioactivity after administration of ^{14}C-labeled fluphenazine enanthate	Single dose	Dreyfuss et al. [17]
Fluphenazine	Base	Sesame oil	0.1	0.3	Female beagle dogs	Determination of plasma fluphenazine by HPLC with coulometric detection	Single dose	Luo et al. [18]
Fluphenazine	Decanoate	Sesame oil		6 and 10[b]	Female beagle dogs	Determination of plasma fluphenazine and fluphenazine decanoate by HPLC with coulometric detection	Single dose	Luo et al. [18]
Zuclopenthixol	Acetate	MCT[c]	1–2		Volunteers, patients and beagle dogs	Determination of serum zuclopenthixol by HPLC	Single dose	Aaes-Jørgensen [19]
Zuclopenthixol	Decanoate	MCT	4–7		Volunteers, patients and beagle dogs	Determination of serum zuclopenthixol by HPLC	Single dose	Aaes-Jørgensen [19]
Zuclopenthixol	Decanoate	MCT	4–7	11–14	Patients	Determination of serum zuclopenthixol by HPLC	Multiple injections every third week	Viala et al. [20]
Zuclopenthixol	Decanoate	MCT	4–7	19	Patients	Determination of serum zuclopenthixol by a fluorimetric method	Multiple injections every fourth week	Jørgensen and Overø [21]

(continued)

Table 7.2 (continued)

Drug	Ester	Oil vehicle	t_{max} (days)	$t_{½}$ (days)	Model	Method of analysis	Dosage	References
Haloperidol	Decanoate	Sesame oil		10–20	Male Wistar rats	Determination of total plasma radioactivity after administration of ^{14}C-labeled haloperidol decanoate and estimation of plasma haloperidol and haloperidol decanoate after separation by TLC	Single dose	Matsunaga et al. [22]
Haloperidol	Decanoate	Sesame oil	7	28	Patients	Determination of plasma haloperidol by radioimmunoassay after extraction procedure	Multiple injections every fourth week	Wiles et al. [23]
Haloperidol	Decanoate	Sesame oil	7	27	Patients	Determination of plasma haloperidol by HPLC using electrochemical detection	Multiple injections every fourth week	Chang et al. [24]

MCT medium-chain triglyceride (see Table 7.3)

[a] Radioactivity in plasma declined rapidly at first (2–12 h) and then more slowly (2–14 day) revealing two half-lives

[b] The half-lives for fluphenazine decanoate and fluphenazine, respectively

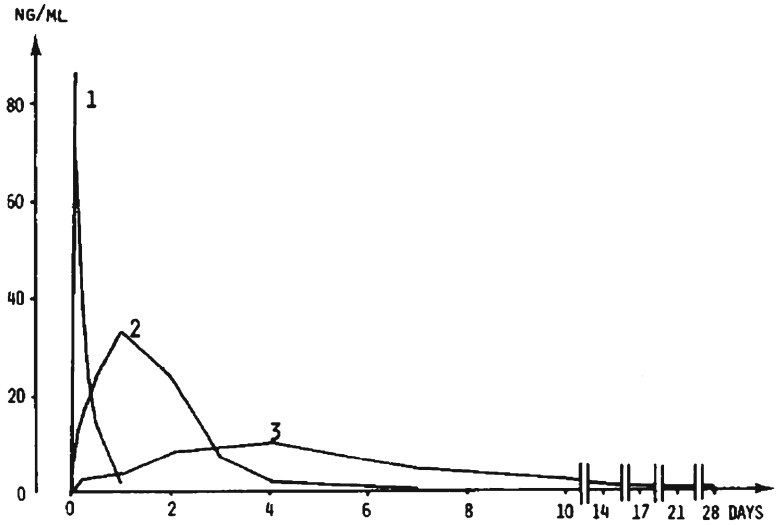

Fig. 7.1 Mean serum concentrations of zuclopenthixol in humans after intramuscular administration of: 1, zuclopenthixol dihydrochloride in aqueous solution; 2, zuclopenthixol acetate in fractionated coconut oil (medium-chain triglycerides); and 3, zuclopenthixol decanoate in fractionated coconut oil (reprinted by permission, from Aaes-Jørgensen [19])

7.2.2 Vehicle Viscosity

Syringeability of oil-based injectables might be considered inversely related to the viscosity of the oil vehicle [25]. A micro-capillary rheometer has been developed to evaluate the flow and injection characteristics of parenteral formulations in a quantitative manner [26]. Good injectability of Newtonian oil-based solutions with viscosities up to about 60 mPa s are expected since commercial parenteral solutions have viscosities in the range 29–60 mPa s [26]. Achievement of a desirable vehicle viscosity might be accomplished by mixing different oil vehicles. Various models to describe relationships between oil mixture viscosities (η_{mix}) and intrinsic viscosities of the pure oils ($\eta_{pure\ oil}$) have been reported [27, 28]. The exponential relationship given by (7.1) [29] might be applied to predict mixture viscosities in binary systems:

$$\log \eta_{mix} = v_1 \times \log \eta_{pure1} + v_2 \times \log \eta_{pure2}, \tag{7.1}$$

where v_1 and v_2 refer to volume fractions of the pure oils. Compared to other oils, the drug solubility in castor oil is often enhanced. Syringeability, however, might be considered poor owing to the high viscosity of the latter vehicle. By mixing castor oil with the less viscous sesame oil or medium-chain triglyceride (MCT), log linear relationships were found between mixture viscosity and % (v/v) castor oil in the mixtures (Fig. 7.2). A similar Arrhenius relationship (7.1) was observed by mixing castor oil with isopropyl myristate [30].

Table 7.3 Chemical composition, density, and viscosity of commonly used vegetable oils applied for parenteral drug delivery

Oil	Composition[a]		Density (g/ml)	Viscosity (mPa s)
Castor oil (ricinus oil)	C16	1%	0.957–0.965	283 (37°C)
	C18	1%		
	C18:1	3%		
	C18:2	4%		
	C18:1 (OH)	90%		
	Other fatty acids	1%		
Corn oil	C16	13%	0.917–0.925	37–39
	C18	3%		
	C18:1	31%		
	C18:2	52%		
	C18:3	1%		
	Other fatty acids	1%		
Cottonseed oil	C16	24%	0.918–0.926	70 (20°C)
	C18	3%		
	C18:1	19%		
	C18:2	53%		
	Other fatty acids	2%		
Medium-chain triglyceride (MCT, fractionated coconut oil, Viscoleo®, Miglyol 812®)	C8	~60%	0.942	12 (37°C)
	C10	~40%		23 (25°C)
Olive oil	C16	10%	0.910–0.916	36 (40°C)
	C18	2%		84 (20°C)
	C18:1	78%		
	C18:2	7%		
	Other fatty acids	2.0%		
Peanut oil, groundnut oil, arachis oil	C16	13%	0.914–0.917	35–38 (37°C)
	C18	23%		
	C20	1%		
	C22	3%		
	C24	2%		
	C18:1	37%		
	C18:2	41%		
	Other fatty acids	2%		
Sesame oil	C16	9%	0.915–0.923	33 (37°C)
	C18	6%		56 (25°C)
	C18:1	38%		
	C18:2	45%		
	Other fatty acids	2%		

(continued)

Table 7.3 (continued)

Oil	Composition[a]		Density (g/ml)	Viscosity (mPa s)
Soybean oil	C16	11%	0.919–0.925	50 (25°C)
	C18	4%		69 (20°C)
	C20	1%		
	C18:1	22%		
	C18:2	53%		
	C18:3	8%		
	C20:1	1%		
	Other fatty acids	1%		

[a]Composition of the fatty acids in the triglycerides; C8 = caprylic acid, C10 = capric acid, C16 = palmitic acid, C18 = stearic acid, C20 = arachidic acid, C22 = behenic acid, C24 = lignoceric acid, C18:1 = oleic acid, C18:2 = linoleic acid, C18:3 = linolenic acid, C18:1 (OH) = ricinoleic acid, C20:1 = eicosenoic acid

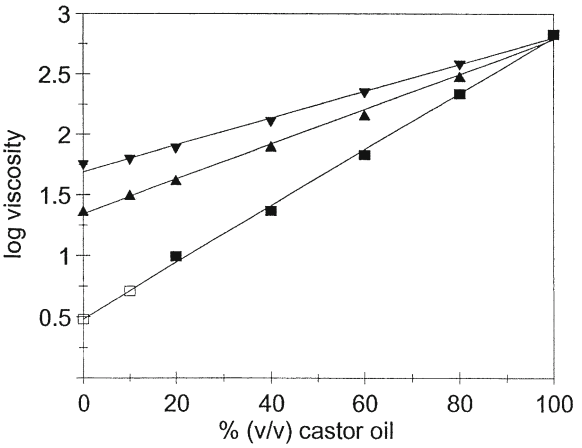

Fig. 7.2 Linear relationships between logarithm of viscosities (mPa s) and % (v/v) castor oil in oil vehicles. *Filled down triangle*, castor oil in sesame oil; *filled triangle*, castor oil in MCT; *filled square*, castor oil in isopropyl myristate and *open square*, theoretically obtained values of castor oil in isopropyl myristate. *Straight lines* are obtained by linear regression (reprinted by permission, from Larsen et al. [30])

While a reasonably low vehicle viscosity favors syringeability, an enhanced vehicle viscosity might under certain conditions result in a decreased drug release rate at the site of injection. This will be the case if drug diffusion in the oil phase to the oil–water interface, instead of drug partitioning into the aqueous phase, becomes the rate-limiting step in the overall release process. The relative contribution of the diffusion step to the overall release process might be augmented by addition of aluminum monostearate to the oil resulting in a gelled and highly viscous mixture [31]. However, comparable in vitro release rates of a model drug from oil vehicles containing different concentrations of aluminum monostearate in MCT have been

Table 7.4 The relative order of oil solubility of various compounds

The order of oil solubility	Drug/prodrug	References
Tributyrin > trioctanoin > ethyl oleate > triolein	Phenytoin prodrugs	Yamaoka et al. [36]
Triacetin > tributyrin > MCT > isopropyl myristate	5-Fluorouracil and 1-alkylcarbonyl prodrugs	Patrick et al. [37]
Tributyrin > MCT > ethyl oleate > soybean oil	Testosterone propionate	Malcolmson et al. [38]
Castor oil > castor oil:MCT (2:1) > MCT	Local anesthetics, NSAIDs and testosterone	Larsen et al. [39]
Castor oil > peanut oil > sesame oil	Steroids	Riffkin and Huber [40]
Castor oil:MCT (1:1) > castor oil:soybean oil (1:1) > MCT > sesame oil and soybean oil:MCT (1:1) > soybean oil	Tetrazepam	Jumaa and Müller [41]
MCT > sesame oil	Flupentixol and probucol	Christensen et al. [42]
MCT > peanut oil > soybean oil and cottonseed oil	Isotretinoin	Nankervis et al. [43]

MCT medium-chain triglyceride

reported [32]. The lack of connection between vehicle viscosity and in vitro drug release was also observed in a study based on pure vegetable oils and mixtures thereof [33]. Likewise, oil viscosity does not seem to significantly influence drug absorbance rate following intramuscular injection [34, 35].

7.2.3 Drug Solubility in Oil

Since the dielectric constant of most vegetable oils is close to zero, the solubility of polar compounds and ions in such vehicles is very low. Solubility is related to the size of attractive intermolecular forces between triglyceride molecules and drug solute molecules. Attraction between such unlike molecules may involve van der Waal's forces (mainly dipole–dipole interactions) and hydrogen bond formation with the triglyceride ester groups acting as hydrogen acceptors. Drug solubility varies with the chemical composition of the individual oils (Table 7.4).

However, variation of solubility in many of the commonly used oil vehicles (excluding castor oil) for each individual drug usually amounts to a factor of 2–3 or less. One explanation for this behavior could be that drug interactions with the triglyceride ester functions influence solubility to a significantly larger extent than drug interactions with the carbon chains. In keeping with this theory, solubility (S) data for phenytoin prodrugs where S(tributyrin) > S(trioctanoin) > S(triolein) for each of the ten investigated derivatives have been reported (Table 7.4) [36]. Calculating the molar concentration of the polar triglyceride functionality (largest for tributyrin) from solvent density and molecular weights of the three solvents, a linear correlation between log S and the

Table 7.5 Melting points (MP, °C) and molar solubilities of various compounds in MCT (S_{MCT}) MCT/castor oil 2:1 (v/v) ($S_{MCT/C}$) and castor oil (S_C) at 37°C (solubilities in mmol/ml)[a]

Compound	MP	S_{MCT}	$S_{MCT/C}$	S_C
Benzocaine	88–90	0.497	0.474	0.784
Bupivacaine	107–108	0.202	0.294	0.286
Flurbiprofen	110–111	0.266	0.282	0.400
Ibuprofen	75–77	0.732	0.848	0.911
Ketoprofen	94	0.111	0.235	0.377
Lidocaine	68–69	1.05	1.13	1.74
Naproxen	155.3	0.0517	0.0912	0.125
Salicylamide	140	0.115	0.159	0.193
Testosterone	155	0.0385	0.0818	0.0784

MCT medium-chain triglyceride
[a]*Source*: Larsen et al. [39]; used by permission

molar concentration of the ester functions was observed for three selected prodrug derivatives. Compared to other vegetable oils, castor oil exhibits enhanced solubilizing effects that can be ascribed to increased hydrogen bonding activities of the hydroxy groups in ricinoleic acids. To this end, it has been shown that addition of aliphatic alcohols to triglycerides resulted in an increase of the oil–buffer distribution coefficients of lidocaine [39]. Also, the addition of hydrogen donating aliphatic fatty acids provided improved haloperidol solubility in the resulting oil mixtures [44]. Other biocompatible cosolvents (such as benzyl benzoate, benzyl alcohol, and ethyl lactate) might be of potential utility in oil mixtures for the provision of feasible drug loads [40].

Linear correlations between melting point and log mole fraction solubility (or log molar solubility) have been reported for (1) phenytoin prodrugs in ethyl acetate and triglycerides [36], (2) various hydrophobic compounds in trioleylglycerol [45], and (3) different small-molecule drugs in castor oil–MCT mixtures (Table 7.5; Fig. 7.3) [39]. Although the linear relationships established in these studies are difficult to explain from a thermodynamic point of view, such correlations might be used to estimate oil solubility of future drug candidates. To this end, it should be mentioned that various strategies of predicting solubility in different lipid formulations have been reviewed by Rane and Anderson [46].

7.2.4 Oil–Buffer Partition Coefficients

The partition coefficient (P) of a drug substance is defined as the ratio of drug activities (at equilibrium) in the two immiscible solvents comprising the partitioning system. In the present context, knowledge of the oil–buffer pH 7.4 distribution coefficient ($D_{oil/7.4}$) is of key interest since the drug release process in vivo seems to involve partitioning of the drug between the oil vehicle and the tissue fluid (see also Sect. 7.2.5). In support of this suggestion, linear relationships between log absorption rate and log oil–water distribution coefficient have been reported [34, 47].

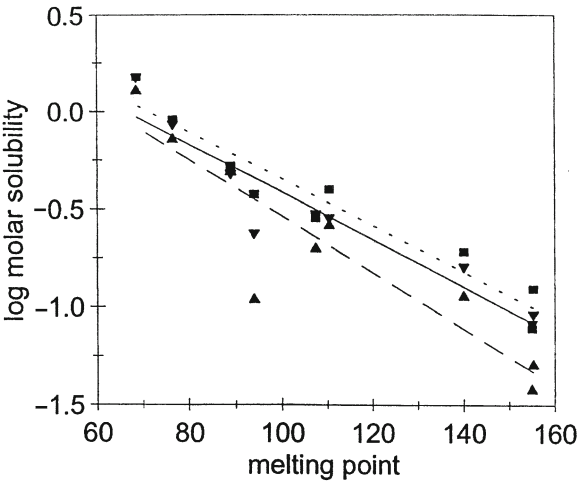

Fig. 7.3 The effect of melting point (°C) on the molar solubility in MCT, MCT/castor oil 2:1 (v/v) and castor oil of different crystalline compounds at 37°C. *Filled triangle*, solubility in MCT; *filled down triangle*, solubility in MCT/castor oil 2:1 (v/v); *filled square*, solubility in castor oil. *Straight lines* are obtained by linear regression (reprinted by permission, from Larsen et al. [39])

In principle, two different approaches might be considered in an attempt to modify the oil–buffer distribution coefficient of a given drug. First, enhanced drug affinity for the oil phase might arise from increasing the hydrogen bonding potential of the nonaqueous vehicle effected by addition of suitable water-immiscible cosolvents containing hydrogen bond acceptor functionalities [48] or hydrogen bond donating groups (Fig. 7.4) [30]. Second, partition coefficients might efficiently be manipulated by transiently masking the drug in the form of a lipophilic prodrug. In fact, most of the marketed parenteral lipophilic solutions contain the dissolved drug in the form of a relatively lipophilic prodrug (Table 7.1). An increase in the oil–buffer distribution coefficient (D) of a drug substance, which can be obtained by using these approaches, will most likely also result in enhanced oil solubility (S_{oil}). Interestingly, the relation between D and S_{oil} has been investigated for three amide anesthetics and a fairly good correlation ($D_{oil/7.4}=0.92\ D_{estim}+5.0$; $n=9$, $r=0.985$) was established between the experimentally determined distribution coefficients ($D_{oil/7.4}$) and those (D_{estim}) calculated from (7.2) (Table 7.6).

$$D_{estim} = \frac{S_{oil}}{S_{7.4}}, \tag{7.2}$$

where S_{oil} and $S_{7.4}$ represent the drug solubility in the oil and buffer phase, respectively [49]. In octanol–water systems, similar correlations have been observed [50]. Thus, the distribution coefficient (determined in dilute solution) might be used to obtain an estimate of the oil solubility of a drug substance and thereby the potential drug load in the formulation.

Correlation of distribution coefficients determined in different partitioning systems (Linear Free Energy Relationships [51, 52]) might be exploited to estimate oil–buffer distribution coefficients of drugs. Since *n*-octanol–water partition coefficients are available for a huge number of chemical entities, this partitioning system

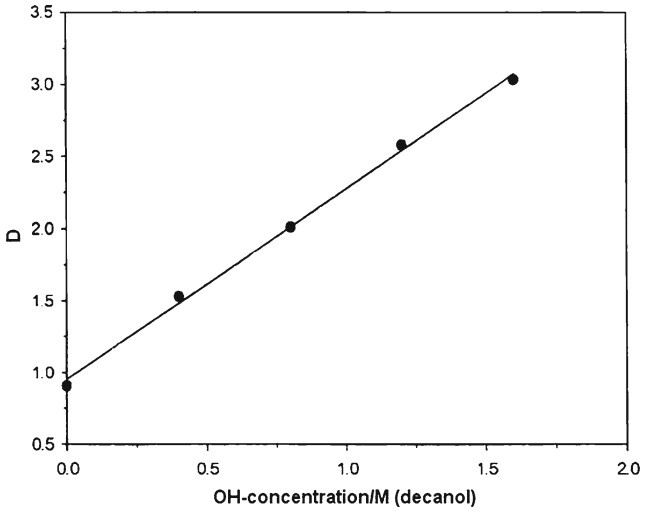

Fig. 7.4 Relationship between oil–phosphate buffer (pH 6.0) distribution coefficients (*D*) for lidocaine and molarity of OH groups (decanol) in sesame oil. The *straight line* is obtained by linear regression (reprinted by permission, from Larsen et al. [30])

Table 7.6 Basic physicochemical properties of some local anesthetics

Drug compound	pK_a	$S_{7.4}$	Oil vehicle	S_{oil}	$D_{oil/7.4}$	D_{estim} [a]
Bupivacaine	8.12	1.08	Sesame oil	140	114	130
			MCT	202	161	187
			Castor oil	331	301	306
Lidocaine	7.9	44.5	Sesame oil	761	16.2	17.1
			MCT	1,050	24.7	23.6
			Castor oil	1,740	48.0	39.1
Ropivacaine	8.16	1.14	Sesame oil	23	38.3	20.2
			MCT	62	58.8	54.4
			Castor oil	136	105	119

The aqueous solubility in 67 mM phosphate buffer pH 7.4 ($S_{7.4}$ in mM), the oil solubility of the free base form (S_{oil} in mM), the experimental ($D_{oil/7.4}$) and the estimated (D_{estim}) oil/phosphate buffer (pH 7.4) distribution coefficient at 37°C
Source: Larsen et al. [49]; used by permission
MCT medium-chain triglyceride
[a] D_{estim} was calculated from (7.2)

is usually used as the reference system in the generation of linear correlations of the type given by (7.3).

$$\log P_{oil} = a \times \log P_{octanol} + b. \tag{7.3}$$

Reasonable linear correlations might be accomplished although the oil vehicles differ significantly from octanol with respect to physicochemical characteristics (Fig. 7.5). Hansch substituent constants (π values) have long been used in the

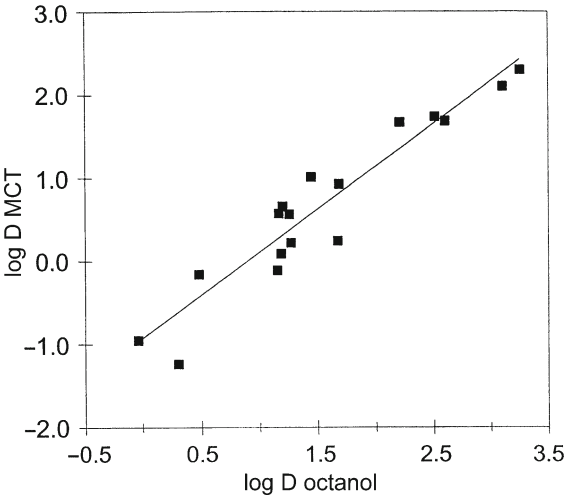

Fig. 7.5 Linear relationship between distribution coefficients (*D*) determined for various compounds in an octanol–buffer system and in a MCT–buffer system. The *straight line* is obtained by linear regression (reprinted by permission, from Larsen et al. [39])

prediction of *n*-octanol–water partition coefficients associated (homologous) series of prodrug derivatives. The substitution constant for a methylene group ($\pi(CH_2)$) amounts to about 0.5–0.6. Interestingly, $\pi(CH_2)$ values of 0.58, 0.57 and 0.54 were derived from oil–buffer partitioning systems of a homologous series of aliphatic ester derivatives of nicotinic acid using MCT, sesame oil and castor oil, respectively (Fig. 7.6) [53].

7.2.5 Utility of In Vitro Release Models

The development of suitable in vitro release models (for quality control as well as formulation development) constitutes a highly critical activity that, preferably, should be initiated in the early depot design phase. Ideally, the outcome of these efforts should be the establishment of an in vitro in vivo correlation (IVIVC). Usually, this requires that drug release from the depot is the rate-limiting step in the absorption and the drug release mechanism is the same in vitro and in vivo. Hence, such point to point relationships are most often linear; however, nonlinear correlations are also acceptable [54]. No regulatory approved standard methods exist for testing drug release from sustained release parenteral products, despite the long-recognized need for such in vitro release methods [55, 56]. In the area of oil-based injectables, release methodologies employed can roughly be divided into three categories (1) models with the lipophilic solution floating on top of the release medium, (2) dialysis techniques, and (3) continuous flow methods [10]. In particular, the rotating dialysis cell model has been used for the study of drug release from lipophilic solutions. This includes establishment of a linear in vitro–in vivo relationship of drug release following subcutaneous injection of an oil solution of bupivacaine in the rat (analgesic effect lasting for about 24 h) [57]. Apparently, strict IVIVCs have

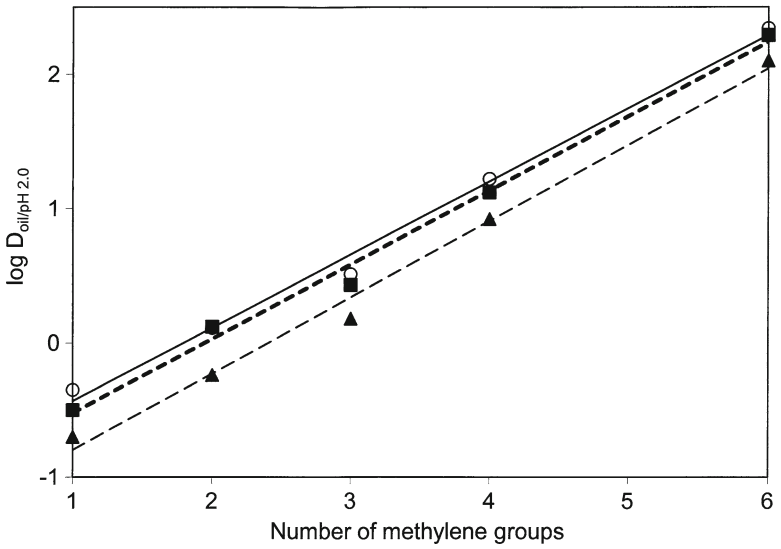

Fig. 7.6 The relationships between log $D_{oil/pH2.0}$ for nicotinic acid esters and the number of methylene groups in the alkyl chain. *Filled triangle*, sesame oil; *filled square*, MCT; *open circle*, castor oil. *Straight lines* are obtained by linear regression (data from Larsen et al. [53]: used by permission)

not been achieved most likely reflecting that current in vitro release models are unable to mimic, in an adequate manner, the rate, equilibrium, and transport processes of the drug substance as well the depot formulation, per se, in the environment of the administration site.

The rotating dialysis cell has been used to study drug release from oil vehicles under two different conditions (1) the depot was added to an empty donor compartment, and (2) the oil depot was added to the donor cell containing an aqueous buffer solution. In the former experimental set up, the existence of a linear relationship between the logarithmic forms of the partitioning pseudo-first-order rate constant and the oil–buffer distribution coefficient of the drug compound was clearly demonstrated (Fig. 7.7) [39]. With aqueous buffer present in the donor cell, the rate constant could be expressed quantitatively by several parameters, including the oil–buffer distribution coefficient and the drug permeability coefficient (transport across the dialysis membrane) [58]. The role of the distribution coefficient to control drug release from oil vehicles opens up the possibility of designing depots with tailored delivery characteristics. In the field of pain control, administration of oil solutions comprising two analgesic agents differing with respect to lipophilicity might be of potential utility to ensure rapid onset of action as well as a desired prolonged duration of action (Fig. 7.8) [59]. It has been suggested that optimal pain relief after minor joint surgery requires analgetic and anti-inflammatory action locally at the site of trauma over about 1 and 7 days, respectively [60]. Such multimodal analgesia might result from intra-articular injection of oil solutions capable

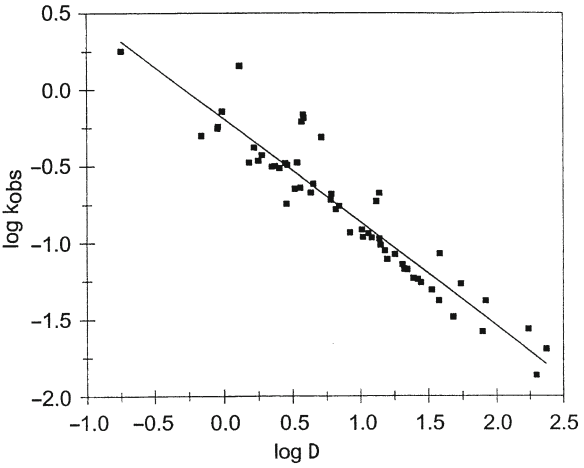

Fig. 7.7 Linear relationship between the logarithmic forms of k_{obs} (pseudo-first-order rate constant related to the attainment of equilibrium between the oil and the aqueous buffer phases) obtained by employing the rotating dialysis cell and *D* (oil–buffer distribution coefficient) for various compounds. The *straight line* is obtained by linear regression (reprinted by permission, from Larsen et al. [39])

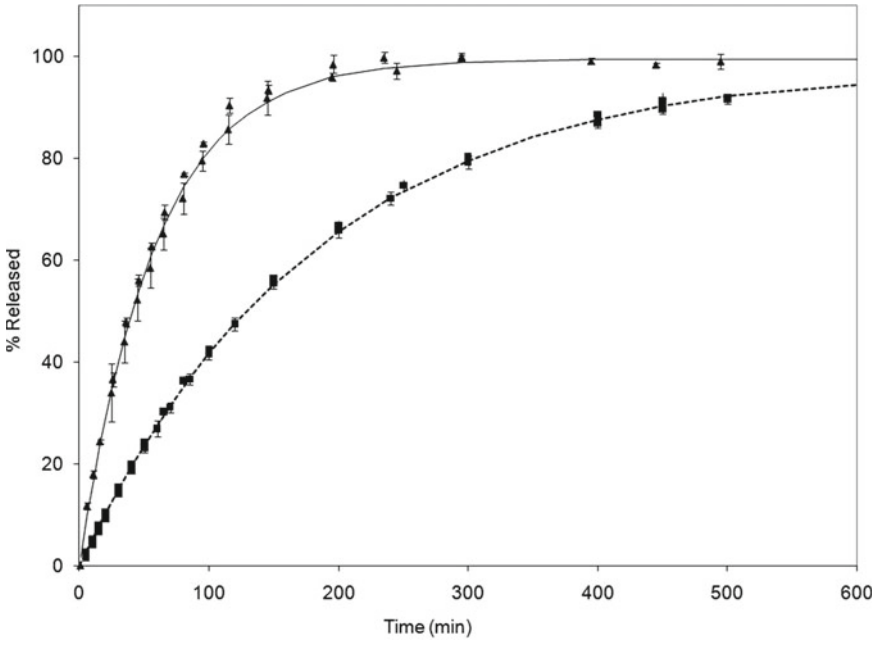

Fig. 7.8 Release profiles of prilocaine and bupivacaine at 37°C in phosphate buffer pH 6.0 employing the rotating dialysis cell model. 1.0 ml MCT containing 6.2 µmol bupivacaine (*filled square*) and 1.3 µmol prilocaine (*filled triangle*) was added to 5.0 ml buffer in the donor compartment. The full lines have been drawn according to calculated apparent first-order rate constants (see Larsen et al. [59] for further details) (reprinted by permission, from Larsen et al. [59])

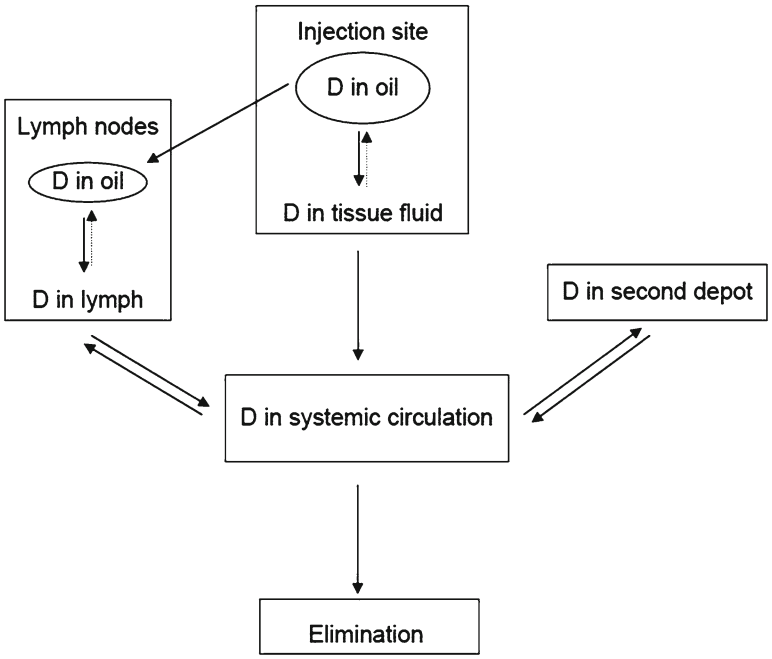

Fig. 7.9 Overview of processes influencing the pharmacokinetic fate of drug substances after injection of lipophilic drug solutions. The drug substance (D) may represent the parent drug compound or a prodrug derivative (reprinted by permission, from Larsen and Larsen [10])

of concomitant release of an analgesic and an anti-inflammatory agent, with the coadministered drugs (or prodrugs) being released at different rates.

Various processes might affect the pharmacokinetic fate of drugs injected intramuscularly in the form of an oil solution (Fig. 7.9).

An in-depth understanding of the importance of such processes taking place in the immediate surroundings of the administration site is a prerequisite for the development of discriminatory in vitro release models. On the other hand, there seem to be in vivo conditions that cannot easily be simulated in vitro. Some of these events, which influence the in vivo fate of the drug, impart variability to the pharmacological response. This might be the case when a so-called "second depot" (e.g., redistribution of the drug compound into fatty tissue) is controlling the drug appearance in the blood [61]. Unpredictable spreading at the injection site may lead to different interfacial areas between the oil and the tissue fluid and therefore result in variability in the overall apparent release process [62, 63]. A third in vivo phenomenon, which cannot be simulated in vitro, is the emergence of a tissue response to the depot instillation. The tissue reaction (intensity as well as duration of the inflammatory response) is dependent on the size, shape, and chemical and physical properties of the injected biomaterial [64]. For long acting depot formulations, the disappearance of the oil vehicle containing the drug from the injection site (and potentially other drug release

processes) contributes to the overall rate of drug release from the local administration site [62, 63]. The latter in vivo conditions are difficult to simulate by use of an in vitro drug release model and may become prohibitive for the establishment of an IVIVC.

7.3 Oil Suspensions

A significant challenge in the development of parenteral lipophilic solutions might be the achievement of sufficient oil solubility of novel drug candidates. Design of lipophilic prodrug derivatives, as mentioned in the previous section, constitutes a means to enhance the oil solubility. Alternatively, the solid drug particles could be dispersed in the oil vehicle (oil suspension) and consequently high drug load can be achieved. Despite the apparent simplicity of suspension-based injectables, parenteral oil suspensions, like aqueous suspension (see Chap. 8), pose certain challenges to the manufacturing process and physical stability [65]. A priori, oil suspensions might be considered particularly attractive for water-soluble drug compounds and therapeutic agents suffering from poor aqueous stability [49, 66]. A few reports have dealt with injectable oil suspensions providing sustained drug release. A bovine growth hormone releasing factor analog dispersed in MCT was able to effectively elevate serum somatotropin levels for at least 14 days following subcutaneous injection in Holstein steers [67]. The latter article also contained a brief overview over patents related to the dispersion of proteins, peptides, and small-molecule drugs in oils to yield depot formulations. Seemingly, the provision of delayed drug release is not a universal property of oil suspensions. A thixotropic suspension of cytosine arabinoside in arachis oil containing aluminum stearate was unable to delay the release of the anticancer agent following subcutaneous injection in both rabbits and patients [68]. Compared to an aqueous artimisinin suspension, an oil suspension of the antimalarial drug afforded much faster drug appearance in the blood after intramuscular injection in man. Furthermore, the bioavailability of artimisinin from the latter formulation far exceeded that observed for the aqueous suspension [69]. Several other studies have been devoted to the investigation of the in vivo performance of oil suspension-based injectables in animal models and man [70–80]. These studies, however, have not lead to the elucidation of the mechanism(s) of drug release from such particulate systems. Sustained release properties of oil suspensions might be explained by a mechanism where solid drug particles have to dissolve in the surrounding oil phase prior to release into the aqueous tissue fluid by partitioning [81]. Alternatively, comparable release rates from aqueous and oil suspensions are to be expected in case the disperse phase, due to gravity, is transported to the oil–water interface or directly into the aqueous phase [82, 83]. In keeping with the latter findings, it has been reported that the oil film surrounding solid drug particles (ropivacaine and bupivacaine) is instable in an aqueous environment [49].

7.4 Summary

Parenteral long acting oily (lipophilic) solutions have been in clinical use for many years in the field of schizophrenia and hormone replacement therapy. In addition to uncomplicated manufacture and favorable long-term stability, these simple oil-based solutions open up the possibility of designing depots with tailored delivery characteristics. For the lipophilic drug solutions, three basic physicochemical parameters: (1) vehicle viscosity; (2) drug solubility in oil vehicle, and (3) drug partitioning between oil and aqueous buffer, affect syringeability as well as degree of drug load and rate of drug release from the vehicle. Modification of the oil vehicle with respect to viscosity, solubilization capacity, and oil–buffer partition coefficient can be obtained by using mixtures of triglycerides/synthetic oils or by addition of excipients (e.g., hydrogen bond donating compounds). Another approach for optimization of the oil–buffer partitioning and/or the oil solubility of drug substances involves the use of prodrug derivatives. In order to enhance the drug load in the oil formulation even further, solid drug particles could be dispersed in the oily vehicle. In the design of lipophilic drug formulations and for quality control purposes, development of in vitro release models based on in vivo drug release mechanism constitutes an important activity. The rotating dialysis cell model has particularly been applied to study drug release from oil solutions. In this model, the oil–buffer distribution coefficient of the drug substance is one of the major parameters controlling the release rate from oil solutions. Thus, the drug partitioning process from oil vehicle into tissue fluid might be simulated in an in vitro model; however, other factors, such as spreading of the formulation at the injection site, drug uptake into second depot, in vivo fate of the oil vehicle, and host responses are not easy to predict in vitro.

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Chapter 8
Aqueous Suspensions

Susan M. Machkovech and Todd P. Foster

Abstract A good formulation of coarse particles dispersed in a liquid vehicle is facilitated by an understanding of the theory of particle technology and methodologies to assess physical properties of the resulting product. Formulation parameters include particle–vehicle interactions, sedimentation/flocculation kinetics, particle size and crystal form identity and stability, and appropriate suspension rheology to aid flocculation and minimize caking. The vehicle and stabilizers added must allow for creation of a flocculated system, prevent caking, and promote rapid redispersion. Additional delivery considerations include irritation and pain potential, in vitro vs. in vivo release methodologies, and the targeted delivery profile.

Symbols

ΔA	The change in surface area
d	Mean particle diameter
F	Sedimentation volume
F_∞	Sedimentation volume of a totally deflocculated suspension
g	Acceleration constant associated with the force of gravity
ΔG	Change in surface free energy
V	The velocity of the fall of an average particle in the suspension
V_o	Original volume of the suspension before settling
V_u	Ultimate volume of sediment
V_∞	Ultimate volume of sediment of a completely deflocculated, or caked, suspension
β	A measure of the degree of flocculation of a system
γ_{sl}	The interfacial tension between the liquid medium and the particles

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ρ Density of the particles
ρ_o Density of the medium
η Newtonian viscosity of the system
η_o Viscosity of the dispersion medium
φ Relative volume concentration of the dispersed particles (volume of the particles divided by the total volume of the dispersion)

8.1 Introduction

Sustained release formulations consisting of sterile aqueous suspensions are generally intended for injection by the subcutaneous, intramuscular, or intra-articular route. The formulations can be relatively fast releasing or sustained release, compared with the aqueous solution delivery. Currently marketed sterile aqueous suspensions are identified in Table 8.1. An understanding of the physicochemical properties of the active and its formulation coupled with knowledge of the rate-limiting step in the presentation of the dissolved active to the systemic circulation is

Table 8.1 Marketed sterile aqueous suspensions

	Formulation concentration	Depot dose	Duration or dosing interval
Dexamethasone acetate (Decadron-LA)	8 and 16 mg/mL	8 and 16 mg	1–3 weeks
Medroxyprogesterone acetate (Depo-Provera®)	150 mg/mL	150 mg	13 weeks
Methylprednisolone acetate (Depo-Medrol®)	20, 40, 80 mg/mL	40–120 mg	1 week
Triamcinolone acetonide (Tac®)	3, 10, 40 mg/mL	40–100 mg	6 weeks
Triamcinolone diacetate (Aristocort Forte®)	25, 40 mg/mL	40–80 mg	1 week
Insulin with zinc chloride	40, 80 U/mL	Variable	Hours
Penicillin G procaine injectable suspension (Bicillin)	150,000–600,000 U/mL	Variable	10 days
Cortisone acetate (Cortone)	50 mg/mL	Variable	Daily
Aurothioglucose (Solganal)	50 mg/mL	50 mg	1 week
Betamethasone phosphate and acetate (Celestone Soluspan)	6 mg/mL	Variable	3–14 days
Program 6-month injectable for cats (Lufenuron)	100 mg/mL	0.4–0.8 mL	6 months
Isoflupredone acetate (Predef 2X) – vet	2 mg/mL	5–20 mg	12–24 h
Betasone dipropionate and sodium phosphate	7 mg/mL	Dog: 2–4 mg/20# Horse: 15–35 mg/joint	3 weeks

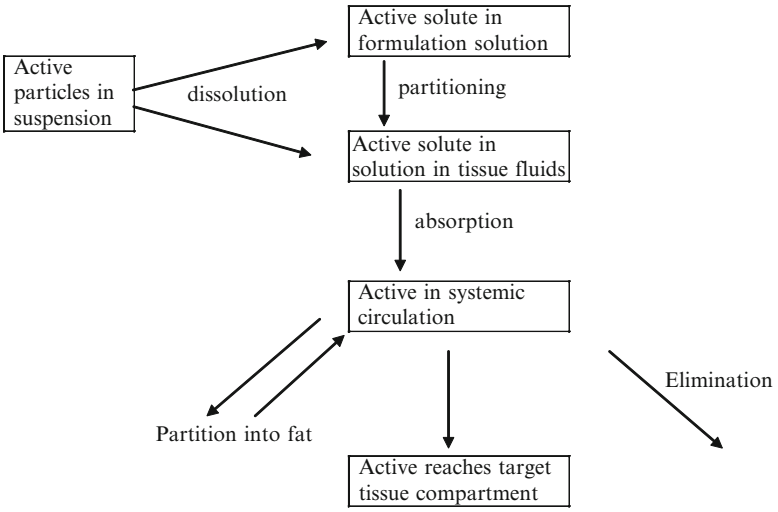

Fig. 8.1 Pathway for drug absorption from an aqueous suspension

required to optimize the formulation and the delivery of the active. The formulation parameters available for manipulation include:

1. Particle size/surface area of the active ingredient
2. Crystal habit of the active ingredient
3. pH of the formulation
4. pK_a of the active ingredient
5. Vehicle composition
   (a) Attenuation of partition coefficient (vehicle/tissue fluid) of the active ingredient
   (b) Affects rate of dissolution of active ingredient in vehicle/tissue fluid milieu
   (c) Diffusivity of the active ingredient
   (d) Movement of the active particles to tissue fluid
   (e) Excipients that interact with the active ingredient
6. Prodrugs, salts, complexes to alter lipophilicity of the active ingredient

The rate of delivery of the active to the systematic circulation, through the general pathway shown in Fig. 8.1, is controlled by the slowest or rate-limiting step.

The particles in a suspension have diameters for the most part greater than 0.1 μm. The individual particles generally range in size from about 0.5 to 5 μm. Aggregates or flocculation of particles may attain effective sizes of 50 μm or larger. The formulation, preparation, and stabilization of pharmaceutical suspensions are challenging tasks, requiring considerable skill and knowledge. Some considerations in the design and the preparation of a suitable injectable suspension for sustained release are:

1. Introduction of the powdered material into the vehicle.
2. Utilization of parenterally acceptable excipients.
3. Prevention of caking and promotion of rapid redispersion.

Table 8.2 Characterization techniques for assessment of physical stability of aqueous suspensions

Technique	Parameter evaluation	Example references
Zeta potential	Surface charge and stability	Kayes [1]
Centrifugation	Simulation for settling and caking	Kuentz and Roethlisberger [2]
Settling rate	Visual or turbidity scans	
Rheometry	Viscosity	Briceno [3], Tripathi et al. [4]
Freeze/thaw and temperature cycling	Accelerate particle growth and caking	Graham and Pomeroy [5]
Shaking	Simulate shipping conditions	Deicke and Suverkrup [6]
	Accelerate aggregation and flocculation	
Laser diffraction light-scattering, lasentec, microscopy	Particle size assessment with time	Han et al. [7], Wong et al. [8]
Syringeability	Impact of high shear and narrow needle bore considerations	

4. Prevention of particle growth by the use of appropriate stabilizers.
5. Consideration of injection site dynamics.
6. Achieving product sterility through terminal sterilization or aseptic manufacturing.

An understanding of the fundamentals describing particle–vehicle and particle–particle interactions, sedimentation, and rheology provides the basis for designing well-constructed, stable suspensions. Table 8.2 shows a listing of characterization techniques used to evaluate the physical stability of suspensions.

This chapter reviews the information that has been learned based on theory and practice. The review starts with the parameters of interest to produce, or at least influence, a physically stable aqueous suspension followed by a focus on those features of the formulation that affect the delivery in vivo. A successful product often times requires compromises between physical stability attributes and drug delivery attributes.

8.2 Suspension Attributes

The general objectives in assessing physical properties of suspensions are to (a) observe changes with time that could be indicative of unacceptable formulations; (b) attempt to predict long-term stability and understand mechanism(s) of instability; and (c) set specifications and establish expiration dating. A common feature of suspensions is an intrinsic thermodynamic instability giving rise to a natural tendency for flocculation (reversible) and sedimentation (reversible or irreversible). Particles are subject to certain kinetic changes due to external forces (a) sedimentation or creaming in a gravitational field and (b) electro-kinetic effects for charged

particles in an electric field. Critical physical results of any dispersion instability are particle growth, nonuniformity of dosing, and significant changes in rheological properties. The goals in formulating suspensions are to optimize physical stability and functionality through the introduction of appropriate particle size and charge. This includes developing strategies to prevent change in particle size and sedimentation, and the monitoring and control of rheological properties. Therefore, the major physical parameters to address include particle size distribution, particle surface charge, solubility of the dispersed phase, dispersion rheological properties, and drug release.

8.2.1 Particle–Vehicle Interactions

Particles with diameters less than 5 μm show Brownian movement and remain distributed uniformly throughout the suspension, provided that the collisions do not result in agglomeration. The small particles that settle may pack into a dense sediment, or cake, and thereby become difficult to redisperse. On the other hand, because of their size, particles that tend to flocculate or group together in the form of light, fluffy clumps, settle more rapidly than do the individual particles. These loosely packed, flocculated sediments do not cake and can be redispersed easily. Thus it is the goal to create suspensions with flocculated particles which will settle to form lose cakes instead of non-flocculated or agglomerations which tend to settle and are not easy to redisperse.

When a solid is reduced by comminution to small particles, the surface area increases and the associated free energy of the surface becomes correspondingly greater. The highly energetic particles tend to regroup in such a way as to reduce the total area and the surface free energy. The tendency to agglomeration into larger masses in order to attain a minimum free energy is the systems response to the thermodynamic instability. The increase in surface free energy, ΔG, which results from dividing a substance into fine particles and dispersing it in a liquid medium, can be described by a simplified equation (disregarding the electrical potential at the surface of the particles):

$$\Delta G = \gamma_{s1} * \Delta A, \tag{8.1}$$

where γ_{sl} is the interfacial tension between the liquid medium and the particles, and ΔA is the increase in surface area resulting from the decrease in particle size. The suspension approaches a thermodynamically stable condition as ΔG approaches zero. According to this equation, this requires either the lowering of the interfacial tension or the reduction of the interfacial area. The interfacial tension can be reduced by a surface-active agent, as shown by [9], but cannot ordinarily be made equal to zero. It is a challenge for the formulation scientist to correctly balance particle size, particle charge, vehicle, and other added excipients to yield a system that flocs but does not agglomerate.

8.2.2 Sedimentation Rate

Stoke's law describes the initial velocity of particle settling in a suspension:

$$V = \frac{d^2 (\rho - \rho_o) g}{18 \eta_o}, \tag{8.2}$$

where V is the velocity of the fall of an average particle in the suspension, d the mean particle diameter, ρ the density of the particles, ρ_o the density of the medium, g the acceleration constant associated with the force of gravity, and η_o the suspension medium viscosity. Stokes equation, based on ideal uniform, noninteracting spheres, is an approximation of a pharmaceutical suspension with its nonuniform sized and shaped particles that interact with one another and with the suspending medium. Hence, the equation merely provides an indication of the factors that contribute to settling and that can be adjusted to improve the formulation.

Parameters of influence are the difference between the density of the suspended particles and the medium, viscosity of the Newtonian medium, and the particle size. Adjustment of the density of the medium to approach that of the particles is generally impractical. A Newtonian medium with high viscosity is not practical due to dosing constraints (i.e., too difficult to inject). Rather, a vehicle with sufficient structure to support the particle network but does not prevent dosing via syringe and needle is preferred. Reducing the particle diameter will slow down the sedimentation rate (squared term in the numerator of Stokes's law), but will not necessarily give a better product. Very small particles, with high surface energy, tend to form a compact cake which is difficult to resuspend. In fact, particles that take months to settle may appear to provide initially an acceptable product. However, if you require a 2-year shelf-life or a product able to handle the detrimental vibrations of truck or ship transportation, merely reducing particle size will likely be inadequate. A better solution is to promote flocculation of the particles and allow the large flocs to be built up into a scaffold-like network throughout the suspension.

8.2.3 Sedimentation Parameters

The volume of a sediment may be used as a measure of flocculation in a suspension. The sedimentation volume, F, is defined as the ultimate volume of the sediment (V_u) divided by the original volume (V_o) of the suspension before settling:

$$F = \frac{V_u}{V_o}. \tag{8.3}$$

The sedimentation volume (F) of a product may have a value of less than 1, equal to 1, or, uncommonly, greater than 1. An F value of less than 1 occurs when the sediment settles to some ultimate volume that is less than the original volume of the suspension. An ideal, pharmaceutically elegant product will have an $F = 1$, where the sediment is equal to the total volume of the suspension because it shows no sediment or clear supernatant upon standing. A completely deflocculated, or caked, suspension will have a relatively small ultimate volume of sediment, designated as V_∞. The sedimentation volume of a totally deflocculated suspension can be written as:

$$F_\infty = \frac{V_\infty}{V_o}. \tag{8.4}$$

The ratio of F to F_∞ is given the symbol β:

$$\beta = \frac{F}{F_\infty}. \tag{8.5}$$

Thus, substituting for F_∞ and F,

$$\beta = \frac{V_u / V_o}{V_\infty / V_o}, \tag{8.6}$$

where (V_u/V_o) is the ultimate sediment volume of the flocculated suspension, (V_∞/V_o) the ultimate sediment volume of the deflocculated suspension, and β a measure of the degree of flocculation of a system. A suspension consisting of floccules held loosely in an open scaffold-like arrangement will be characterized by a large β value. Conversely, a suspension containing highly condensed sediment has a small β value.

8.2.4 Particle Size and Crystal Form

The crystal form of the particle must be controlled over the product's shelf life. This attribute is important to monitor for aqueous suspensions as crystal form change is more likely than if using purely nonaqueous suspensions (e.g., oil). The crystals in a suspension may change their size, while not changing their habit (external shape of a crystal), because of variations or cycling of the storage temperature. In dilute suspensions, small particles will be preferentially dissolved with subsequent crystal growth on exposed surfaces of the larger particles (Ostwald ripening). Another possibility, noted by de Villiers et al. [10] for nicolsamide, is a change in the polymorphic or solvate form of the drug particle which will likely alter the rate of dissolution

and the solubility of the active and, hence, the availability of the active or alter the physical stability of the product. For example, Macek [11] provides evidence of changes in the crystal form of cortisone acetate when the unstable form was dispersed in an aqueous suspending media with subsequent form change involving changes in particle size and unacceptable caking. There are literature examples citing the influence of differences in particle size of the suspended particles on the onset and duration of the biological response. Buckwalter and Dickison [12] report that large particles in the IM procaine penicillin G suspensions extend the effectiveness because of slower dissolving action. Butterstein and Castracane [13] provide data that danazol suspensions (160 mg/mL in vehicle of 10% alcohol in peanut oil) had equal onset of activity, but the suspension of particles of 2 μm was only effective for 25 days, whereas the 5-μm suspension was effective for greater than 35 days.

8.2.5 Rheology of Suspensions

A consideration of the rheology of suspensions is important in the design of products to prevent settling of the dispersed particles, to promote resuspension of the material when the container is shaken, and to provide proper flow properties for ease of injection and resulting injection site conformation. When a suspension remains at rest on the shelf, only a negligible shear is produced in the product as the particles slowly settle (gravitational forces). When the product is passed through a colloidal mill or forced through a syringe, a high rate of shear is produced. Rheology can be quite helpful in designing the formulation and in determining whether a flocculated system exists or not. Generally a Newtonian system will be nonflocculated and excessive particle settling causing compaction will occur which is not desirable. A non-Newtonian system where the greater shear or energy input into the system (e.g., by shaking) creates a less viscous suspension is the desired product.

For Newtonian flow, the Einstein equation relates the viscosity of a very dilute colloidal suspension of spherical particles to the volume occupied by the dispersed phase

$$\eta = \eta_o \left(1 + 2.5\varphi\right), \tag{8.7}$$

where η is the Newtonian viscosity of the system, η_o the viscosity of the dispersion medium, and φ the relative volume concentration of the dispersed particles (volume of the particles divided by the total volume of the dispersion).

In systems containing lypohilic molecules and colloidal and microparticulates, the swollen, solvated, irregularly shaped masses make a greater contribution to the consistency of the system than is accounted for by their concentration alone. Suspensions containing even moderate concentrations of dispersed solids, uncomplicated by the presence of viscous suspending agents, ordinarily show a yield value and plastic flow behavior, due in part, to the flocculated nature of such suspensions.

If the particles interact to yield a three-dimensional scaffold-like structure, thixotropy becomes evident. When a viscous suspending agent is added to the product, the plastic viscosity of the simple particle–vehicle system can be modified considerably by the pseudoplastic character of the suspending agent.

In the selection of a rheological instrument for the analysis of a pharmaceutical product, it is desirable to choose a viscometer which operates within a shear range, roughly comparable to that produced by the treatment that the product will undergo in practice. Besides the brochures of the various instrument makers, a number of rheological instruments and their ranges of speed have been reviewed and are not covered here [14–17]. Furthermore, the components of a product should be chosen on the basis of the rheological properties that they exhibit under various conditions of flow. Ideally, a suspending agent should have a high viscosity at negligible shear when the suspension remains at rest during storage, and it should exhibit low viscosity at high shear rates when it is being withdrawn from the container and when it is being injected.

8.2.6 Vehicle Composition

Considerations in the design and preparation of successful pharmaceutical suspensions include:

1. Introduction of the active into the vehicle
2. Creating a flocculated system to prevent caking/agglomeration and promotion of rapid redispersion
3. Prevention of excessive sedimentation by the use of the appropriate vehicle, particle size, and appropriate stabilizers

8.2.6.1 Wetting of Particles by Vehicle

Lyophilic powders (e.g., magnesium carbonate in water where there is good attraction between the solid dispersion and vehicle) are easily wetted by the vehicle and have no difficulty in the initial dispersion stage. Lyophobic powders (e.g., magnesium stearate in water) tend to clump and float on the surface of the waters due to layers of air adhering to the particle surface. Passing a lyophobic powder through a colloid mill in the presence of a wetting agent improves the displacement of air, dispersing the particles, and allowing penetration of the vehicle into the powder mass.

8.2.6.2 Promotion of Flocculation

Brownian motion is the dominant force for bringing together particles of less than 5 μm. Larger particles, which include large flocs, require more energy. Therefore,

initial flocculation in suspensions is relatively rapid, but the equilibrium state takes a longer time to achieve. Indeed, mechanical agitation and even thermal convection can disrupt flocs, either reversibly or irreversibly. Flocculating agents added to formulations to stabilize suspensions include electrolytes, detergents, and polymers. The ions of electrolytes probably reduce the electrical barrier between the particles as well as form a bridge between the particles to produce a loosely arranged structure. Both ionic and nonionic surfactants (e.g., polyoxyethylated compounds) have been used to bring about flocculation of suspended particles [18].

8.3 Irritation and Pain

Formulation techniques can be used to overcome irritation and pain at the injection. Crystal shape will play an important role. Speiser [19] describes crystals with sharp edges or long, needle shapes causing pain and discomfort. Discomfort due to osmotic effects can be minimized by injection of a larger volume of a more dilute formulation. Cosolvents may induce irritation. Apelian et al. [20] provide irritation examples for the use of greater than 30% *N*-methyl-2-pyrrolidone or 2-pyrrolidone. More importantly, sometimes it is the active in solution that is causing unacceptable irritation. Oftentimes, complexation can ameliorate the irritation. To decrease irritation caused by the active, Patterson and Holmes [21] obtained a patent for oxytetracycline complex formulation and Mosher and Thompson [22] obtained a patent for cyclodextrin as a complexing agent.

8.4 Water-Miscible Cosolvents

Although water is the ideal carrier, physical or chemical instability may require changing to a nonaqueous system. Oil-based formulations are discussed elsewhere (Chap. 7). Water-miscible cosolvents should be nontoxic, nonirritating, and nonsensitizing. Because no cosolvent has been identified as being as good as water in these properties, compromises are required. Spiegel and Noseworthy [23] reviewed pharmaceutically acceptable cosolvents for injection including glycerol formal, glycofurol, dimethylacetamide, *N*-(β-hydroxyethyl)-lactamide, polyethylene glycols, glycerin, ethanol, propylene glycol, and 1,3-buylene glycol. The use of a cosolvent has been found to alter solubilization [24] and/or chemical stabilization or produce complexes (e.g., phenobarbital with PEGs as reported by Higuchi and Lach [25]).

The preparation of a concentrated solution of the active, for example, using suitable water-miscible solvent(s) which are diluted or absorbed from the injection site causing precipitation of the active in vivo was the formulation approach used for the antibiotics oxytetracycline [26] and florfenicol. This formulation method allows for a fairly large driving force from the dissolved active at early times, followed by the lower driving force and concomitant sustained delivery of the precipitated active.

8.5 Polymeric Micelles

Polymeric micelles are smaller than liposomes, but have similar drug carrier functionality including the ability to avoid interaction with cells of the reticuloendothelial system [27, 28]. This added feature allows sustained release and tissue targeting beyond that delineated in Fig. 8.1. The polymeric micelles function as dispersants, emulsifiers, and wetting agents, generally with high solubilization capacity and low critical micelle concentration which makes them useful upon strong dilution. The active moiety can be covalently linked to one of the blocks of the copolymer (examples include cyclophosphamide reported by Bader et al. [29] or doxorubicin) or solubilized without covalent linking in the hydrophobic core of the micelle; for example, as reported by La et al. [30] for indomethacin. Partitioning and biodegradation as affected by pH, ionic strength, and hydration/hydrolysis can be attenuated by the location of the active in the micelle.

8.6 In Vitro Release

This is an area of considerable interest and opinions. Several meetings have addressed in vitro and in vivo options and considerations for parenteral sustained release products as summarized by Burgess et al. [31, 32] and Martinez et al. [33]. Caution has been exercised in the use of accelerated testing in vitro because it may cause a change in the rate-determining mechanism of release and therefore not provide an appropriate model for in vivo results. There is general consensus that an in vitro method may be unique to each formulation, requiring wide latitude for both formulators and regulators. In general, the ability to predict the impact of formulation or processing changes on the delivery of the active through in vitro testing allows faster and more thorough investigations as well as a quality assessment tool, once a clear correlation exists between in vivo changes and measured in vitro changes. Ostergaard et al. [34] utilized a rotating dialysis cell model to monitor release characteristics from aqueous solutions and suspensions of bupivacaine salts intended for intra-articular presentation. Although applicable for simulating release from a small local compartment such as the joint cavity and where the rate of drug release is governed by drug dissolution, this membrane diffusion model has limited application to other sites where perfect biological sink conditions prevail.

8.7 Prodrugs

Sometimes the duration of an active agent in vivo can be extended by modifying the active agent via a prodrug approach. The prodrug can be designed to have a different aqueous solubility, partition coefficient, and/or dissolution rate which in turn will

Table 8.3 Delivery of the antibiotic, ceftiofur, to cattle via two salts, one formulated in aqueous solution and the other as a suspension in oil (mean of $n=6$; data from [36])

	Aqueous solution: ceftiofur Na		Oil suspension: ceftiofur HCl	
	IM	SC	IM	SC
Maximum plasma concentration (μg/mL)	14.5 ± 3.3	13.8 ± 5.3	11.0 ± 1.69	8.56 ± 1.89
Time of maximum plasma concentration (h)	0.67	1–1.5	1–4	1–5
Area under the time-course curve (μg h/mL)	108.4 ± 41.9	95.8 ± 27.0	160 ± 30.7	95.4 ± 17.8
Elimination half life (h)	10.3 ± 1.7	9.7 ± 2.0	12.0 ± 2.63	11.5 ± 2.57

alter the release rate of the active moiety, the rate of absorption from the injection site, and/or alter the tissue distribution. In addition to these changes in the physical and chemical properties of the drug, a potential mechanism for a controlled or sustained release via prodrugs includes controlling the conversion from prodrug to drug at the site of absorption, in plasma, or at the target site of action. One variant is to produce lipophilic prodrugs, with good oil solubility, to allow a high dose of the prodrug to be administered IM or SC in a relatively small volume of oil. Several such products are able to provide sustained plasma levels of the active entity for over a month [35].

Another tact is to make relatively aqueous insoluble salt forms for use in aqueous suspensions. Perusal of Table 8.1's listing of commercially available aqueous suspension formulations of poorly soluble active drug moieties highlights the fact that steroid molecules were most successfully esterified to form acetate prodrugs. Their resulting low aqueous solubility provides prolonged plasma levels.

One caution must be stated. When looking at different salts of an active ingredient, the availability of an active agent as salt #1 from a solution may not be significantly different than that from salt #2 as a suspension. For example, the pharmacokinetic data for both the SC and IM administration illustrate that the availability of the sodium salt of ceftiofur from an aqueous solution is similar to that of the HCl salt from an oil suspension in cattle (Table 8.3, with the exception of the higher blood plasma maximum concentration for the solution [36]). Similar findings of therapeutic equivalence were noted in swine [37]. This further illustrates the complex interplay of factors at the site of injection including the solubility and the rate of dissolution.

8.8 Delivery Site

The intra-articular injection of corticosteroids in suspensions (methylprednisolone and triamcinolone) appeared to maximize the anti-inflammatory effects [38] with duration of benefit reported for several months relative to the soluble hydrocortisone succinate preparation [39]. In addition, the intra-articular route of delivery

circumvented the need for systemic corticosteroid delivery and its associated side effects. The pharmacokinetics of an aqueous-based suspension of rimexolone provided by Derendorf et al. [40] describes a slow release from the knee joint with detectable levels in the plasma over 3 months. Nieuwenhuyse and Lewis [41] have shown that the rate-limiting step for steroid disposition is the dissolution rate in the synovial fluid.

8.9 Conclusions

Sustained release aqueous suspensions are useful dosage forms to provide long-term drug action in humans and animals. The active ingredient must be stable in an aqueous medium and sufficiently insoluble to provide slow dissolution at the injection site. During formulation design and development particular attention to physical stability is important.

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Chapter 9
In Situ Forming Systems (Depots)

Jeremy C. Wright, Michael Sekar, William van Osdol, Huey Ching Su, and Andrew R. Miksztal

Abstract In situ forming systems transform into semi-solids (or viscous masses) upon injection and provide sustained release of pharmacological agents, including small molecule drugs, peptides and proteins. These formulations can be utilized for systemic or site specific delivery and generally comprise a polymer or carrier and a solvent. This chapter reviews examples including the Atrigel® (PLGA + water miscible solvent), SABER™ (sucrose acetate isobutyrate + solvent), ALZAMER® (PLGA + solvent) and ReGel® systems (PLGA/PEG copolymer + water), among others. In vivo delivery durations for in situ forming systems range from days to months.

9.1 Introduction

In situ forming systems are fluid formulations that can be injected into the body in a minimally invasive manner prior to transforming into a semi-solid within the desired tissue, organ, or body cavity. They are applicable in fields such as drug delivery, tissue repair and cell encapsulation. For drug delivery applications, these systems offer several advantages over solid implantable systems or devices and microspheres, including ease of administration, potential for local/site specific delivery, potential for improved drug stability, and simplified manufacturing.

In many instances, targeted delivery to the local site can provide advantages over systemic delivery and potentially yield optimum drug-specific effects. High local drug concentrations can be achieved and maintained in the target tissue without significant systemic levels, thereby avoiding systemic side effects or toxicity. In addition, drug

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metabolism and excretion and permeability barriers can be circumvented by local delivery. Delivering drug directly to the target tissue via an in situ forming system can improve the drug's efficacy at a fraction of the doses that are required for a systemic administration.

Most frequently, in situ forming systems comprise a natural or synthetic polymer or carrier and a solvent. In situ formation is achieved through strategies such as solvent removal, cross-linking and temperature-responsive polymers. Solvent removal is associated with the processes of non-solvent induced phase separation and polymer membrane formation. Cross-linking can be covalent or ionic; the former achieved via thermal- or photo-induction of free radical reactions, the latter typically mediated by small cations. Temperature-responsive polymers have been incorporated into thermoplastic pastes and thermally induced gelling formulations. Thus, development of these technologies has been fostered by research and development of new materials. The choice of solvent is largely dictated by the polymer or carrier and the mechanism of forming the depot.

It is beyond the scope of this chapter to review all the wide range of mechanisms and specific formulation strategies being investigated in industry and academia. Instead, the chapter will emphasize those systems that have been investigated preclinically or clinically and have generated commercial interest. Several other comprehensive reviews on these in situ forming systems have recently been published ([1–3]).

9.2 Development Considerations for In Situ Forming Systems

Drug can be present in in situ forming depots in either solution or suspension (dispersion) form. Solution formulations tend to be simpler in design, development and manufacturing than suspension formulations; however, the drug may be less stable. The challenge for suspension formulations is that the drug must be uniformly dispersed at the time of administration. Thus, either the suspension must be physically stable throughout its shelf life (e.g., in a prefilled syringe), or it must be readily resuspendable (which mandates a vehicle of low viscosity), or the drug and vehicle must be mixed and uniformly dispersed immediately prior to administration. One option for mixing just prior to injection consists of coupling two syringes (one containing the drug substance and the other containing the vehicle) and passing the material back and forth between the syringes [4]. Another option is the use of dual chambered syringes.

Small needle sizes are desirable for injection but the viscosity of some formulations can preclude the use of standard syringes and fine needles. In these cases advanced syringe designs or mechanically assisted injection systems can facilitate the administration of formulations through smaller needles. These approaches, however, may impact their commercial viability.

9.3 In Situ Forming Systems

9.3.1 Atrigel® System

One of the earliest systems in this class was developed by Dunn and coworkers at Atrix Laboratories and is known as Atrigel® [5–7]. Atrigel comprises a water-insoluble biodegradable polymer (e.g., PLGA) dissolved in a biocompatible, water-miscible organic solvent (e.g., N-methyl-2-pyrrolidone, NMP) to which a drug is added, forming a solution or suspension. When this formulation is injected subcutaneously the solvent migrates into the surrounding tissues and water penetrates into the organic phase. This leads to phase separation and precipitation of the polymer at the injection site.

The polymer (PLGA) plays a major role in controlling the delivery. The phase inversion dynamics of PLGA from organic solvents are affected by solvent properties [8]. In addition to NMP, several other organic solvents such as DMSO, tetraglycol, glycolfurol, propylene carbonate, triacetin and ethyl acetate have been studied. Delivery of drug is strongly influenced by M_w and the lactide:glycolide (L:G) ratio of the PLGA. The effects of polymer concentration have also been studied [6].

The most advanced product using Atrigel technology is Eligard®, for palliative treatment of advanced prostate cancer [4, 7, 9]. Eligard contains PLGA dissolved in NMP and the LHRH agonist leuprolide acetate. Depending on the intended duration of leuprolide delivery, the L:G ratio in the PLGA varies from 50:50 to 85:15 and the polymer concentration varies from 34 to 50% (individual injection durations of 1–6 months). Clinical studies demonstrated that a depot containing 22.5 mg leuprolide maintained an effective suppression of serum testosterone below the medical castrate level (50 ng/dL) for more than 3 months (Fig. 9.1).

There are other marketed products based on the Atrigel technology: Atridox® periodontal treatment for subgingival delivery of doxycycline; Atrisorb® GTR barrier without any drug for guided regeneration of periodontal tissue, and Atrisorb D with doxycyline for periodontal tissue regeneration.

9.3.2 SABER™ System

SABER™ (Sucrose Acetate isoButyrate Extended Release) depot technology has been utilized for the controlled release of small molecules, peptides and proteins over periods of days to several months, for both veterinary applications and human therapy [10–13]. The system consists of a biodegradable, high viscosity, non-polymeric liquid carrier material formulated with one or more pharmaceutically acceptable solvents and other excipients.

Sucrose acetate isobutyrate (SAIB) is a mixture of fully esterified sucrose derivatives, with its main components bearing six isobutyrates and two acetates (Fig. 9.2).

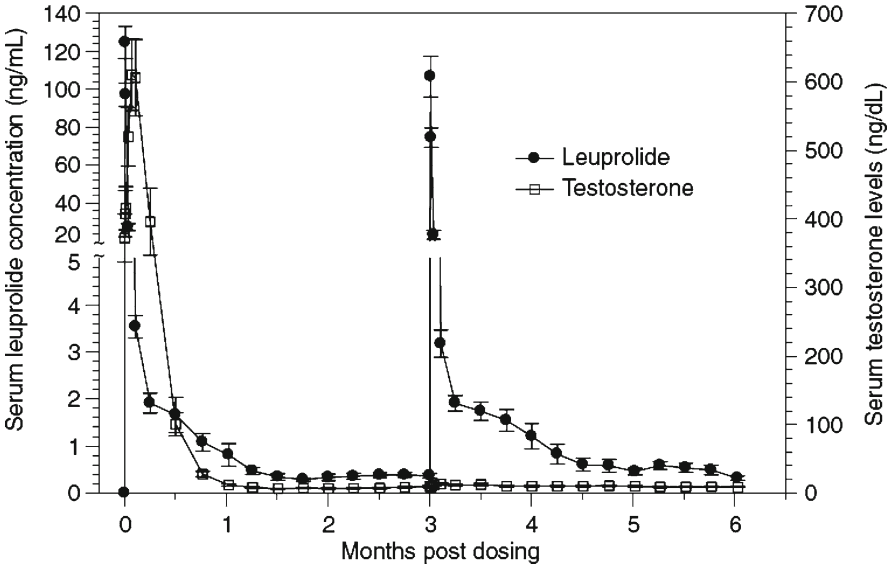

Fig. 9.1 Serum leuprolide and serum testosterone profiles for the 3 month ELIGARD® 22.5 mg product (reproduced from Dadey [7])

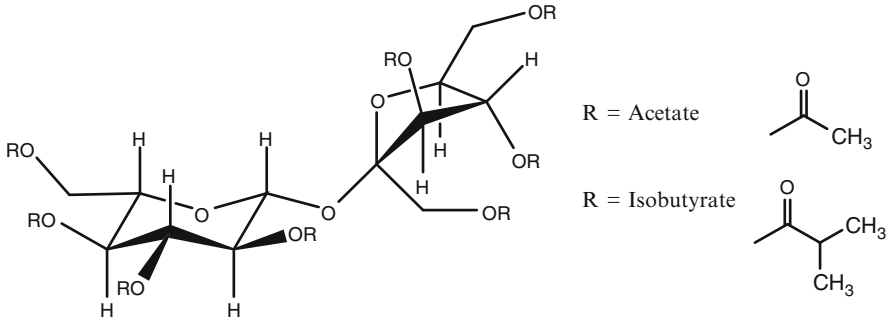

Fig. 9.2 Chemical structure of sucrose acetate isobutyrate (SAIB)

It does not crystallize but exists as a highly viscous, hydrophobic liquid. The viscosity of SAIB can be lowered by combining it with one or more pharmaceutically acceptable solvents, resulting in a vehicle that can be injected through standard needles and syringes. SABER formulations that are either solutions or suspensions have been described [13].

SAIB is a direct food additive approved in over 40 countries [14]. As a pharmaceutical excipient, SAIB is a component of several investigational drug products.

Pharmaceutically acceptable solvents utilized in the formulation of the SABER system include ethanol, NMP, DMSO, benzyl alcohol and benzyl benzoate, among others. When injected, water-miscible solvents such as ethanol or DMSO diffuse out

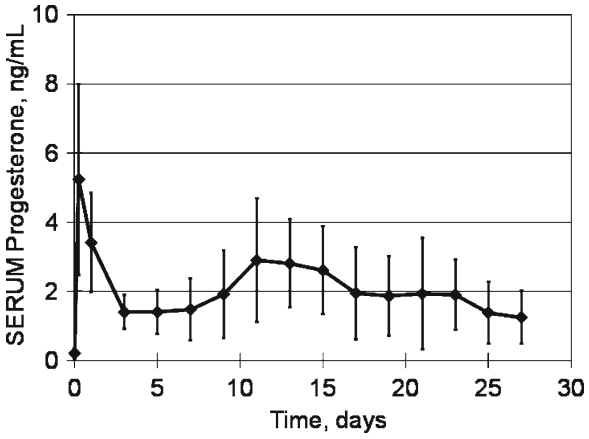

Fig. 9.3 Serum progesterone levels following SABER™ administration in horses (©DURECT Corp., reprinted by permission)

of the depot and the viscosity of the resulting depot increases. Since diffusivity and viscosity are inversely related, diffusivity within the depot decreases. Solvents that are water immiscible (e.g., benzyl benzoate) diffuse from the depot more slowly in vivo and the diffusivity decreases more slowly. Additives utilized to moderate the delivery rate from the SABER depot include the PLA and PLGA polymers. PLA has been explored in SABER systems designed to control the delivery of biomolecules [11].

Figure 9.3 shows the progesterone plasma levels resulting from the parenteral (IM) administration of a SABER formulation to mares. Sustained delivery is observed over 28 days. The formulation consisted of 20% progesterone in SAIB/ethanol 85:15 [10, 12].

POSIDUR™, a SABER™ bupivacaine formulation, is currently in Phase III clinical trials for the treatment of post-operative pain. Bupivacaine is a long acting amide local anesthetic/analgesic. The system is instilled within the surgical site and provides up to 48–72 h of local delivery of the drug. The formulation consists of bupivacaine dissolved in a SAIB/benzyl alcohol formulation. In initial clinical trials, the POSIDUR product was administered subcutaneously. The plasma levels resulting from subcutaneous administration are shown in Fig. 9.4. A rapid onset of delivery (without initial burst) and sustained drug levels for 48–72 h were observed. Administered volumes ranged from 2.5 to 7.5 mL. Over 400 patients have been dosed to date and the system has been well tolerated.

SABER depot formulations of recombinant human growth hormone (rhGH) have been investigated for both systemic and local delivery. Systemic delivery of rhGH from SABER depots is a potential treatment for growth hormone deficiency. Sustained delivery of growth hormone from subcutaneously administered SABER depots has been demonstrated over periods of weeks to 1 month in rats [11, 13] and in monkeys, where elevated IGF-1 levels confirmed the activity of the released rhGH [15]. Additionally, intra-articular delivery of growth hormone is a potential treatment for osteoarthritis or traumatic damage to a joint. SABER formulations of rhGH (150 μL at 50 mg/mL protein) were administered in the right, hind knees of

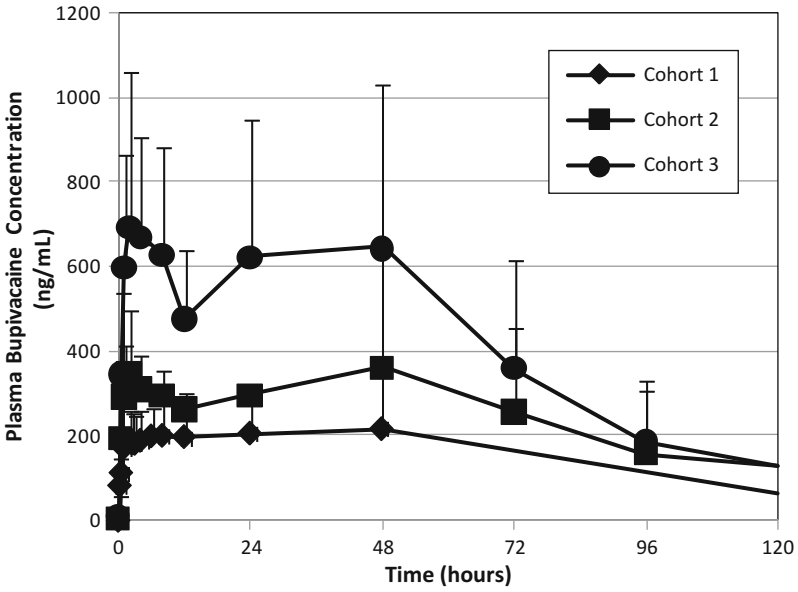

Fig. 9.4 Plasma bupivacaine levels following administration of POSIDUR™ formulation. Cohort 1: 2.5 mL, 330 mg bupivacaine; Cohort 2: 5.0 mL, 660 mg bupivacaine; Cohort 3: 7.5 mL co-administered with 50 mg of commercial bupivacaine–HCl, total dose of 1,040 mg bupivacaine (©DURECT Corp., reprinted by permission)

beagle dogs. Synovial fluid samples were collected at various time points and analyzed for drug concentration, which demonstrated sustained release of the protein for at least 14 days [16].

Beyond the examples cited above, delivery of other steroids, antineoplastic agents, peptides and additional proteins via the SABER system have been reported [10–12, 17, 18]. Sustained drug plasma levels for 3 months have been observed [12]. The SABER system has also been investigated for local chemotherapy of brain tumors, with systems being injected into the cerebral cortex of adult and neonatal rats [19].

9.3.3 ALZAMER® System

ALZAMER® Depot technology is an in situ forming system for the controlled release of APIs following subcutaneous or intramuscular injection. The formulations comprise a biodegradable polymer, biocompatible solvent and engineered particles of the API. Thus, the formulation is most commonly a suspension. The technology has been evaluated for the controlled delivery of low molecular weight compounds, peptides and proteins, advancing to preclinical investigation but not as yet to clinical development [20].

The kinetics of the platform upon injection is governed by the loss of solvent and the concurrent ingress of water or interstitial fluid. There appears to be two dynamical regimes, depending on the aqueous solubility of the solvent used in the formulation and on the polymer characteristics. Solvents such as ethanol and NMP, which are miscible with water, are lost rapidly from the depot after injection into an aqueous medium, but facilitate the uptake of water which provokes polymer precipitation through a process of non-solvent induced phase inversion [21, 22]. Alternatively, if the solvent is hydrophobic, such as ethyl or benzyl benzoate, the rates of its loss, water ingress, and phase separation decline tremendously, converting the membrane thus formed from a coarse, asymmetric blend of polymer-rich and -poor domains to a much finer, symmetric, sponge-like morphology. Among the broad range of biodegradable polymers with which to formulate, poly-lactide-*co*-glycolides (PLGA) have been favored. In addition to being approved for parenteral use, these polymers offer several characteristics that can be varied independently to achieve a target delivery profile: molecular weight (M_w), lactide:glycolide ratio (L:G), initiator chemistry, and degree of branching.

The polymer molecular weight distribution can affect in complex ways the dynamics of in situ gelling upon injection into aqueous media, as it influences intra- and inter-chain interactions. The L:G ratio affects solubility and the rate of biodegradation. The preferred M_w and L:G are ~15 kDa and 50:50, respectively.

An alternative gelling mechanism for certain PLGAs, in hydrophobic solvents such as benzyl benzoate, has been suggested by Wang et al. [23, 24]. This involves the accumulation of chain–chain interactions among sufficiently long glycolide repeats that arise statistically as polymerization conditions vary.

Initial release is largely governed by the solubility of the API in solvent and aqueous solubility of the solvent. APIs having substantial aqueous solubility will attract water to the depot upon injection, accelerating the rate of release. This can be controlled by particle engineering: for example, forming API–counterion complexes [25] or coating the API with hydrophobic material [20], and optimizing the particle size distribution.

By judicious choice of the polymer, solvent and API particle characteristics, nearly zero-order delivery can be achieved for durations of days to months, with what is considered to be acceptable systemic and local tolerability.

9.3.4 ReGel® System

The ReGel® system is a block copolymer system based on PLGA and PEG (A-B-A or B-A-B), resulting in a water soluble, biodegradable thermal gel, which is made up of biocompatible polymers that demonstrate reverse thermal gelation properties (Fig. 9.5). The gel formation results from the hydrophobic interaction between the polymer molecules (but without any chemical cross-linking). An increase in viscosity of approximately four orders of magnitude accompanies the sol–gel transition [26]. Thermally gelling systems based on amphiphilic block or graft copolymers exhibit

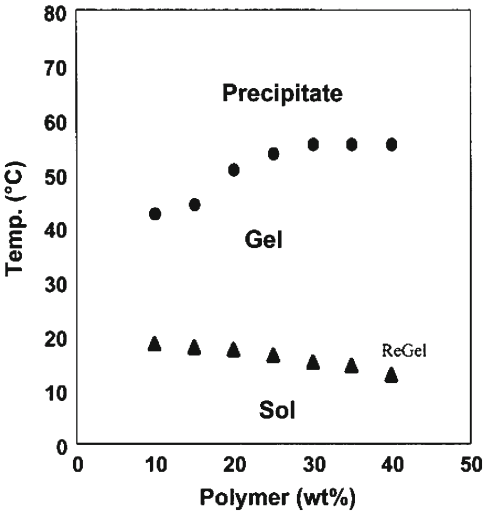

Fig. 9.5 Phase diagram for ReGel® (reprinted with permission from Zentner et al. [26])

the behavior that the components are soluble in water at room temperature (resulting in a low viscosity solution) but form a gel at body temperature. This reverse gelation behavior was originally observed with some members of the poloxamer family of polymers (PEO–PPO–PEO) within specific concentration ranges. Poloxamer based delivery systems have been reported for protein drugs including human growth hormone [27]. Conceptually, the performance of the reverse gelling poloxamer systems led to the development of the ReGel system.

A number of different molecular weights have been investigated for the constituents of the ReGel system. Reported data generally involve PEG 1000 (or PEG 1450) and overall copolymer molecular weights are on the order of 4,000 Da. ReGel is formulated as 23 wt% solution of the copolymer in water or buffer. At 37°C in water (in vitro) the copolymer degrades to final products of lactic acid, glycolic acid and PEG 1000 over a period of 6–8 weeks. In vitro release of a number of drugs from ReGel has been investigated, including paclitaxel, porcine growth hormone, glucagon-like peptide-1 (GLP-1), interleukin 2 (IL-2) and G-CSF. Release was observed over periods of 1–6 weeks. Because of the hydrolysis of the copolymer, ReGel systems must be stored refrigerated (5°C) or frozen (−20°C) [26, 28].

OncoGel™ (ReGel/paclitaxel) is being investigated for the treatment of esophageal cancer in combination with commonly used treatments such as surgery, chemotherapy and radiotherapy. OncoGel™ is injected directly into the tumor. In non-clinical models paclitaxel release has been observed for up to 6 weeks with concentrations of the drug several orders of magnitude higher at the tumor site than in the blood. In a small Phase IIa study of OncoGel™ in patients with late stage inoperable esophageal cancer, 70% of patients had a reduction in tumor volume when OncoGel™ was administered in combination with radiotherapy. Biopsy samples were reported not to contain tumor cells in almost 40% of patients [26, 29].

9.3.5 *Other Depot Systems*

A number of other depot systems have been described in the literature, including those based on amine-modified graft polyesters, those based on poly[sebacic-*co*-(ricinoleic acid)] and those based on polyorthoesters [1, 2, 30]. A 36 h duration polyorthoester system containing mepivacaine has been developed for post-surgical pain relief and is in Phase II testing. An injectable polyorthoester system containing granisetron has been developed for the treatment of chemotherapy induced nausea and vomiting. In addition to the polyorthoester polymer, the system also contains methoxy poly(ethylene glycol) with a molecular weight of 550 Da. The system has a duration of 5 days and a US NDA has been submitted [31, 32].

9.4 Mathematical Models for Release Kinetics from In Situ Forming Systems

Many mathematical models have been developed to describe the release of API from in situ forming depots. The essential task is to relate API release to physical properties that are determined by composition, and thus, ultimately to simplify the formulation process. Because a detailed survey is beyond the scope of this chapter, two of the simplest physically based models are reviewed and several other models that are particularly relevant are described.

Upon injection into an aqueous medium in situ forming depots initially undergo a transient dynamic phase of solidification, cross-linking or gelation, during which large changes in their transport properties can occur over short length and time scales. Moreover, at later times, API release may be substantially affected by surface or bulk erosion. The complex interplay of factors can make it quite difficult to describe API release from first principles. This has tended to foster reliance on simple, readily applicable models that nonetheless provide some insight into formulation behavior.

If the formulation is a suspension or forms one upon injection, the Higuchi model [33] may adequately describe API release from the system. The model assumes constant solubility and diffusivity of API in the formulation and treats the external medium as an infinite sink. The cumulative mass of API released from the depot is given as

$$M_t = A\{DC_s(2C_0 - C_s)t\}^{1/2}, \tag{9.1}$$

with the fractional release given by

$$M_t / M_\infty = 2\{DC_s(2C_0 - C_s)t^{1/2}\} / C_0 l. \tag{9.2}$$

C_0 is the total concentration, D is the diffusivity and C_s is the solubility of API in the formulation. The surface area and thickness of the depot are denoted by A and l, respectively. Equations (9.1) and (9.2) describe release from a rectangular slab, so

that $M_\infty = Al\ C_0/2$. Typically, the approximation for $C_0 \gg C_s$ is made. Equations for cylindrical and spherical geometries are also available, involving implicit equations that are slightly less convenient to use.

In this model, release increases only as $t^{1/2}$, reflecting the declining concentration gradient that drives API transport within the system. In addition, as C_0 increases, the absolute rate of API release increases, but the fractional API release rate decreases. Conversely, smaller depots (reduced A or l) have lower absolute release rates, but higher fractional API release rates. Note that the Higuchi model has been refined to include a finite mass transfer resistance at the depot surface [34].

In situ forming depots may share some characteristics of solutions of hydrophobic compounds in oily depots, for which Hirano has developed a model of release. In this model [35], release is controlled by a boundary layer in the depot coupled to an external boundary layer. The release rate is given by

$$dM_t / dt = Ak_0(M_\infty - M_t) / V(1 + Kk_0 / k_a), \tag{9.3}$$

where V is the depot volume, k_0 is a mass transfer coefficient (permeability) in the depot, k_a is a mass transfer coefficient in the external boundary layer adjacent to the depot, K is the depot/external medium partition coefficient, and the other symbols have their previous meanings. The model assumes a constant thickness of boundary layer/concentration gradient zone inside the depot.

Equation (9.3) is solved by noting that a characteristic time for API release is defined by

$$\tau = V(1 + Kk_0 / k_a) / Ak_0, \tag{9.4}$$

then separating variables and integrating, with the result that

$$M_t = M_\infty(1 - e^{-t/\tau}). \tag{9.5}$$

The qualitatively different temporal behavior of the Higuchi and Hirano models stems from the different state of API in each case.

The Hirano model is an approximation of the partial differential equations for diffusion of dissolved API from a sphere to an external medium. The infinite series solution depends on two parameters [36] and can be applied via a spreadsheet. Short and long time approximations are available and easily evaluated numerically.

There are more purely mathematical expressions to which cumulative release profiles can frequently be fitted. Among the better known are the Weibull and Hill equations,

$$M_t = M_\infty(1 - e^{-(t/\tau)^\alpha}), \tag{9.6}$$

$$M_t = M_\infty t^\beta / (t_{50}^\beta + t^\beta). \tag{9.7}$$

in which α, β and τ are constants, and t_{50}^β is the time required to release half the API payload. The physics underlying these equations is less direct than in the Higuchi

and Hirano models, but note the similarity of (9.5) and (9.6), and see Shlesinger and Montroll [37].

These models can be readily applied to in vitro release data. The Higuchi and Hirano models are one parameter fits; the Weibull and Hill equations involve two parameters. Thus, it may be possible to distinguish among the models statistically, although several may fit a particular set of release data equally well. Of course, they may also be distinguished by the predictions they make about the sensitivity of release to changes in formulation parameters.

A model of API release from depots formed by non-solvent induced phase separation (e.g., ALZAMER) has been developed by Raman and McHugh [38] in terms of coupled convection–diffusion equations. This complements earlier phase diagram calculations and transport models for the phase inversion process [39, 40].

Lastly, it is noted that models for the release of API from swelling polymers (e.g., [41]) may be relevant to hydrophilic in situ forming depots.

9.5 In Vivo–In Vitro Correlation

The availability of a "universal" in vitro drug release test that predicts in vivo drug release kinetics would be of enormous utility in the development of in situ forming depots. Unfortunately, no such test exists, although the authors are aware of efforts to overcome this limitation.

A moment's reflection suggests reasons such a test is technically challenging. It may be difficult to mimic in vitro the essential details of the subcutaneous space; in particular, the biological reaction to the presence of a foreign body. Also, the shape (and thus surface area) assumed by the depot upon administration in vivo may be unknown and highly variable; hence difficult to reproduce in vitro. There is also a temporal problem. As the duration of an in vitro release method lengthens, the effect of small, possibly randomly generated differences in release rate tend to be magnified. Of course, the same is true of delivery in vivo.

In some cases, it is possible to develop a method that correlates well with in vivo release kinetics for a specific depot formulation of a specific drug. But, over the long run, it might be faster and less expensive to rely on animal models for formulation selection. Once selected, in vitro drug release methods would be established to characterize the final product to satisfy regulatory requirements.

9.6 Preclinical Testing of In Situ Forming Systems (Including Excipients)

In situ forming systems must be safe and biocompatible. While each individual system must be evaluated separately, those systems that are in advanced development or commercialized may generally be considered biocompatible. However, a

finding of biocompatibility may be dependent on the drug and therapeutic application and the site of application to the body. Some excipients present in in situ forming systems have an extensive history of parenteral use and the history may be relied upon to qualify the excipient. Novel proprietary excipients utilized in in situ forming systems must be tested for safety and biocompatibility either separately or as part of the depot system. Regulatory guidances specify the expected preclinical testing for new excipients or excipients utilized for the first time in parenteral applications or excipients utilized in larger amounts than in approved products [42]. Such testing may include safety pharmacology studies, acute toxicology studies, ADME studies, genetic toxicology studies, repeat dose toxicology studies, reproductive toxicology studies, carcinogenicity studies and hemolysis studies. The inclusion of a "new" drug in an in situ forming system may require new safety and preclinical testing even though the delivery system has previously been used or approved and even though the drug has been previously approved, as the combination of the "new" drug and the delivery system might have new or unexpected adverse effects. Such testing might include pharmacokinetic studies (including determination of local drug concentrations for site specific delivery), evaluation of acute and/or chronic reactions at the injection site and acute or chronic systemic toxicology studies.

9.7 Regulatory Considerations

In situ forming systems are regulated as drug products. Regulatory authorities will expect preclinical testing (see above) and clinical testing to demonstrate the safety and effectiveness of the system. These products can be either terminally sterilized or manufactured aseptically. Testing must demonstrate that the products are sterile and pyrogen-free. In situ forming systems are specified by total drug content and delivery rate (duration). Testing for drug release rate is generally required for lot clearance, with intervals for initial release, an extended or steady state release period and an end of delivery portion (generally, ≥80% drug release). Because of the long duration of action of these depots, manufacturers usually attempt to develop accelerated release rate test methods.

9.8 Summary and Conclusions

In situ forming depots represent a versatile class of systems for the sustained systemic and local delivery of the full range of pharmaceutically active chemicals and biologicals. The duration of delivery can last from days to months. There are a variety of applications for these systems and a range of system types has been investigated. The biocompatibility of novel materials used in these systems must be established through rigorous testing programs. In situ forming systems can offer better control of systemic drug levels, and less frequent and more convenient administration, thus leading to greater efficacy, reduced adverse events and better patient compliance. They can

also provide advantages (e.g., efficiency and cost savings) to the provider and the health care system. In the future, further development and commercialization of in situ forming systems is expected, especially for the delivery of biomolecules.

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Chapter 10
Microsphere Technologies

Yan Wang and Diane J. Burgess

Abstract This chapter, divided into three sections, discusses important developments in microsphere technology for parenteral drug delivery. In the first section, microsphere systems are briefly described and polymers including both natural and synthetic polymers used in microsphere technology are reviewed. In the second section, various conventional microsphere fabrication methods as well as recent advances in microsphere preparation methods are discussed. Specific examples of microsphere applications, including microspheres for small molecules, protein therapeutics and vaccines are provided. In the final section, some of the challenges associated with developing microsphere products and means to overcome these challenges are discussed.

10.1 Rationale for Using Microspheres for Systemic/Local Delivery of Drugs

Controlled drug delivery systems are designed to delivery drugs to their therapeutic sites of action at a controlled rate and dose regime to optimize efficacy. The route by which drugs are administered can significantly alter their effectiveness. Oral administration is the most popular route for the majority of drugs due to its noninvasive nature and convenience of self-administration. However, there are many instances where oral medication is not desirable, appropriate, or technically feasible, for example, when the drugs are rapidly metabolized ("first pass" effect) and eliminated from the body following oral administration. In the case of conventional parenteral

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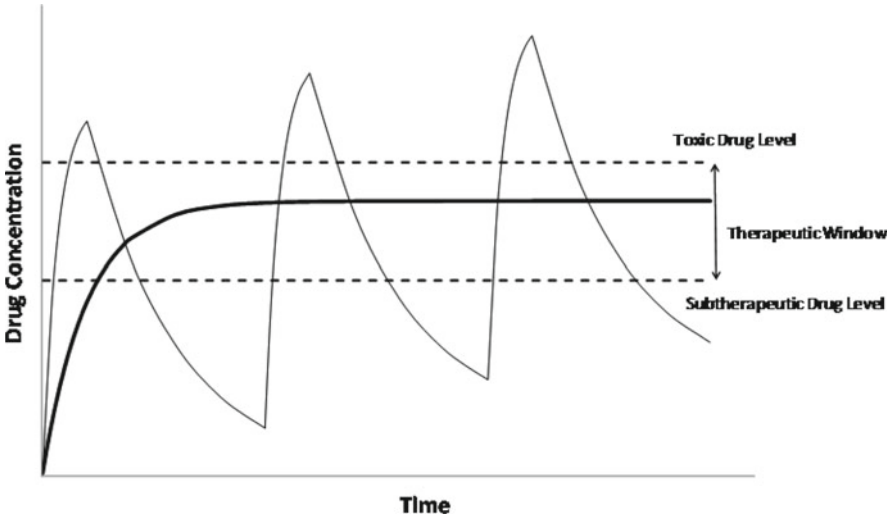

Fig. 10.1 Drug concentrations at the site of therapeutic action after delivery as a conventional injection (*thin line*) and as a controlled release system (*bold line*)

dosing, the drug concentration peaks as the dose enters the bloodstream and goes down very quickly after the peak. This causes drug concentration fluctuation and necessitates frequent injections. Parenteral controlled drug delivery systems are a promising strategy to overcome these problems. As shown in Fig. 10.1, controlled drug delivery systems are capable of maintaining the drug concentration within the therapeutic window (the dosage range of a drug between the minimum effective dose and the amount that gives side effects) over an extended duration compared to conventional parenteral dosage forms [1]. Accordingly, controlled drug delivery systems not only improve drug efficacy but also reduce potential toxic side effects (Fig. 10.2).

Microsphere technology is one type of controlled drug delivery system, which has been successfully developed from the bench scale to products approved by the Food and Drug Administration (FDA). The first use of microspheres can be traced to the 1960s. Currently, there are ten commercially available parenteral products (Table 10.1). Considerable research effort has been conducted on microsphere technology over the past four decades to: (1) establish the basic principles of drug release from solid matrices; (2) develop mathematical models of controlled drug release; (3) synthesize new biocompatible polymers for preparation of these controlled delivery systems; (4) identify the parameters affecting the physical and chemical properties of microspheres; and (5) determine the release profiles of the incorporated drugs. Many excellent reviews have been published to describe the progress that has been made and the challenges that remain in microsphere technology [2].

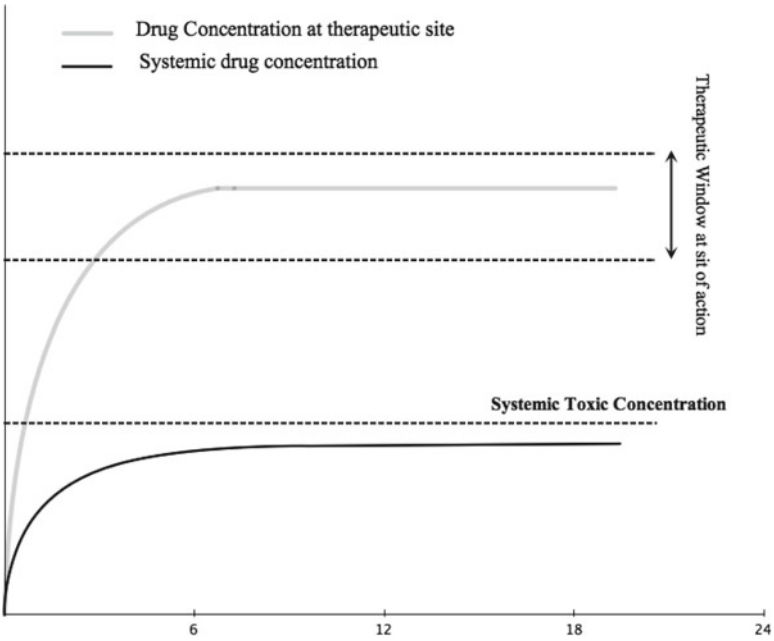

Fig. 10.2 Drug concentrations at the site of therapeutic action (*grey bold line*) and drug systemic concentration (*blank thin line*) after delivery as a controlled release system

Table 10.1 FDA approved microsphere products

Product	API	Route of administration	Indication
Leupron Depot®	Leuprolide	Intramuscular	Prostate cancer
Sandostatin Depot®	Octreotide	Intramuscular	Acromegaly
Trelstar Depot®	Triptorelin	Intramuscular	Prostate cancer
Somatuline LA®	Lanreotide	Subcutaneous	Acromegaly
Neutropin Depot®	Somatropin	Subcutaneous	GH deficiency
Risperdal Consta®	Risperidone	Intramuscular	Bipolar mania and Schizophrenia
Arestin®	Minocycline	Sub gingival	Periodontitis
Retin-A Micro®	Tretinoin	Topical	Acne
Zmax®	Azithromycin	Oral	Sinusitis
SIR-Spheres®	Yttrium-90	Through hepatic artery	Liver tumor

Microspheres have some specific advantages over other controlled drug delivery systems, such as: (1) the rate of drug release as well as the duration of drug release can be tailored by altering the materials and fabrication techniques [2]; (2) microspheres possess better stability compared to other controlled drug delivery systems, such as liposomes; and (3) patient compliance is enhanced due to lower dosing frequency. This chapter focuses on the most important aspects of

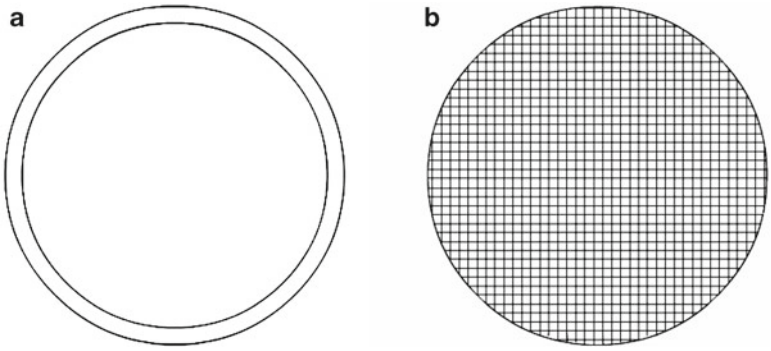

Fig. 10.3 Schematic diagram illustrating microspheres: (**a**) microcapsule consisting of a drug reservoir/core; and (**b**) micromatrix consisting of dispersed drug phase

microsphere technology: polymer types, fabrication techniques, encapsulation methods, limitations associated with the technology, and recent technological advancements.

10.2 General Description of Microspheres

Microspheres are solid spherical particles ranging in size from 1 to 999 μm. They consist of natural or synthetic polymeric materials in which the drugs or other biologically active agents are entrapped or encapsulated. In general, microspheres can be suspended in an aqueous vehicle and parenterally administered through a small gauge needle without the need for anesthesia. The particles have either a continuous polymeric matrix where the drugs are homogeneously dispersed throughout (also known as micromatrices) or a shell-like wall surrounding the drug reservoir/core (as indicated in Fig. 10.3). Microspheres consisting of a drug core are also named microcapsules. The terms microspheres and microcapsules are often used synonymously.

10.3 Materials Used in Microsphere Technology

The very first step in designing a microsphere formulation is choosing an appropriate polymeric material as the drug/bioactive agent carrier. The physical, chemical and biological properties of the polymers, such as biocompatibility and biodegradability, play a key role in the final product performance. Various polymers have been investigated for microsphere technology and it is very difficult to classify polymers in a simple way. Based on the resource materials used, polymers can be classified as either natural or synthetic.

10.3.1 Natural Polymers

Research on natural polymers used for microsphere technology has focused on two types of the materials: proteins (e.g., albumin, collagen and gelatin) and polysaccharides (e.g., chitosan, alginate and dextran). Natural polymers are derived from both plants and animals and possess intrinsic advantages over synthetic polymers: they are readily available and have little/no toxicity. However, natural polymers tend to result in relatively rapid drug release and may be immunogenic.

10.3.1.1 Proteins

Albumin: Albumin, a major plasma protein constituent, is one of the most commonly utilized proteins in controlled release drug delivery systems. It is known that albumin can be used to conjugate with small molecule drugs to enhance their stability as well as their in vivo circulation half-life. Albumin microspheres are often injected directly to the site of action, such as joints for anti-arthritic drug delivery [3]. Thakkar and his co-workers have reported on the use of albumin microspheres for targeting the drug celecoxib to arthritic joints following intravenous administration [4]. The results showed that the concentration of celecoxib in the inflamed joints was much higher than in the non-inflamed joints. Blood kinetic studies revealed that the celecoxib loaded albumin microspheres exhibited prolonged circulation half-lives compared to a celecoxib i.v. solution.

Collagen: Collagen is a major component of the extracellular matrix and has been used for decades in tissue engineering, surgical medicine and drug delivery vehicles [5]. A number of bioactive agents have been incorporated into collagen microspheres including: insulin, growth hormone, vascular endothelial growth factor, antibiotics, heparin and various cells [6]. For example, human mesenchymal stem cells loaded into collagen microspheres have been used as a scaffold for bone regeneration and have shown good osteogenic ability to enhance bone matrix deposition [7].

Gelatin: Gelatin is another intensively studied natural polymer that is produced by controlled hydrolysis of collagen. Gelatin is highly water soluble, and therefore in order to achieve sustained drug release from gelatin microspheres, chemical cross-linking techniques are used. For instance, gelatin microspheres, containing lactic acid, have been formed by cross-linking with glutaraldehyde [8]. The in vitro release profile of lactic acid from gelatin microspheres showed that the release rates were inversely proportional to the cross-linking density and duration.

10.3.1.2 Polysaccharide

Chitosan: Chitosan is a cationic polysaccharide with molecular weights over one million Daltons. It is produced via the alkaline deacetylation of chitin (a combination

of sugar and protein that mostly comes from the shells of crab, shrimp and krill). Chitosan microspheres have been extensively investigated for the delivery of various classes of drugs, such as cardiac agents (diltiazem hydrochloride, nifedipine and propranolol hydrochloride), anticancer drugs (fluorouracil, cisplastin, mitoxantrone and taxol), and anti-inflammatory drugs (diclofenac sodium, indomethacin, prednisolone, ketoprofen and ibuprofen) [9–12]. Chitosan has been considered as a good candidate for gene delivery since it is cationically charged, and therefore can easily complex with negatively charged nucleic acid therapeutics (e.g., plasmid DNA or siRNA).

Dextran: Dextran is a complex, branched glucan polysaccharide composed of chains of varying lengths. The straight chain consists of α-1,6 linkage with branches in varying proportions of α(1–4), α(1–2) and α(1–3) linkage types. The average molecular weight of dextran varies from 10 to 2,000 kDa.

In spite of their excellent biocompatibilities, there are some drawbacks associated with natural polymers, which significantly limit their applications. Most natural polymers degrade by enzymatic hydrolysis. Accordingly, their in vivo degradation is highly tissue specific and very hard to control which makes it difficult to achieve desirable drug release kinetics. In addition, these polymers are hydrophilic in nature, and therefore drug release rates tend to be rapid due to fast water influx following administration. Some natural polymers, such as collagen, have very poor dimensional stability due to significant swelling in vivo as a result of their hydrogel nature.

10.3.2 Synthetic Polymers

Many polymers have been synthesized and investigated to promote the development of microsphere technology since the 1970s. Synthetic polymers are often associated with toxicity problems in comparison with natural polymers, which is the major problem limiting their application. At present, only two classes of synthetic polymers have been used in FDA approved medical products: polyesters and polyanhydrides. With the development of biomaterial science, more and more novel polymers have been shown to have enormous potential for use in commercial products, such as poly(ortho esters), poly(amino acids) and poly(ethylene glycol).

Poly(esters): Poly(esters) are the most extensively utilized synthetic, degradable polymer group in the pharmaceutical area [2, 13–17]. Examples include poly(lactic acid) (PLA), and poly(glycolic acid) (PGA), as well as their copolymers, poly(lactic-*co*-glycolic acid) (PLGA). These are usually synthesized by ring-opening polymerization of the cyclic lactone monomers [1]. Depending on the ratio of monomers used and the extent of polymerization, various forms of PLGA with different hydrophobicities can be obtained.

Degradation of poly(esters) occurs through hydrolysis resulting in the scission of the ester linkages. Compared to PGA, PLA is more hydrophobic due to the methyl group present on the β carbon of lactic acid and its greater degree of crystallinity.

Therefore, PLA microspheres have slower rates of water uptake, and thus slower degradation rates [15]. The degradation rates of PLGA copolymers can be modified by varying the lactic to glycolic acid ratios. PLGA copolymers prepared with a higher percentage of lactic acid will have slower degradation rates compared to those with a higher percentage of glycolic acid. In addition, changes in pH or temperature of the in vitro release medium/in vivo sites of action will significantly affect the degradation rates of poly(esters) [13, 18, 19].

The biocompatibility of degradable polymers is mainly defined by the biocompatibility of the monomers. Poly(esters) are considered biocompatible since their monomers are natural metabolites of the body. However, many studies have reported increased local acidity following the injection of poly(ester) microspheres, and this causes localized tissue irritation (inflammation and fibrosis).

Polyanhydrides: Polyanhydride was first synthesized in 1909, by the polymer scientists Carothers and Hill [20]. At that time, only low molecular weight polymers that were hydrolytically labile were synthesized. The hydrolytic liability made this polymer impractical for many applications. In the late 1980s and early 1990s, Langer and his colleagues conducted a systematic study to determine the mechanism of polymerization of polyanhydrides and successfully synthesized high molecular weight polyanhydrides, which are much more stable compared to low molecular weight polyanhydrides [20, 21]. Since then, these polymers have received more and more attention in the pharmaceutical industry.

Polyanhydride polymerization involves two individually prepared prepolymers: poly(sebacic anhydrides), p(SA), and poly(bis carboxyphenoxyropane), p(CPP). p(SA) is a linear aliphatic polymer that degrades within days. p(CPP), on the other hand, is a hydrophobic, aromatic polymer, which degrades much slower. Therefore, by varying the ratio of these two prepolymers, polyanhydrides with different degradation rates can be obtained to provide desired drug release profiles [22].

The hydrophobicity of polyanhydrides and the hydrolytic liability of the anhydride bonds make polyanhydride copolymers very unique in terms of their biodegradability. The hydrolysis rate of the anhydride bonds are much faster than the rate of water penetration through the surface into the center of the microspheres, therefore, unlike poly(ester) micorspheres, polyanhydride copolymer microspheres erode from the surface inwards [23–25].

10.4 Conventional Fabrication Methods and Techniques of Microsphere Preparation

10.4.1 Coacervation

Coacevation, also known as phase separation, is a process where a polymer solution is separated into two immiscible liquid phases: a dense coacervate phase concentrated in polymer, and a dilute polymer phase. These two phases are in equilibrium [11].

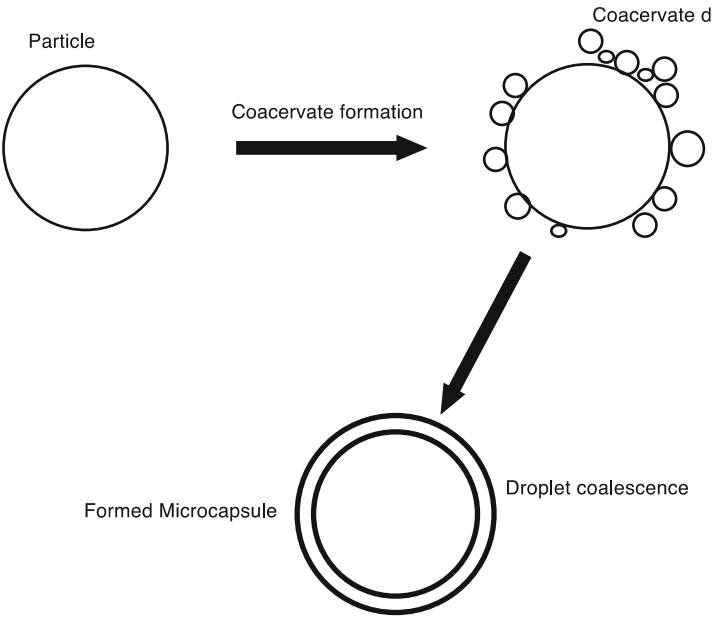

Fig. 10.4 Schematic diagram of the formation of a coacervate droplet around a drug particle

When only one polymer is present in the system, this phenomenon is called simple coacervation [26–28]. When more than one polymer is present in the system, it is referred to as complex coacervation. Simple coacervation can be initiated by change in temperature, change in ionic strength or addition of a non-solvent [28–30]. All of these changes promote polymer–polymer interactions over polymer–solvent interactions, which result in dehydration of the polymer. In the case of complex coacervation, electrostatic interaction between two or more polymers is the driving force of this phenomenon [31].

Coacervation is a useful method to entrap solid particles to form microcapsules. When solid drug particles are present, the coacevate droplets form around these as shown in Fig. 10.4. These tiny coacervate droplets tend to coalesce and sediment; therefore, agitation of the system is needed to maintain emulsification and prevent coalescence and sedimentation [32]. In addition, physical or chemical cross-linking techniques, such as the application of heat or the addition of a chemical cross-linking agent, are required to stabilize the coacervate emulsion droplets to form microcapsules.

Although coacervation has been successfully employed in many microsphere preparations, there are a number of drawbacks associated with this method. Phase separation can only occur at specific pH values. Heat or chemical cross-linking agents are needed to stabilize the coacervate droplets, which may be harmful to entrapped drugs. The retention of the encapsulants relies on the extent of cross-linking, which may cause batch-to-batch variance. Burgess and Singh have developed a stable

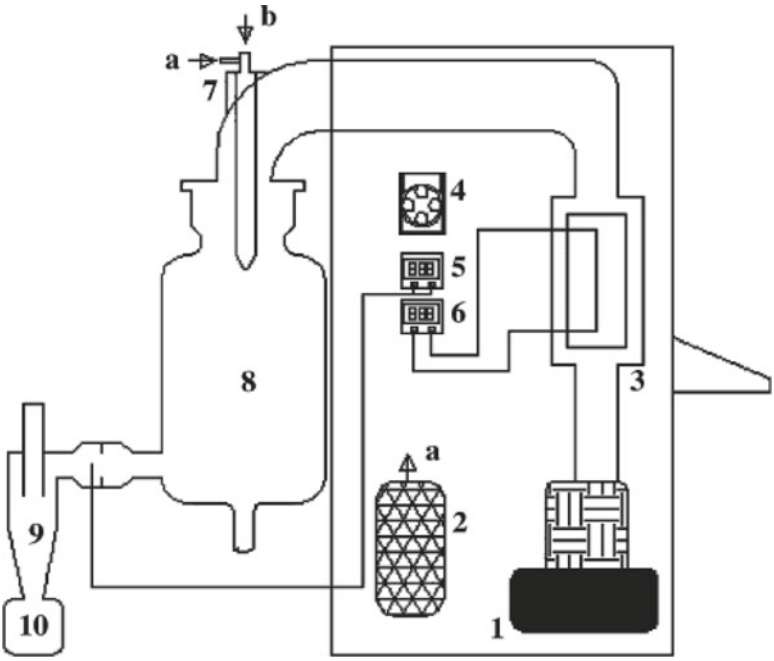

Fig. 10.5 Schematic diagram of a spray dryer: (1) blower + air filter; (2) air compressor; (3) heater; (4) peristaltic pump; (5) temperature control; (6) inlet thermocouple; (7) atomizer: (a) compressed air; (b) feed microencapsulating composition; (8) drying chamber; (9) cyclone; and (10) dry product collector (this figure is reproduced from publication [33] with permission)

coacervate system, without the need for chemical cross-linkers or the application of heat. This system is potentially useful for the delivery of sensitive drugs, such as proteins and peptides [31].

10.4.2 Spray Drying

Spray drying is a single step, closed system process used to transform a material from the fluid state into the solid state by spraying the liquid through a hot drying gas medium. It is applicable to various materials including heat-sensitive materials and is ideal for the production of sterile materials. Figure 10.5 is a schematic diagram of a spray drying instrument [33]. Spray drying is very popular in industry due to its ease of use, cost effectiveness, relatively short processing time and compatibility with most drugs and drug products.

The drug is either dissolved or suspended in an organic or aqueous solvent containing the polymer(s), depending on the properties of the polymer(s) and the drug [34]. For instance, polylactide (PLA) microspheres containing hydrophobic drugs can be prepared by dissolving the drug and the PLA in methylene chloride.

All spray dryers use an atomizer or spray nozzle to disperse the solution or suspension into controlled size droplets, which are rapidly dried by a heated gas carrier. The size of the resulting microspheres is heavily dependent on the rate of spraying, the nozzle size, the drying temperature, the feed rate of the polymer drug solution/suspension, as well as the temperature and size of the collecting chamber. In general, microspheres prepared by this method are in the size range of 1–15 μm or larger [12]. Care needs to be taken during manufacturing to prevent particle agglomeration. Plasticizers, that promote polymer coalescence and increase the formation of spherical and smooth-surfaced microparticles, are used to improve the quality of spray-dried products.

10.4.3 Solvent Evaporation

The solvent evaporation method is one of the earliest and most commonly used microsphere preparation methods [35–37]. Typically, the polymer is dissolved in a volatile organic solvent such as methylene chloride [19, 38]. When a hydrophobic drug is used, it can be added directly to the polymer solution. This mixture is emulsified in an aqueous solution containing an emulsifying agent, such as poly(vinyl alcohol) (PVA). The resulting emulsion is stirred for hours until most of the organic solvent is removed. The hardened microspheres are collected by filtration and washed with water following either vacuum drying or freeze drying. When a hydrophilic drug is used, it is usually first dissolved in water, then added to the polymer solution or dispersed as fine solid particles. Figure 10.6 is a schematic depiction of microsphere formation by solvent evaporation. In order to facilitate solvent evaporation, the system is often under vacuum during the solidification process.

10.4.4 Solvent Extraction

The solvent extraction method is very similar to the solvent evaporation method. The only difference between these two methods is the properties of the solvents used [36]. The solvent extraction method does not require the use of solvents with high vapor pressures. Non-volatile solvents can be removed by increasing the solvent concentration difference between the dispersed and continuous phases, or by the addition of a third solvent into the continuous phase to facilitate polymer precipitation.

10.4.5 Cross-Linking Technique

Physical/chemical cross-linking is often employed to produce microspheres from hydrophilic polymers, such as chitosan, gelatin, starch, dextran, albumin and

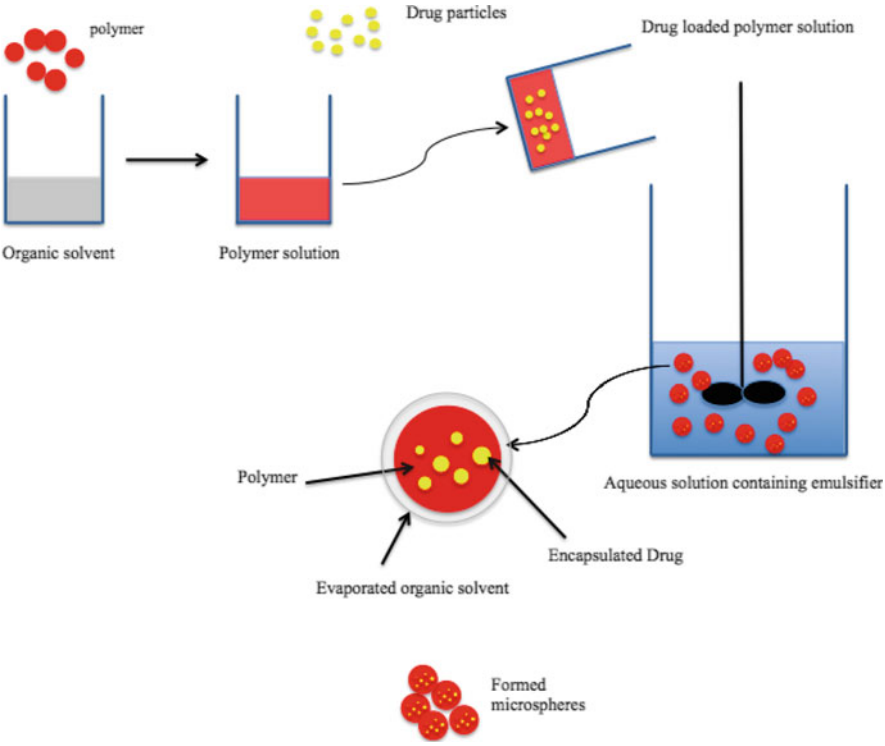

Fig. 10.6 Depiction of microsphere formation by solvent evaporation. The drug containing polymer droplets are dispersed inside the continuous aqueous phase forming solvent–polymer spheres; the spheres harden as the organic solvent evaporates

hyaluronic acid [39, 40]. These microspheres possess excellent tissue compatibility, as well as ease of control of microsphere swelling and solute permeability.

A water-in-oil (w/o) emulsion is prepared by emulsifying the drug polymer solution in an oil phase (such as vegetable oil, or an oil/organic solvent mixture) containing a surfactant as an emulsifier. The emulsion is then either heated or cross-linking agents are added to cross-link the polymer droplets and form solid microspheres. Many different cross-linking agents have been utilized [41]. Glutaraldehyde is the most commonly used chemical cross-linking agent for protein microspheres, however, its toxicity remains an obstacle for pharmaceutical applications [40].

10.4.6 Ionic Gelation

Ionic gelation refers to cross-linking of polyelectrolytes in the presence of multivalent counter ions. For instance, spraying a sodium alginate solution into a calcium

chloride solution generates rigid gel particles. Complexation of oppositely charged polyelectrolytes is often applied following ionic gelation to form a membrane on the surface of the gelled particles to enhance their mechanical strength. In the case of calcium alginate gelling, polylysine is usually utilized for this purpose.

This method was first developed by Lim and Sun for the encapsulation of live cells [42]. It has also been widely applied to drugs and other bioactive agents. Various polymer systems, such as chitosan/alginate chitosan/triphosphate, pectin/calcium and polyphosphazene/polylysine have been utilized.

10.4.7 Interfacial Polymerization

Microspheres can be formed via interfacial polymerization [43]. This involves dispersing a monomer solution into a non-solvent, such as dispersing an oil solution of organic monomers into an aqueous media or dispersing an aqueous solution of water soluble monomers into organic medium. Polymerization occurs at the interface of the two immiscible liquids. Many methods, such as emulsion, suspension dispersion and sedimentation, can be used in this process. Different techniques result in different final products with particle sizes ranging from 0.01 to 500 μm.

10.5 Recent Advances in Microsphere Technology

10.5.1 Double-Walled Microspheres

Microspheres containing hydrophilic drugs or proteins/peptides often show high initial burst release, which can be undesirable and may cause serious side effects in vivo due to a large amount of drug administered in a short period of time. Surface bound drug is responsible for the initial burst release. Microspheres prepared with a double-layered structure can have the advantage of no or reduced burst effect [44]. There are several methods of preparation of double-walled microspheres [45]. One method involves encapsulating drugs into microspheres using one of the conventional methods mentioned above and then coating the microspheres with a second polymer layer. The additional coating can be applied via a dip or pan coating process or via an air suspension technique. However, there are several disadvantages associated with these coating techniques: (1) uneven or incomplete polymer coating around the microspheres; (2) low yield; and (3) relatively large size of the resulting microspheres.

Alternatively, double-layered microspheres can be made by polymer–polymer phase separation of a binary blend of polymer solutions [46, 47]. A mixture of two polymer solutions, such as poly(ortho ester) (POE) and PLGA, is emulsified in an aqueous non-solvent phase [48]. As the solvent slowly evaporates, the droplets of

the polymer–polymer solution become more and more concentrated. At a certain point the polymers are no longer mutually soluble, and they start to phase separately into one phase rich in one polymer, and the other phase rich in the second polymer.

10.5.2 Self-Healing Polymeric Microsphere System

Till now, the double emulsion method is the most commonly used to form microspheres containing proteins and peptides. The presence of organic solvent is the main factor causing protein instability during the preparation process. A new microencapsulation method called polymer self-healing has been developed based on the natural "self-healing" capacity of the polymer. In this method, the protein encapsulation process is conducted in aqueous media without the use of organic solvent, which potentially can enhance protein stability during preparation [49]. Briefly, blank porous microspheres are prepared using a conventional method (e.g., the solvent evaporation method). Porosigens (such as trehalose dehydrate and magnesium carbonate) are required to make the blank microspheres highly porous. Once the blank microspheres are prepared, they are placed into a concentrated protein solution at 4°C for 24–48 h on a rocking platform to allow protein penetration through the pores. The temperature of this system is increased for 30–24 h in order to close the pores and encapsulate the protein. The pore closing temperature has to be slightly higher than the glass transition temperature of the polymeric system used in order to ensure that the polymer chains have enough mobility for pore closure. Microspheres containing proteins prepared using the "self-healing" method have been shown to have lower initial burst release compared to microspheres formed in the traditional way. This new drug encapsulation method has potential application to various biomacromolecular drugs. It also has some shortcomings: in order to obtain an acceptable drug encapsulation efficiency, concentrated protein solution is required which potentially can induce protein aggregation; and residual porosigen may have an impact on drug stability and release profiles.

10.5.3 Solvent Exchange Method

The solvent exchange encapsulation method is based on interfacial mass transfer between an aqueous drug solution and a water-insoluble polymer organic solution upon contact to form reservoir-type microcapsules. The surface tension difference and the incompatibility between the drug aqueous phase and the polymer solution phase are the reaction driving forces. Figure 10.7 shows one method of forming microcapsules using this method. Aqueous microdrops containing drugs and microdrops containing polymers are produced simultaneously using ink-jet nozzles controlled by a piezoelectric transducer (Fig. 10.7a) [50]. The two ink-jet nozzles are aligned to cause a mid-air collision between the two microdrops (Fig. 10.7b) [50].

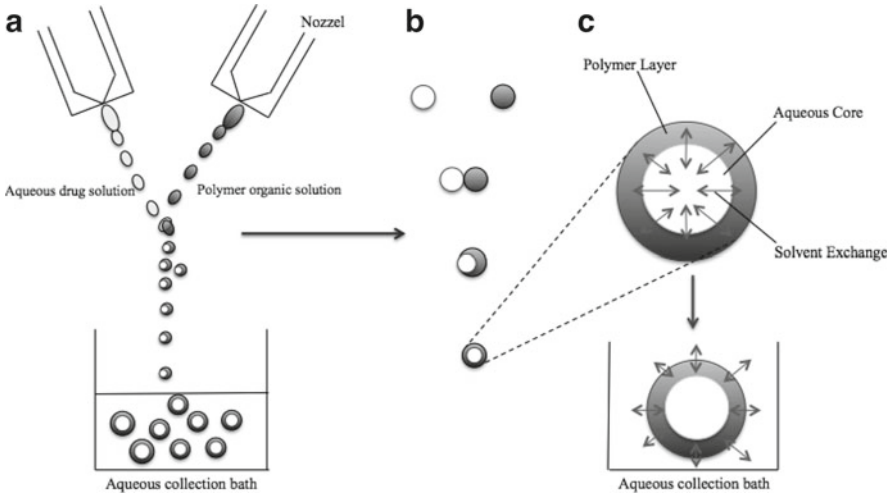

Fig. 10.7 Formation of microcapsules by the solvent exchange method. (**a**) Simultaneous generation of microdrops containing drugs in soluble form and microdrops containing polymers using ink-jet nozzles. (**b**) Mid-air collision between the two microdrops. (**c**) Solvent exchange at the interface upon contact of the two liquids to form a polymer layer on the aqueous droplet. Formed microcapsules are collected in the aqueous bath

Solvent exchange proceeds upon contact of the two microdrops resulting in reservoir-type microcapsules which are collected in an aqueous bath (Fig. 10.7c) [50].

There are several potential advantages of this method. First, intensive physical stress is not involved in the preparation process (e.g., shear stress or elevated temperature), which can cause drug instability. Second, for protein/peptide encapsulation, undesirable exposure to organic phase is limited.

10.5.4 Microfluidic Flow-Focusing Method

The particle size distribution has a significant influence on the kinetics of drug release from microspheres. Microspheres prepared using conventional emulsification techniques, such as sonication or mechanical homogenization, usually have a very broad size distribution, which results in: (1) potential batch-to-batch variations, (2) different polymer degradation rates, and (3) different drug release profiles. Microfluidic flow-focusing produces uniform-sized drug loaded droplets to obtain microspheres with narrow size distribution as indicated in Fig. 10.8 [51]. Figure 10.9 is a schematic illustration of the procedure used to fabricate monodisperse polymer microspheres via this method [51].

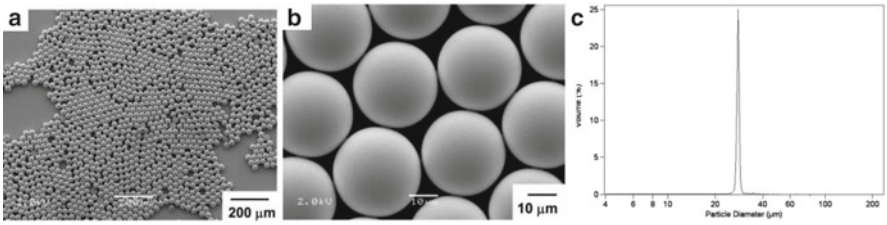

Fig. 10.8 Size distribution of microparticles generated by the microfluidic flow-focusing technique (this figure is reproduced from publication Xu et al. [51]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission)

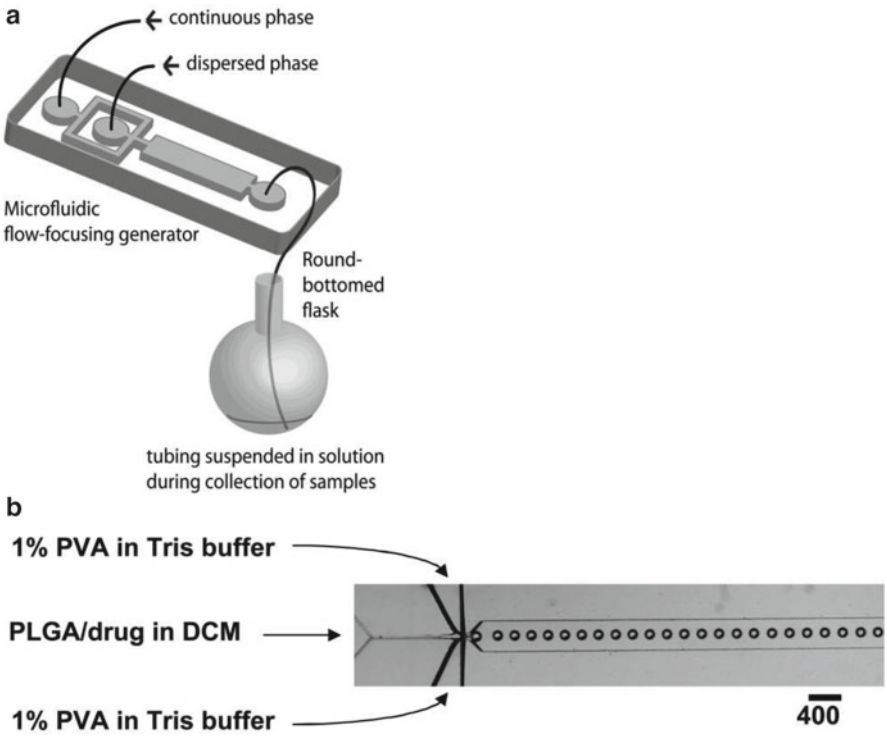

Fig. 10.9 (**a**) Schematic illustration of monodisperse polymer microparticle fabrication using a microfluidic flow-focusing generator. (**b**) Optical microscopy image of flow-focusing region (this figure is reproduced from publication Xu et al. [51]. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission)

10.5.5 Supercritical Assisted Atomization

Supercritical assisted atomization (SAA) is an alternative to the conventional jet-milling process. During the process, supercritical carbon dioxide is dissolved in a liquid drug loaded solution; this mixture is then sprayed through a nozzle [52].

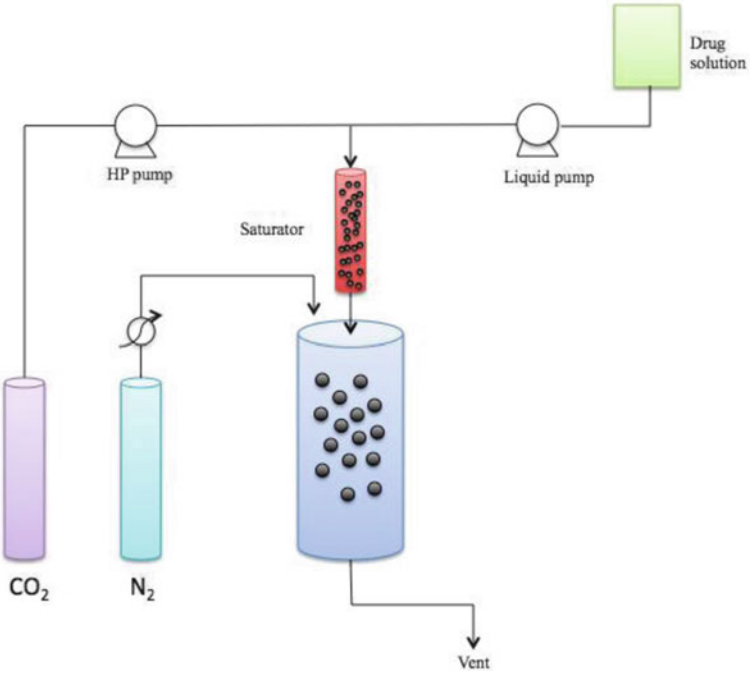

Fig. 10.10 Schematic presentation of SAA apparatus layout

Microspheres are formed as a result of atomization. Figure 10.10 is a schematic representation of the SAA apparatus. Compared to the conventional jet milling or spay drying technology, SAA is more suitable for thermo-labile compounds because the operation temperature is very close to room temperature. In addition, SAA provides better control over the particle size.

10.6 Characteristics of Microspheres

10.6.1 Drug Encapsulation Efficiency

Drug encapsulation efficiency is a parameter indicating how much drug has been encapsulated into the microspheres following preparation. It is calculated using the formula: $EE = D_L/D_{T,}$ where D_T is the total amount of drug weighed at the beginning and D_L is the actual amount of drug loaded into the microspheres. Analytical techniques, such as HPLC, are needed to determine D_L. It is sometimes difficult but always very important to ensure that the entire drug loading is determined.

A good microsphere preparation method should result in high drug encapsulation efficiency, thus minimizing the waste of materials. Many studies have been conducted to determine the key formulation parameters that significantly affect drug

encapsulation efficiency. It has been shown that the physical and chemical properties of the encapsulation polymers and solvents, as well as any drug–polymer interactions, can influence the drug encapsulation efficiency. Which formulation parameter is the most important depends on the individual formulation and the selected method of preparation. For example, in the solvent evaporation method, it has been shown that the preparation temperature can have a significant effect on the encapsulation efficiency. Within a temperature range, the two ends of this range might result in higher encapsulation efficiency. At the lowest temperature, the immiscibility between the polymer and water phase is increased resulting in quicker formation of the microsphere wall. At the highest temperature, the solvent evaporation rate is elevated, which has a similar result. Many studies need to be performed to obtain a better understanding of the impact of various parameters on encapsulation efficiency.

10.6.2 Particle Size

Microsphere particle size plays a very important role in the drug release profile and varies with different microsphere preparation methods. The smaller the particle size, the higher the surface area exposed to release media. Therefore, the rate of flux of drug out of smaller microspheres is faster compared to larger microspheres. The erosion rate of water-insoluble polymers is also greater in the case of smaller sized microspheres. Particle size distribution is more meaningful than average particle size when comparing different microsphere preparation methods. Microspheres with the same average particle size but different size distributions may show very different drug release profiles.

Many factors need to be taken into account to produce microspheres with desirable particle size/size distribution. These factors include but are not limited to: polymer type and concentration in the organic phase, the volume fraction of the dispersed phase, the identity and amount of excipients, the rate of stirring during hardening, the temperature during preparation, the speed of homogenization and the identity as well as the amount of surfactant.

Particle size can be measured by various methods including: optical microscopy, resistance and light blockage methods, light scattering, laser diffraction analysis, scanning electron microscopy (SEM) and photon correlation spectroscopy.

10.6.3 Porosity

Porosity is a very important characteristic of microspheres, as it significantly affects the drug release profiles. Pores maybe present only on the surface or in the internal polymeric matrix, or both. Porosity can be determined using electron microscopy. High porosity usually leads to undesirable high initial burst release, which may cause drug side effects due to a large dose of drug administered in a relative short period. In the case of water-soluble drugs prepared via the solvent evaporation process, the drug will tend

to diffuse into the external aqueous phase during preparation, generating channels in the internal polymeric matrix as well as pores on the microsphere surface. The size of these pores can vary up to 2,000 nm. Therefore, the entrapped drug will leach out quickly through the pores when the microspheres are exposed to an aqueous environment.

Many studies have been conducted to decrease or control porosity. For example, porosity can be decreased through the addition of salts, such as sodium chloride, into the continuous aqueous phase during the solvent evaporation process. The resultant increase in the osmotic pressure of the external phase will decrease the diffusion of internal aqueous phase, thus reducing the porosity.

10.7 Applications of Microsphere Technology

10.7.1 Controlled Delivery of Small Molecules

Many small molecules have been delivered using microsphere technology, such as immuno-suppressants, antagonists, antibiotics [53], local anesthetics [54], anti-cancer drugs [55], anti-inflammatory agents and steroids [56, 57]. These drugs are usually associated with high systemic side effects and/or have very short half-lives. Localized administration of microspheres is able to maintain the drug concentration within the theoretical window for an extended duration at the site of action, which reduces the dosing frequency, and thereby enhances patient compliance. For example, localized delivery of gentamicin, an antibiotic, in microsphere systems has been successfully used to prevent or cure acute and chronic bone infections post surgery in animal models. Some sites of action (such as joints, inner ears and cancerous tissues) are very difficult to reach following systemic drug administration. Localized parenteral delivery using microsphere systems can achieve adequate therapeutic drug concentrations at such local sites for a prolonged period of time. For instance, localized delivery of paclitaxel to joints has been successfully used for the treatment of arthritis [58].

Chemotherapeutic drugs are highly toxic and their natural distribution following systemic administration results in these drugs encountering all rapidly dividing cells, stopping their proliferation, and thus causing major side effects that prohibit further treatment. Localized delivery in microsphere formulations can be utilized to avoid systemic toxicity and ensure adequate drug levels at the tumor site [59].

10.7.2 Controlled Delivery of Macro Molecules

10.7.2.1 Protein Therapeutics

The bioavailability of orally administered proteins is very poor due to degradation in the gastrointestinal tract (GIT) as a result of the low pH in the stomach and the

presence of peptidases throughout the GIT. Currently, parenteral delivery using microsphere technology is a suitable way to deliver such molecules. Many protein therapeutics have been successfully encapsulated into microspheres and have shown therapeutic efficacy following administration [60]. There are five protein/peptide microsphere products on the US market. In addition, there is considerable research ongoing into the development of other protein microspheres. Three examples of protein microspheres under extensive investigation are described below.

10.7.2.2 Insulin

Insulin is the most important regulatory hormone that controls glucose homeostasis. According to the WHO, there are approximately 19 million diabetic patients in the United States alone and over 220 million around the world [61]. These patients depend on multiple daily insulin injections. It is very important to maintain a basal insulin level close to the normal range. Insulin loaded microspheres for long-term delivery could be very useful in maintaining insulin levels for extended periods of time without the necessity of frequent painful injections. Poly(lactic acid), poly(glycolic acid) and poly(lactic-*co*-glycolic acid) are the most commonly utilized polymers to encapsulate insulin within microspheres. In order to protect the bioactivity of insulin during the encapsulation processes, different strategies have been investigated. For example, Schmidt et al. have investigated PEGylated insulin in PLGA microspheres both in vitro and in vivo. This study showed that insulin retained full biological activity after PEGylation and PLGA encapsulation. A single injection of the PEGylated insulin loaded microspheres successfully lowered the serum glucose levels of diabetic rats to less than 200 mg/dL for approximately 9 days [62].

10.7.2.3 Heat Shock Protein

Heart failure is one of the leading causes of mortality worldwide. Heat shock protein is used to treat myocardial infarction, which is a chronic heart disease that can lead to heart failure. However, due to its low cell membrane permeability and short half-life, conventional dosage forms cannot provide efficient delivery of heat shock proteins. Therefore, frequent dosing is required to maintain the drug concentration within the therapeutic window. Encapsulation of heat shock protein within microspheres protects it from enzymatic degradation and reduces the dosing frequency. A PLGA microsphere/alginate hydrogel combination system was developed by Lee et al. to deliver heat shock proteins over prolonged time periods [63]. Different ratios of PLGA microspheres and alginate hydrogels were shown to result in a range of different release behavior. This parental controlled delivery system is an example of a minimally invasive manner to treat myocardial infarction.

10.7.2.4 Vascular Endothelial Growth Factor

Vascular endothelial growth factor has been studied extensively in animal models and human clinical trials for the treatment of vascular ischemia [64]. Like most proteins, VEGF has a very short half-life and undergoes very rapid clearance after administration. Therefore, the amount of VEGF present at the ischemic site following systemic administration is minimal. Consequently, localized controlled delivery using microspheres is an alternative method to overcome these problems. A pharmacokinetic study has been conducted comparing subcutaneous injection of VEGF microspheres with a VEGF solution [65]. The T_{max} was extended approximately 36-fold and the C_{max} was reduced by approximately 1,000 for the microsphere formulation compared to the VEGF solution. In addition, the concentration of VEGF at the subcutaneous site following microsphere injection was 36 times higher than that in the blood. However, a subcutaneous injection of VEGF solution was cleared from the site within 4 h and the majority of the VEGF was located in the blood, liver, spleen, lung and kidney. The microsphere formulation pharmacokinetic data were consistent with one absorption and single elimination phase and the VEGF profile at the subcutaneous site was modeled using a one-compartment open model with first-order absorption and elimination. Considerable research is ongoing to develop microsphere formulations containing VEGF for the treatment of vascular diseases. VEGF microspheres are also investigated to promote vascularization, and therefore ensure an adequate supply of analytes at the site of glucose biosensors. Patil et al. have shown neovascularization at the sensor site as early as 2 weeks following VEGF microsphere implantation. The number and size of the new blood vessels continued to grow over a 1 month study period [66].

10.7.3 Vaccine Delivery

Due to the advances in biotechnology, several new types of vaccines have been developed: protein subunits, peptides and plasmid DNA. Compared to whole-cell vaccines, subunit vaccines are generally safer but are usually very poorly immunogenic when administered alone. Usually several boosters along with standard adjuvants (aluminum salts and MF59) are required to ensure complete and long lasting immunity. A drug delivery device such as polymer microspheres is a promising alternative to traditional vaccination [67, 68]. These formulations are designed to release the entrapped antigens over a period of weeks to months, and thus there is no need to provide booster shots following the initial administration. Consequently, a single-shot vaccine containing microspheres would be sufficient to fully vaccinate an individual [16, 69]. Such microsphere vaccine delivery systems offer many advantages. First, microspheres are capable of protecting the encapsulated antigens from enzymatic degradation and rapid elimination. Second, prolonged release of microencapsulated or surface conjugated antigens can elicit both humoral and cellular immune responses. Third, microspheres with diameters smaller than 10 μm are readily phagocytosed by dendritic cells.

Fourth, the microspheres also act as an adjuvant attracting and activating macrophages at the site. Fifth, microspheres can also co-deliver other adjuvants (such as IL_2) along with the antigens for further potentiation of the immune system. Currently, PLGA is the most commonly used polymer for vaccine microsphere delivery.

10.7.4 DNA Delivery

Gene therapy holds tremendous potential for the treatment of disorders such as cancer and vascular disease. Although many studies have demonstrated proof of concept, the efficacy of gene therapy needs to be enhanced. Rapid nuclease degradation and short half-lives present considerable challenges. Cationic liposomes and polymers are being investigated as gene delivery vectors and much success has been achieved. These nano-sized vesicles have been engineered to promote uptake into the cells as well as endosome escape. In addition, for plasma DNA delivery, features have also been added to these vectors to facilitate nuclear uptake. Although microspheres are too large to enter cells, they are being investigated as an additional vesicle entrapping DNA complexes in order to achieve sustained delivery and therefore, prolong gene expression [70, 71].

Gene therapeutics may be susceptible to physical stress that is involved in the microsphere preparation processes. Accordingly, care should be taken to stabilize DNA during processing to preserve the bioactivity. Cationic polymers are typically used to complex DNA prior to microsphere fabrication.

10.8 Issues Associated with Microsphere Technology

In this section, issues or obstacles associated with microsphere technology are summarized.

10.8.1 Drug Instability During Microsphere Preparation

Microspheres are used to deliver both small molecules and large molecules (such as proteins, DNAs and peptides). Stability issues associated with microsphere technology more often occur in the case of protein delivery [72]. Due to their extreme sensitivity to various physical and chemical stresses, proteins often lose stability during microsphere processing, which may result in an unpredictable drug release profile such as incomplete release. In addition, toxicity and/or immunogenicity may arise as a result of the presence of protein degradation/aggregation products. Processing conditions which lead to protein instability include: (1) exposure to organic/aqueous or air/liquid interfaces [73, 74]; (2) shear stress [75]; as well as (3) local high temperature.

Until now, most microsphere preparation methods involve organic solvents. Proteins tend to undergo configurational changes upon exposure to highly hydrophobic environments [76]. In addition, as a result of their surface activity, proteins tend to adsorb at the organic/aqueous or air/liquid interfaces which are generated during the emulsification process, and this can cause irreversible aggregation through hydrophobic interactions as well as disulfide bond reshuffling. Moisture is another factor that induces protein aggregation by providing a medium for thiol-disulfide exchange. Therefore, it is important to remove water and any residual organic solvent from the formed microspheres [77]. Freeze-drying is often used to remove water, however, the resultant dehydration of proteins can cause significant irreversible conformational changes. Sugars may be used to protect proteins during freeze-drying [78].

Care must be taken to enhance protein stability during microsphere preparation. Relatively hydrophilic solvents (such as ethyl acetate) and protective excipients or anhydrous microencapsulation processes can be utilized to reduce or avoid protein aggregation [79].

10.8.2 Heterogeneous Drug Distribution

Heterogeneous drug distribution inside the microsphere polymer matrixes may cause undesired release profiles, such as unexpected high burst release. Drug migration, which may occur during the drying process and/or the storage period, is considered a main reason for heterogeneous drug distribution within microspheres. During air or vacuum drying processes, water and other residual solvents diffuse towards the matrix surface. At the same time, encapsulated drugs, especially hydrophilic drugs, also move towards the surface. Factors such as the length of the inner channels, the microsphere porosity and the location of the entrapped drug contribute to the heterogeneous drug distribution in the polymer matrix. Freeze drying has been used to quickly fix the location of hydrophilic drugs within microspheres post solvent evaporation.

10.8.3 High Burst Release Phase

Burst release is the term given to the phenomenon where an initial large bolus of drug is released immediately upon placement of microspheres in the release medium. High burst release may be desirable for certain applications, such as wound treatment. However, burst release can be pharmacologically dangerous and economically inefficient in many instances.

There are many potential factors that contribute to burst release in microsphere systems: (1) processing conditions [80, 81], (2) polymer molecular weight, (3) polymer concentration, (4) copolymer composition, (5) polymer hydrophobicity,

(6) polymer/drug interactions (surface adsorption) [82], (7) drug properties [83] and (8) morphology and porosity of the formed microspheres [84].

In the case of hydrophilic drug loaded microspheres, the high affinity/solubility of the encapsulated drug for the continuous aqueous phase results in an accumulation of drug close of the surface of the microspheres. This surface associated drug will result in a high burst release phase. In order to minimize the burst release phase, many strategies have been investigated: (1) addition of a small fraction of inert water-soluble compound into the discontinuous phase which can enhance the affinity between the drug and the organic phase thus reducing drug diffusion to surface; (2) addition of a hydrophilic polymer (e.g., PEG) into the encapsulating polymer system to facilitate internalization of the drug within the polymer phase [85]; (3) increase in polymer concentration, since high polymer concentration leads to high viscosity of the discontinuous phase which prevents/slows down the migration of the internal aqueous phase toward the continuous phase; and (4) utilization of high molecular weight polymers, which are less soluble in the organic solvent compared to low molecular weight polymers [1] which results in reduced porosity due to faster polymer precipitation; and (5) increase in the osmotic pressure across the polymer phase through the presence of salts in the external continuous phase.

10.8.4 Formulation Variations

Formulation variations often occur in microsphere preparations. The same formulation prepared by different researchers may result in different drug loadings and release profiles. Slight variation in the equipment used may translate into significant variation in the final product. For example, microsphere particle size is dependent on homogenization as well as solvent evaporation/extraction processes. These processes can differ depending on the instrumentation used.

Another issue is when a microsphere formulation platform is used for a different drug. Differences may arise due to drug properties, such as solubility, particle size, crystallinity, as well as any drug/polymer interactions. Therefore, a series of extensive tests are needed to develop and optimize the new drug formulation to achieve acceptable drug encapsulation efficiency as well as desired release profile.

10.9 Conclusions

Microsphere systems have been successfully used to deliver a wide range of pharmaceutical agents. In recent years, new technologies have been developed to overcome problems traditionally associated with microsphere drug delivery, such as burst release of drug and broad particle size distribution. These advances have allowed a wider application of this technology. There has also been significant research into understanding the design space and overcoming challenges associated with the

development of microsphere products. With the trend towards more biotechnology products together with the advances in polymer science, it is anticipated that the science in this area will continue to grow. Accordingly, it is expected that more pharmaceutical microsphere products will reach the market in the near future.

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Chapter 11
Liposomes as Carriers for Controlled Drug Delivery

Xiaoming Xu and Diane J. Burgess

Abstract Important recent technological developments in liposome research for parenteral drug delivery are discussed. The chapter is divided into two major sections. The first section provides an overview of liposome physicochemical properties and the mechanism of vesicle formation. Various liposome preparation methods are reviewed, including film hydration method, organic solvent injection method, and reverse phase evaporation. Then several drug encapsulation/loading techniques are discussed. In the second part, the use of liposomes for parenteral drug delivery is discussed with specific examples of marketed products as well as those that are currently under investigation. In particular, the in vivo fate of drug containing liposomes and several successful examples of formulation approaches to alter the drug/liposome in vivo disposition are discussed.

Abbreviations

DMPC	Dimyristoyl-phosphatidylcholine
DMPG	Dqqqimyristoyl-phosphatidylglycerol
DOPE	Dioleoyl-phosphatidylethanolamine
DPPC	Dipalmitoyl-phosphatidylcholine
DSPC	Distearoyl-phosphatidylcholine
DSPG	Distearyl phosphatidylglycerol
GV	Giant vesicles
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
MPS	Mononuclear phagocyte system

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MVV Multivesicular vesicles
RES Reticuloendothelial system
REV Reverse-phase evaporation vesicles
SUV Small unilamellar vesicles

11.1 Introduction

Since the discovery of liposomes in 1965 by Bangham and his colleagues [1, 2], scientists have applied these systems to various fields including chemistry, physics, biology, and medicine. There are well over 114,000 scientific articles describing various applications of liposomes, which indicates the interest in and importance of liposomes to the scientific society. Being composed predominantly of phospholipids and therefore resembling cellular membranes, liposomes were considered a good model to investigate membrane structure and functionality [3]. The structural similarity to cellular membranes has intrigued scientists to explore the potential of using liposomes as drug carriers to deliver therapeutics with different properties to specific parts of the body since the early 1970s [4–7]. The structural versatility of liposomes allows for the incorporation of lipid- and water-soluble compounds into different regions of liposomes [8–12] (Fig. 11.1). Recently, more and more effort has been put into understanding the in vivo fate of drug containing liposomes [13], potential applications of liposomes as drug carrier systems, as well as development of appropriate preparation methods suitable for large-scale manufacturing [14–18]. Twelve parenteral liposomal drug products have been commercialized, and more have progressed into advanced clinical trials. Currently, liposome research focuses on the following therapeutic areas: (1) systemic delivery of antifungal agents [19, 20]; (2) localized delivery for anticancer therapy [21, 22]; (3) stimulation of immune response and vaccination [23]; (4) antimicrobial/antiviral therapy [24, 25]; and (5) gene therapy [26]. Most of these therapeutic applications are intended for parenteral delivery. In addition, liposomes have also been used as carriers for the delivery of dyes to textiles [27], as biosensors to detect pesticides [28], as delivery agents for medical imaging [29], as carriers for cosmetics (sunscreens) and cosmeceuticals (antiaging agents), etc.

In this chapter, important recent technological developments in liposome research for parenteral drug delivery are discussed. The chapter is divided into two major sections. The first section is an overview of liposome physicochemical properties, the mechanism of vesicle formation as well as various drug encapsulation/loading techniques. In the second part, the use of liposomes for parenteral drug delivery is discussed with specific examples of marketed products as well as those that are currently under investigation. In particular, the in vivo fate of drug containing liposomes and several successful examples of formulation approaches to alter the drug/liposome in vivo disposition are discussed.

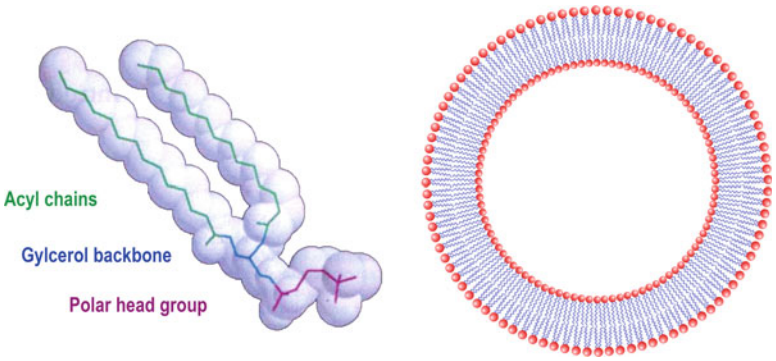


Fig. 11.1 Chemical structure of dipalmitoylphosphatidylcholine (DPPC) and schematic presentation of a liposome with a single bilayer

11.1.1 Liposome: A "Fat Body"

The name liposome comes from two Greek words: "*Lipos*" meaning fat and "*Soma*" meaning body, which describes the chemical composition and physical structure of a lipid vesicle. Chemically, liposomes are made predominantly from amphiphiles, a class of surface-active agents, which are characterized by possessing both hydrophilic (water-soluble) and hydrophobic (water-insoluble) groups on the same molecule. A typical liposome-forming amphiphile, such as lecithin, has two hydrocarbon chains (also known as hydrophobic tails) and one hydrophilic group (often named hydrophilic head) linked to three hydroxyl groups on a glycerol backbone at positions 1, 2, and 3, respectively (Fig. 11.1). Physically, liposomes are usually spherical, self-closed structures composed of one or more curved lipid bilayers (lamella) that entrap part of the solvent into their interiors. Hydrophilic groups of the lipids are on the outside and inner surfaces of the lamella, and hydrophobic groups are in the interior of the lamella. Their size may range from 20 nanometers (nm) (due to geometric constraints, liposomes <20 nm cannot exist) to several dozen micrometers (μm), while the thickness of the bilayer is around 4–5 nm.

Liposomes are generally classified based upon their lamellarity, size, charge, and functionality. A suspension of lecithin in water typically yields liposomes in the size range of 200 nm to several microns with concentric bilayers. Each bilayer is separated from the next one by an aqueous region. These liposomes are termed multilamellar vesicles (MLV). When small vesicles are trapped within the large vesicles (similar structures to multiple emulsions), the complexes are known as multivesicular vesicles (MVV). Liposomes consisting of only one lipid bilayer are named unilamellar vesicles. Depending on the particle size, the unilamellar vesicles can be further subcategorized into small unilamellar vesicles (SUV) with a diameter of 20–100 nm, and large unilamellar vesicles (LUV) with a diameter of 100–1,000 nm. Vesicles above 1,000 nm are known as giant vesicles (GV).

The advantages of liposomes used as drug carriers result from their unique biological and physicochemical properties. Most lipids used to prepare liposomes are biocompatible and biodegradable. Because of their colloidal nature, systemically administered liposomes are quickly cleared by the reticuloendothelial system (RES), which makes them an ideal vehicle for the delivery of agents to the RES. For applications that need to by-pass RES uptake, e.g., long circulating liposomes targeting to tumors, the surface of the liposomes can be chemically modified with special hydrophilic moieties, such as polyethylene glycol (PEG), thus allowing the liposome particles to be "invisible" to the natural defense systems of the body and to exhibit prolonged residence times in the blood circulation. From a physicochemical point of view, the advantage of liposomes lies in the fact that they are kinetically stabilized systems rather than thermodynamically stabilized systems [30]. In contrast to the common belief, this makes an important difference: since systems in thermodynamic equilibrium (such as micelles and microemulsions) are rapidly affected by changes in the environment, e.g., dilution, whereas kinetically stabilized systems such as liposomes are not. In addition, liposomes can be formulated as solutions, dry powders, aerosols, creams or lotions, and therefore practically all conventional administration routes can be employed, such as parenteral, oral, topical, pulmonary, etc.

11.1.2 Thermodynamic Basis of Liposome Formation

Most liposome-forming amphiphiles are not soluble in water, and instead they hydrate and form colloidal dispersions. This is because water molecules have a unique capacity for forming hydrogen bonds in addition to ordinary dipole–dipole interactions. A hydrogen bond is formed when a hydrogen atom in one water molecule is attracted by a nonbonding pair of electrons from the oxygen atom of a neighboring water molecule. In general, these hydrogen bonds are constantly formed and broken in liquid water (so-called loose hydrogen bond network), leading to a very dynamic equilibrium [31, 32]. For pure water, this network is strongly stabilized by entropy. However, in the presence of a strong hydrophobic material, e.g., phospholipid, the hydrogen bond network is disrupted and the entropy of the system is significantly reduced due to the lack of bonding between the hydrocarbon tails of the lipid with water. To counter the loss of hydrogen bonding, and to maximize the entropy, various structures of lipid aggregate are adopted [1, 33, 34] (hydrophobic effect). One of the most frequently encountered aggregate forms is the lipid bilayer: on the surface of either side are polar heads, which shield nonpolar tails in the interior of the bilayer from water. Obviously, the open ends of the bilayers cannot be tolerated, and the lipid bilayers have to close onto themselves and form closed objects.

The fundamental thermodynamic properties dictate the formation of the dispersed liposome particles, as shall be discussed in detail below. During the liposome formation process, the Gibbs energy change can be described as,

$$\Delta G = \gamma \Delta A - T\Delta S, \tag{11.1}$$

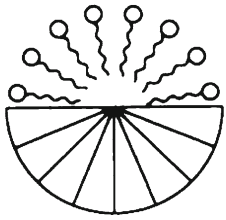
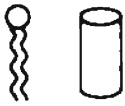
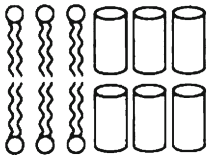
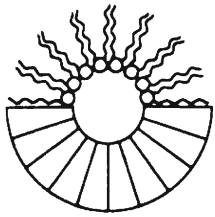
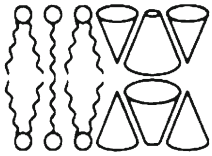
where γ is the interfacial tension, A is the interfacial area, T is the absolute temperature, and ΔS is the entropy gain of the system. After transforming from dry lipid films to liposome particles, a large number of surfaces are created. This is energetically unfavorable due to the increase of the Gibbs free energy (mostly the surface free energy). Despite the fact that most lipids are surface active, an enormous amount of Gibbs energy still exists since the reduction in interfacial tension is insufficient to compensate for the surface area increase. For example, dipalmitoylphosphatidylcholine (DPPC) only reduces the air/water interfacial tension from ~72 dyne/cm to about 25 dyne/cm. Therefore, the only way to make the transformation to liposomes energetically favorable is through the gain in entropy ($T\Delta S$) resulting from the decrease in contact between the lipid and water molecules.

Various forces are involved during this process, including hydrophobic, hydration, and electrostatic forces as well as van der Walls forces between hydrocarbon chains. Among them, hydrophobic and van der Walls forces (London dispersion forces) are attractive in nature, while hydration and electrostatic forces are normally repulsive. Typically, for each transfer of $–CH_2–$ and $–CH_3$ groups from polar to nonpolar phase, hydrophobic forces contribute ~700 and 2,100 cal/mol to the system entropy, respectively [35]. In addition to this entropy gain, the attractive dispersion forces between hydrocarbon chains and the hydration effect of the polar head group also significantly lower the ΔG of the system. If the polar head groups are charged, the contribution is even larger. The two repulsive forces, hydration forces and electrostatic forces (only in systems with nonzero surface charges), have the most influential effect on the shape of the formed liposome particles. This can be demonstrated using the packing parameter, P, which is defined as:

$$P = \frac{v}{a \cdot l}, \tag{11.2}$$

where v is the volume of the molecule, a is the polar head group area, and l the length of hydrocarbon chain(s). Table 11.1 shows some examples of surfactants with different packing parameters. In the absence of any chemical reaction, the molecular volume and length of the lipids generally exhibit a near zero change under isothermal conditions. Consequently, the packing parameter is mostly decided by the surface area of the polar head group. Since both the hydration forces (through the volume exclusion effect) and electrostatic forces (through columbic repulsion of the ion spheres) can alter the lipid polar head group area, the surface packing pattern of liposomes is therefore very sensitive to media and/or environmental changes (e.g., ionic strength, pH, and temperature). This indeed becomes the basis of using liposomes as nonviral vectors to deliver DNA and RNA intracellularly. These gene therapeutics must be delivered into the cells and must escape the endosomes to avoid degradation. Endosomal escape is realized by membrane fusion facilitated by fusogenic lipids, such as dioleoylphosphatidylethanolamine (DOPE) [36], which are pH sensitive and transform from lamellar phase ($P \approx 1$) to hexagonal phase II ($P > 1$) after entering the endosome, where the pH drops from 7.4 to 5.

Table 11.1 Effect of lipid molecular shape on the formation of colloidal aggregates

Lipids	Shape	Organization	Phase
Soaps Detergents Lysophospholipids	 Inverted cone ($P < 1/3 \sim 2/3$)	 Micelles	Hexagonal I
Phosphatidylcholine Phosphatidylserine Phosphatidylinositol Sphingomyelin	 Cylinder ($P \sim 1$)	 Bilayer	Lamellar
Phosphatidylethanolamine Phosphatidic acid Cholesterol Cardiolipin	 Cone ($P > 1$)	 Inverted micelles	Hexagonal II
Mixtures	 ($P > 1$)		Lamellar

Although the formation of liposomes is energetically favored, the system is not in a true thermodynamic equilibrium. Firstly, the key properties of liposomes (e.g., size and lamellarity) depend on the processing conditions (path dependent). For example, hydration of dry lipids in the medium for a short period of time with gentle shaking normally generates large MLV, whereas prolonged hydration under static conditions results in much smaller and less lamellar vesicles. Secondly, after liposome formation a reasonable amount of energy is locked into the vesicle bilayer as bending energy due to membrane curvature (~10 $k_B T$). As mentioned earlier, most of the lipids have a packing parameter close to one,

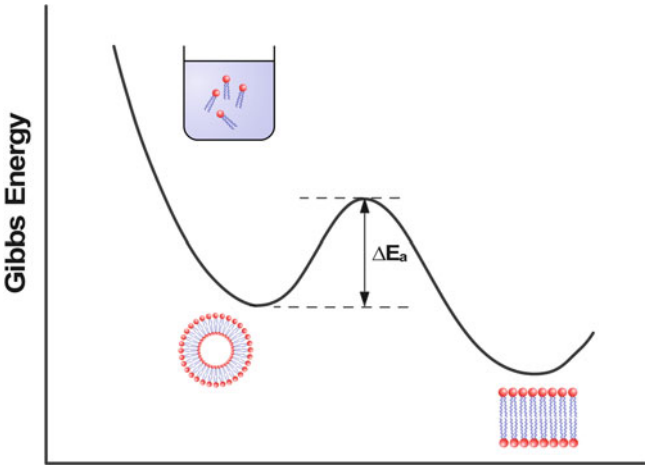


Fig. 11.2 Schematic presentation of Gibbs energy change during the formation of liposomes

and hence would favor a near zero curvature. Therefore, any form of a spherical structure requires energy input to bend the membrane, which eventually is kinetically locked into the system. As depicted in Fig. 11.2, for liposomes to reach a true thermodynamic equilibrium (flat lipid molecule aggregates with zero membrane curvature [37–39]), an energy barrier has to be crossed. This energy barrier comes from the fact that any changes in the vesicle structure would inevitably involve the process of exposing hydrocarbon chains to water, which is entropically prohibited.

11.1.3 Choice of Lipids

A wide variety of lipids are available for liposome preparation, for example, phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylglycerols (PG), sphingomyelines, etc. The lipids can be cationic, anionic, and zwitterionic as well as modified phospholipids (such as lipids capped with PEG for stealth liposomes [37]). The addition of excess cholesterol [38, 39] in the bilayers and substitution of unsaturated phospholipids with the high-melting distearoylphosphatidylcholine (DSPC) or with sphingomyelin can result in vesicles of greatly improved in vivo physical stability. It is generally believed that the greater the bilayer stability (in terms of drug retention by the vesicles), the longer the vesicle half-life in the circulation. With respect to chemical instability (hydrolysis and oxidation) of liposomes, this can be overcome by measures such as proper selection of lipids, addition of antioxidants, optimization of pH, and the addition of metal chelators [40].

11.1.4 Liposome Preparation Method

Generally, a drug containing liposome preparation process involves the following four steps: (1) formation of liposomes, (2) sizing, (3) encapsulation of drugs, and (4) purification. These four steps can be arranged in different orders or combined together to suit different purposes. In this section, the discussion is thus divided into these four parts: formation, sizing, drug encapsulation, and purification.

11.1.4.1 Formation of Liposomes

Certainly, liposomes can be prepared in a variety of ways as reviewed in numerous papers [41–44]. In this chapter, an attempt is made to categorize various preparation methods into two groups, based on the initial physical state of the lipids: hydration process (lipids initially in solid state) and precipitation process (lipids initially in solution state). The first group includes the film hydration method (from lipid bilayers to MLVs). The second group includes organic solvent injection (from true solution), detergent removal (from mixed micelles), and reverse-phase evaporation (from w/o emulsions) methods. Some of these methods are discussed in more detail below.

Film Hydration

This is still considered the simplest and also the oldest method of preparation of MLVs. Typically, dry lipids are dissolved in chloroform and added into a pear shaped flask. In case of solubility issues, up to 20 vol% methanol can be added to chloroform. Evaporation of the solvent is then performed (a rotary evaporator is commonly used to achieve this) under room or elevated temperature. This process can be assisted using a steady stream of nitrogen gas over the lipid surface [45]. After the solvent is removed (typically via high vacuum overnight), the film is hydrated by appropriate buffer solutions. The hydration process is always performed at temperatures above the phase-transition temperature, T_m, of the lipid, accompanied by agitation and vortexing. Liposomes formed by the film hydration method are predominantly large MLVs of very heterogeneous size distribution, which require further treatment to reduce the size as well as to increase homogeneity. Normally, charged lipids yield smaller and less lamellar vesicles as compared with neutral lipids. In addition to the lipid composition, processing conditions, such as pH, ionic strength, and agitation speed also vary the size distribution as mentioned earlier. For large-scale preparations (beyond 20 liters), several small batches can be mixed together. Alternatively, one can start the preparation with high lipid concentrations (up to 400 mM [16, 46]) and dilute the sample to lower concentrations to obtain larger volumes.

Organic Solvent Injection

This method involves the injection of lipid containing organic solutions (can be either water miscible such as ethanol, or water immiscible such as ether) into the aqueous medium under rigorous agitation. The lipids undergo precipitation in the form of unilamellar vesicles. The size and polydispersity of the liposomes are affected by lipid concentration, relative percentage of organic solvent to aqueous phase, and agitation speed [47]. Newer methods utilizing similar principles, such as microfluidic techniques, can generate SUVs with much greater homogeneity [48]. However, despite the fact that this method is readily scalable to large-scale preparation, some technical challenges limited its application. For example, when using water-miscible solvents such as alcohols, the resulting lipid vesicle concentration is extremely low (<5 mM) due to the limited organic solvent volume as compared to aqueous phase (<10 vol%). At higher lipid concentrations, the liposomes produced are much larger, multilamellar, and heterogeneous and in most of the applications they require additional treatment. When using water-immiscible solvents such as ether, higher liposome concentrations can be obtained (up to 10 mM), and the formed liposomes are predominantly LUVs. A very slow injection speed is required to introduce ether into the aqueous medium to avoid phase separation. In addition, regardless of solvent miscibility this method suffers from organic solvent residue issue, which may require a very long period of dialysis or diafiltration to remove the solvent.

Detergent Removal

Developed early for topical liposomal formulation, this technique utilizes the solubilization effect of lipids with detergent, forming mixed micelles [49, 50]. The detergents are then removed by methods such as dialysis, gel filtration, ultrafiltration, etc. This leads to the disruption of the mixed micelles and the precipitation of the phospholipids, in the form of polydispersed unilamellar vesicles. Commonly used detergents include bile salts, such as sodium cholate, sodium taurocholate, and sodium deoxycholate, and other ionic and nonionic surfactants such as sodium dodecyl sulfate and dodecyl maltoside. However, this technique is limited by the potential of incomplete detergent removal, which may compromise liposome stability.

Reverse-Phase Evaporation

Similar to the injection method, in this technique the lipids are also introduced into an aqueous medium from an organic solvent. However, the organic solvent is not removed immediately, and instead the two-phase system is sonicated to form a temporary stable water-in-oil (w/o) emulsion [14]. Due to their amphiphilic

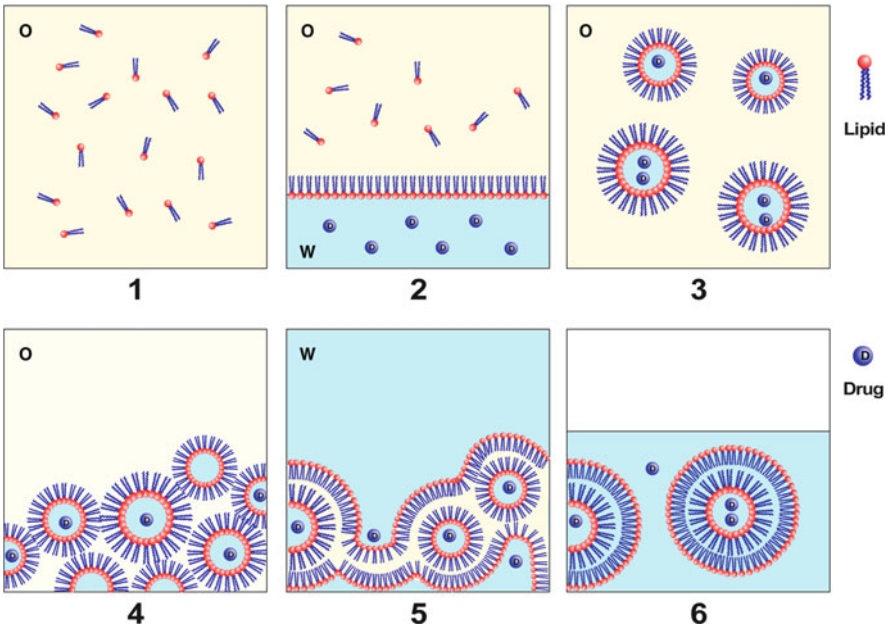


Fig. 11.3 Diagram of the formation of reverse-phase evaporation vesicles (REV)

properties, lipid molecules concentrate at the organic solvent/water interface, stabilizing the formed emulsions. Typical preparation (see Fig. 11.3) starts by drying the appropriate lipid mixtures from the chloroform solution. The dry lipids are then re-suspended in freshly washed diethyl ether or isopropyl ether (chloroform can be added if there are lipid solubility issues), at 1 ml of solvent per ml of liposomes (step 1). Then approximately one third of the aqueous phase is added and the mixture sonicated in a bath sonicator to produce stable small emulsion droplets (step 2 and 3). The solvent is carefully removed at 300–400 Torr and 20–25°C using a rotary evaporator (step 4). As the majority of the solvent is evaporated, the material first forms a viscous gel (step 5) and subsequently it becomes an aqueous suspension [14] (gel broken or phase inversion) (step 6). The remaining two thirds of aqueous medium is added at this point and vortexed gently. The residual solvent is usually removed after an additional 15 min of evaporation at 20°C. Liposomes formed by this method are predominantly polydispersed LUVs or MLVs, which may require further treatment to reduce the size as well as to increase homogeneity. The biggest advantage of this technique is the capability of producing vesicles with larger internal volume, and higher drug encapsulation efficiency (up to 65% at the size of ~300–400 nm). However, the drawback of this method is clear: the exact endpoint of phase inversion (gel broken) depends on various experimental conditions and may require practice to obtain a more reproducible process.

11.1.4.2 Size Reduction Techniques

Liposomes prepared using any of the above methods (except the microfluidic approach) typically generate heterogeneous polydispersed vesicles, either unilamellar or multilamellar. For pharmaceutical applications, the product should possess a reasonable level of homogeneity and reproducibility. Therefore, processing involving size reduction of the prepared "raw" liposomes is necessary. The following paragraphs describe various methods that are widely used. The advantages and limitations of each method are discussed.

Sonication

By applying very high energy, in the form of ultrasonic waves, to a dispersion of liposome vesicles, lipid bilayers are broken apart into small pieces. Following the same mechanism as liposome formation (Sect. 11.1.2), small fragments of bilayers rearrange to generate vesicles. The energy input can be either direct, using an ultrasonic probe, or indirect, using a bath sonicator. The potential of metal contamination is a problem with the probe method, and therefore bath sonication is preferred. However, the energy generated from the bath sonicator is too low (35–80 W) to effectively produce homogeneous SUVs.

Extrusion

This method is based on forcing large MLV through filters (polycarbonate membrane) of defined pore sizes under medium pressure (50–800 psi, normally by an inert gas) to achieve sample size reduction. In addition to size reduction, an additional benefit is that extruded liposomes are predominantly unilamellar vesicles (after 5–10 consecutive passes) [16]. Extruders are available in a range of sizes, from 1 ml (syringe extruder) to 100 ml (robust stainless steel extruder that is capable of extruding concentrated lipids of up to 400 mM). Extrusion techniques can be easily scaled up for industrial production and can be compliant to cGMPs and other regulatory requirements.

Microfluidization/Homogenization

This type of technique presses the sample through a small and adjustable orifice at extremely high pressure (up to 20,000 psi) [51], in a closed loop. After 10–100 passes, the size and lamella reduction can be easily achieved for formulations with lipid concentrations up to 300 mM. Due to the heat generated during the pressing process and the extremely high pressure, this technique may be inappropriate for some applications, such as proteins and heat sensitive drugs.

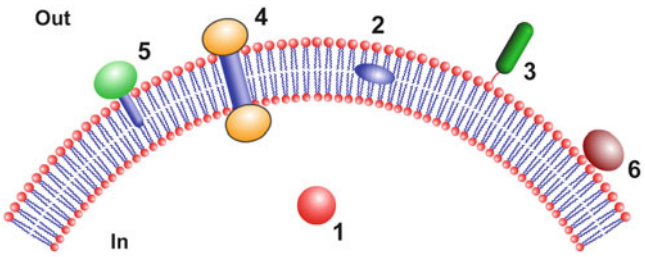


Fig. 11.4 Schematic presentation of the liposome and drug interactions: (1) entrapment in the inner aqueous core, (2) entrapment in between bilayers, (3) chemical binding to the polar head groups, (4) association with lipid bilayer while penetrating through, (5) intercalation in the polar head group region, and (6) physical adsorption onto the polar head groups

Freeze–Thaw Cycling

Repeated freezing and thawing of MLVs results in physical disruption of the liposomal phospholipid bilayers, probably due to ice crystals formed during the freezing process [46, 52]. This also serves to break apart the closely spaced lamellae of the vesicles, thereby raising the encapsulation efficiency by increasing the ratio of aqueous solute to lipid as well as promoting the solution equilibration across the bilayers. Extrusion of frozen and thawed MLVs results in production of unilamellar liposomes more readily than those made by conventional techniques [46]. Using 5,6-carboxyfluorescein as an aqueous marker, Elorza et al. showed [53] that extrusion of freeze–thaw cycled liposomes resulted in a mono-disperse population with a high internal volume. The internal volume of these liposomes was higher than that of LUVs prepared solely by extrusion through membranes with the same pore size.

11.1.4.3 Encapsulation/Loading of Drug Molecules

Drug molecules can interact with liposomes in several different ways, as shown in Fig. 11.4. Water-soluble agents can be physically entrapped in the inner aqueous compartment(s), intercalated in the polar head region(s) [8, 9], and physically or chemically attached to the polar head groups [11, 12]. These interactions depend on the solubility and polarity of the drug. In contrast, for water-insoluble drugs the only possibility of entrapment, in the absence of chemical modifications, is in the hydrophobic region (bilayers) of the liposomes. Liposomal drug encapsulation efficiency is defined as the percentage of drug encapsulated inside liposomes compared with the total amount of drug (equation 11.3). As a critical quality attribute, drug encapsulation efficiency is widely used for water-soluble drugs, and is less frequently used for water-insoluble drugs. During liposome formation, water-insoluble drugs are solubilized in the lipid bilayers and any excess is present as precipitates which are difficult to separate from the liposomes. Therefore, encapsulation efficiency is often

incorrectly reported as 100%. In addition, in some cases (e.g., drug–lipid complexes), the surface complexed drug is also considered part of the encapsulated drug.

$$EE\% = \frac{Drug_{Encapsulated}}{Drug_{Total}} \times 100\%. \tag{11.3}$$

Liposomal drug loading is defined as the percentage of drug encapsulated compared with the lipid used (drug-to-lipid ratio). For water-soluble drugs, drug loading is much less a challenge as compared with water-insoluble drugs. This is because for water-soluble drugs drug loading can be easily improved by increasing the drug concentration. On the other hand, for water-insoluble drugs, drug loading depends on various formulation variables, including the solubility of the compound inside the lipid bilayers, morphology of the liposomes (lamellarity), and processing conditions. It is therefore a critical formulation parameter that requires optimization.

Encapsulation of Water-Soluble Actives

Water-soluble drugs are encapsulated spontaneously (passively) into the inner aqueous core of the liposomes or in between the adjacent bilayers. However, the efficiency of passive drug encapsulation is low. This is because the encapsulation process is predominantly a result of the drug diffusion (distribution) process, and is dependent on the volume of internal and external aqueous phases. To achieve a higher drug encapsulation, at least three approaches can be used: (1) increasing the internal aqueous volume while maintaining the same lipid concentration; (2) increasing the liposome vesicle population while keeping the liposome particle size constant; and (3) facilitating the distribution (diffusion) of the drug through the bilayers (e.g., using freeze–thaw cycling which repeatedly breaks and re-forms the vesicles during which the solute can become homogenized [17, 52]). Generally, these approaches can be combined together to achieve optimal drug encapsulation.

In addition to the passive drug encapsulation, pH-induced transmembrane mass transport of the drug (also known as the pH remote loading technique) has been developed [21, 54]. The most successful example of this technique is the commercial product Doxil®. As shown in Fig. 11.5, this approach is based on the observation that the deprotonated form of doxorubicin has a higher permeability across the liposome bilayers relative to the protonated form. This process typically involves the formation of MLV liposomes via film hydration and extrusion in the presence of 250 mM ammonium sulfate. The external ammonium sulfate is subsequently replaced with a nonelectrolyte solution of sucrose via diafiltration. This creates a transmembrane gradient for ammonium ions. A solution of the hydrochloride salt of doxorubicin is then added to the external medium to establish an equilibrium between the protonated and deprotonated form of the primary amine group of doxorubicin. Owning to its greater hydrophobicity, the deprotonated form of doxorubicin rapidly diffuses through the bilayers. Once a molecule of doxorubicin

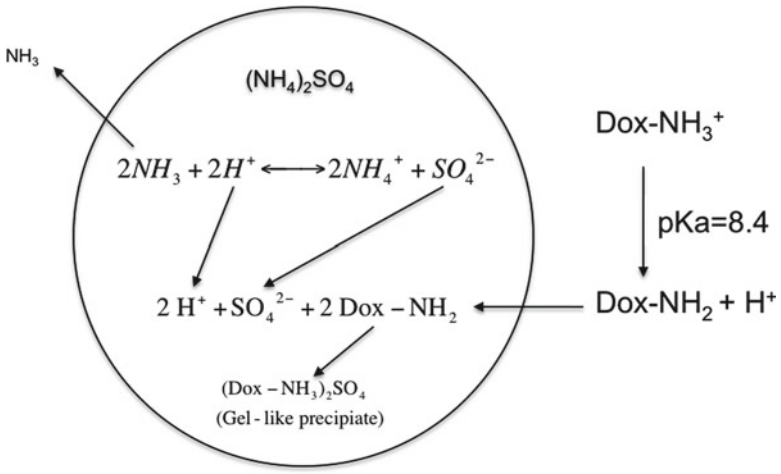


Fig. 11.5 Schematic presentation of pH remote loading method for encapsulating doxorubicin into Doxil

enters the liposome and complexes with sulfate ions, an insoluble sulfate salt forms and precipitates as a gel-like structure. This process continues until virtually all of the doxorubicin is transported to the inside of liposomes. However, this active loading approach does not apply to all drug candidates, and is only suitable for the compounds that have a primary amine group (p*K*a > 8) and are capable of forming precipitates inside liposomes.

Encapsulation of Water-Insoluble Actives

Hydrophobic drugs are normally dissolved in an appropriate solvent system together with the lipids and deposited onto the flask wall to form a mixed lipid-drug film. Upon hydration of the film, hydrophobic drugs are easily entrapped into the lipid membranes. Generally, MLVs are preferred due to the high lipid-to-volume ratio, which allows more drug entrapment per given volume.

The most critical parameter for the entrapment of hydrophobic drugs into liposomes is the drug partition coefficient between lipid and water, typically expressed as LogP or LogD (if the drug is ionizable at different pH conditions). For LogP values above 3, the entrapment process is very straightforward. For instance, a liposome dispersion consisting of 10% (w/w) lipid will retain 99.9% of the drug if the LogP of this drug is 3.

It is difficult to retain the drugs with LogP values smaller than 2 inside liposome bilayers. The LogP of hydrophobic drugs also affects the stability of the liposome preparations on dilution. In the blood, liposomes are typically diluted up to 6,000 times and drugs with low LogP values will rapidly leak out during the circulation.

Table 11.2 Comparison of five different purification techniques

Method	Mechanism	Advantages	Disadvantages
Dialysis	Membrane separation	• High efficiency (>99%) • Easy sample collection	Time consuming
Centrifugation	Centrifugal force	• Easy operation	Low efficiency
Ultra-centrifugation	Centrifugal force facilitated membrane separation	• High efficiency (>90%) • Less processing time	Difficult sample collection
Diafiltration	Membrane separation	• High efficiency (>99%) • Easy sample collection	Tangential flow rate needs to be optimized
Gel filtration	Size exclusion	• High efficiency (90–99.99%) • Less processing time	Sample dilution

11.1.4.4 Purification Method

Freshly prepared drug containing liposomes always contain some un-encapsulated free drug. Free drug may cause liposome stability issues during storage due to drug–liposome interactions at the surface (e.g., charge–charge interactions). Also free drug may result in unwanted systemic toxicity upon administration to patients due to high drug exposure. Therefore, it is crucial to remove any free drug following preparation to obtain purified liposomal drug product to maintain product quality and safety as well as preparation consistency. Currently, at least five techniques are suitable for purification processes. Table 11.2 summarizes the advantages and disadvantages of these five techniques.

11.2 In Vivo Fate of Liposomes

Drug loaded liposomes can be administered via many different routes, such as parenteral injection, oral [55, 56], topical [57, 58], pulmonary [59, 60], etc. The most common route is parenteral administration with the intravenous (i.v.) being used more often than intramuscular (i.m.), subcutaneous (s.c.), or intraperitoneal (i.p.). In this section, only the i.v. injection route is described. However, the fate of s.c., i.m., or i.p. injected liposomes is very similar since liposomes drain from these injection sites into the blood circulation predominantly via lymphatics.

After liposomes enter the blood stream, there are predominantly two reactions: lipid exchange with the blood lipoproteins and adsorption of marker macromolecules (opsonins). The lipid exchange results in removal of lipids from the liposome surface by blood lipoproteins, mostly the plasma high-density lipoproteins (HDL) [61, 62].

This leads to a significant reduction in liposome membrane stability and consequently substantial amount of drug leaks. Various factors, including lower lipid concentration (higher degree of dilution), larger particle size, and use of lipids with lower phase transition temperatures (containing unsaturated bonds and/or shorter chains) have been reported to contribute to a faster lipid exchange rate. This results in a higher degree of membrane instability and consequent loss of drug content. The addition of excess cholesterol (20–50 mol%) [38, 39] as well as substitution of unsaturated phospholipids with the high-melting saturated lipids (such as distearoyl phosphatidylcholine (DSPC)) have both been demonstrated to significantly reduce lipid exchange and result in vesicles with greatly improved in vivo stability.

Opsonization involves the binding of opsonins (such as immunoglobins and mannose-binding lectins) to liposome surfaces via either electrostatic interaction or simple adsorption. The attached opsonins further interact with the receptors on macrophages, causing them to endocytose the liposomes [63, 64] and clear them from the blood stream. There are many different types of macrophages, residing in the liver, spleen, lungs, gastrointestinal tract, skin, and close to other portals of entry. They also circulate as monocytes in blood and are commonly referred to as the RES or as mononuclear phagocyte system (MPS).

11.2.1 Passive Targeting of Liposomes to Tumor Tissues

As well as the passive targeting to RES as described earlier, liposomes can also be passively targeted to tumor tissues and sites of inflammation. For intravenous injected liposomes intended for extravascular targets, the biggest challenge is to cross the vascular wall, which is composed of a continuous layer of endothelial cells supported by a base membrane. This barrier virtually excludes extravasation of any particles including liposomes. Therefore, under normal physiological conditions, extravasation is restricted to a few selected sites where the endothelial lining is discontinuous, such as in the liver and spleen tissues. In areas where there is significant neovascularization (such as tumors and tissues under inflammatory conditions), the vasculature is uneven and the intercellular spaces are abnormally wide [65]. This allows small particles approximately 100 nm or less, which includes small liposomes, to exit these “leaky” blood vessels, allowing selective accumulation in these tissues. This phenomenon is known as the enhanced permeability and retention (EPR) effect [66]. It serves as the basis for passive targeting of long circulating anticancer liposomes to tumor tissues (refer to Sect. 11.3.2).

11.2.2 Active Targeting

Liposomes (mostly PEGylated liposomes) are modified with surface target-specific moieties, such as antibodies [67], immunoglobulins [68, 69], lectins [70], transferrin [71], folate [72], etc. to facilitate recognition and internalization into the target cells.

For example, the IgG monoclonal antibody (34A) has been linked to liposomes to target to the surface glycoprotein receptor (gp112) on the pulmonary endothelial cells of the mouse lung. It has been shown that more than 50% of the total dose could be found in the lungs after 30 min [73]. It is known that in various cancer tissues, some of the receptors are overly expressed. These receptors can interact with specific ligands which can be bound onto liposome surfaces to achieve tumor targeting. For example, prostate-specific membrane antigen (PSMA) (a class II transmembrane glycoprotein) is known to be over-expressed in cancerous prostate tissue [74]. Human epidermal growth factor receptor 2 (HER-2) has been shown to be over-expressed in various cancers including breast cancer, ovarian cancer, stomach cancer. PSMA and HER-2 can be used to achieve active targeting of liposomes. Anti-HER-2 containing liposomes, with encapsulated doxorubicin, exhibited significantly increased therapeutic efficacy towards four different breast cancer xenograft models when compared to naked PEG–liposomes [75].

Several critical issues exist which significantly hinder the therapeutic use of actively targeted liposomes. These include: (1) targeting moiety bearing liposomes may bind to the first line of target cells (after accumulating in the interstitial compartment), which may obstruct the pathway for accumulation of subsequent liposomes [76]; (2) antibody attachment may destabilize the steric protection thus exhibiting enhanced liposome clearance [77]; and (3) after internalization by endocytosis the drug has to escape the endosomes/lysosomes before being degraded. Overall, compared with the passive targeting technique, active targeting is at a much less advanced stage of development, and so far there are no products on the market related to active targeting.

11.3 In Vivo Applications of Liposomes

11.3.1 Liposomes for Treatment of Infectious Diseases

Infectious diseases typically result from the invasion of pathogenic microorganisms, such as bacteria, viruses, fungi, protozoa, multicellular parasites, and prions (aberrant proteins) to the body of the host [78, 79]. Depending on the type of pathogens as well as the location of the infected tissues, various drug candidates have been developed for the treatment of these diseases. While there has been significant progress and success in the treatment and prevention of bacterial infections, current options for antifungal and antiviral therapy are still much less satisfactory, mostly due to the following two reasons: (1) limited access of small molecules to the intracellular tissues where the pathogens reside; and (2) severe toxicities associated with nonspecific distribution of therapeutics to unwanted organs and tissues [80, 81]. As foreign particles, liposomes are taken up by phagocytic cells (which also engulf bacteria, viruses, and fungi), and hence they offer a unique opportunity to deliver drug molecules directly to the site of infection (passive targeting). In this section, liposomes intended for antifungal and antiviral therapy are discussed.

11.3.1.1 Antifungal Liposomes

The number of systemic fungal infections reported in immunocompromised patients has continued to grow as a result of (1) increasing numbers of bone marrow and solid organ transplantations; (2) the acquired immunodeficiency syndrome (AIDS) epidemic; and (3) the intensive use of antineoplastic therapies [82]. To date, the most widely used fungicidal agent is amphotericin B, a polyene antibiotic, which was isolated from *S. nodosus* found in the vicinity of the Orinoco River in Venezuela in 1955 [83]. The mechanism of the fungicidal activity of amphotericin B originates from the fact that it specifically binds to sterols (primarily ergosterol) in fungal cell membranes. This causes leakage of intracellular contents of fungi and subsequent cell death due to changes in membrane permeability. Despite being very effective, amphotericin B suffers from severe dose-dependent toxicities, including fever, chills, rigors, nausea, vomiting, and hypokalemia. In addition, because the drug is insoluble in water, its administration becomes very difficult and some of the commercial formulations of amphotericin B utilize deoxycholate micelles to overcome the solubility issue.

The observations above make amphotericin B an ideal candidate for liposome encapsulation. Liposomes do not accumulate in the kidneys and central nervous system thus avoiding the most severe side effects associated with this drug. In addition, amphotericin B is readily solubilized within liposome bilayers. Most importantly, amphotericin B liposomes can accumulate in the same cells where fungi reside, thus substantially increasing the drug's therapeutic index.

Currently, three lipid based amphotericin B products are available for the treatment of systemic fungal infections. These three formulations differ markedly in terms of formulation design but all have demonstrated reduced toxicity while maintaining the drug's antifungal efficacy.

Abelcet® is a lyophilized drug–lipid complex product, containing fluid phospholipids, such as dimyristoylphosphatidylglycerol (DMPG) and dimyristoylphosphatidylcholine (DMPC). It is a suspension of ribbon-like structures in water and closely resembles the ribbon-like lyotropic mesophases reported in several phase diagrams by Luzzati and coworkers in the early 60s. The biggest challenge associated with the preparation of drug–lipid complexes is control of the particle size (1–10 μm).

Amphotec® (or Amphocil®) is another lyophilized drug–lipid complex product, developed based on the observation that amphotericin B binds strongly to sterols (such as cholesterol). It is prepared by weighing equimolar amounts of amphotericin B and cholesterol sulfate, dissolving them in DMSO at 50°C and isothermally injecting the solution into a buffer at pH 7.4. The DMSO is subsequently removed via diafiltration. Lactose is added as a cryoprotectant before sterile filtration and lyophilization. The structure of the complex has been confirmed to be disk-like, with extremely high thermodynamic stability [84].

Despite the fact that both of the above two products originated from the concept of using liposome's solubilization, passive RES targeting, and site avoidance properties, they are not considered true liposomal drug products. The only antifungal liposomal product commercially available is AmBisome®. AmBisome is a lyophilized

SUV liposome product, consisting of phosphatidylcholine, cholesterol, distearylphosphatidylglycerol (DSPG), and amphotericin B (in a molar ratio of 2:1:0.8:0.4). The size of the SUVs is around 80 nm. Because of their extremely small size, these SUVs exhibit prolonged blood circulation times and hence require less frequent dosing. This is another advantage of this product (in addition to the enhanced solubility and reduced toxicity as discussed earlier).

11.3.1.2 Antiviral Liposomes

Current therapy copes rather well with viruses located in the blood stream. However, for viruses preferentially distributed in the lymphocytes and macrophages, the existing drug delivery regimen lacks adequate efficiency and suffers from dose-dependent toxicity. Some of the most common viruses include the human immunodeficiency virus (HIV), which is the virus that causes AIDS; the herpes simplex virus, which causes cold sores, smallpox, and multiple sclerosis; hepatitis viruses which can develop into a chronic viral infection that leads to liver cancer [85]; and the human papilloma virus, now believed to be a leading cause of cervical cancer in women [86, 87].

Based on their ability to passively target to RES and deliver the encapsulated drugs directly across the cellular membranes, liposomes hold promise as a potential antiviral drug delivery system.

The effect of azidothymidine (AZT) containing liposomes in mice was determined in the early studies [88, 89]. Unlike free AZT, liposomal encapsulated AZT demonstrated no bone marrow toxicity with normal erythrocyte and leukocyte profiles and exhibited reduced hematopoietic toxicity. In addition, increased accumulation of AZT liposomes in the liver, spleen, and lung was observed and resulted in enhanced antiretroviral activity. Other examples of using conventional liposomes to target drugs to the RES include didanosine (ddI) [90], zalcitabine (ddC) [91], stavudine (D4T) [25], tenofovir (PMPA) [92], etc. All of these have demonstrated enhanced liver and spleen tissue accumulation and most have resulted in elevated antiviral activity relative to the free drug.

11.3.2 Long Circulating Liposomes for Anticancer Therapy

The development of long circulating liposomes, also known as Stealth™ liposomes, represents a milestone in liposomal delivery research [93, 94]. This type of liposome can circumvent immune surveillance and recognition by the body as foreign and hence avoid opsonization and phagocytic uptake. They can circulate in the blood stream for a prolonged time before accumulating at the target regions, with half-lives of up to several days compared with just minutes for conventional liposomes. This effect is mostly attributed to the steric stabilization induced by the incorporated PEGylated lipids, a class of synthesized lipids composed of polyethylene

glycol-phosphatidylethanolamine (PEG-PE). These lipids typically have PEG chains of molecular weights between 1,000 and 2,000, extending approximately 5–6 nm from the liposome surface, and generally are used at a density of about 5–8% of total lipid in a formulation [95]. The most notable example of long circulating liposomes is Doxil®, a PEGylated liposome product containing doxorubicin. The size of the Doxil liposome is around 100 nm, and compared with the free drug, liposome formulations of doxorubicin exhibited much less toxicity and a higher efficiency as a result of passive tumor targeting. The reader is referred to the separate chapter in this book on PEGlyation of nanoparticles.

11.3.3 Liposomes for Gene Therapy

Gene therapy is emerging as one of the most promising strategies for the treatment of human diseases. More than 1,300 gene therapy protocols have either been approved or implemented [96]. However, despite such promising developments, the complete potential of gene therapy remains to be exploited. Lack of safe and efficient delivery systems for the targeted transfer of DNA into cells is one of the major obstacles in developing pharmaceutical treatments based on gene therapy.

Both viral and nonviral vectors have been developed for in vivo delivery of DNA-based therapeutics (such as plasmids for gene expression, antisense oligonucleotides, siRNAs, ribozyme, etc.). Generally viral vectors, such as attenuated replication-defective viruses, have high gene transfection efficiency due to the natural mechanism of targeted introduction of DNA into cells. However, a number of potential problems have limited the use of viral vectors in pharmaceutical applications. These include potential to provoke mutagenesis and carcinogenesis, possibility of inducing immune response, high cost and difficulty in manufacturing, as well as low shelf life. For these reasons, nonviral vectors have been widely investigated and used in clinical trials. However, they have much lower transfection efficiencies compared with viral vectors. Among the various nonviral vectors, cationic liposomes (lipoplexes) have been extensively investigated and through the incorporation of various agents their uptake and transfection efficiencies have been improved [97–100]. For example, fusogenic, neutral lipids, such as DOPE has been added to enable endosomal escape [101, 102]. Targeting moieties such as transferrin (Tf) for receptor-mediated endocytosis and antibodies for tumor targeting have been added to enhance cellular uptake [103–105]. PEGylated liposomes have been used to prolong circulation times and thereby increase the possibility of cellular uptake [106–109]. Lipofectamine™2000 is reportedly the most efficient and widely used of the marketed cationic liposomes for in vitro transfection [110]. Cationic liposomes as well as the cationic polymers that have been used as nonviral vectors can cause cellular toxicity due to interaction between the positive charge and the negatively charged cellular membrane. Recently our group [111, 112] and others [113, 114] have reported the use of anioinic lipoplexes as an alternative to cationic lipoplexes and polymers. These anionic lipoplexes are a ternary complex of DNA and anionic lipids mediated via microcations. Similar

or better transfection efficiencies and significantly improved cell viability has been demonstrated using optimized Ca^{2+}/DNA/lipid formulation in a CHO-K1 cell model when compared to Lipofectamine™2000 [112].

11.4 Conclusion

After nearly five decades of research effort, liposomal drug products have progressed beyond the experimental stage and started to emerge as reliable and clinically viable systems for the delivery of a wide range of pharmaceutical actives. In several areas, they have already proven to be much more effective delivery systems including anticancer therapy, antifungal and antiviral therapy. In some other areas, they have begun to show promise such as in gene therapy and vaccination (the reader is referred to the chapter in this book on delivery of vaccines). As the understanding of the impact of human genetics in disease and the knowledge necessary for producing quality products evolve, this type of novel drug delivery system will become even more prevalent, and will play a crucial role in achieving the goal of personalized medicine.

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Chapter 12
Micro- and Nanoemulsions (Controlled Release Parenteral Drug Delivery Systems)

Jacqueline M. Morais and Diane J. Burgess

Abstract Parenteral emulsions may be utilized to overcome major formulation challenges such as solubilization of poorly aqueous-soluble drugs and/or protection of drugs susceptible to hydrolysis. In addition, they can be used for the purposes of controlled and sustained drug release, drug targeting, and/or reduction of toxic effects to sensitive tissues. Emulsions are thermodynamically unstable and do not form spontaneously; however, emulsion stability can be substantially improved using suitable emulsifiers and viscosity enhancing agents. Drug release rates from emulsion systems are determined by both the carrier and the drug characteristics. It is not an easy task to characterize in vitro drug release from emulsions due to the physical obstacles associated with separation of the dispersed and continuous phases; however, various techniques have been successfully used. This chapter provides an overview of the physicochemical properties, methods of preparation, stability, and application of emulsion formulations.

12.1 Introduction

Emulsions are well-accepted carriers for both hydrophilic and lipophilic substances and have been widely used in cosmetic and pharmaceutical fields [1]. An emulsion is a heterogeneous system consisting of at least two immiscible or partially immiscible liquids, one of which is dispersed (internal phase) in the other (external or continuous phase) in the form of droplets, with a third component (emulsifier) used to stabilize the dispersed phase droplets [2–4]. Emulsions for parenteral administration were not developed until after World War II, when they were used to supply calories and essential fatty acids to patients unable to tolerate an oral diet. However,

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the use of emulsions as drug delivery systems started only in the 1970s with the incorporation of drugs such as barbituric acid into o/w emulsions [5–7]. For many drugs intended for the parenteral delivery route, insufficient aqueous solubility and/or water hydrolysis are major formulation challenges. The use of an emulsion delivery system can reduce or even overcome these problems by incorporating the drug into the internal phase [8–10]. Recent progress in controlling droplet size distribution and understanding both preparation processes and stabilization mechanisms has resulted in renewed attention to these liquid dispersed systems. In addition, their potential to provide sustained, controlled or targeted release has been recently investigated [1, 11–13].

Another important parenteral application of emulsions is perfluorocarborn (PFC) emulsions as artificial blood substitutes [5, 10]. These emulsions contain submicron fluoro-organic particles in water. They are capable of dissolving large amounts of oxygen and were developed as oxygen carriers or blood supplements, in order to reduce patient exposure to hazards that can be associated with human blood products [14]. Examples of PFC emulsions are Fluosol DA® (Green Cross Osaka, Japan) and Oxygent® (Alliance Pharmaceutical Corp., San Diego). Unfortunately, neither of these products is currently on the market due to stability issues and high production costs.

12.2 Parenteral Nutrition

The term parenteral has a Greek origin, para meaning besides or other than, and enteron meaning gut. Therefore, any method of administration of substances to the body that does not include passage through the gastrointestinal (GI) tract is correctly referred to as parenteral delivery [5, 7, 10]. Parenteral nutrition (PN) is a means of providing intravenous nutrition to patients who are unable to ingest and absorb nutrients via the GI tract. The majority of marketed IV emulsions for total parenteral nutrition (TPN) administration consist of vegetable oils dispersed in aqueous solutions using egg phospholipids as emulsifier. Usually, they are composed of soybean oil (~10–20%) emulsified into glycerol solutions, for example Liposyn™ II (Abbot) utilizes a blend of safflower and soybean oils (50:50) as the internal phase. Other TPN commercial formulations are Intralipid 10%, Intralipid 20% (Kabi Vitrum), Lipofundin, and Lipofundin S (Braun) [7, 8, 15].

12.3 Types of Emulsions and Emulsion Stability

Emulsions are thermodynamically unstable and do not form spontaneously [2–4, 12]. In accordance with the second law of thermodynamics they are subject to instability processes such as flocculation, creaming, coalescence, and even eventual phase separation. However, emulsion stability can be substantially improved using suitable emulsifiers that are capable of forming a mono- or multilayer coating film (mechanical barrier) around the dispersed droplets, and/or increasing the

droplet/droplet repulsion (electrosteric barrier) [3, 16, 17]. Depending on the component concentrations; oil/water phase ratio, emulsifier(s) used and the temperature, macroemulsions (oil-in-water (o/w) or water-in-oil (w/o)), nanoemulsions (o/w or w/o), or multiple emulsions (o/w/o and w/o/w) may form. The processing conditions also affect emulsion formation and depending on the particle size, emulsions may be classified as coarse emulsions, microemulsions, submicron, or nanoemulsions.

An emulsion is considered stable when there are no significant changes in certain parameters such as: the number of droplets of the disperse phase; mean droplet size and droplet size distribution; total interfacial area; chemical composition and other related parameters [16, 18]. Emulsion stability is also related to the rate of change of droplet size. Other methods used to determine emulsion stability include accelerating the separation process, which normally takes place under storage conditions [2, 9, 19, 20]. These methods include high and freezing temperatures, freeze–thaw cycling, and centrifugation. Burgess et al. in a series of publications have reported a method to predict emulsion stability based on evaluation of interfacial properties (elasticity, tension, and charge) [21–26].

Rearrangements of the emulsion structure can occur in order to reduce the interfacial area and can lead to instability [3, 16, 27]. Emulsions can be destabilized through one of the following mechanisms: (1) *Creaming*. Creaming occurs due to the density difference between the dispersed and continuous phases, the lighter phase has a tendency to concentrate on the top. In addition, the creaming rate is dependent on the particle size as well as the volume fraction of the dispersed phase (Φ). Creaming is dominant at Φ values between 0.1 and 0.5; (2) *Flocculation*. Flocculation is defined as a process by which two or more droplets aggregate without losing their individual identity and is dependent on the interdroplet forces. Accordingly, the rate of flocculation can be affected by the pH and ionic strength of the aqueous environment [3, 4]. Droplets >2 μm tend to flocculate rapidly [27]. Bridging flocculation occurs as a result of adsorption of macromolecules (when present at relatively low concentrations) on two or more emulsion droplets; (3) *Coalescence*. Coalescence is the process in which two or more droplets collide and form a larger droplet. Coalescence involves breaking the interfacial film around emulsion droplets, is irreversible and dominates when the Φ value is high. Various factors, such as emulsifier solubility, pH, presence of salts, emulsifier concentration, phase–volume ratio, temperature, and interfacial film properties, affect coalescence [12, 27, 28]; and (4) *Ostwald ripening.* Ostwald ripening occurs in emulsions with polydispersed droplets and results from differences in the mobility of molecules at the droplet interfaces. It is easier for smaller droplets to give up interfacial molecules and for larger droplets to adsorb molecules. As a result, small droplets become smaller and eventually disappear, while larger droplets continue to grow until there is complete phase separation [16, 20].

In vivo stability of emulsions (biocompatibility) can be correlated to the net charge on the droplet surfaces. In general, a more electronegative surface exhibits a reduced tendency to aggregate in the presence of blood proteins. An ideal biocompatible emulsion is also isotonic, i.e., containing 280–300 mOsm/kg [29, 30].

12.3.1 Nanoemulsions

Submicron emulsions and nanoemulsions have gained increasing attention as drug and other bioactive carriers not only for parenteral and ocular but also for topical, dermocosmetic (e.g., vitamins and antiaging agents), and transdermal applications [1, 20, 31]. Nanoemulsions consist of very fine droplets with a typical size range of 20–200 nm and are usually prepared via high-pressure homogenization [19, 31]. Nanoemulsions are generally more stable than macroemulsions, since their small size makes them less susceptible to creaming or sedimentation phenomena [16, 19]. Nanoemulsions also have the advantage that they can be produced using more practical surfactant concentrations e.g., 5.0%, compared to ~50% that is typically used to prepare microemulsions [20]. It is important to mention that the surfactant concentration in emulsion formulations should be as minimal as possible irrespective of its nature, origin, and type [32]. For example at high concentrations, CremophorEL® (PEG35-castor oil) can cause anaphylactic shock and histamine release [33], whereas polyoxyethylene alkyl ethers can cause hemolysis [34] following parenteral administration.

Nanoemulsions are expected to penetrate deeper into tissues through fine capillaries and even cross the fenestrations present in the epithelial lining in liver. This allows efficient delivery of therapeutic agents to target sites in the body. Nanoemulsions have recently been applied as adjuvants to enhance the potency of DNA vaccines. Ott et al. [35] prepared a cationic o/w submicron emulsion based on MF59 (commercially termed as Fluad®), squalene in water and the cationic lipid DOTAP (1, 2-dioleoyl-*sn*-glycero-3-trimethylammonium-propane). This formulation resulted in a higher antibody response in mice compared to the naked DNA at the same dose.

12.3.2 Multiple Emulsions

Multiple emulsions are complex and heterogeneous systems in which both types of simple emulsions (O/W and W/O) coexist simultaneously, forming either W/O/W or O/W/O emulsions in the presence of two stabilizing surfactants, one hydrophilic and another lipophilic [36–38]. They have potential applications as pharmaceutical and cosmetic delivery vehicles due to their ability to entrap and slowly release different agents [39–42]. However, their use has been restricted due to their intrinsic instability and complex structure [36, 43, 44].

There are few reports on in vivo drug release from w/o/w emulsions for the parenteral administration route, which could be explained by both the poor long-term stability and the low drug entrapment efficiency of these systems. In addition, the exact mechanisms of drug release from multiple emulsions and following parenteral administration are not completely understood [45].

12.3.3 Microemulsions

In contrast to macro-, nano-, and multiple emulsions, microemulsions form spontaneously and are thermodynamically stable. These systems consist of at least four components: oil phase, water phase, surfactants, and cosurfactants. When a mixture of surfactant and cosurfactant is added to a biphasic oil/water system, a thermodynamically stable, optically transparent or translucent, low viscosity, and isotropic mixture spontaneously forms [46, 47].

Microemulsions occur only under select circumstances (specific areas of the phase diagram). Accordingly, it is necessary to fine-tune process and formulation space variables to obtain optimum conditions for the microemulsion formation. The use of high surfactant concentrations and cosurfactants is typically required [47].

The transparency of these systems arises from their small droplet diameter (~10–100 nm). However, microemulsions cannot be structurally considered as only a dispersion system of very small droplets, but rather as a single percolated phase containing micelles or reverse micelles, water, or oil droplets and bicontinuous structures in which there is no internal or external phase [47, 48]. The term "microemulsion" is somewhat deceiving, though, as the structures often diverge from the static spherical droplet shapes of regular emulsions. Microemulsions are dynamic systems in which the interface is continuously and spontaneously fluctuating [46, 47, 49].

Microemulsions have demonstrated potential as vehicles for parenteral delivery of hydrophobic drugs. These systems can be an alternative to cosolvent-based parenteral formulations for delivery of cytotoxic, antifungal, and anti-inflammatory agents [32]. He et al. [50] developed a microemulsion containing paclitaxel, lecithin, ethanol, poloxamer 188, and CremophorEL® for parenteral delivery. In addition, the potential of this formulation to produce hypersensitivity reactions was evaluated in vivo. Paclitaxel microemulsions demonstrated significantly less hypersensitivity reactions when compared to Taxol® due to its lower CremophorEL® content, demonstrating the tolerability improvement of the therapeutic agent. Moreno et al. [51], in a series of investigations, evaluated the impact of microemulsions on the acute toxicity, efficacy, and in vivo tolerability of amphotericin B and indicated the advantage of microemulsion structures over the micellar formulation (Fungizone®). Wang et al. [52] investigated submicron lipid emulsions for parenteral drug delivery using nalbuphine as model drug. Submicron emulsions were prepared using egg phospholipid as the main emulsifier with Brij 30, Brij 98, and stearylamine as coemulsifiers. The authors showed that the loading of nalbuphine into lipid emulsions resulted in slower and sustained release of nalbuphine.

12.4 Preparation of Emulsions

Emulsification is usually achieved by the application of mechanical energy, where the interface between the two phases is deformed and subsequently broken. In order to prepare emulsions, the following aspects should be considered: (1) material chosen,

(2) addition of the emulsifier, (3) addition of phases, (4) temperature, and (5) agitation technique [2, 3, 12]. Commercial emulsions are produced by a wide range of agitation techniques, from simple mechanical mixing to the use of propeller or turbine mixers, so-called static mixers, homogenizers, colloid mills, and ultrasonic devices.

Depending on the preparation method, different droplet size distributions can be achieved, explaining why the method of preparation can have an influence on emulsion stability. Usually, the smaller the droplet size, the more energy and/or surfactant required, making the preparation of very small droplets unfavorable for industrial applications [19]. In addition, injectable emulsions must meet many of the same requirements that relate to all parenteral products, such as: (1) physicochemical stability; (2) sterile; (3) endotoxin free; (4) biological stability (nonantigenic, low incidence of side effects, all components metabolized, and/or excreted); and (5) reasonable cost to both the manufacturer and the patient [7, 10, 53, 54]. Additionally, some parameters are unique to parenteral emulsions including: (1) controlling droplet size and size distribution; (2) surface charge; (3) oil type; (4) emulsifier type and (5) pH [5, 53].

12.4.1 Nanoemulsions

Nanoemulsions are usually prepared via: (1) high-pressure homogenization; (2) ultrasound generators (both based on droplet disruption) and (3) low-energy emulsification methods [20, 31]. Nanoemulsion formation using low-energy methods is attributed to the kinetics of the emulsification process during transitional or catastrophic phase inversion phenomena as a consequence of the spontaneous rearrangement and change in the curvature of the interfacial surfactant molecules [18–20]. This process requires neither, high energy input nor a multistep processing method and therefore is economical in terms of time and cost. Phase inversion processes (transitional and/or catastrophic) can occur in response to an appropriate perturbation of a liquid/liquid dispersion, where the continuous phase becomes the dispersed phase and vice-versa [55]. Transitional inversion can occur when there is a change in the affinity of the surfactants from one phase to another and this can be induced by factors such as temperature, hydrophilic–lipophilic balance (HLB) value, water phase salinity, and oil phase polarity [18, 56]. The transitional phase inversion phenomenon has been particularly promoted when nonionic emulsifiers are used. *Shinoda* and collaborators postulated that the system temperature can influence the behavior of nonionic polyethylene oxide surfactants [57, 58].

The phase inversion temperature (PIT) method is an example of transitional inversion phenomenon. At the PIT, the surfactant system exhibits strong solubilizing power and such characteristic properties as ultralow interfacial tension [18, 59–61]. A catastrophic inversion occurs as a result of a change in the volume fraction (i.e., increasing the volume fraction of the dispersed phase). This type of inversion is irreversible and can occur over a wide range of volume fractions. The designation catastrophic is used since there is a sudden change in the behavior of the system as a result of a gradual change in the processing conditions [18, 60, 62]. The emulsion inversion point is the point at which the emulsion changes from a

W/O to an O/W or vice-versa at constant temperature. For example, on successive addition of water into oil, water droplets are initially formed in the continuous oil phase. However, once the water phase reaches a certain critical value, spontaneous change in interfacial curvature occurs, driven by rearrangement in the effective geometry of the surfactant molecules [18, 59].

12.4.2 Multiple Emulsions

Multiple emulsions can be obtained by either the one- or the two-step emulsification methods [63]. Typically, multiple emulsions are prepared by the two-step method, where a simple emulsion (O/W or W/O) is prepared and then re-emulsified to form O/W/O and W/O/W emulsions, respectively. The one-step emulsification method has been directly related to multiple droplet formation (named as *mesophase* or intermediate phase) during the phase inversion processes (as described above) of simple emulsions [24]. *Mesophase* formation has been particularly promoted when nonionic emulsifiers are used [57, 58]. It has been reported that during the emulsion inversion process an ultralow interfacial tension point is reached where system stability is at a minimum [24, 56, 64].

The two-step method is complex and introduces possible destabilization pathways (e.g., solubilization of the primary surfactant may take place as a result of the excess surfactant that is typically introduced in the re-emulsification step; and osmotic pressure differences between the internal and external water phases are often created) [22–24]. Such destabilization pathways can be avoided when the one-step method is used. In addition, the one-step emulsification method is more efficient in terms of time and cost production [24, 44, 65].

Different research groups have devoted significant effort in order to elucidate the parameters involved in the two-step emulsification process as well as in the stability of and release from these systems [37, 40, 63]. Considerable progress has been made in practical adaptation of the two-step multiple emulsion process for different industrial applications. On the other hand, the one-step emulsification process has been reported to be extensively shear and temperature sensitive, with low yield of multiplicity, poor reproducibility, potential conversion into simple O/W emulsions and no long-term stability [24]. However, a novel one-step method whereby stable W/O/W multiple emulsions are spontaneously produced has recently been reported by our group [24]. The authors constructed a pseudoternary phase diagram using the following conditions: emulsification temperature of 76–80°C; HLB value of 9.3; and surfactant blend (Span80® and CremophorRH40®) added to the oil phase (canola oil) (Fig. 12.1). The phase diagram allows determination of the optimum area (surfactant/oil/water ratio) where different emulsion systems were formed and is provided here as an example. The results of the macro- and microscopic evaluations are as follows: (1) stable and unstable (temporary) W/O/W emulsions and O/W nanoemulsions were observed for the right-hand bottom corner; (2) W/O macroemulsions were observed for the left-hand bottom corner area; (3) microemulsions were observed for the middle left area; whereas (4) gels (but not liquid crystalline phase)

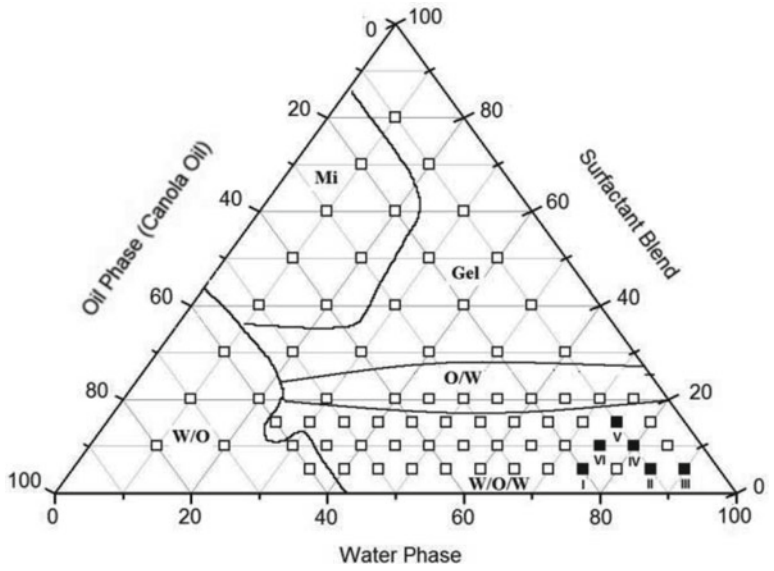

Fig. 12.1 Pseudo ternary phase diagram, showing the areas where different types of emulsions were observed. *Mi* microemulsion

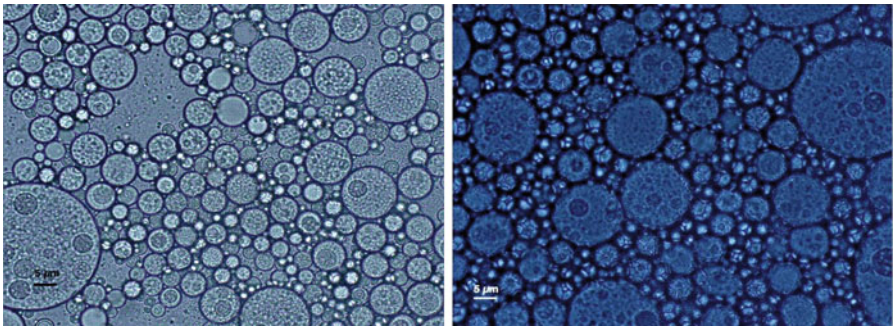

Fig. 12.2 Photomicrographs of W/O/W emulsion: freshly prepared (*left*) and 30 days after preparation (*right*). Emulsions were maintained at 25 ± 2°C. The magnification was 200×

were observed for the central area, right-hand middle area, and upper corner. Moreover, a dispersed/continuous phase ratio of 1:16 to 1:18 was required (right-hand bottom corner of the phase diagram), which is far from the dispersed/continuous phase ratio of 1:1 that is usually required for coarse emulsions. The high surfactant concentration and the high amount of continuous phase compared to dispersed phase are aspects that favor the formation of nano- and/or multiple emulsions via catastrophic phase inversion. These results confirmed that the oil/water phase ratio was critical to determine the morphology of this emulsion system.

Figure 12.2 shows representative photomicrographs of the one-step multiple emulsion indicated in Fig. 12.1 phase diagram (75.0% w/w water/20.0% w/w canola

oil/5.0% w/w surfactants). These micrographs show the stability of these one-step multiple emulsions, studied over a 1-month period.

12.4.3 Microemulsions

Depending on the component properties, microemulsions can potentially be formed over a wide range of oil–water–surfactant composition ratios. Emulsion system formation is typically presented in pseudoternary phase diagrams that show the ratios of oil, water, and surfactant/cosurfactant mixtures [46, 47]. The primary determinant for the region where microemulsion formation occurs is the physicochemical properties of the aqueous phase, oil phase, and surfactants [66]. The physicochemical interaction between the components is too complex to provide a functional general mathematical guideline for prediction of microemulsion formation as a function of component properties; however, a few essential conditions have been described by Schulman et al. [67]: (1) the production of a very low interfacial tension at the water–oil interface; (2) the formation of a highly fluid interfacial surfactant film; and (3) the penetration and association of the molecules of the oil phase with the interfacial surfactant film. Lowering of the interfacial tension and fluidization of the interfacial film are usually done by introducing a short chain co-surfactant to the surfactant film [46, 47, 66].

12.5 Emulsions as Vehicles for Parenteral Delivery

Many candidate drugs for the parenteral route, especially those for intravenous administration have poor water solubility. Several approaches can be used to increase the aqueous solubility of a drug such as: (1) water-soluble salt formation; (2) pH adjustment for ionizable drugs; (3) use of water-miscible cosolvents; (4) use of surfactants; and (5) complexing agents [5, 7, 53]. In addition, dispersed systems for parenteral use may be developed if a drug cannot be dissolved and administered in solution [6, 68, 69]. Accordingly, the use of an o/w emulsion can reduce or overcome problems with poorly water soluble drugs, by incorporating the drug into the dispersed phase [7, 10]. Moreover, an emulsion (especially microemulsions) can be an alternative to conventional cosolvent-based formulations that are generally associated with precipitation of the drug at the injection site [53] (Table 12.1).

12.5.1 Emulsion Formulation as Sustained, Controlled, or Targeted Delivery

Emulsion formulations can allow sustained and/or prolonged release based on the liophilicity of the drug incorporated into the dispersed phase. Hydrophilic drugs are incorporated into the inner phase of a w/o emulsion system, whereas hydrophobic

Table 12.1 Examples of parenteral emulsion formulations available on the market. Non-exhaustive or selected list of marketed emulsions

Product	Manufacturer	Drug	Application	Market
Diazepam-Lipuro®	Braun Melsungen	Diazepam	IV	Europe and others
Diprivan®	Zeneca	Propofol	IV	US and Europe
Diazemuls®	Dumex	Diazepam	IV	Europe and others
Dizac®	Ohmeda	Diazepam	IV	US
Etomidat-Lipuro®	Braun Melsungen	Etomidate	IV	Europe
Fluad (MF59)®	Chiron	Adjuvant	SC	Europe
Liple®	Green Cross	Prostaglandin E1	IV	Japan
Lipotalon®	Merckle	Dexamethasone palmitate	IA	Europe and others
Stesolid®	Dumex	Diazepam	IV	Europe and others
Vitalipid N®	Kabi	Vitamins	IV	Europe and others

IA intra-articular, *IV* intravenous, *SC* subcutaneous

drugs (which are either insoluble or sparingly soluble in water) are incorporated into the oil phase of an o/w system and as a consequence, the drug dispersed/dissolved in the inner phase must partition into the outer phase/medium during the release process [6, 10, 14, 15, 70].

Moreover, the release of a drug from an emulsion can also be prolonged by incorporation into a multiple emulsion [71]. Multiple emulsions provide an extra barrier that the drug must cross before being exposed to the body compartment for release. Another advantage of drug incorporation into, and slow release from, emulsions is that only low concentrations of the drug contact the body fluids and tissues an any time, reducing the toxicity of some drugs [40, 72, 73].

For targeted delivery, both the droplet size and the lyophilic aspect of emulsions can be exploited [13, 30, 74, 75]. The particulate nature of emulsions can be harnessed to deliver drugs to phagocyte cells of the reticuloendothelial system (RES), where they are taken up when injected intravenously. This property makes them useful for the delivery of agents against parasitic and infectious diseases. For example, emulsion systems with particle size in the range of 1–2 μm are especially useful for the delivery of antifungal agents and immune-modulators [7, 8, 76, 77]. When injected intramuscularly or subcutaneously, emulsion droplets tend to migrate and accumulate in the lymph nodes avoiding the RES. These characteristics of emulsions are exploited in the delivery of vaccines, where emulsions act as good adjuvants for potentiating the immune response toward antigens. It is believed that emulsions allow slow release of antigens from the site of injection as well as presentation of antigens in a way that enhances both the cellular and humoral responses [7, 29, 30, 76]. However, in vivo drug targeting is difficult for most exogenous colloidal systems. The critical problem is the nonspecific uptake of these systems by the cells of RES, such as the liver and spleen [7, 15, 77]. Accordingly, polyethyleneglycol derivatives have been successfully used to avoid this phenomenon and increase the half-life of the carrier in the blood stream [78, 79].

12.5.2 Bioavailability

Absorption of drugs encapsulated in carrier systems after s.c. and i.m. administration is more complicated than absorption from conventional formulations. Not only absorption of free drug but also absorption of the carrier and release of the drug from the carrier should be considered [9, 30, 74]. After release, the drug will behave similarly to a drug administered in conventional formulations and general biopharmaceutical principles will be applicable. However, if the drug is not released from the carrier and the carrier is absorbed from the injection site as an intact entity, the drug will follow the kinetics and biodistribution of the carrier, which is generally very different from the kinetics of the free drug [30]. Drug release rates are determined by the administration route, as well as by the drug and the carrier aspects.

Absorption of drugs from the s.c. site is generally slower than from the i.m. site, due to the lower level of vascularization of the s.c. tissue. Therefore, sustained release injections are better placed in the s.c. or lipomatous tissues. Upon i.v. injection or intact absorption from local injection sites, particulate carrier systems are mainly eliminated by accumulation in organs rich in cells belonging to the RES system, accordingly the major sites of accumulation are the liver and spleen. Following local administration, large molecules and drugs encapsulated in small particles can enter the blood circulation only via the lymphatic capillaries. Absorption of particles from the injection site after local parenteral administration depends mainly on the carrier particle size, particles smaller than 100 nm can be absorbed, whereas larger particles remain at the injection site [5, 7, 53, 77]. The size-dependent time of retention at the s.c and i.m. injection sites is likely to be related to the process of particle transport through the interstitium. The structural organization of the interstitium dictates that larger particles will have more difficulty to pass through the interstitium and will remain at the site of injection to a large, almost complete extent. Accordingly, if sustained drug release is intended, larger particles that remain at the site of injection are preferred. These disintegrate or release their contents gradually resulting in low but prolonged plasma concentrations [30, 68, 69, 74].

Emulsions for intravenous use should have particle size and size distributions below 1 μm containing droplets ranging from 0.5 to 1.0 μm. Droplet size also directly affects both toxicity and stability. Droplets larger than 4–6 μm are known to increase the incidence of emboli and can cause changes in blood pressure [53, 80]. Given the key physiologic functions of the RES immunologically as well as its role in the clearance of triglycerides, all vital organs of the RES (i.e., liver, lungs, bone marrow, and spleen) can be adversely affected by the intravenous infusion of lipid emulsion with an excessively high population of large-diameter fat globules. Since these droplets will accumulate in the RES organs following phagocytoses, large quantities of large-diameter fat globules can lead to impairment of RES function in animals and humans [29, 81]. However, since it has been demonstrated that any possible blockade of blood capillaries in the lung may be reversible due to the biodegradation of fat droplets [82], Koster et al. [80] discussed the emphasis laid on the

5 μm as an upper size limit for fat emulsions. Moreover, it has been reported that lipid particles with diameters even greater than 7.5 μm can deform and pass through the pulmonary vasculature without difficulty [83].

In addition, the absorption rate appears to be inversely related to molecular weight. It has been shown that relatively small molecules are absorbed primarily via the blood capillaries. Whereas compounds with molecular weights larger than approximately 16 kDa and particulate matter, such as drug carriers, appear to be absorbed mainly by the lymphatics, which results in a lower rate of absorption [30, 68, 69, 75].

Drug solutions in oil are often thought to be sustained release preparations. However, rapid absorption is often observed. Most likely, slow release is not a property of the oily vehicle but it is the result of the relatively high lipophilicity of the drug or interactions between the drug and the vehicle [5, 53, 74]. Oily vehicles are absorbed slowly and remain present at the injection site for several months. As long as the oily vehicle is present at the site of injection and contains drug in solution, the drug will be released and absorbed from the injection site. Drug release and absorption from an oily vehicle into the blood circulation depends mainly on the lipophilicity of the drug. At the oily vehicle/tissue fluid interface, the transition of drugs from oily vehicles into the aqueous phase is controlled by the oil/water partition coefficient. More lipophilic drugs will transit slowly into the aqueous tissue fluid and consequently will be released and absorbed slowly. Lipophilic drugs will be more easily absorbed from hydrophilic tissue than from lipophilic tissue [30]. The fraction of the drug available for absorption also depends on the phase volume ratio between internal and continuous phases in the delivery system [70, 74, 84]. For example for o/w emulsions, if the volume of the aqueous phase (continuous phase) is much larger than that of the oil phase (dispersed phase), a high partition coefficient will result in a small fraction of the drug being available for absorption and consequently in a sustained release effect.

Drug solubility also affects its bioavailability when administered via subcutaneous (s.c.) and intramuscular (i.m.) routes. Hydrophilic drugs in solution injected i.m. and s.c. are generally absorbed from a local depot [10, 68, 69]. Absorption only takes place as long as enough vehicle or essential elements of the vehicle are presented to keep the drug in solution or to drive the absorption process. Hydrophilic drugs are usually absorbed completely. In contrast, aqueous solutions and suspensions of relatively lipophilic drugs are often absorbed incompletely within a therapeutically relevant time. After s.c. injection, relatively hydrophilic drugs present higher absorption when compared to lipophilic ones. This is a result of fast transition of the drug into the hydrophilic tissue fluid. More lipophilic drugs, which transit slower into the aqueous phase, are absorbed slower. As a result of the slower absorption process, the aqueous vehicle may be absorbed before drug absorption is complete, which consequently results in reduced bioavailability [5, 7, 30, 74].

12.6 Stability of Emulsions

Emulsion formulations should be characterized physically, chemically, and microbiologically during the intended shelf life period of the product [29, 53]. Accordingly, the following tests should be performed: (1) physical evaluation (creaming, coalescence, oil separation, and color change); (2) chemical analysis, determining drug, oil, emulsifier, and adjuvants, as well as degradation products; (3) pH; (4) globule size; (5) surface charge; (6) pyrogen test; and (7) sterility test [13, 53].

12.7 Emulsion Testing Release Methods

It is technically difficult to characterize in vitro drug release from emulsions due to the physical obstacles associated with separation of the dispersed and continuous phase. Various techniques can been used: (1) sample-and-separate; (2) membrane barrier; (3) in situ, and (4) continuous flow-through method (USP 4). The sample-and-separate method is not ideal since it is difficult to preserve the physical integrity of emulsion droplets during the separation process. Filtration and centrifugation should be used to separate the released drug present in the continuous phase from the releasing dispersed phase. These techniques involve the application of external energy, which can destabilize the emulsion system [85–87]. For membrane barrier techniques, the dispersed phase is separated from the receiver phase by a semiporous membrane. Submicron droplets have a large surface area compared to their volume and to the membrane surface, which can lead to rapid transport from the oil to the continuous phases and to potential violation of sink conditions. In addition, another disadvantage of the barrier methods is the limited volume of the continuous phase available to solubilize the released drug in the donor chamber [88, 89]. A novel technique for parenteral submicron emulsion formulations using a reverse dialysis sac was developed by Chibambaram and Burgess [89]. Phenylazoaniline and benzocaine were used as model drugs. According to the authors, biphasic release profiles were obtained due to an initial rapid release from the donor to the receiver chambers followed by slow release from the oil droplets. For in situ methods, the drug released is analyzed without separation from the receptor media and additionally there is not violation of sink conditions. However, the method of analysis should allow separation and identification from the drug incorporated into the dispersed phase [90]. The use of the continuous flow-through method for testing of colloidal disperse systems has been considered not feasible, since the formulation could either block the filter in USP apparatus 4 or pass through it. However, Bhardwaj and Burgess [88] have developed a novel dialysis adapter that can be used with the compendial USP dissolution apparatus 4 (flow-through). Different colloidal formulations such as solution, suspension, and extruded and nonextruded liposomes containing dexamethosone as a model drug were successful evaluated. Other research groups have also reported the

use of USP 4 system with different types of dialysis adaptors for semisolid emulsion formulations intended for topical/dermatological route [91, 92].

12.8 Conclusions and Perspectives

The development of nano-, micro-, and multiple emulsions has a significant role in the formulation and delivery of drugs. The use of parenteral emulsion formulations should be considered not only to improve drug efficacy, but also to reduce drug toxicity. Recent progress in controlling droplet size distribution and understanding both preparation processes and stabilization mechanisms provides new insights for the improvement of pharmaceutical emulsion formulations. Selection of materials, optimization of process and formulation variables, characterization techniques, and adequate stability tests are important requirements regarding to the development of stable parenteral emulsions. However, their potential to provide sustained, controlled, or targeted release should be further investigated.

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Chapter 13
Nanosuspensions

Sumit Kumar and Diane J. Burgess

Abstract A large proportion of new drug candidates are poorly soluble in water, and therefore have poor bioavailability. A promising approach to overcome solubility problems is the production of nanoparticles (i.e., nanosuspensions). Parenteral nanoparticulate formulations provide an effective way of achieving high drug concentrations with low toxicity and can be administered via the intravenous (IV) route. Major advantages of this technology include ease of scale up and applicability to most drug candidates. Abraxane® was the first FDA approved (2005) IV nanoparticulate product available on the market. This chapter reviews various methods of nanoparticle production and characterization. In addition, formulation considerations and ongoing research specific to parenteral nanoparticles/nanosuspensions are described.

13.1 Introduction

The goal of controlled and targeted drug delivery is to provide an appropriate dosage of active pharmaceutical ingredients (APIs) at a desired rate to a specific region in the body. Thirty to forty percent of the new chemical entities discovered or synthesized in pharmaceutical companies have poor solubility and hence, poor bioavailability. The poor solubility of new chemical entities makes their formulation very challenging and is often cited as a main reason for the discontinuation of development of these new compounds [1, 2]. In recent years, nanotechnologies have been used to overcome poor solubility and bioavailability, as well as to achieve site-specific drug delivery [3–5]. Nanotechnology in parenteral drug delivery is a fairly new concept.

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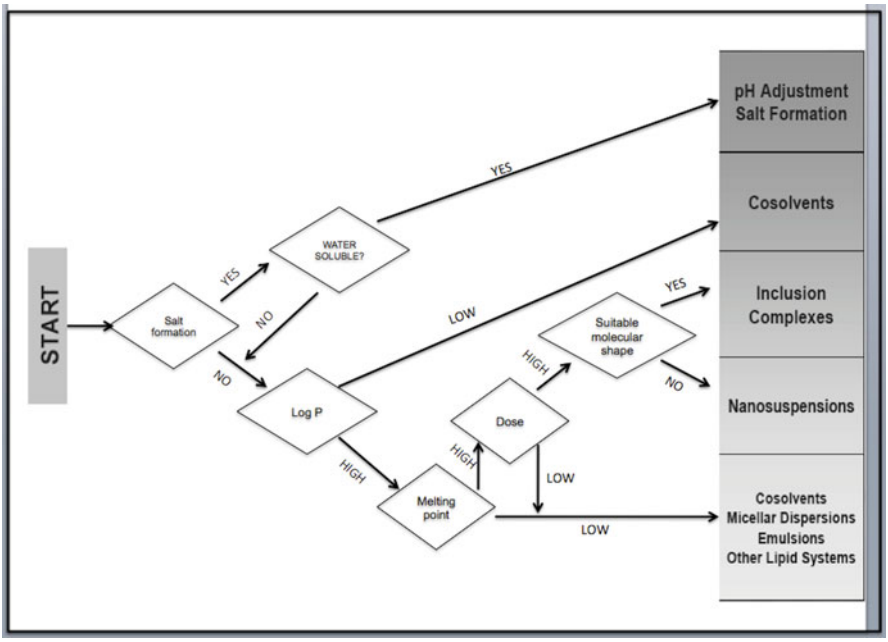

Fig. 13.1 Decision tree on different ways of solubility enhancement of poorly soluble drugs

For many decades, coarse solid suspensions have been produced for the parenteral delivery of poorly water-soluble drugs. For example, a range of coarse suspensions of insulin have been formulated for subcutaneous delivery (such as, HUMULIN, ILETIN, LENTE etc., manufactured by Elli Lily). Other examples are Bicillin® L-A manufactured by Wyeth-Ayerst and Decadron-LA manufactured by Merck, which are administered intramuscularly. Parenteral nanosuspensions are used to reduce irritancy and control the rate of drug delivery [6].

The term nanotechnology was first used by the scientist Norio Taniguchi (1974), at the University of Tokyo, Japan, for any material in the nanometer size range. The prefix *nano* is derived from the Greek word for *dwarf* or small. Nanosuspensions consists of drug nanoparticles, stabilizers (such as, surfactants and polymers, etc.) and dispersion medium. The dispersion medium can be aqueous or non-aqueous in nature. The FDA (The US Food and Drug Administration) has not yet established a precise definition/size range for pharmaceutical nanosuspensions. However, pharmaceutical nanosuspensions are generally considered as consisting of particles with mean diameters below 1,000 nm or 1 μm.

A major question in formulation of an API is when to select nano-sized formulations over conventional formulations. This can depend on drug solubility, as well as the desire for controlled and/or localized delivery. In the case of poorly soluble drugs, solubility may be enhanced by salt formation, use of co-solvents, micellization and incorporation within emulsion formulations, as well as size reduction to nanoparticulates [4, 5]. Refer to Fig. 13.1 for a decision tree on different ways of solubility enhancement of poorly soluble drugs.

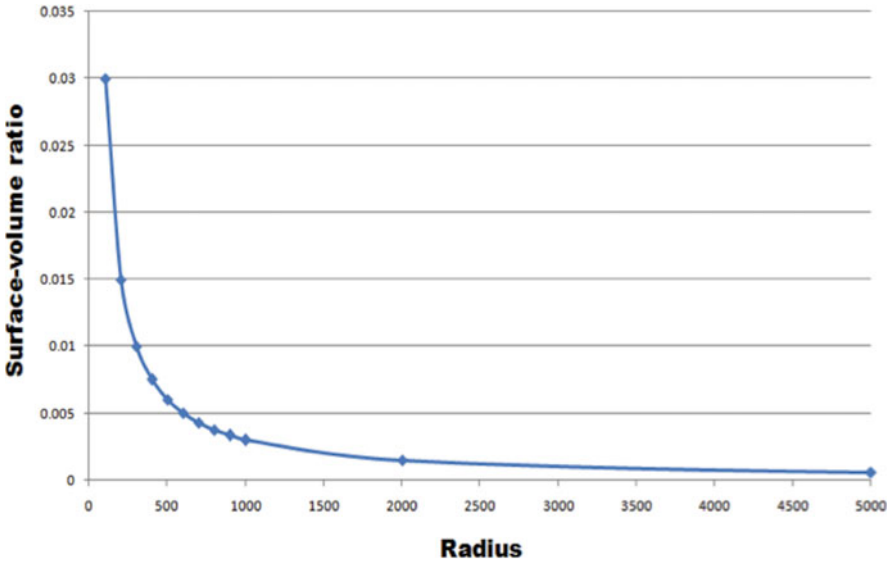

Fig. 13.2 Surface-to-volume ratio decreases with increase in the radius of spherical drug particles

13.1.1 Saturation Solubility and Dissolution Rate

Nanosuspensions have widespread interest as a drug delivery system due to their relatively high saturation solubilities and dissolution rates. Nanosuspensions differ significantly from all other formulations due to the following two major factors:

- *Surface-to-volume ratio*: Surface-to-volume ratio is the amount of surface area per unit volume of an object/drug particle. The surface area-to-volume ratio is measured in units of inverse of the length. For example, in the case of spheres, it is measured as surface area/volume of a sphere ($4\pi r^2 / (4/3\pi r^3)$, where, r is radius of the particle) i.e., $3/r$. Thus in the case of drug particles, as the size decreases the surface-to-volume ratio increases (Fig. 13.2). The solubility dependence on particle size is described by a modified form of the Kelvin equation:

$$\frac{S}{S(0)} = \exp\left(\frac{\gamma V}{RTd}\right), \tag{13.1}$$

  where, S = solubility, $S(0)$ = solubility of bulk material, R = gas constant, V = molar volume, T = temperature, d = diameter of particle and γ = surface free energy.
- *Higher dissolution rate*: Surface-to-volume ratio is directly proportional to the dissolution rate, i.e., the higher the surface-to-volume ratio, the higher the

dissolution rate of the drug. The kinetics of the drug particle dissolution can be described by the Noyes Whitney equation.

$$\frac{dC}{dt} = \frac{DS}{Vh}(C_s - C), \tag{13.2}$$

where, dC/dt is change in concentration, D is the diffusion coefficient, S is the surface area of the drug particle, h is the thickness of the diffusion layer, C_s is the saturation solubility of the drug particle, C is the concentration of the drug in solution with V as total volume of the solution [7].

13.1.2 Clinical Performance

Increase in surface-to-volume ratio and dissolution rate of nanoparticles/nanosuspensions improves their pharmacokinetic properties in terms of: increased rate and extent of release and absorption [8, 9]; rapid onset of action; reduced side effects and improved clinical performance [10, 11]. Nanoparticles are generally well tolerated via IV delivery. The inner diameter of the smallest blood vessels is in the range of 5–7 μm. Large quantities of inert polystyrene-divinylbenzene particles even up to 25-μm particles have been shown to be tolerated, if administered slowly over 1 h [12]. Whereas, hemodynamic effects, such as hypotension were observed when 3-μm size inert polystyrene beads were administered intravenously [13]. The concentration and rate of infusion play are important with respect to the hemodynamic effects. For example, reducing the concentration to 5% and the infusion rate from 1 to 0.5 ml/min reduced hemodynamic effects in anesthetized dogs. The hemodynamic effect appears to be mediated by histamine release, but further elaboration is required.

Another major advantage of nanoparticles as parenteral formulations is delivery of large quantities of drug with lower toxicity than would otherwise be possible by drug solutions, micellar solutions, co-solvent systems, etc. Nanosuspensions generally require low amounts of stabilizers (i.e., surfactants and/or polymers). Other approaches to increase drug solubility (such as, the use of high levels of surfactants, co-solvents, cyclodextrin complexes, etc.) may cause hypersensitivity reactions in certain individuals [14, 15] when administered parenterally. The commercial product SPORANOX® IV (Janssen Pharmaceutica Products, L.P.) contains 400 mg of excipient (2-hydroxy propyl-β-cyclodextrin) per 10 mg of itraconazole (Janssen, SPORANOX package).

13.2 Theory

Manufacturing of nanosuspensions involves the generation of a large number of small particles with enormous surface area. This significantly increases the Gibb's free energy of the system and, due to the high interfacial tension, these systems are thermodynamically unstable. Accordingly, nanoparticles will tend to minimize their

total energy by undergoing agglomeration. The increase in free energy is given by the Gibb's free energy equation:

$$\Delta G = \gamma \Delta A - T\Delta S, \tag{13.3}$$

where, ΔA is the change in surface area, γ is the surface tension, T is the absolute temperature and ΔS is the change in entropy.

The process of agglomeration depends on the activation energy, which is influenced by the addition of stabilizers to the system (such as, surfactants and polymers). These stabilizers reduce the interfacial tension between the particles and the dispersion medium. To achieve maximum stability, stabilizers are added at the early stages of nanosuspension preparation. The first and foremost requirement of these stabilizers is to reduce interfacial tension and act as wetting agents. The second requirement is to provide a barrier between the drug particles to prevent agglomeration. Possible mechanisms for providing a barrier are:

- Electrostatic repulsion
- Steric stabilization

13.2.1 Electrostatic Repulsion

The concept of electrostatic repulsion can be explained by the DLVO theory. The DLVO theory is named after Derjaguin and Landau, Verwey and Overbeek. According to the DLVO theory, the interaction of solid particles in liquid medium can be described by: (a) attractive or Lifshitz-van der Walls interaction; (b) repulsive, electrostatic repulsive forces due to overlap of electrical double layers; and (c) structural forces due to solvent molecules around the drug particle that can be attractive or repulsive in nature. When a drug particle is suspended or dispersed in a liquid medium, an electrostatic double layer forms around it. This electrostatic double layer arises as a consequence of the charge at the solid–liquid interface, which arises due to adsorption of ions, dissociation of ionizable groups, isomorphic substitution or accumulation of electrons on the surface, etc. Counter ions present in the liquid medium are attracted towards the charged particle surface and form a double layer of ions which consist of: (a) a tightly bound layer and (b) a diffuse layer of ions. The ions in the tightly bound layer are determined by the charge on the drug particle, whereas ions in the diffuse layer are distributed around the drug particle due to diffusive forces associated with random motion. Accordingly, the diffuse layer includes both ions of the opposite charge and ions of the same charge. At the outside of the diffuse layer the charge on the drug particle is neutralized (Fig. 13.3). The total energy of the interaction (V total) between drug particles is given by:

$$V_{\text{total}} = V_{\text{repulsion}} + V_{\text{attraction}}, \tag{13.4}$$

where, $V_{\text{repulsion}}$ is calculated using the approximation approach (called Derjaguin approximation).

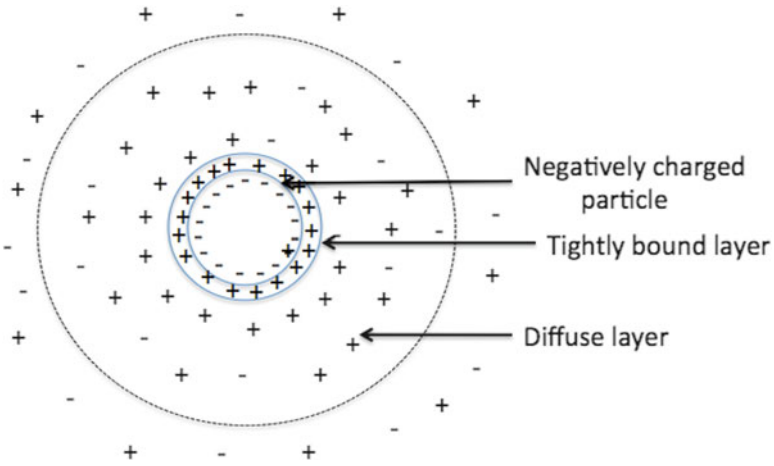

Fig. 13.3 Schematic representation of "*Electrostatic stabilization*" – a double layer of charge surrounds the drug particle

$$V_{\text{repulsion}} = 64a\pi n_{\infty} Y^2 \kappa^{-2} k_B T \exp(-\kappa H), \tag{13.5}$$

where, a is the radius of the particle, n_{∞} is the bulk concentration of the ions, $1/\kappa$ is the Debye screening length, K_B is the Boltzmann constant, T is the absolute temperature and H is the separation distance between the particles.

$$\gamma = \frac{\exp(ze\psi / 2K_B T) - 1}{\exp(ze\psi / 2K_B T) + 1}, \tag{13.6}$$

and where, Ψ is the potential associated with double layer and z is the valency of the ions.

Whereas the attractive forces between the two dispersed particles/sphere of equal radius and separated by distance H (for $a >> H$) is calculated as:

$$V_{\text{attraction}} = -\frac{Aa}{12H}, \tag{13.7}$$

where, A is the Hamaker constant, and a is the radius of the particles.

These attractive and repulsive forces can be easily explained using a potential energy diagram (Fig. 13.4). The attractive forces dominate at very small and large distances. At intermediate distances, the repulsive forces dominate resulting in net repulsion between particles which prevents agglomeration.

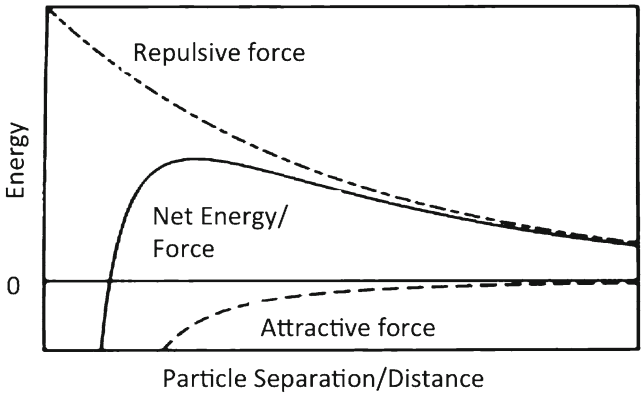

Fig. 13.4 Potential energy diagram of interacting nanoparticles

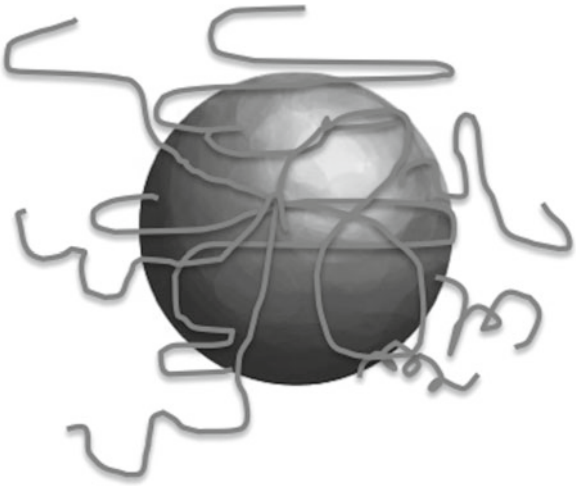

Fig. 13.5 Schematic representation of "*Steric stabilization*" – drug particle with adsorbed polymer chains extending into the medium

13.2.2 Steric Stabilization

Another approach/mechanism for the stabilization of nanosuspensions is "steric stabilization," using polymeric additives such as, HPMC, PVP, etc. In this approach, a high concentration of polymers is added to the colloidal system. These polymers are adsorbed onto the drug particle and their long hydrophilic chains (extend into the water) preventing the particles from agglomerating (Fig. 13.5). This approach has advantages over other stabilization methods such as, relative insensitivity to the presence of the electrolyte and equal efficacy in both aqueous and non-aqueous media (the polymer used should have a good affinity for the external medium, as well as for the insoluble drug particle to provide good surface coverage and even distribution on the nanoparticles).

13.3 Different Types of Nanoparticles

Pharmaceutical nanoparticles can be classified into four main categories:

(a) Crystalline drug nanosuspensions stabilized using polymers and/or surfactants.
(b) Polymeric nanoparticles such as, poly(lactic co-glycolic) acid (PLGA) containing entrapped API.
(c) *Solid lipid nanoparticles* – Solid lipid nanoparticles are submicron particles made up of an oily core, surrounded by a solid or semi-solid shell. In lipid nanoparticles, the drug is encapsulated within the lipid matrix. Lipid nanoparticles are usually produced via high-pressure homogenization techniques.
(d) *Liposomes* – Liposomes are small spherical shaped vesicles, made up of lipid bilayers. The drug can either be dissolved or dispersed in the inner aqueous compartment or the lipid bilayer depending on the hydrophobicity of the drug molecule.

13.4 Methods of Preparation

13.4.1 Top Down Processes

The top down approach consists of reducing the particle size from larger to smaller particles using different techniques such as, high pressure homogenization, media milling, etc. In these processes, heat is generated during particle size reduction; therefore, auxiliary cooling systems are required to prevent degradation of thermolabile compounds or to prevent any polymorphic changes. Different top-down methods are explained below.

13.4.1.1 Media Milling

Media milling processes are commonly used for the production of ultrafine particles. Media milling processes involve attrition of the particles in a mill using milling media such as, glass, zirconium oxide, etc. This process involves feeding the milling chamber with milling media, stabilizer and drug particles and rotating the milling shaft at a desired speed (Fig. 13.6). The high forces generated during the process cause the particles to break along weak points and finally smaller particles are produced. Milling media can be selected from a variety of dense and hard materials such as, silicon carbide, ceramics, zirconium silicate, glass, alumina, titanium and polymers (e.g., cross-linked polystyrene). The media milling process is a patented technology of Elan® drug technologies (known as Nanocrystals®). This technology was first used to prepare and market an inflammatory drug nanosuspension formulation, Rapamune®. Some of the major advantages of this process are reproducibility, cost-effectiveness and control over drug particle size. The major disadvantage of this technique is contamination of the final product with milling media or machine parts as a result of erosion during milling [16].

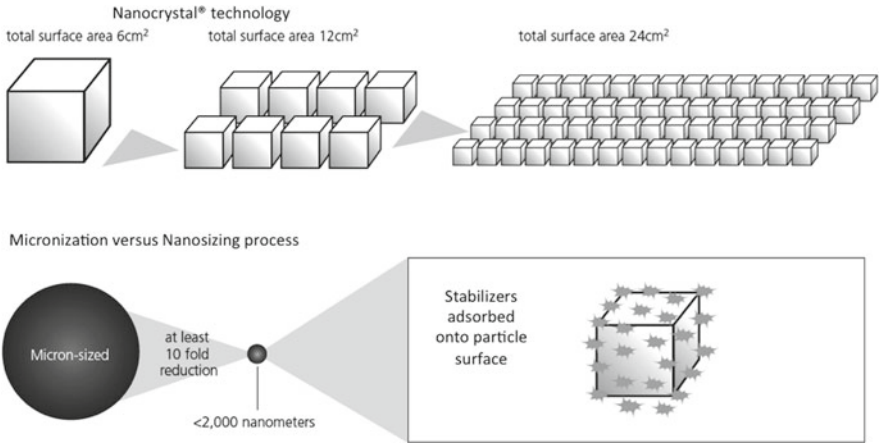

Fig. 13.6 Schematic representation of NanoCrystal® Technology by Elan drug technologies (picture reproduced by kind permission of Elan pharma International Ltd.)

13.4.1.2 High Pressure Homogenization

High-pressure homogenization has emerged as a powerful and reliable technique for the preparation of nanosuspensions. It was first developed and patented by Muller and Becker [17] and is now owned by Sykepharma LLC, known as Dissocubes®. This technique has been used for the production of nanoemulsions for parenteral nutrition. Scaling up is easier as compared to other techniques.

The high-pressure homogenization process can be divided into two broad categories:

- Hot homogenization
- Cold homogenization

Generally, nanosuspensions are prepared via cold homogenization, where temperature regulation is required to prevent any degradation of the drug. Hot homogenization is generally used for the preparation of microemulsions. The main disadvantages of these methods are strict temperature control and pre-micronization of macro-suspensions to prevent any blockage during homogenization.

Further, high-pressure homogenization can be classified into three different technologies:

Microfluidics® Technology

In this technology, nanoparticles are generated by high shear stress using a jet steam homogenizer. The microfluidizer was originally designed by the Arthur D. Little Co., but was later taken over by the Microfluidics Corp. The principle of particle size reduction is based on the collision of two fluid streams under high-pressure,

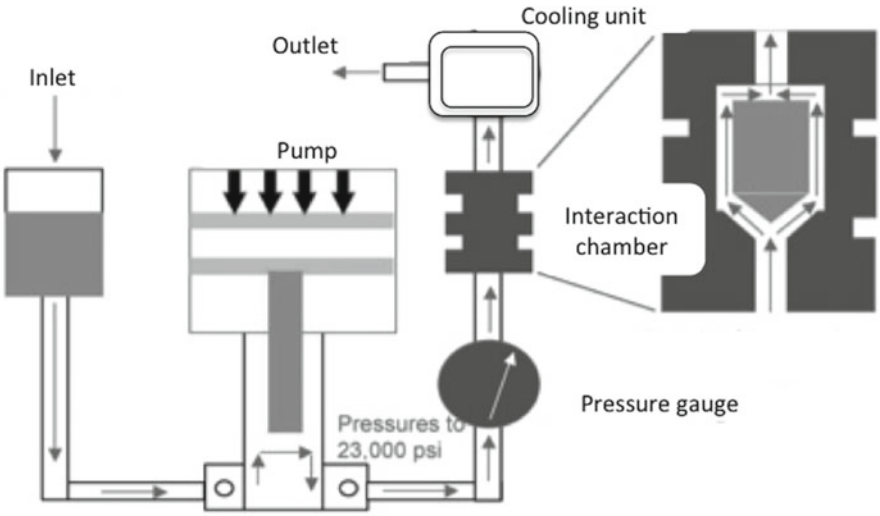

Fig. 13.7 Schematic representation of microfluidizer and its interaction chamber (picture reproduced by kind permission of Microfluidics®)

which leads to the generation of high shear and cavitation forces (Fig. 13.7) [18]. In this process, macro suspensions are passed through specialized chambers under high pressure. These chambers consist of narrow openings, which divide the macro suspensions into several parts. At high pressure and velocity, different streams of liquid collide against each other inside the interaction chamber, which causes the drug particles to fracture and break. The major disadvantage of this method is production time. The advantage of this method is narrower distribution of the particles as compared with other homogenization methods.

Piston-Gap Homogenization in Water (Dissocubes® Technology)

This technique involves the passage of macro suspensions through a small slit/aperture under high pressure (100–2,000 bar). Depending on the viscosity and concentration of the particle suspension, the width of the slit is in the range of 5–20 μm. The fluid accelerates to a high velocity and the pressure reduces tremendously at the small slit/aperture. When the suspension emerges from the aperture there is a drop in velocity and an increase in pressure as per Bernoulli's law, which creates high-energy shock waves. These high-energy shock waves are mainly responsible for the particle size reduction or fracture of the drug particles (Fig. 13.8) [19, 20].

Nanopure® Technology

Nanopure® Technology was developed and owned by PharmaSol GmbH/Berlin. In this process, homogenization is conducted using low vapor pressure dispersion

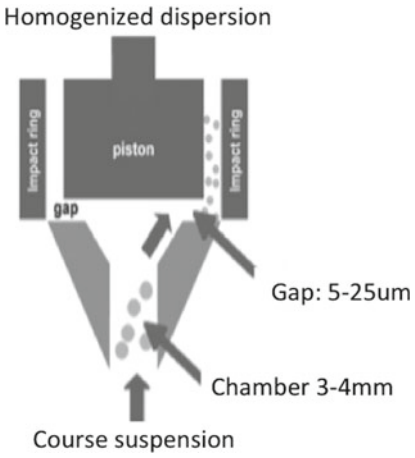

Fig. 13.8 Schematic representation of Piston-Gap homogenization (Dissocube®) (modified from Skype pharma website)

media at very low temperature (e.g., 0°C). Drug nanocrystals are produced using pure water as a dispersion medium. The turbulent flow and shear forces generated during homogenization are responsible for breaking the drug particles into the nano-size range. Non-aqueous homogenization is beneficial if the nanosuspensions are to be finally converted into solid dosage forms using spray drying, fluidized bed drying, etc. Another advantage of this method is that thermo-labile drugs can be used, since the process is performed at low temperature.

13.4.2 Bottom Up Processes

13.4.2.1 Solvent–Anti-solvent Technique or Precipitation Method

List and Sucker have utilized this method for the formulation of poorly soluble drugs, which was patented as Hydrosol technology in 1988 [21, 22] (owned by Sandoz, now Novartis). Hydrosols are colloidal particles in a size range of a few nanometers to 10,000 nm. In this technique, the poorly soluble drug is dissolved in the organic solvent (water-miscible) such as, ethanol. After dissolving the drug, the anti-solvent or non-solvent is poured or mixed slowly with the previously made drug–solvent solution. This leads to precipitation of drug particles from the solvent–anti-solvent mixture. These nanoparticles tend to grow bigger in size, driven by the "Oswald Ripening" phenomenon. To prevent or preserve the size of these particles different approaches have been utilized. For example, freeze-drying or spray drying is conducted immediately after precipitation to preserve the particle size [23]. In another approach, polymeric growth inhibitors are used in the system to preserve the size of the precipitated particles.

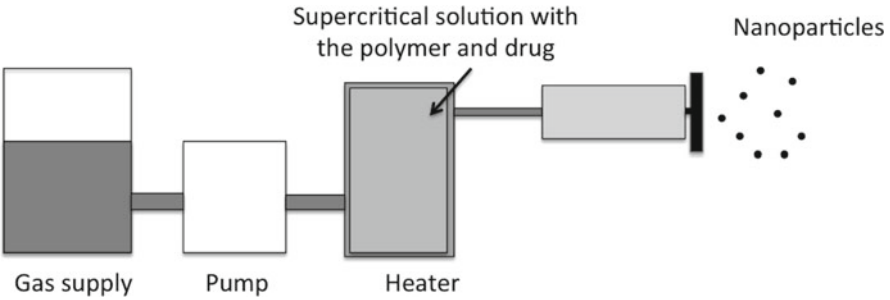

Fig. 13.9 Schematic representation of supercritical fluid technology

Another variation of this process, was developed by Sarkari et al. [24], and is called "evaporative precipitation." In this process, a heated solution of drug–water immiscible phase is atomized into a stabilizer containing aqueous solution, causing precipitation of the nanoparticles. In another approach, change in temperature and pH has been used to prepare a drug as a nanoparticle dry powder [25].

13.4.2.2 Supercritical Fluid Process

In this technology, both drug and polymer/stabilizer are dissolved in the organic solvent and then atomized through a nozzle into supercritical CO_2, where CO_2 acts as an anti-solvent (Fig. 13.9). As the dispersed organic phase containing drug and polymer comes into contact with CO_2, both phases diffuse into each other. CO_2 is only miscible with the solvent, thus the solvent is extracted and expelled from the outlet causing the insoluble solid to precipitate and fall out as nanoparticles [26].

13.4.2.3 Emulsion-Solvent Evaporation

An emulsion-solvent evaporation technique can be used to prepare polymeric self-assembled nanoparticles. These polymeric self-assembled nanoparticles offer many advantages such as, their hydrophobic core serves as a reservoir for poorly soluble drug and the hydrophilic shell reduces their interaction with plasma proteins. These self-assembled nanoparticles are in the size range of 150–500 nm. In this technique, drug and polymeric amphiphiles are suspended in an appropriate buffer solution and then an organic solvent (such as, chloroform) is added to form an emulsion. This emulsion is sonicated to reduce the particle size. Chloroform is evaporated using a rotary evaporator and then the product is passed through a syringe filter to achieve a nanoparticle suspension [27] (Fig. 13.10). Later it can be mixed with appropriate sugars to undergo freeze-drying.

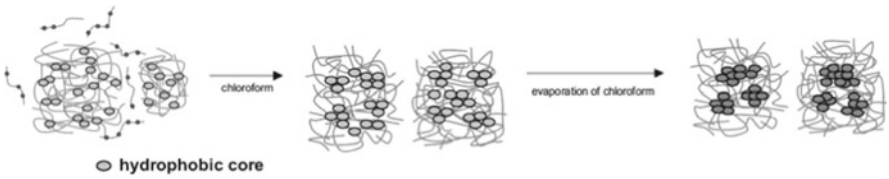

Fig. 13.10 Schematic representation of generation of polymeric nanoparticles (reprinted with permission, from, Lee [27])

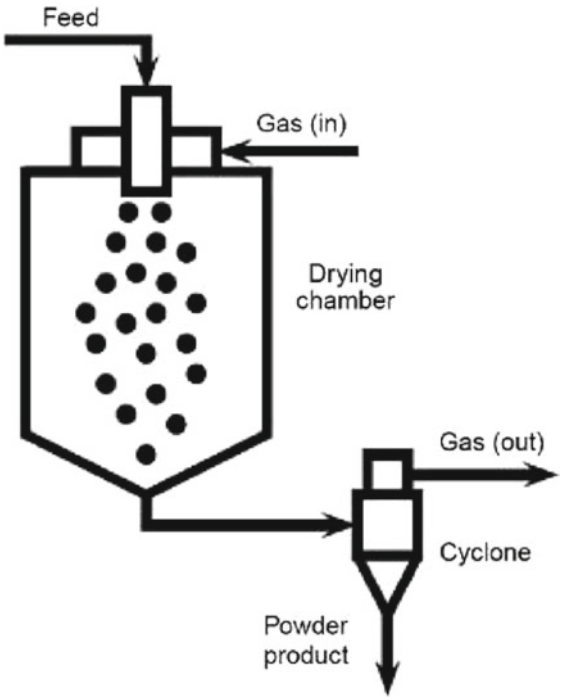

Fig. 13.11 Schematic representation of spray dryer

13.4.2.4 Spray Drying

Spray drying processing is utilized for a number of applications in the pharmaceutical industry such as, drying of solutions and emulsions, coating, nanoparticle manufacture, etc. In spray drying, drug-containing macro-suspensions are forced through an atomizer or nozzle, producing tiny droplets or mists, which are then dried in the drying chamber to obtain fine particles (Fig. 13.11). The spray-dried powder can easily be re-suspended in water and used when needed. This process has several advantages over other methods such as: it is a continuous process, it is less time consuming than other processes, it is easy to scale-up and it is cost effective. However, the one-droplet-to-one-particle mechanism during spray drying sets the lower size limit; therefore, it is difficult to generate particle sizes below 200 nm.

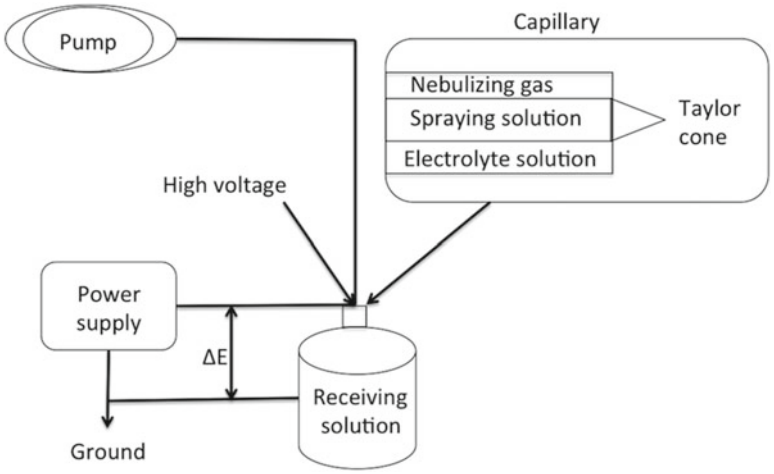

Fig. 13.12 Schematic representation of electrospraying

13.4.2.5 Electro Spraying

Electro spraying is a technique in which a micro-capillary electrospray atomizer and high voltage is used to generate small droplets of macro-suspensions/emulsions [28]. The application of high voltage causes the potential of the solution to increase due to the accumulation of electrostatic charges. The increase in potential increases the electrostatic forces and thus decreases the effect of surface tension on the droplets at the interface. When the surface tension and the applied electrostatic charge are equal, a Taylor cone is formed at the micro-capillary interface (Fig. 13.12). Further application of electrostatic charges disturbs the cone and breaks the suspension into smaller droplets at the tip of the cone. To achieve a required droplet size, the ratio of flow rate and conductivity should be controlled. These small charged droplets then travel in the gas phase under the electric field towards a counter electrode, where the solvent evaporates and this leads to further size shrinkage. The main advantages of this method are its versatility, inexpensiveness and simplicity to operate.

13.4.3 Other Techniques

13.4.3.1 Nanoedge Technology

BAXTER owns Nanoedge technology and this process relies on the combination of a micro-precipitation technique with a subsequent annealing step either by applying high shear or thermal energy. Nanosuspensions are formed using the solvent–anti-solvent technology (as explained above) and depending on the precipitation conditions,

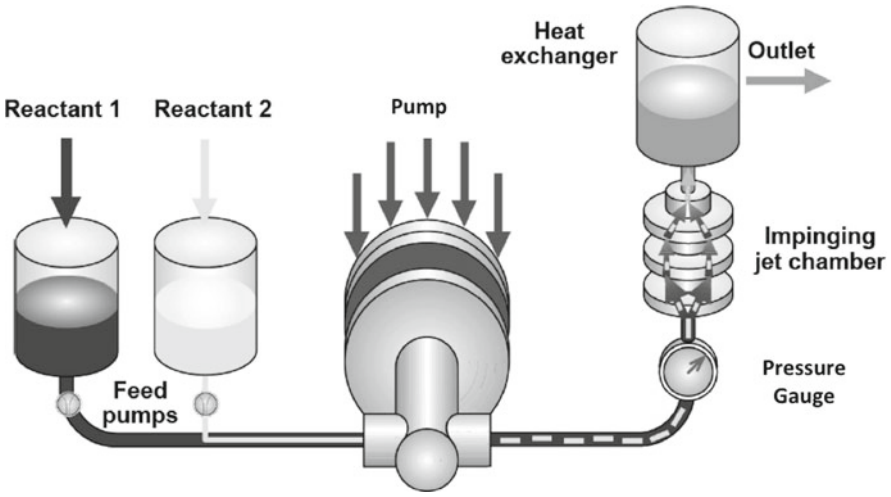

Fig. 13.13 Schematic representation of MRT technology by Microfluidics® (picture reproduced by kind permission of Microfluidics®)

either amorphous or crystalline drug particles are formed. A subsequent annealing process will preserve the size of the drug particles without changing the mean diameter. Particle sizes in the range of 400–2,000 nm can be easily obtained using this technology [4, 5].

13.4.3.2 Microfluidization Reaction Technology

Microfluidization reaction technology (MRT) is a continuous and scalable microreactor system, which is an amalgamation of the "bottom up" and "bottom down" approaches. In MRT pressurized solutions of drug and anti-solvent are pumped through a Microfluidizer® reaction chamber. In the reaction chamber, streams of the liquids collide with each other at supersonic speeds up to 300 m/s (Fig. 13.13). The particle size reduction achieved in the interaction chamber is mainly due to cavitation and high shear forces produced during particle collision.

13.5 Characterization of Nanosuspensions

Nanoparticles are characterized by both chemical and physical methods. Chemical methods are specific for APIs such as, analysis of API, as well as their degradation products (including HPLC, UV, Mass Spectrometry, etc.).

13.5.1 Physical Methods

13.5.1.1 Particle Size and Shape

Different approaches have been used to measure the particle size of nanoparticles. Photon correlation spectroscopy (PCS) and laser diffraction (LD) are the most powerful and popular techniques to measure nanoparticle size. Dynamic light scattering (also called as PCS) measures the intensity of scattered light caused by particle movement in the solution/suspending agent. Laser diffraction (LD) utilizes a correlation between the intensity of scattered light and its diffraction angle from the particles.

Particle size techniques can be divided into three major categories for easy explanation:

1. *Ensemble methods*, such as, laser diffraction assess bulk property.
2. *Counting methods*, individual particles are counted, for example electron microscopy.
3. *Separation methods*, classify particles in different size ranges depending on their behavior such as, centrifugation and chromatography.

13.5.1.2 Zeta Potential

Zeta potential, also known as "electrokinetic potential," is a measure of the electric potential at the interface of the electrical double layer. Zeta potential provides a way of expressing the stability of colloidal dispersions such as, nanosuspensions. There are several instruments available for measuring zeta potential, based on the electrophoretic mobility of particles suspended in a medium.

13.5.1.3 Re-suspendability

Many pharmaceutical formulations are aqueous suspensions or nanosuspensions of poorly water-soluble drug together with appropriate excipients. Their sedimentation characteristics during storage are of significant importance, since these can give rise to non-uniform distributions of drug and hence failure and/or side effects due to overdosing. There are many techniques/methods available to study sedimentation (e.g., optical analysis, ionizing radiation absorption, electrical sensing, etc.).

13.5.1.4 Dissolution

The dissolution rate of a drug is dependent upon its crystalline/amorphous form and particle size distribution. There are different apparatus/methods available to test the dissolution profile of different formulations. For nanosuspension dissolution, there is no current compendial method. Their rapid release and small particle size restrict

the use of various compendial methods. The most commonly used method for dissolution testing of nanosuspensions is USP II apparatus (paddle type). However, non-compendial methods such as, the dialysis sac method have been investigated for dissolution testing of nanosuspensions. Recently a dialysis sac adapter for the USP IV apparatus has been developed for liposome release testing and is under investigation for use with nanosuspensions [29]. Another method used for dissolution testing of nanosuspensions involves measurement of the %-transmitted light using a UV-spectrophotometer [30]. This method is based on the principle that the nanosuspensions being colloidal in nature block/refract light. Accordingly, by measuring the % of transmitted light, a dissolution profile can be obtained.

13.5.1.5 Polymorphs/Crystallinity

Drugs may exist in numerous solid forms (or nanoparticle generation process can yield different polymorphs), which may feature different physical and chemical properties. It is important to understand the solid form a nanosuspension, as this affects both the solubility and the stability of the product. These solid forms include polymorphs (true), solvates (pseudo-polymorphs), desolvates and the amorphous state. This phenomenon is known as "polymorphism." There are different techniques used to characterize polymorphs such as, crystallography, spectroscopy, microscopy and thermal techniques. Microscopic (light or electron) characterization is based either on the thermal technique or on the morphological properties of the drug. Alternative techniques are also available to characterize drugs that are not perfectly crystalline or pure, including X-ray diffraction and solid-state spectroscopy.

13.5.1.6 Excipients

Excipients play a major role in nanosuspension stability. The tendency of smaller particles in nanosuspensions to dissolve and re-grow on bigger particles, termed Ostwald ripening, is one mode of nanosuspension instability. The speed of Ostwald ripening is controlled by molecular diffusion and surface reaction [31] and happens as a result of the Kelvin effect [32]. Accordingly, faster ripening can be expected for smaller particle size suspensions such as, nanosuspensions. Addition of excipients can delay or totally prevent this phenomenon. Thus selection of excipients is an important concern for the stability of nanosuspensions. In one study, an AFM technique has been used to explain the type of adsorption of different stabilizers on nanosuspension formulations and this method is suggested as a rapid screening method for nanosuspension stabilizer selection [33].

Different stabilizers are used for nanosuspension stabilization either alone or in combination. The most popular are non-ionic surfactants such as, poloxamers, Tween 80, sodium lauryl sulfate, etc. Polymers are also used for stabilization such as, cellulosics (such as, HPMC, HPC, etc.) and polyvinyl alcohol.

Table 13.1 Overview of stabilizing excipients used in nanosuspension formulation

Stabilizer	Compound	References
Multiple stabilizer combinations		
Surfactant combinations		
Lecithin (20%)–Sodium cholic acid (16.7%)	Prednisolone	Muller and Peters [36]
Lecithin (20%)–Sodium cholic acid (16.7%)	RMKP 22 (3%)	Muller and Peters [36]
Lecithin (20%)–Sodium cholic acid (16.7%)	RMKP 23 (3%)	Muller and Peters [36]
Lecithin (20%)–Tyloxapol (20%)	Budesonide (1%)	Jacobs and Muller [37]
Poloxamer 188 (20%)–Lecithin (10%)	Azithromycin (1%)	Zhang et al. [38]
Poloxamer 188 (100%)–Lecithin (50%)	Bupravaquone(1%)	Muller and Jacobs [39]
Tween 80 (20%)–Lecithin (10%)	Azithromycin (1%)	Zhang et al. [38]
Tween 80 (2.5–5%)–Potassium oleate (5–10%)	RMKK99 (10/20/30%)	Krause and Muller [40]
Polymer–surfactant combination		
Carbopol 974 (2.5%)–Tween 80 (12.5%)	Albendazole (4%)	Kumar et al. [41]
HPC–Sodium lauryl sulfate (1.6%)	Cilostazol	Jinno et al. [9]
HPC (80%)–Sodium lauryl sulfate(1.6%)	MK-069 (5%)	Wu et al. [42]
Polyvinyl alcohol (100%)–Poloxamer 188 (200%)	Budesonide (1%)	Muller and Jacobs [37]
PVA VA (23%)–Sodium lauryl sulfate (1.67–3.33%)	Undisclosed	Deng et al. [43]
Single stabilizer systems		
Surfactants		
Lecithin (6.7%)	RMKP 22 (9%)	Muller and peters [36]
Lecithin (20/40/167%)	RMKP 22 (3%)	Peters et al. [44]
Poloxamer 188 (20%)	Bupravaquone (2.5%)	Muller and Jacobs [39]
Poloxamer 407	Itraconazole	Mouton et al. [45]
Poloxamine 908 (20%)	Ethyl diatrizoate (20/30%)	Na et al. [46]
Tween 80 (12.5%)	Albendazole (4%)	Kumar et al. [41]
Tyloxapol (50%)	Budesonide (1%)	Jacobs and Muller [47]
Polymers		
Acacia gum (2%)	ucb-35440-3 (5%)	Hecq et al. [48]
HPC (60 kDa; 16.7%)	Undiscolsed	Lee and Cheng [50]
HPMC (Methocel E15; 10–200%)	Nifedipine	Hecq et al. [49]
Polyvinyl alcohol (30–70 kDa; 50%)	Beclomethasone	Wiedmann et al. [51]
Povidone K15 (30%)	Danazol (5%)	Liversidge [52]

Additionally, natural excipients (such as, lecithins, cholic acid derivative, etc.) are frequently used. Table 13.1 provides an overview of different excipients used in nanosuspension stabilization. Few examples are included, since the list is too extensive (for example, Elan's NanoCrystal® technology has over 700 patents on file).

Table 13.2 Marketed and developmental formulations based on solid nanoparticulates

Drug	Indication	Route	Status	Company
Emend	Anti-emetic	Oral	Marketed	Merck/Elan
Rapamune	Immuno-suppresant	Oral	Marketed	Wyeth/Elan
Megace ES	Eating disorder	Oral	Marketed	Par/Elan
Tricor	Lipid regulation	Oral	Marketed	Abbot/Elan
Trigilde	Lipid regulation	Oral	Marketed	Sciele Pharma/ Skyepharma
Abraxane	Anti-cancer	IV	Marketed	Abraxis Bioscience/ AstraZeneca
Paliperidone palmitate	Schizophrenia	IM	Phase III	J&J/Elan
NPI 32101	Atopic dermatitis	Topical	Phase II	Nucryst
Panzem NCD	Glioblastoma	Oral	Phase II	Entremed/Elan
BioVant	Vaccine adjuvant	IM	Phase I	BioSante
Undisclosed multiple	Anti-infective Anti-cancer	Oral/IV	Preclinical to Phase II	Cytokine PharmaSciences/ Elan
Diagnostic agent	Imaging agent	IV	Phase I/II	Photogen/Elan
Thymectacin	Anti-cancer	IV	Phase I/II	Newbiotics/Elan
Busulfan	Anti-cancer	Intrathecal	Phase I	Supergen/SkyePharma
Budesonice	Asthma	Pulmonary	Phase I	Supergen/SkyePharma
Silver	Eczema, atopic, dermatitis	Topical	Phase I	Sheffield/Elan
Insulin	Diabetes	Oral	Phase I	BioSante

13.6 Nanosuspensions for Drug Delivery

Nanoparticulates can be used for compounds that are water insoluble and have high log *P* values to deliver large amount of drug with minimal or no toxicity. Their small size and increased surface-to-volume ratio leads to an increase in dissolution rate and bioavailability. The particulate nature of nanosuspensions can be useful for drug targeting (such as, targeting to the monocyte phagocytic system). Marketed products and those that are currently in clinical trials, which include nanoparticulates, are listed in Table 13.2. Nanosuspension performance can be further improved by controlled surface modification of the drug nanoparticles. To create targeted nanoparticles with desired surface properties, specific surfactants or polymeric stabilizers are used. The degree of modification can be measured in terms of surface charge (i.e., zeta potential) or hydrophobic interaction chromatography (HIC) analysis.

13.7 Nanosuspensions for Parenteral Delivery

The approval of Abraxane® in 2005, as an IV nanoparticle dosage form (130 nm amorphous particles entrapped in an albumin matrix) for the treatment of breast cancer has resulted an increase in activity in the area of nanoparticles for parenteral delivery. IV drug infusion/injection provides the most rapid delivery of drug to the body.

For other parenteral routes such as, intramusular (IM), absorption of drug must take place and this can take minutes to months. Hence, this route can be utilized for controlled and delayed drug delivery. Release of drug from nanosuspensions following IM delivery includes two major steps: (a) dissolution of the nanosuspensions and (b) diffusion of dissolved drug. Dissolution is generally a rate-limiting step for absorption of poorly soluble drugs and nanosuspensions provide better solubility and faster dissolution.

Factors to be considered in injectable nanoparticle formulation:

1. *Excipients*: Different types of stabilizers are used to stabilize nanosuspensions for parentral use. There are only a limited number of excipients that have been approved for parenteral use, these include poloxamers and phospholipids. In the case of surfactants, only non-ionic and anionic surfactants are preferred since cationic surfactants can cause hemolysis and cell toxicity. In addition, drug nanoparticles can be coated with special coating materials to avoid capture by the reticuloendothelial system. For example, phospholipid–PEG coatings on the drug particle can be used to increase the half-life of the nanoparticles [34].
2. *Particle size*: Particle size distribution and morphology are the major parameters for characterizing nanoparticle formulations and their safety upon administration. Different methods are used to determine the particle size of nanoparticles (as described above) such as, dynamic light scattering, photon correlation spectroscopy, etc.
3. *Syringeability*: Syringeability is an important factor to consider for nanosuspension used for IV delivery. Syringeability is measured as the pressure associated with injection using a needle of predetermined gauge and length. A method to measure syringeability using a specific apparatus has been proposed [35]. For non-aqueous suspensions, syringeability is given by a following equation:

$$\text{Syringeability} = \frac{\pi d^4}{128 \mu l_n}, \tag{13.8}$$

   where, d = diameter of the needle, l_n = length of the needle, μ = viscosity of suspension/solution.
4. *Sterility and pyrogenicity*: One of the most important requirements for IV and other parenteral nanosuspensions is sterility. It has been shown that the process of crystallization can entrap bacterial spores and these entrapped spores may be resistant to chemical sterilization [36, 37], as well as moist and dry heat [38, 39] sterilization. Sterilization of the final product at 121°C for an extended period of time and then cooling can promote physical and chemical instability such as, Ostwald ripening, drug degradation, etc. If the particle size of the IV nanosuspensions is small enough, then sterile filtration may be performed. For example, sterile filtration is used for NanoCrystal® iodipamide of mean particle size 98 nm with all particles <220 nm [40].

Aseptic manufacturing of IV nanosuspensions is another way to achieve sterile formulation. The risk factors associated with sterility assurance include personnel, facility, aseptic process, quality assurance, etc. and have been described by the FDA.

Another requirement of IV injectable nanosuspensions is that they are free from endotoxins and pyrogens. A bacterial endotoxin test is required to check the endotoxin levels in the final formulation. An alternative to the endotoxin test is the USP pyrogen test.

13.8 Concluding Remarks

Drug nanoparticulates represent a technology to overcome solubility and bioavailability problems of poorly soluble drugs. Nanoparticles offer various advantages such as, an increase in drug-to-volume ratio and saturation solubility that lead to an increase absorption and bioavailability. Other major advantages offered by nanoparticulates are high drug loading and minimal or no side effects. Over the last several years, there has been rapidly growing interest in nanotechnology for parenteral delivery. Selection of excipients plays an important role in the stability, as well as the targetability of nanoparticles. Most of the marketed products are solid dosage form. Drying (such as, freeze or spray drying) of the nanosuspensions can be used to prevent both physical and chemical instabilities (such as, Ostwald ripening and drug degradation) associated with them.

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Chapter 14
PEGylated Pharmaceutical Nanocarriers

Vladimir Torchilin

Abstract Surface coating of various pharmaceutical nanocarriers with polyethylene glycol (PEGylation) is the most popular and elaborated method to prepare drug delivery systems capable of prolonged circulation time in the blood. Prolonged circulation is often required to provide a sufficient time for effective accumulation of drug-loaded nanocarriers in target organs or tissues. This chapter considers key properties of PEG and some other polymers, which can be used to prepare long-circulating nanocarriers, and discusses the most important biological and pharmacological consequences of PEGylation and prolonged circulation. Special attention is paid to the preparation, properties, and application of long-circulating PEGylated liposomes, a popular and clinically approved drug delivery system. PEGylated polymeric nanoparticles, iron oxide nanoparticles, dendrimers, and other pharmaceutical nanocarriers are also described. The combination of longevity and specific targeting ability (attachment of both protecting polymer and targeting ligand to the surface of nanocarriers) is discussed as the next step in the development of effective drug delivery systems.

14.1 Introduction

Pharmaceutical nanocarriers, such as liposomes, micelles, polymeric nanoparticles, solid lipid nanoparticles, and many others, are widely used in research and practical medicine for the delivery of therapeutic and diagnostic agents as well as genes and related products (DNA, oligonucleotides, and siRNA) [1–4]. Since nanoparticulate

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pharmaceutical carriers are often rapidly cleared from the body, are unstable at physiological conditions, are taken up by the mononuclear phagocytic system (MPS), and do not specifically target the site of pathology, surface modification is often used to adjust the carrier properties. Frequent surface modifiers include certain synthetic polymers to achieve carrier longevity and stability in the circulation; specific ligands to provide targeting effects; pH- or temperature-sensitive moieties to impart stimuli-sensitivity; and contrast moieties for visualization. Moreover, different modifiers can be combined on the surface of the same nanoparticle drug carrier providing it with a combination of useful properties. Preparation of modified nanocarriers requires chemical or physical conjugation of different molecules to the carrier surface. Amphiphilic polymers or hydrophobically modified proteins can absorb onto the hydrophobic surfaces of some polymeric nanoparticles [5] or they can be incorporated into the phospholipid membrane of liposomes [6] or into the hydrophobic core of polymeric micelles [7]. Chemical conjugation can occur between reactive groups generated on the carrier surface and groups in the ligand to be attached.

The study of long-circulating pharmaceuticals and pharmaceutical nanocarriers is an area of active biomedical research [6, 8–12]. There is a frequent need to maintain pharmaceutical agents in the blood for extended periods of time. Long-circulating diagnostic agents are of primary importance for blood pool imaging. Artificial blood substitutes also require long-circulation times [13]. Long-circulation times allow drug-containing microparticulates or large macromolecular aggregates to slowly accumulate in pathological sites with compromised and leaky vasculature (such as tumors, areas of inflammation, and infarcted areas) [14–16]. This "passive" targeting effect is also known as the enhanced permeability and retention (EPR) effect. Prolonged circulation times can also improve the targeting of specific ligand-modified drugs and drug carriers by allowing more time for target interaction [11]. The reader is referred to the excellent review on gene delivery using long-circulating nanocarriers [17].

Prolonged circulation times are usually achieved by surface modification with synthetic hydrophilic polymers that help to protect drugs and drug carriers from undesirable interactions with biological milieu components [18]. The term "steric stabilization" has been introduced to describe this phenomenon of polymer-mediated protection [19].

14.2 Polyethylene Glycol and Other Polymers Used to Achieve Long Blood Circulation Times

The most popular and successful method to obtain long-circulating biologically stable nanoparticles is via coating with hydrophilic and flexible polymers, primarily polyethylene glycol (PEG) [20–24]. On the biological level, coating nanoparticles with PEG sterically hinders interactions between blood components and particle surfaces (Fig. 14.1). Of special importance is the role of the surface charge and

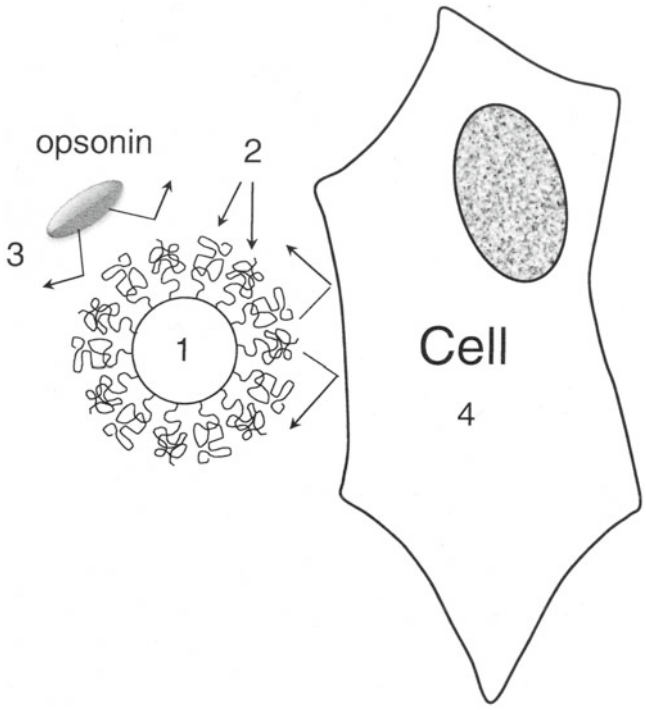

Fig. 14.1 Surface modification of pharmaceutical nanocarrier (1) with PEG (2) prevents its opsonization with opsonins (3) from the blood and simultaneously hinders its interaction with phagocytic cells (4)

hydrophilicity of PEG-coated nanoparticles [25] in decreasing/preventing plasma protein (opsonin) adsorption on the nanocarriers and thus decreasing their ability to interact with phagocytic cells [26]. The flexible nature of the PEG protective layer (free rotation of individual units around interunit linkages) is also important. PEG effectively forms a polymeric hydrogel layer over the particle surface even at low polymer concentrations and this renders the surface of the particle to look almost like water [27, 28]. This prevents interaction between the particles and opsonins and therefore slows down their fast capture by the RES [29]. Summarizing, the mechanisms of PEG in preventing opsonization include shielding of the surface charge, increased surface hydrophilicity [25], enhanced repulsive interactions between polymer-coated nanocarriers and blood components [30], and the formation of a polymeric layer (hydrogel) over the particle surface which, even at relatively low polymer surface concentrations, is impermeable to other solutes [25, 27].

These considerations have been confirmed experimentally, by studying the efficacy of liposome-incorporated fluorescent marker quenching by macromolecular quenchers present in the solution [27]. To study the interaction of polymer-modified and *N*-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]-dioleoyl-phosphatidylethanolamine (NBD)-containing liposomes with soluble rhodamine-modified ovalbumin (Rh-OVA),

plain NBD-liposomes and NBD-liposomes lipid-conjugated with either PEG (flexible polymer) or dextran were treated with increasing quantities of Rh-OVA. NBD fluorescence diminished identically for Rh-OVA-treated "plain" NBD-liposomes and NBD-liposomes with 0.8% mol of dextran. About 50% of the initial fluorescence was quenched, which suggests even distribution of NBD between outer and inner monolayers of the liposome membrane (only "outer" NBD is susceptible for quenching). However, NBD fluorescence quenching was drastically hindered in NBD-liposomes containing 1% mol of PEG or even less. Even at maximal Rh-OVA concentration, about 80% of the initial fluorescence was observed. Since the whole quenching process is limited only by Rh-OVA diffusion from the solution to the liposome surface, it is evident that the presence of PEG on the surface created diffusional hindrances. Another experimental proof was obtained using liposome surface-incorporated fluorescein derivative, *N*-[glutarylamido-(5-aminoacetamido-fluorescein)]-*sn*-glycero-3-phospho ethanolamine-1,2-dioleoyl (GFl-PE), and anti-fluorescein antibody [27]. In this case, fluorescence quenching with antibody was equal for "plain" GFl-liposomes and for GFl-liposomes with 1% mol of dextran. This indicates that the presence of dextran in the quantities used did not create any diffusional limitations for antibody-to-fluorescein interactions. The presence of 1% mol PEG on the liposomes noticeably decreased both the rate of fluorescein quenching and the quantity of fluorescein residues accessible for interaction with the antibody. This was due to the diffusional limitations for antibody penetration imposed by PEG.

Although quite a few polymers have been investigated as steric protectors for nanoparticular drug carriers [12] (which are discussed below), the majority of research on long-circulating drugs and drug carriers uses PEG due to its very attractive properties. These include excellent solubility in aqueous solutions and ability to bind a significant amount of water molecules; high polymer chain flexibility; very low toxicity, immunogenicity, and antigenicity; lack of accumulation in the RES cells; and minimal influence on biological properties of modified pharmaceuticals [31–34]. It is also important that PEG is not biodegradable and does not form any toxic metabolites. On the other hand, PEG molecules with low-molecular weight (i.e., below 40 kDa) are readily excretable from the body via kidneys. From a practical point of view, PEG is easily commercially available in a variety of molecular weights. PEGs which are normally used to modify drugs and drug carriers have molecular weights in the range of 1,000–20,000 Da. Single-terminus reactive (semitelechelic) PEG derivatives are often used for modification of pharmacologically important substances without the formation of cross-linked aggregates and heterogenic products. Currently, there exist many chemical approaches to synthesize activated derivatives of PEG and to couple these derivatives with a variety of drugs and drug carriers. An extensive review of these methods and their applicability toward different situations has been conducted by several authors [34–36].

Despite the well-developed chemistry of PEG coupling, the search for alternative sterically protecting polymers is quite active. This may be mainly explained by the patent situation around PEG and its derivatives. Through understanding the mechanism underlying the stabilizing effect of PEG onto liposomes, a number of other polymers that may be used as effective steric protectors for various nanocarriers

have been suggested [11, 12]. These polymers must be biocompatible, soluble, hydrophilic, and have highly flexible main chains. Synthetic polymers of the vinyl series, such as poly(acryl amide) (PAA) and poly(vinyl pyrrolidone) (PVP), are examples of other potentially protective polymers. It has been shown that amphiphilic derivatives of PAA and PVP can be synthesized by radical polymerization of the corresponding monomers in the presence of a hydrophobic growing chain terminator and that products obtained provided effective protection of liposomes in vivo [12, 27, 37]. PAA and PVP were prepared by radical polymerization of monomers in dioxane. Using different quantities of growing chain terminator, both polymers were prepared with MW values 6,000–8,000 (low-molecular-weight polymers, PAA-L and PVP-L) and 12,000–15,000 Da (high-molecular-weight polymers, PAA-H and PVP-H). These polymers have been made amphiphilic and membranotropic by chemical attachment of hydrophobic acyl groups of different length to a single terminus of a polymer molecule. The following products have been used in further experiments: PAA-L with terminal dodecyl group (PAA-L-D); PAA-L with terminal palmityl group (PAA-L-P); PAA-H with terminal palmityl group (PAA-H-P); and PVP-L and PVP-H with terminal palmityl group (PVP-L-P and PVP-H-P, respectively) [38].

Liposomes have been prepared via the detergent (octyl glycoside) dialysis method from the mixture of egg phosphatidylcholine and cholesterol (7:3 molar ratio) with the addition, when necessary, of 2.5 or 6.5% mol of the corresponding amphiphilic polymer. Liposomes were labeled with 111In-diethylenetriaminepentaacetic acid stearylamine prepared as in [39]. The liposomes obtained were sized by passing through polycarbonate filters 0.6, 0.4, and 0.2 μm. The final liposome size in all preparations was between 165 and 190 nm with narrow size distribution. The biodistribution of PAA- and PVP-coated liposomes has been studied in BALB/c mice.

Liposomes modified with amphiphilic derivatives of PAA and PVP change their biodistribution in a fashion similar to that of PEG-liposomes. When modified with the same palmityl residue, PAA, PVP, and PEG of similar molecular weight (ca. 6,000–8,000) and at similar concentrations, all provide steric protection and sharply increase the residence time of liposomes in the circulation. Half-clearance times for PVP-L-P-, PAA-L-P-, and PEG-liposomes with 2.5% mol of protective polymer were ca. 45, 80, and 80 min, and for PVP-L-P-, PAA-L-P-, and PEG-liposomes with 6.5% mol of protective polymer – ca. 120, 140, and 170 min, respectively, whereas half-clearance time for “plain” liposomes of the same size was about 10 min [38].

The protective activity of PAA-L-D and of both PAA-H-P and PVP-H-P was, however, much lower. Despite a definite increase in the circulation time, these polymers are much less effective steric protectors than polymers of similar molecular weight, but with longer acyl anchors, or polymers with the same long acyl anchor, but with smaller molecular weight hydrophilic moieties (compare PAA-H-P- and PVP-H-P-liposomes with PAA-L-P, PVP-L-P, and PEG-liposomes). This can be explained considering the energy of interaction between the fatty acyl anchor and the hydrophobic part of the liposomal membrane. From a thermodynamic point of view, a relatively short dodecyl group is unable to keep a 6–8 kDa polymer molecule on the liposome surface: the energy of the polymeric chain motion is, probably,

comparable to (or even higher than) the energy of the dodecyl group interaction with the phospholipid surroundings within the liposomal membrane. As a result, PAA-L-D can be easily removed from the liposomal membrane and demonstrate only slight and transient protective effect. The longer palmityl anchor provides firmer polymer binding with the liposome (higher energy of interaction with the hydrophobic membrane core due to the larger number of membrane-embedded CH_2-groups) and thus much better liposome steric protection. On the other hand, even the length of the palmityl anchor might be insufficient to provide firm fixation of 12–15 kDa polymer on the liposome surface because of the much higher energy of the polymer chain motion in solution compared with that of the shorter polymers [40]. Accordingly, the liposome surface gradually loses the protective polymer coat, and therefore, becomes opsonized and is taken up by the RES.

Similar regularities have been found following liposome accumulation in the liver. Plain liposomes were captured by the liver very rapidly (more than 50% in 45 min and ca. 70% in 240 min). The longest circulating liposomes containing 6.5% mol of PAA-L-P, PVP-L-P, or PEG demonstrated much slower liver uptake: less than 20% of these liposomes are captured in 45 min, and less than 40% in 240 min. Liposomes with 2.5% mol of protective polymer demonstrated an intermediate liver uptake.

Other amphiphilic polymers with highly soluble and flexible hydrophilic moieties, such as amphiphilic poly(acryloyl morpholine) (PAcM), have been successfully used as liposome steric protectors [12, 41]. Amphiphilic PAcM and PVP were prepared by synthesizing carboxyl-terminated polymers (PAcM-COOH, PVP-COOH) [42–44], which were then conjugated with PE.

Biodistribution and blood clearance experiments with polymer-modified 111-In-labeled liposomes in CD-1 mice clearly demonstrated that amphiphilic phosphatidylethanolamine (PE)-containing derivatives of PAcM, PAA, and PVP provide effective protection to liposomes in vivo similar to PEG–PE. This agrees well with theoretical considerations and other experiments [27, 28, 41, 45, 46]. The extent of protective activity for different polymers toward liposomes in vivo depends on the length of the hydrophobic anchor, the polymer molecular weight, and the structure and quantity of the protecting polymer on the liposome surface [12, 27].

Liposomes containing 5 mol% of distearoyl-PE covalently linked to poly(2-methyl-2-oxazoline) or poly(2-ethyl-2-oxazoline) also exhibit extended blood circulation times and decreased uptake by the liver and spleen [47]. Similar observations have been made with phosphatidyl polyglycerols [48]. Prolonged circulation times of doxorubicin-loaded liposomes in rats were observed when the liposome surfaces were modified with polyvinyl alcohol [49] (see Table 14.1).

Some special methods have been designed to attach various sterically protective polymers to the surface of nanocarriers (see below). Thus, for example, to make PEG capable of incorporation into the liposomal membranes, the reactive derivative of hydrophilic PEG is single terminus-modified with a hydrophobic moiety (usually, the residue of PE or long chain fatty acid is attached to PEG-hydroxysuccinimide ester) [21, 50]. In the majority of protocols, PEG–PE is used, which must be added to the lipid mixture prior to liposome formation. Alternatively, it has been suggested

Table 14.1 Polymers successfully tested as steric protectors for pharmaceutical nanocarriers after being single terminus-modified with hydrophobic groups

Poly(ethylene glycol)
Poly(acryl amide)
Poly(vinyl pyrrolidrone)
Poly(acryloyl morpholine)
Poly(vinyl alcohol)
Poly(2-methyl-2-oxazoline)
Poly(2-ethyl-2-oxazoline)
Poly(phosphatidyl glycerol)

to synthesize single end-reactive derivatives of PEG able to be coupled with certain reactive groups (such as maleimide) on the surface of already prepared liposomes, referred to as the postcoating method [51]. The preparation and properties of polymer-modified liposomes have been well reviewed in several important books [9, 52, 53]. Spontaneous incorporation of PEG–lipid conjugates into liposome membranes from PEG–lipid micelles has also been shown to be very effective and did not disturb the vesicles [54].

The most important biological consequence of nanocarrier modification with protecting polymers is a sharp increase in circulation times and a decrease in RES (liver) accumulation [9, 21, 27]. From the clinical point of view, various long-circulating liposomes of relatively small size (100–200 nm) were shown to effectively accumulate in many tumors via the "impaired filtration" mechanism [14–16, 55]. As a result, PEG-coated and other long-circulating liposomes have been prepared containing a variety of anticancer agents, such as doxorubicin, arabinofuranosylcytosine, adriamycin, and vincristine [56–59]. The biggest success was achieved with PEG-liposome-incorporated doxorubicin, which has demonstrated very good clinical results [14, 60, 61]. An analysis of the pharmacokinetics of long-circulating nanocarriers (using PEG-liposomes) was performed by Allen et al. [62]. In general, the association of drugs with nanocarriers has pronounced effects on their pharmacokinetics: delayed drug absorption, restricted drug biodistribution, decreased volume of drug biodistribution, delayed drug clearance, and retarded drug metabolism [63]. All these effects are determined by hindered interstitial penetration of the drug and lesser drug accessibility in the biological milieu because of entrapment in the drug carrier. The presence of a protective polymer on the carrier surface changes all these parameters still further [21, 24]. Thus, while "plain" liposomes have nonlinear, saturable kinetics, long-circulating liposomes demonstrate dose-independent, nonsaturable, and log-linear kinetics [64–66]. All pharmacokinetic effects depend on the route of liposome administration and their size and composition, and always are less expressed for sterically protected PEG-carriers [67–70].

An additional function can be added to long-circulating PEGylated pharmaceutical carriers, which allows for the detachment of the PEG chains under the action of certain local stimuli characteristic of pathological areas, such as decreased pH value or increased temperature which usually occurs in inflamed and neoplastic areas. The rationale for this additional detachment feature is that the stability of PEGylated

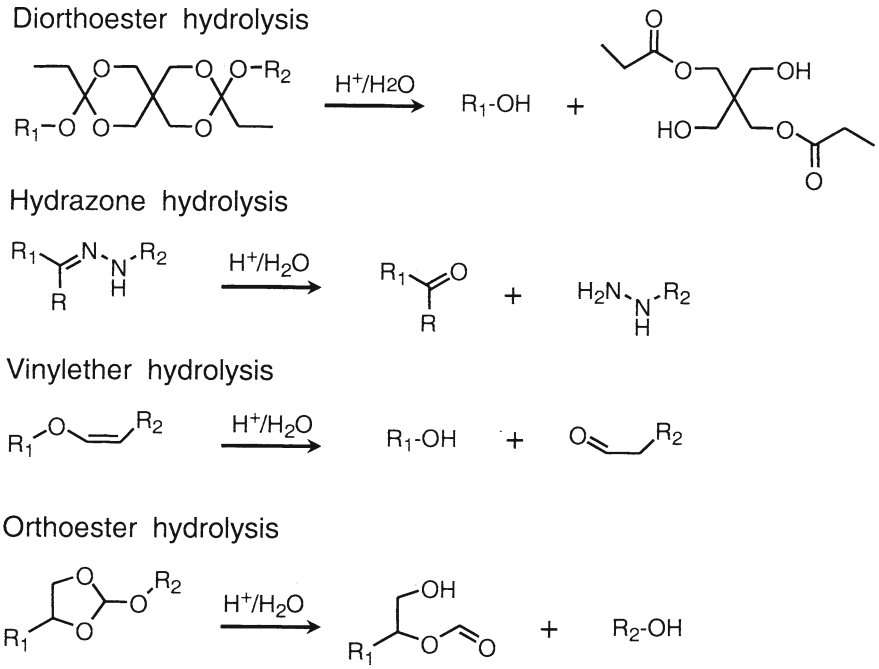

Fig. 14.2 Different types of acid-sensitive linkers used in detachable coatings and their proposed hydrolysis scheme. R1 contains the hydrophilic part (PEG); R2 contains the hydrophobic anchor

nanocarriers may not always be favorable for drug delivery. In particular, although drug-containing nanocarriers may accumulate inside a tumor, they may be unable to easily release the drug to kill the tumor cells. Likewise, if the carrier has to be taken up by a cell via an endocytic pathway, the presence of the PEG coat on its surface may preclude the contents from escaping the endosome and being delivered into the cytoplasm. In order to solve these problems, for example, in the case of long-circulating liposomes, chemistry has been developed to detach PEG from the lipid anchor in the desired conditions. The labile linkage can be designed to degrade only in acidic conditions characteristic of an endocytic vacuole or tumor mass. Such linkages can be based, e.g., on diortho ester acid-labile chemistry [71], or vinyl ester chemistry [72]. The latter reference describes the preparation of an acidic medium-cleavable PEG–lipid. Cystein-cleavable lipopolymers have also been described in [73]. When the PEG brush is cleaved (for example, from the liposome surface), membrane destabilization should occur, and the liposome contents will be delivered to its target (e.g., by escaping from a primary endosome into the cell cytoplasm). Polymeric components with pH-sensitive (pH-cleavable) bonds are widely used to produce stimulus-responsive drug delivery systems that are stable in the circulation or in normal tissues. However, they acquire the ability to degrade and release the entrapped drugs in body areas or cell compartments with lowered pH, such as tumors, infarcts, inflammation zones, or endosomes [74–76]. In “acidic” sites, the

pH drops from the normal physiological value of 7.4 to pH 6 and below. Chemical bonds used to prepare acidic pH-sensitive carriers have included vinyl esters, double esters, and hydrazones that are quite stable at pH values around 7.5 but are hydrolyzed relatively rapidly at pH values of 6 and below [71, 77, 78]. By now, a variety of liposomes [79, 80] and micelles [81–83] have been described that include components with the above-mentioned bonds as well as a variety of drug conjugates capable of releasing drugs such as adriamycin [84], paclitaxel [85], doxorubicin [86], and DNA [87–89] in acidic cell compartments (e.g., endosomes) as well as pathological body areas under acidosis. New detachable PEG conjugates are also described in [73], where the detachment process is based on the mild thiolysis of the dithiobenzylurethane linkage between PEG and amino-containing substrates (such as PE) (see some examples in Fig. 14.2). Serum stable, long-circulating PEGylated pH-sensitive liposomes have been prepared using a combination of PEG and pH-sensitive terminally alkylated copolymers of *N*-isopropylacrylamide and methacrylic [75].

14.3 Long-Circulating PEGylated Liposomes

To render PEG capable of incorporation into liposomal membranes, the reactive derivative of hydrophilic PEG is a single terminus-modified with a hydrophobic moiety (usually, the residue of PE or a long chain fatty acid is attached to PEG-hydroxysuccinimide ester) [21, 50]. In the majority of protocols, PEG–PE is used, which must be added to the lipid mixture prior to liposome formation. Alternatively, it has been suggested to synthesize single end-reactive derivatives of PEG able to be coupled with certain reactive groups (such as maleimide) on the surface of already prepared liposomes, referred to as the postcoating method. Numerous studies on the preparation and properties of polymer-modified liposomes have been reviewed [9, 52, 53].

An analysis of the pharmacokinetics of long-circulating PEG-liposomes was performed by Allen et al. [62]. In general, the association of drugs with nanocarriers has pronounced pharmacokinetic effects: delayed drug absorption, restricted drug biodistribution, decreased volume of drug biodistribution, delayed drug clearance, and retarded drug metabolism [63]. All these effects are determined by hindered drug interstitial penetration and reduced drug accessibility into the biological milieu due to entrapment into drug carrier. The presence of the protective polymer on the carrier surface changes all these parameters [21, 24]. Thus, while “plain” liposomes have nonlinear, saturable kinetics, long-circulating liposomes demonstrate dose-independent, nonsaturable, and log-linear kinetics [64–66]. All pharmacokinetic effects depend on the route of liposome administration as well as their size and composition, and the presence of sterically protective PEG-carriers [67–70, 90].

Current research on PEG-liposomes focuses on new methods of attaching PEG onto liposome surfaces in a removable fashion in order to facilitate liposome capture by the cell after the PEG-liposomes accumulate within the target site via the

EPR effect [91], and in order for the PEG to be detached under the action of local pathological conditions (pH decrease in tumors). The spontaneous incorporation of PEG–lipid conjugates into liposome membranes was shown to be very effective and did not disturb the vesicles [54]. The relative importance of the PEG chain length and lipid anchor length on the stability of PEG surface-modified liposomes has been investigated [92]. New detachable PEG conjugates have been described [73], where the detachment process is based on mild thiolysis of the dithiobenzylurethane linkage between PEG and amino-containing substrates (such as PE). Serum stable, long-circulating PEGylated pH-sensitive liposomes have also been prepared using a combination of PEG and pH-sensitive terminally alkylated copolymers of *N*-isopropylacrylamide and methacrylic [75]. The attachment of the pH-sensitive polymers to liposome surfaces might facilitate liposome destabilization and drug release in compartments with decreased pH values, such as tumors or intracellular organelles. Refer to review on long-circulating liposomes [93].

Continuing studies on the in vivo therapeutic effects of PEGylated doxorubicin-containing liposomes have confirmed that vascular permeability in tumors is the limiting factor for their activity [94]. Studies also continue with long-circulating hemoglobin-containing liposomes, which are considered as blood substituents [95]. Interestingly, hemoglobin-loaded long-circulating liposomes were found not to induce accelerated blood clearance in mice [96]. PEGylation of cationic liposomes resulted in enhanced uptake by cancer cells and increased cytotoxicity, evidently, due to the fact that PEG cannot completely shield the cationic charge on liposomes facilitating both uptake and cytotoxicity [97].

Some new ideas in using long-circulating liposomes include their application to prolong circulation lifetime of tissue plasminogen activator [98]. With the increased interest in carriers for delivering siRNA, liposomal carriers have attracted much attention, and PEG-protected pH-sensitive liposomes have been successfully used for the triggered release of siRNA [99]. The combination of siRNA-loaded PEGylated liposomal bubbles and ultrasound has also been successfully used for siRNA delivery into the cell cytoplasm [100–103].

Contemporary design protocols are used to optimize the properties of drug-loaded PEGylated liposomes. Factorial design has been applied to prepare topotecan-loaded PEG-liposomes that are optimized in terms of size and stability [104]. Topotecan-loaded PEG-liposomes have also been described by [105].

PEGylated liposomes with a pH-sensitive detachable PEG coat attached to their surface via a hydrazone bond have been used for enhanced transfection of tumor cells in vitro and in vivo (the plasmid encoding for the Green Fluorescent Protein was used) [106, 107]. PEG coating detachment has also been achieved using ester bonds (cleaved by esterases) [108].

The phenomenon of accelerated blood clearance of PEGylated liposomes is under continuous investigation, and there is evidence that complement activation caused by anti-PEG IgM raised by the first dose of PEG-liposomes plays a major role in accelerated clearance of subsequent doses by the liver macrophages [109, 110].

14.4 PEGylated Polymeric Nanoparticles

Synthetic amphiphilic polymers have been used for steric stabilization of particles with hydrophobic surfaces in order to prolong their circulation in the blood and to alter their biodistribution. These polymers demonstrate the ability to be easily adsorbed on the surface of particulate carriers, due to hydrophobic interactions. The hydrophilic part of these molecules is exposed to the solution and effectively protects the particulates from interactions with plasma proteins. The mechanism of protection is essentially the same as for PEG-containing liposomes – a conformational "cloud" of flexible polymer chains protects the particle hydrophobic core from contact with opsonizing proteins [27, 28]. A detailed description of polymer-modified nanoparticulates can be found in several reviews [10, 111–116]. There are several principal types of amphipathic polymers used so far for coating injectable nanoparticulate carriers. All of them consist of a hydrophobic moiety, which easily adsorbs on the surface and performs an anchoring function, and hydrophilic moiety, which protrudes into the solution and protects the particulates from interactions with plasma proteins in the blood.

Surface modification of particles can be achieved by one of the following two methods: (1) absorption of a polymer on a particle surface; and (2) chemical grafting of polymer chains onto a particle. Possible examples of the first case include the absorption of a series of polyethylene oxide and polypropylene oxide copolymers (Pluronic/Tetronic™ or Poloxamer/Poloxamine™ surfactants) on the surface of polystyrene latex particles via a hydrophobic interaction mechanism. Interestingly, the absorption of Poloxamer-type copolymers takes place only on solid particles with clearly hydrophobic surfaces. No interaction is detected, for example, between such copolymers and liposome surfaces [117]. There have been numerous studies on blood clearance and biodistribution of these particles, including the use of surfactants for particle protection from uptake by the MPS upon intravenous injection [118] and enhanced delivery to lymph nodes after subcutaneous administration [119].

Upon i.v. administration, hydrophobic particles in the submicron size range are opsonized with macrophage-recognizable serum proteins [120]. Similar to liposomes, protecting the particle surface with hydrophilic flexible polymeric chains results in a substantial decline in phagocytosis by liver macrophages and subsequent prolongation of circulation times. Porter et al. have demonstrated that the absorption of the above copolymers leads not only to a decrease in particle uptake by resident macrophages in the liver, but, after coating with some specific copolymers, the injected nanoparticles can be redirected to other organs. For example, the coating of 60 nm polystyrene latex with Poloxamer 407 results in increased particle accumulation in bone marrow [121], while the coating with certain Poloxamer/Poloxamine copolymers results in their increased accumulation in regional lymph nodes. The optimal length of the copolymer polyoxyethylene block has been demonstrated to be 5–15 oxyethylene units. Noncoated particles have a tendency to stay at the injection site, while particles coated with longer polyoxyethylene-containing copolymers are not retained in the nodes and eventually appear in the systemic circulation [119].

The hydrophobic surface of commercially available polystyrene nanospheres can be coated with various polymers of amphiphilic nature, such as hydrophilic linear polymers with terminal lipid or fatty acyl group. Amphiphilic polymers can be synthesized either directly by linking polymer to lipid (like for PEG–phosphatidylethanolamine, PEG–PE, MW 5 kDa) [21], or by using a fatty acyl moiety as a chain terminating agent during the radical polymerization of some vinyl monomers [38]. Thus, acylpolyacrylamide capped with C16 saturated fatty acid residue (APAA, MW 12 kDa) was prepared. To investigate the behavior of coating polymers on the surface of nanoparticulate carriers, polystyrene latex particles with diameters of 100 nm were used. Two already mentioned amphiphilic polymers, APAA and PEG–PE, have been used to coat the surface of latex particles. The incubation of nanospheres with the polymers in water resulted in polymer attachment to the surface, which can be confirmed by the measurement of particle size before and after incubation with the polymer. The particle diameter increase upon polymer adsorption was found to be ca. 5 nm for PEG–PE and ca. 20 nm for APAA. PEG–PE-coated particles stayed in the circulation for a long time ($t_{1/2}$=4 h).

Another important type of amphipathic polymers includes copolymers in which in the water media the hydrophobic block is able to form a solid phase (particle), while the hydrophilic part remains as a surface-exposed protective "cloud." PLAGA–PEG copolymers have been used to prepare long-circulating particles with an insoluble (solid) PLAGA core and water-soluble PEG shells covalently linked to the core [122]. Several polymer preparations have been synthesized where a PEG block (molecular weight 20 kDa) was connected to a PLAGA block with 1:9, 1:5, and 1:4 PLAGA:PEG w/w ratios. Such nanospheres have been labeled by incorporation of hydrophobic 111In-diethylenetriaminepentaacetic acid stearylamide into the PLAGA core during the solvent evaporation procedure. Blood clearance and biodistribution experiments in BALB/c mice have demonstrated that the protective effect of PEG in this system depends on the content of its block. The clearance and liver accumulation patterns reveal one basic feature of preparations under discussion: the higher is the content of the PEG block, the slower the clearance and the better the protection from liver uptake. The splenic uptake also reflects the particle size-dependent filtering effect. As it has been already demonstrated for liposomes, vesicles with diameters exceeding 250 nm can be nonspecifically detained in the spleen [50]. The 1:5 and 1:4 PEG–PLAGA nanoparticles were 265 and 315 nm in size, respectively, so the elevated splenic uptake for these preparations can be explained by their passive retention in the spleen.

Similar effects on longevity and biodistribution of microparticle drug carriers might be achieved by direct chemical attachment of protective polyethylene oxide chains onto the surface of preformed particles [123].

The surfaces of PLAGA microspheres have been modified by adsorption of polylysine–PEG copolymers, and this has resulted in a dramatic decrease of plasma protein adsorption onto modified nanoparticles [124]. Similarly, when the surface of polycyanoacrylate particles was modified with PEG, it also resulted in their increased longevity in the circulation allowing even for their diffusion into the brain tissue [125, 126].

Drug (fluorouracil)-containing dendrimer surface-modified with PEG demonstrated better drug retention and less hemolytic activity [127]. Comparative studies on the modification of the surface of colloidal polycaprolactone carriers with PEG or chitosan demonstrated that the PEG coating accelerated the transport of nanoparticles across the epithelium of the ocular mucosa, while chitosan coating favored the retention of modified nanoparticles in the superficial layers of the epithelium [128]. This observation opens an opportunity to control certain biological properties of nanocarriers by properly chosen surface modification.

Similar to liposomes, protecting nanoparticle surfaces with hydrophilic flexible polymeric chains results in a substantial decline in phagocytosis by liver macrophages and subsequent prolongation of circulation time. However, there exist certain differences in the biological behavior of hydrophobic nanoparticulates and liposomes coated with the same polymers. Thus, although PEG–PE-coated Polybeads particles stayed in the circulation for a long time, PAA-coated nanospheres, unlike PAA-coated liposomes [38], cleared from the blood as fast as noncoated particles with similar patterns of liver accumulation. At the same time, spleen accumulation of PAA-coated particles was substantially reduced compared to noncoated ones. Different biological effects of the same amphiphilic polymer coating on the behavior of different particulates in vivo have also been reported previously. It has been shown that Poloxamer 407 could protect latex nanospheres from RES uptake, but not liposomes of the similar size [117]. This difference in the in vivo behavior has been explained by the different orientation of PAA chains on the particle surfaces compared to the liposome surfaces, which results in different degrees of carrier protection from absorbing plasma proteins. In [129], the method of polymer attachment to the surface was pointed out as the major difference between liposomes with poloxamers or PEG–PE attached. With poloxamers, the hydrophobic region lies perpendicularly to the acyl chain region of the bilayer, whereas for PEG–PE the hydrophobic anchor is parallel to the acyl chains. As a result, the thickness of the coating is considerably less for poloxamers on liposomes than for poloxamers on nanoparticles [130, 131]. For liposomes, PEG–PE works better than Poloxamer F-108 [131]. Nevertheless, the behavior of a hydrophilic moiety in the surrounding aqueous media remains pretty much the same in all cases.

Similar to the liposome case, the use of other soluble polymers, such as PVP, can also render polymeric particles with an increased stability against opsonization and clearance, although the spectrum of proteins eventually adsorbed on PEG-coated nanoparticles and PVP-coated nanoparticles slightly differs [132]. Other polymers used for steric stabilization of PLGA particles include hydroxyethyl starch and pluronics [133].

PEGylation was performed with poly(epsilon-caprolactone) nanoparticles and significantly suppressed their opsonization and phagocytosis [134]. For a variety of polymeric nanoparticles, the increase in the content of PEG results in increased blood circulation times and decreased liver uptake [134, 135]. The use of terminus-activated PEG for coating PLGA nanoparticles provides a convenient platform for the further derivatization of nanoparticles, for example, with targeting ligands [136]. Additionally, PEGylation enhances the diffusion of PLGA nanoparticles into mucus (human cervical

mucus) [137]. Triple-layered nanoparticles consisting of PLGA, lecithin, and PEG have been described as a promising controlled release system [138].

PEGylated PLGA nanoparticles loaded with tacrolimus have demonstrated improved lymphatic targeting [139]. PEGylated polycaprolactone nanoparticles have been loaded with tetradrine and suggested for local drug delivery [140]. Similar nanoparticles have also been used as carriers for honokiol in cell culture experiments [141]. PEGylated PLGA nanoparticles loaded with betamethasone phosphate were used for the treatment of experimental arthritis in rats [142]. Particles of a similar composition have been used for controlled adriamycin release [143]. Injectable PEGylated chitosan nanoparticles have been suggested for sustained protein (insulin) release [144]. Similar nanoparticles have also been used to enhance nasal insulin adsorption [145].

14.5 PEGylated Iron Oxide and Other Nanoparticles

Recently, many studies have been performed with surface modification of superparamagnetic nanoparticles, which are considered as promising agents for drug delivery into the regional lymph nodes and also for diagnostic imaging purposes [146]. Similar to other nanocarriers, PEG-modified magnetite nanospheres have demonstrated increased colloidal stability and improved localization in lymph nodes [147]. Cell culture experiments have confirmed that surface PEGylation changes the interaction of modified iron oxide particles with fibroblasts [148]. PEG-gallol-modified iron oxide nanoparticles, which can be additionally modified with a targeting ligand, have been suggested as MR imaging agents [149]. Monodisperse magnetite nanoparticles have been modified with PEG using silane-functionalized PEG [150]. Iron oxide nanoparticles have been loaded in PEG–PE-based micelles and additionally modified with tumor-specific antibodies for targeted drug delivery and imaging [151]. Nanomagnetite particles have been prepared by deposition of polycaprolactone–PEG copolymer on their surfaces [152].

Grafting PEG onto the surface of gold particles via mercaptosilanes expectedly has resulted in decreased protein adsorption onto modified particles and less platelet adhesion [153]. In general, PEGylation of gold nanoparticles has been proven to be an effective means of reducing MPS uptake and clearance from the body with the efficiency of protection being dependent on PEG density [154, 155]. An effective "click" chemistry has been developed for PEGylation of gold nanoparticles [156] and a fluorescence-based assay has been developed to control the quantity of grafted PEG [157]. PEG–polyamine copolymer and PEG–peptide conjugates have been used to prepare completely dispersible PEGylated gold nanoparticles [158, 159]. Similar technologies have been applied to nanoparticles made of palladium, platinum, and ruthenium [160].

PEGylated gold nanoparticles have been suggested, among other applications, for photothermal tumor therapy (ablation) following accumulation in the tumor via the EPR effect because of their long-circulation property [161]. PEGylated gold nanoparticles

with a size of 3.7 nm have been shown to penetrate into the nuclei of HeLa cells [162]. PEG-coated gold nanoparticles have been suggested as contrast media for computed tomography (CT) [163]. Fluorescent dye-modified PEGylated gold nanoparticles have been used as markers of intracellular delivery and transport [164].

Dendrimers, which are currently considered as promising drug delivery carriers, have been modified with PEG to increase their longevity and accumulation [165]. PEGylated methotrexate-conjugated poly-L-lysine dendrimers have been shown to accumulate efficiently in solid tumors via the EPR affect [166]. It has been shown that the pharmacokinetics of PEGylated dendrimers depends on the structure of the dendrimer, as well as on the quantity and length of grafted PEG. The higher generation dendrimers with longer PEG chains attached and higher density of attachment have been shown to demonstrate greater blood retention [167–172]. PEGylated polylysine dendrimers have been found to demonstrate good lymphatic targeting following subcutaneous administration in rats and can be used for lymphatic delivery of drugs and imaging agents [173]. Importantly, PEGylating reduces the toxicity of positive charge-bearing dendrimers, such as PAMAM [174, 175].

As potential pharmaceutical carriers, PEGylated dendrimers were used in vitro and in vivo to deliver adriamycin [176], antiarhytmic quinidine [177], photosensitizers for photodynamic therapy (rose Bengal and protoporphyrin IX) [178], antimalarial chloroquine phosphate [179], H_2 receptor agonist [180], siRNA [181], genes [182, 183]. PEGylated dendrimers have also been suggested for oral drug delivery (because of their enhanced transepithelial transport) [184], and for pulmonary delivery of low-molecular-weight heparin [185]. PEGylated dendrimers encapsulating gold nanoparticles have been tested as agents for photothermal therapy [186].

Biomedical application of carbon nanotubes also depends on PEGylation and this can be achieved by simple sonication of single-wall nanotubes in the presence of PEG–PE [187]. PEGylated nanotubes have demonstrated longer circulation in mice upon intravenous administration [188]. Fluorescently labeled PEGylated carbon nanotubes have been shown to penetrate cells and even cell nuclei and can be used for drug delivery inside cells [189]. PEG–doxorubicin conjugates adsorbed onto carbon nanotubes have been suggested for local cancer chemotherapy, since they accumulate in the lymph nodes [190]. PEGylated carbon nanotubes have been suggested as vehicles for delivery of antisense oligonucleotides [191].

Quantum dots have been modified by PEGylation, and it has been demonstrated in mice that PEGylated quantum dots, similar to other nanoparticulates, show a lesser uptake in MPS organs (spleen and liver) and prolonged blood circulation [192]. In general, PEGylation increases the biocompatibility of quantum dots and decreases their toxicity [193, 194], the extent of the effect being dependent on PEG length and density [195]. Universal linkers for attaching PEG to quantum dots have been suggested [196], and PEGylated quantum dots have been successfully used as an intracellular labeling agent [197].

There exists examples of PEGylation of solid lipid nanoparticles both for drug delivery (vinorelbine bitartrate) [198] and for transendothelial penetration [199]. Gene-loaded PEGylated lipid particles have been additionally modified with cell-penetrating oligoarginine for better gene delivery [200].

The surface of red blood cells has been covalently modified with methoxy-PEG, which significantly diminished the immunologic recognition of surface antigens on these cells [201]. Other blood cells as well as some tissue cells have also been surface-modified with PEG derivatives and an effective immunocamouflage has been observed both in vitro and in vivo [202, 203].

14.6 Combination of Targeting Ligands with Protecting Polymers

The further development of the concept of PEGylated pharmaceutical carriers involves attempts to combine the properties of long-circulating carriers and targeted carriers in one preparation, i.e., simultaneously attach both a protecting polymer and a targeting moiety on the surface of a nanocarrier, such as a liposome [204–206]. To achieve better selective targeting of PEG-coated liposomes, it is advantageous to attach the targeting ligand to the particles via a PEG spacer arm, so that the ligand is extended outside of the dense PEG brush to exclude steric hindrances for binding to the target receptors. Currently, various advanced technologies are used, and the targeting moiety is usually attached above the protective polymer layer, by coupling with the distal water-exposed terminus of activated liposome-grafted polymer molecules [205, 207] (see Fig. 14.3). For this purpose, a number of derivatives of end-group functionalized lipopolymers of general formula X-PEG–PE [34, 208], where X represents a reactive functional group-containing moiety, have been introduced, such as *p*-nitrophenylcarbonyl-PEG–PE (pNP-PEG–PE) [207, 209, 210]. pNP-PEG–PE readily adsorbs on hydrophobic nanoparticles or incorporates into liposomes and micelles via its phospholipid residue, and easily binds any amino group-containing compounds via its water-exposed pNP group forming stable and nontoxic urethane (carbamate) bonds.

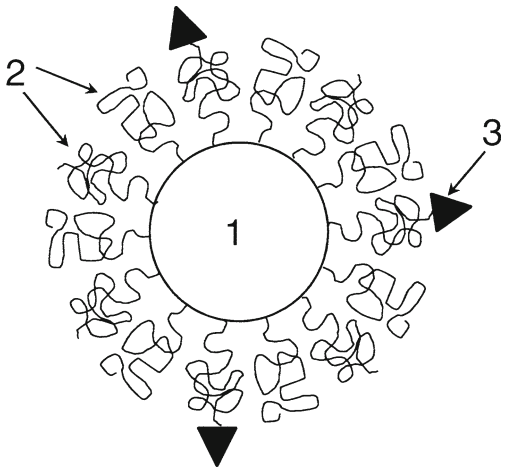

Fig. 14.3 To make pharmaceutical nanocarrier (1) surface-modified with PEG (2) "actively" targeted, the targeting ligand (3) (antibody, transferrin, folate) is usually chemically attached to water-exposed distal tips of PEG chains

Three general strategies have been employed to assemble ligand-bearing long-circulating PEGylated nanocarriers [208, 211]. The first approach involves modification of preformed liposomes. Since this approach involves conjugation after the formation of liposomes, some of the reactive end groups might remain on the outer surface creating a possibility of crosslinking through multiple attachments to a single ligand molecule. According to the second approach, ligand–PEG–lipid conjugates have been mixed with other liposomal matrix-forming components, and then made into unilamellar vesicles [211–214]. In the third approach, a spontaneous insertion strategy has been utilized, where ligand–PEG–DSPE conjugates are incubated with preformed loaded liposomes [211]. The insertion methodology allows for the achievement of the same external surface densities of both PEG-tethered ligands and mPEG chains as by the lipid mixing/extrusion process [211].

A broad variety of targeting ligands have been attached to PEGylated pharmaceutical nanocarriers to achieve higher delivery efficiencies. An interesting concept has been developed to target HER2-overexpressing tumors using anti-HER2 PEGylated doxorubicin-loaded liposomes [215, 216]. Antibody CC52 against rat colon adenocarcinoma CC531 attached to PEGylated liposomes provided specific accumulation of liposomes in a rat model of metastatic CC531 [217]. Nucleosome-specific antibodies capable of recognition of various tumor cells via tumor cell surface-bound nucleosomes improve Doxil® targeting to tumor cells in vitro and in vivo and increase its cytotoxicity [218, 219]. Surface modification with antibodies has also been applied to achieve targeting of other pharmaceutical nanocarriers, in particular cancer-targeting; see [220] for review. Nanoparticles made of poly(lactic acid) have been surface-modified with PEG and with anti-transferrin receptor monoclonal antibody to produce PEGylated immunoparticles with a size of about 120 nm and containing ca. 65 bound antibody molecules per single particle [221]. PEGylated gold nanoparticles have been additionally conjugated to monoclonal F19 antibodies as targeted labeling agents for human pancreatic carcinoma tissues [222]. Similarly, PEGylated carbon nanotubes were additionally modified with antibodies specifically targeting the cytoplasmic compartment [223]. Mammalian cells (NIH3T3, 32D, Ba/F3, hybridoma 9E10) have been surface-modified with distal terminus-activated oleyl-PEG and various proteins (streptavidin, EGFP, and antibody) have been successfully attached to the activated PEG termini [224] producing potentially interesting drug delivery system.

Since transferrin (Tf) receptors (TfR) are overexpressed on the surface of many tumor cells, antibodies against TfR as well as Tf itself are among popular ligands for targeting various nanoparticulate drug carriers including liposomes to tumors and inside tumor cells [225]. Recent studies have involved the coupling of Tf to PEG on PEGylated liposomes in order to combine longevity and target ability for drug delivery into solid tumors [226]. A similar approach has been applied to deliver to tumors agents such as hypericin [227, 228] for photodynamic therapy. Another example is the intracellular delivery of cisplatin into gastric cancer tumors [229]. Tf-coupled doxorubicin-loaded PEGylated liposomes have demonstrated increased binding and toxicity against tumors in rats [230]. Tf-PEG-liposomes have been used

for the delivery of borocaptate to malignant glioma for neutron capture therapy [231]. Interestingly, increase in the expression of TfR has been discovered in postischemic cerebral endothelium, and accordingly Tf-modified PEG-liposomes have been used to deliver to postischemic brains in rats [232]. To enhance Tf interaction with TfR on TfR-overexpressing tumor cells and increase the efficiency of transfection, Tf can be attached to the surface of nanoparticles via a PEG spacer [233].

Targeting tumors with folate-modified nanocarriers including liposomes represents a very popular approach, since the folate receptor (FR) is frequently overexpressed in many tumor cells. After early studies demonstrated the possibility of delivery of macromolecules [234] and then liposomes [235] into living cells utilizing FR endocytosis, which could bypass multidrug resistance, the interest to folate-targeted drug delivery by liposomes has grown rapidly (see important reviews in [236, 237]). Various biodegradable nanoparticles have been surface-modified with folate to enhance tumor targeting. Folate has been attached to the surface of cyanoacrylate-based nanoparticles via activated PEG blocks [238]. Similarly, PEG–polycaprolactone-based particles have been surface-modified with folate and, after being loaded with paclitaxel, have demonstrated increased cytotoxicity [239]. Superparamagnetic magnetite nanoparticles have been modified with folate (with or without PEG spacers) and have demonstrated better uptake by cancer cells, which can be used for both diagnostic (magnetic resonance imaging agents) and therapeutic purposes [240, 241]. Folate-modified solid lipid nanoparticles loaded with paclitaxel have demonstrated improved properties (longevity and drug release) in vivo [242]. Folate-modified PEG-grafted hyperbranched PEI has been used for tumor-targeted gene therapy [243]. Docetaxel-loaded PEGylated PLGA nanoparticles have been modified with folate to increase their cellular uptake and anticancer cytotoxicity [244]. Folate-modified PEG-dendrimers loaded 5-fluorouracil also demonstrated enhanced activity in tumor-bearing mice [245]. Folate has also been used to target PEGylated gold particles to tumor cells [246]. Folate-modified PEGylated quantum dots have been used for the visualization of receptor-mediated endocytosis [247]. Folate-modified carbon nanotubes have been suggested for the delivery of platinum drugs to tumor vasculature [248, 249].

The search for new ligands to be attached to the surface of PEGylated nanocarriers for targeting purposes has concentrated around specific receptors overexpressed on target cells (cancer cells, first of all) and certain specific components characteristic of pathologic cells. Thus, vasoactive intestinal peptide (VIP) has been attached to PEG-liposomes with radionuclides to target them to VIP tumor receptors. This has resulted in an enhanced breast cancer inhibition in rats [250]. PEG-liposomes containing doxorubicin and labeled with RGD peptide targeting moieties have demonstrated increased efficiency against C26 colon carcinoma in murine model [251]. PEGylated liposomes modified with a fibronectin-mimetic peptide have been used to target colon cancer [252] and prostate cancer [253]. Albumin-coated PEG-liposomes have shown enhanced tumor delivery of doxorubicin compared to nonlabeled PEG-liposomes [254]. Lactoferrin-conjugated PEG–polylactic acid nanoparticles have demonstrated improved brain delivery [255]. PEGylated particles have also

Table 14.2 Some examples of targeting ligands attached to PEGylated nanocarriers

Monoclonal antibodies
Fragments of monoclonal antibodies
Transferrin
Folate
RGD peptide
Lactoferrin
Vasoactive intestinal peptide
Cell-penetrating peptides

been modified with cell-penetrating peptides for better intracellular delivery [200] (see some examples in Table 14.2). Specific attention has been paid to how longevity and targeting moieties should be assembled on the surface of the nanocarriers for the optimal performance [256].

14.7 Conclusions

PEGylation has become an important procedure to improve and adjust pharmacological properties of a variety of pharmaceutical nanocarriers for the delivery of drugs, genes, and diagnostic agents. The combination of PEGylation with various targeting ligands can enhance the properties of pharmaceutical nanocarriers still further.

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Chapter 15
Protein PEGylation

Francesco M. Veronese and Gianfranco Pasut

Abstract The covalent linking of poly(ethylene glycol) (PEG) has become the leading approach to improving the therapeutic efficacy of proteins. This highly hydrophilic synthetic polymer possesses unique properties that allowed PEG to emerge as the best candidate for protein modification. Beside the proven success of PEG conjugates already on the market, it should be noted that other derivatives are presently under advanced clinical trials. The increased half-life of the conjugates is probably the main reason for performing PEGylation. In addition, the possibility to greatly reduce the immunogenicity of a given protein is also a strong and determinant incentive. This last advantage offers the possibility to safely use, in the clinic, heterologous proteins that otherwise might trigger dramatic immunogenic responses or even anaphylactic reactions. This chapter introduces the reader to PEGylation strategies showing in detail its potential and the achievements obtained in the recent years. Furthermore, the future perspectives of the technique are also discussed.

15.1 Introduction

The interest in proteins as therapeutic agents started a long time ago but only recently, thanks to the genetic engineering techniques, the optimized procedures of expression in bacteria cells and purification from expression media, have these complex molecules entered effectively into clinical practice [1]. Usually, proteins have a strong and selective biological activity, which in many cases is counterbalanced or

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Table 15.1 Marketed PEG conjugates

Conjugate	Indication	Approval year
PEG-Adenosine Deaminase (Adagen®)	Severe Combined Immuno-deficiency Disease (SCID)	1990
PEG-Asparaginase (Oncaspar®)	Leukaemia	1994
PEG-Interferon-α2b (Peg-Intron®)	Hepatitis C	2000
PEG-Interferon-α2a (Pegasys®)	Hepatitis C	2001
PEG-Human growth hormone mutein antagonist (Pegvisomat; Somavert®)	Acromegaly	2002
PEG-G-CSF (Pegfilgastrim Neulasta®)	Neutropenia after cancer chemotherapy	2002
PEG-anti-VEGF aptamer (Pegaptanib, Macugen™)	Wet age related macular degeneration	2004
PEG-Erytropoietin (Mircera®)	Anaemia associated with chronic kidney disease	2007
PEG-Anti-TNF Fab' (Cimzia®)	Rheumatoid arthritis and Crohn's disease	2008
PEG-Uricase (Pegloticase; Krystexxa®) (R)	Chronic Gout	2010

regulated by Nature through different mechanisms, such as fast kidney clearance, enzymatic degradation or modification with a range of post-translational reactions. In an effort to copy Nature, in the last decades molecular biologists and pharmaceutical chemists have tried to tune the in vivo behavior of proteins by exploiting different chemical or enzymatic modifications (e.g., coupling to natural or synthetic polymers [2], fusion to albumin or other proteins [3] or point mutation of the primary amino acids sequence [4]). The aim of these modifications is to alter or shield the sites recognized by proteolytic enzymes or antibodies, and in general to prolong the protein in vivo half-life.

PEGylation, the covalent linking of poly(ethylene glycol) (PEG) to proteins or biomaterial surfaces, has become one of the best strategies for protein modification and it has so far brought into the market several conjugates with great therapeutic performance (Table 15.1). Ideally, the polymer chains have to be linked to amino acids not essential for protein activity (e.g., those far from the enzymatic active site or the receptor recognition area of ligand proteins) while they should address the sequences sensitive to proteolytic enzymes or recognized by the immunological system (Fig. 15.1). To accomplish this difficult task, a series of PEGylating agents and coupling strategies, as summarized in Table 15.2, have been developed during the 30 year long history of PEGylation. In Table 15.2 are reported the most relevant properties of the formed bonds between these PEGs and the target molecule.

For a description of PEGylation and PEGylation examples more detailed than in this short chapter, the reader might refer to the original research papers, to the numerous reviews on the topic or to a recently edited book on PEGylation [11].

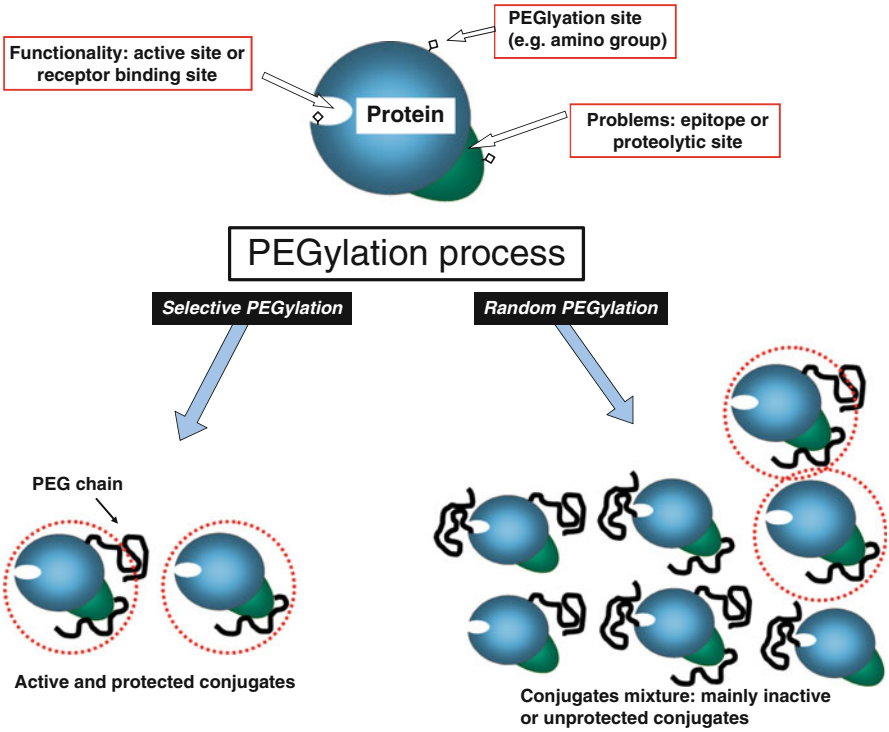


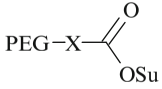
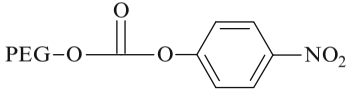
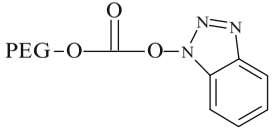
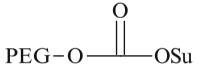
Fig. 15.1 Schematic representation of the different outcomes reached by random or selective PEGylation. The polymer conjugation should be aimed to preserve the protein activity and, at the same time, it should cover potential immunogenic or degradable sites

15.2 General Properties of PEG

The chemical structure of PEG, $CH_3O–(CH_2CH_2O)_n–H$, confers a great flexibility to the polymeric backbone thanks to the absence of bulky substituents along the chain and the easy rotation of the C–C and C–O bonds. This high flexibility, together with the coordination of 6–7 water molecules per oxygen atom, is at the basis of the protein and cell rejecting properties of the polymer [12]. The coordinated water molecules are also responsible for the PEG hydrodynamic volume, which is higher than that of a globular protein of the same molecular weight [13, 14]. This behavior is translated in vivo into reduced kidney filtration. From the point of view of in vitro laboratory characterization, PEG and PEG conjugates have shorter retention time in gel-filtration chromatograph and migrate less in SDS gel electrophoresis compared to a globular protein of the same molecular weight. As with other synthetic polymers, PEG is polydisperse and its Mn/Mw values range between 1.05 and 1.2 depending on the polymer molecular weight.

PEG and its conjugates are cleared from the body mainly by the kidney and the excretion rate has a sigmoidal relationship with the polymer molecular weight, with

Table 15.2 Most frequently used PEGylating agents and their properties in protein conjugation

PEG reagent	Formed bond	Properties	References
 NHS activated PEG carboxylates	PEG–X–CO–NH–protein	The X group (e.g., CH_2, CH_2CH_2, $CH_2CH_2(CH_3)$, etc.) tunes the reactivity. The amide bond formed is very stable and its slightly acidic behavior, opposed to that of the conjugated amine, causes a clear change in the isoelectric point of the conjugated protein	Bailon et al. [5]
 PEG-p-nitrophenyl carbonate	PEG–O–CO–NH–protein	These PEGs are less reactive than NHS activated PEG carboxylates. Also in these cases the isoelectric point of the conjugated protein is decreased	Veronese et al. [6]
 PEG-benzotriazol carbonate			
 PEG-succinimidyl carbonate			Wang et al. [7]

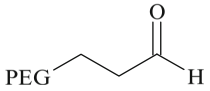
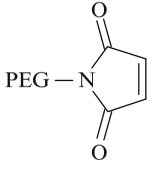
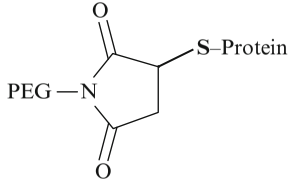
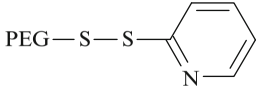
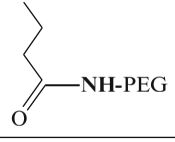
 PEG-propionaldehyde	PEG–CH_2–NH–protein	Very stable secondary amine. This approach yields conjugates with minor difference in isoeletric point with respect to the starting protein To selectively link the N-terminal amino group of proteins the reaction must be carried out at acidic pH values (4.5–6)	Kinstler et al. [8]
 PEG-maleimide		Gives stable thioether linkage. It might also react with amines at high pH values. The maleimide ring might present instability problem by ring opening reactions	Veronese et al. [9]
 PEG-o-pyridyl disulphide	PEG–S–S–protein	The most specific reagent towards thiol but yields a disulphide bridge that can be cleaved by reduction	Veronese et al. [9]
H_2N–PEG Amino PEGs for TGase mediated conjugation	AA–NH–CH–CO–AA 	Stable amide linkage. TGase is highly selective, only one or few glutamines of a protein are proper substrates	Fontana et al. [10]

Table 15.3 Pharmacokinetic prolongation and activity retention of some PEGylated protein conjugates (adapted from [17])

Conjugate	Polymer chains linked and MW	% of retained activity in vitro	In vivo half-life increase (times)	References
PEG-Arginine deiminase	22 × PEG 12 kDa	48	17.8	Wang et al. [18]
PEG-Catalase	65 × PEG 5 kDa	95	10	Abuchowski et al. [19]
PEG-Methioninase	3–7 × PEG 5 kDa	70	36	Yang et al. [20]
PEG-Tricosanthin	1 × PEG 40 kDa	9	6	An et al. [21]
PEG-Lysostaphin	1 × branched PEG 40 kDa	10	30	Walsh et al. [22]
PEG-G-CSF	1 × PEG2 10 kDa	41	7	Tanaka et al. [59]
PEG-Interferon-alpha2a (Pegasys)	1 × branched PEG 40 kDa	7	70	Bailon et al. [5]
PEG-Interferon-alpha2b (Pegintron)	1 × PEG 12 kDa	10	4–5	Wang et al. [7]
PEG-Interferon-beta-1b	1 × branched PEG 40 kDa	24	21.6	Basu et al. [23]
PEG-Interleukin-6	1 × SS-PEG 5 kDa	>10	960	Tsutsumi et al. [24]
PEG-Tumor necrosis factor-alpha	1 × mPEG 5 kDa	100	7	Shibata et al. [25]
PEG-Calcitonin	1 × PEG 2 kDa	74	3.5	Youn et al. [26]
PEG-Human growth factor	1 × PEG 20 kDa	>50	29.4	Cox et al. [27]

a great reduced rate over about 40 kDa [15, 16]. High molecular weights are slowly excreted by liver or permanently entrapped into tissues or cells. Usually, the half-life increase for a given protein, after PEGylation, depends on the total mass of the polymer coupled to the protein, although differences between conjugates with similar size can be ascribed to the protein moiety. In terms of numbers, the increase in half-life spans from a few fold to dozens, but in certain cases an increase of a 100-fold was also observed (Table 15.3). Interestingly, a similar outcome in blood residence time can be reached either by linking several short chains or a single long PEG chain. This last strategy, now commonly preferred, has the advantages of yielding less heterogeneous conjugate isomer mixtures and better retention of the protein activity.

15.3 Chemistry of PEG and Strategy for Conjugation

So far the most used PEG structures for protein conjugation are the linear or the branched methoxy forms. It is important to avoid the presence of diol-PEG as an impurity in batches of PEG used for protein conjugation because it can lead to intra- or inter-molecular cross-linking reactions. Nowadays, for the coupling to various

reactivities (e.g., protein amino or thiol groups), several PEGylating agents are commercially available and easy procedures for the activation of the hydroxyl group are at hand and widely described [28].

The epsilon amino group of lysine is the most exploited for protein conjugation with activated PEG carboxylates or carbonates, yielding amide or urethane linkages respectively (Table 15.2).

The rate of coupling might greatly vary on the basis of both PEG reactivity and nucleophylicity and exposure of the protein amino group. It is worth noting that the reactivity of a given activated PEG can be tuned by changing the chemical groups close to the reactive moiety. For example, the carboxymethylated succinimidyl PEG presents a half-life of hydrolysis in water, pH 8 and 25°C, of 0.75 min, while the butanoate, the propanoate and the alpha-methylbutanoate derivatives have values of 23, 16.5 and 33 min, respectively [28].

Protein amino PEGylation, as coupling strategy, presents the drawback of yielding a mixture of several conjugate isomers with a great heterogeneity, as consequence of the many amines per protein molecules. The most used approach for a selective amino PEGylation is, as below described in detail, the coupling to the lone alpha amino group at the N-terminus, which is feasible because of its lower p*K*a with respect to that of epsilon amines [29].

The thiol group instead, being rarely present in proteins, is an interesting and useful site for PEGylation. The most used thiol reactive PEGylating agent is maleimide PEG, but also the orthopyridyl disulphide PEGs are used, although they form disulphide bridges that might slowly release the protein in vivo under reducing conditions, as in the case of some gene delivery system purposely cross-linked and stabilized with disulphide bridges [30].

In the beginning years of PEGylation, even highly heterogeneous mixtures of conjugate isomers (e.g., PEG-asparaginase and PEG-adenosine deaminase), of which thorough characterization of all isomers was almost impossible, were approved by drug agencies like the FDA. During that time, the need to obtain well characterized conjugates with a high batch-to-batch reproducibility as requested by regulatory agencies stimulated pharmaceutical chemists to develop more specific strategies of protein coupling.

15.4 What Strategies to Use for Site Directed PEGylation

15.4.1 Taking Advantage of Different in pKa of the Amino Acid Residues

When an amino reactive PEG is coupled to a protein at mild alkaline pH values, usually 8–9, all the available protein amino groups are labeled. Differently, when the coupling is performed at acidic pH, usually below 6, only or at least preferentially the α-amino group at the N-terminus is modified. This selectivity is due to the different p*K*a of this group (7.6–8) with respect to the ε-amino group of lysines (10–10.2) [29]; see the section “Examples of PEGylation chemistry” for the PEGylation procedure.

15.4.2 Reversible Protection of Protein Amino Acid Residues Critical for Activity

Sites involved in biological activity can be protected during the modification reaction by non-covalent or covalent masking. In the case of enzymes, substrates or non-covalent inhibitors can be used to prevent PEGylation at the enzyme active site through steric hindrance. Alternatively, chemical approaches of reversible protection can be used for small polypeptides. For example, insulin was reversibly protected at the Gly-B1 and at the Lys-B29 with the tert-butyloxy carbonyl (BOC) group prior to performing the PEGylation reaction at the unprotected Phe-A1, allowing better preservation of the activity, after BOC removal, because the amino group of Gly is essential for activity [31].

15.4.3 Change of Amino Acids Accessibility

It is known that the solvent exposure of an amino acid impacts remarkably on its reactivity. Low accessibility residues are PEGylated at a slow rate or even not coupled at all. On the other hand, by changing the solvent it is possible to modify the protein structure and solvent exposure of certain amino acids, and consequently the PEGylation outcome. The conjugation can be carried out in mixtures of water and water miscible organic solvents, changing the protein conformation and reactivity. For example, human growth hormone (hGH) releasing factor (hGRF1-29) was PEGylated after dissolution in 2,2,2-trifluoroethanol. This solvent increased the peptide alpha helix content from just 20% in water to 90%, allowing a region-selective modification. The reaction yielded a mixture containing 80% of the Lys-12 conjugated isomer [32], which is a great improvement with respect to the equimolar mixture of the two isomers, at Lys-12 or Lys-21, obtained performing the coupling in water [33].

Differently, a destructuring approach has been carried out to modify the buried and lone free cysteine of granulocyte colony stimulating factor (G-CSF). Selective modification was accomplished by partially unfolding the protein, but preserving the disulphide bridges unreduced, and using a thiol reactive PEG. After coupling, the biologically active protein–PEG conjugate was recovered by removing the denaturant [9].

15.4.4 Use of PEGs with Different Structures

Often the active sites of enzymes are in buried clefts not accessible to large molecules. This characteristic can be exploited to avoid the modification of the active site during the PEGylation reaction by using hindered branched PEGs [34]. Due to their structure the branched PEGs have less accessibility to buried sites with respect to the linear PEGs, thus preserving better the enzymatic activity. Furthermore, the "umbrella like" shape of branched PEGs protects the proteins from degradation to a greater extent, offering a higher degree of surface shielding per each point of attachment

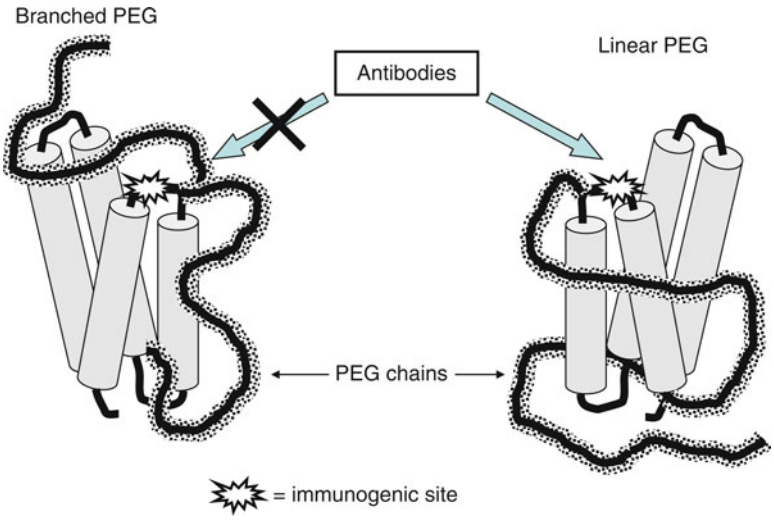


Fig. 15.2 Branched PEGs offer a higher protein surface shielding

(Fig. 15.2). Branched PEGs were successfully used not only for protein modification, with two conjugates already on the market (Pegasys and Cimzia), but also for the coupling of the oligonucleotide aptanib (Macugen) (Table 15.1).

15.4.5 Bridging PEGylation of Disulphides

Generally, disulphide bonds are present in a small number in proteins and sometimes they can be selectively reduced depending upon their solvent exposure. These two characteristics make disulphide bridges attractive sites for specific modification. A specific PEGylating agent was developed. The coupling reaction requires a preliminary reduction, preferably selective, of the protein disulphides followed by a double addition of the PEG to the formed free thiols (Fig. 15.3). This PEG was designed to preserve in the final conjugate the correct distance between the two sulphur atoms as in the native protein. In the literature there are already examples reporting that the disulphide PEGylated proteins, enzymes or cytokines, maintain both structure and activity [35, 36].

15.4.6 Enzyme Catalyzed Conjugation

Enzymes present unique properties of catalysis, specificity and selectivity. So far, their use in chemistry is underexploited and great advances might come in the near future from this approach. For what concern the use of enzymes for selective

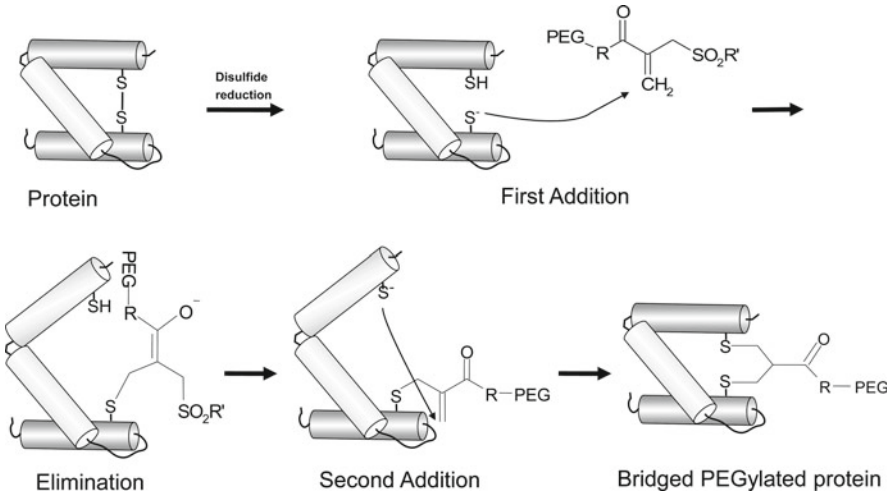


Fig. 15.3 Steps representation of protein bridging PEGylation

PEGylation, so far only one strategy has yielded important results and stimulated several PEGylation studies. Transglutaminase (TGase) was employed to link PEG–NH_2 to glutamine residues of proteins (Fig. 15.4) [37]. It is relevant to highlight that glutamine cannot be modified by chemical methods without also labeling other residues or degrading the protein structure. The selectivity of this enzymatic conjugation resides in the fact that TGase has strict requirements in terms of amino acid sequence and flexibility of the substrate. Therefore, usually only one or two glutamines, among the several glutamines present in a protein, possess the prerequisites for TGase catalysis. The amino acid has to be inserted in a flexible region of the protein backbone in order to allow juxtaposition of the protein sequence with about ten residues in the catalytic sites of TGase, likewise in the case of proteolytic enzyme/substrate recognition [10].

Another example of enzymatic PEGylation has been proposed exploiting in tandem two enzymes: O-(*N*-acetylgalactosamin)-transferase and sialyl-transferase, a technique known as "glycopegylation" [38]. In this case the amino acids involved are threonine and serine.

15.4.7 Strategies Based on Genetic Engineering

Genetic engineering was first exploited to introduce a cysteine residue in convenient positions for a selective PEGylation that could ensure better activity retention. This strategy soon became a standard procedure to achieve selective conjugation for proteins [39].

A different approach has exploited genetic engineering to remove from the protein receptor site those amino acids that can be PEGylated (e.g., lysine when amino

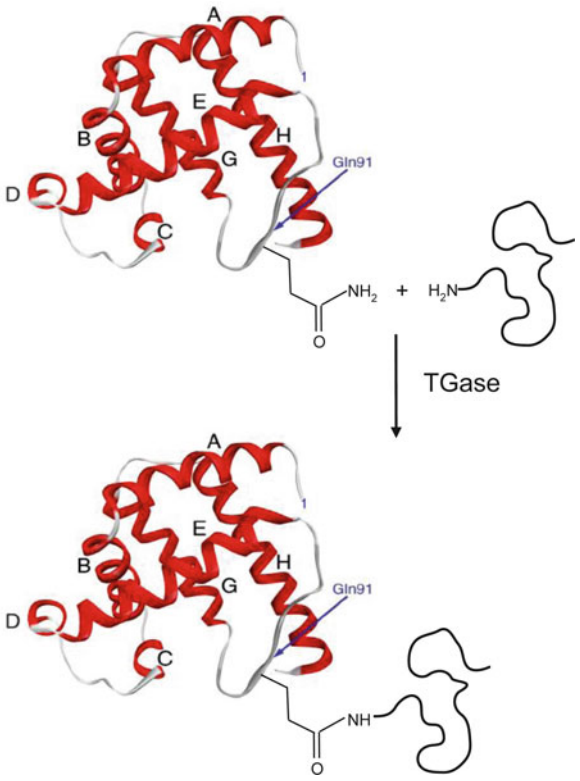


Fig. 15.4 Transglutaminase mediated PEGylation (adapted from [10])

PEGylation is performed) [40]. This reduced the probability that a PEG chain blocks the protein activity by covering its receptor site.

More recently, a strategy known as "RECODE" technology was developed. This concept consists in the introduction of an unnatural amino acid at the desired protein sequence followed by PEG conjugation using click chemistry [41]. The method was successfully verified in several cases and it might open the way to important new applications, although it is not a straightforward procedure because it needs *ad hoc* produced tRNA and RNA synthetase.

15.5 Alternative PEGylation Strategy: The Use of Releasable Linkers

As reported in Table 15.3, protein PEGylation is always accompanied by a loss in protein activity, when tested in vitro. Although this drawback is often counterbalanced by either the increased in vivo half-life or the reduced immunogenicity, it clearly limits the full exploitation of the technique. This fact is even more important when low molecular weight drugs are coupled to the polymer, because in most cases

these drugs are active only when the molecule is in the free form. To circumvent the problem, several researchers have proposed releasable PEGs. These polymers can be detached from the site of conjugation releasing the native and fully active protein. Specific linkers, inserted between PEG and the protein, have been designed to undergo hydrolysis with a controlled rate that ensures both a sufficiently prolonged half-life in vivo and, at the same time, a recovery of the protein activity. Most of the proposed linkers contain an ester bond. The hydrolysis of this group, eventually mediated by plasma esterases, triggers directly or through a cascade of chemical rearrangements of the protein release. Several interesting procedures and results have already been reported in the literature, although none of these systems has yet reached the market [2].

15.6 How to Handle PEG and PEG-Conjugates

Every protein and activated PEGs have their physical–chemical properties and therefore it is difficult either to foresee the results of a PEGylation approach or to develop a general method suitable for all cases. There are, however, some common considerations that can be drawn: (1) PEG, especially in the activated form, is sensitive to temperature and storage conditions and it can be easily deactivated by humidity; each commercial PEG should therefore be tested to evaluate purity and degree of activation just before use; (2) the viscosity of PEG or PEG conjugate solutions might affect the efficiency of chromatographic techniques at the purification or characterization level; (3) ^{1}H-NMR is a convenient method to analyze and evaluate the activated PEGs, (4) PEG does not present UV–Vis absorption, therefore spectrophotometric techniques can be used to characterize the protein moiety; (5) the evaluation of a conjugate molecular weight by SDS electrophoresis or by gel-filtration can be tricky and misleading, thus it is better to use MALDI-TOF mass spectroscopy; (6) ion exchange chromatography is often the preferred procedure for conjugate purification due to the easily scalability and the separation efficacy; (7) to avoid the easy physical and chemical protein degradation, ultra pure solvents devoid of metal contamination must be used, as well as avoidance of prolonged storage of conjugates in aqueous solutions at room temperature.

15.7 Examples of PEGylation Chemistry

In this section we report some examples of PEGylation that, besides being a useful tool for the non-experts in this field, are a clear summary of how this technique has been developed and refined during the years. The first FDA approved PEGylated proteins were two enzymes, adenosine deaminase and asparaginase [42, 43]. The two conjugates were obtained by random PEG coupling at the amino groups, linking several polymer chains per each protein molecule. This approach yielded heavily

heterogeneous mixtures of PEG–protein isomers. High heterogeneity complicates the purification and characterization steps that in turn means increased difficulties for the batch-to-batch production reproducibility. Nowadays, random conjugation yielding multi-PEGylated proteins, although important to establish and prove the potentiality of PEGylation, has been supplanted by more sophisticated and properly designed approaches of coupling as above described. Therefore, following are reported examples of PEGylation that yielded mono-PEGylated protein conjugates either exploiting random or site selective methods.

15.7.1 Random Amino PEGylation: The Cases of Peg-Intron® and Pegasys®

The first PEGylated interferon to reach the market was developed by conjugating interferon α-2b (INF α-2b) with succinimidyl carbonate-PEG (SC-PEG) of 12 kDa. The reaction was performed in sodium phosphate buffer solution at pH 6.5 yielding a mixture of mono-PEGylated isomers, diPEGylated isomers and unreacted protein. The mono-PEGylated fraction was separated and characterized by cationic exchange chromatography. It was determined that approximately 47% of the total mono-PEGylated species was a conjugate at the level of histidine-34 [43]. The overall retained activity in vitro of the mono-PEGylated Peg-Intron mixture is 28% with respect to the native interferon. The relatively high activity retention of this interferon preparation was related to the ability to release free and fully active interferon by slow detachment of the PEG chains coupled to histidine 34 [7], even though there are still doubts about the role of this hydrolysis in vivo [44]. Further studies were also necessary to find adequate formulations to maintain the stability of the conjugate. To prevent de-PEGylation from His-34 the drug is available as lyophilized powder [Peginterferon alfa-2b Product Information. Kenilworth, NJ, USA: Schering Corporation].

Peg-Intron has a serum life about six times longer than that of INF α-2b, thus allowing a less frequent administration schedule while maintaining an efficacy comparable with unmodified interferon [45, 46]. The conjugate reached the market in the year 2000.

Pegasys represents a different approach to interferon PEGylation. INF α-2a was coupled with a branched PEG of 40 kDa, a polymer that presents several advantages over common linear forms. The branched succinimidyl-PEG (PEG2-NHS) was conjugated to the protein using a polymer excess in 50 mM sodium borate buffer pH 9 [5]. The reaction led to a mixture composed of 45–50% mono-PEGylated isomers, 5–10% polyPEGylated isomers and 40–50% unmodified interferon. The purified mono-PEGylated fraction was characterized by combination of high performance cation exchange chromatography, peptide mapping, amino acid sequencing and mass spectroscopy analysis to identify the major positional isomers. In the majority of the isomers (94%) PEG was attached to Lys-31, Lys-121, Lys-131 or Lys-134 [47]. The in vitro activity testing of the purified mono-PEGylated isomers was of concern

because the anti-viral activity was reduced to only 7% of that of INF α-2b. On the contrary, the in vivo activity was higher than that of native protein. This behavior exemplifies the most common mistake that a researcher not familiar with this technique might make. The efficacy of a PEGylated protein cannot be thoroughly evaluated with in vitro experiments only. In fact, in this case one of the most important positive effects, the in vivo half-life prolongation, cannot play a role in the overall evaluation. The positive results of Pegasys are related to the extended blood residence time of the conjugated form, which was increased more than 20-fold [44]. Pegasys was marketed for the treatment of hepatitis C virus (HCV) infection [48].

15.7.2 Selective Amino PEGylation

The best-known example of this approach is the N-terminal reductive alkylation of G-CSF with PEG-aldehyde (20 kDa). The conjugation was conducted in acidic buffer solution at pH 5 to protonate the high p*K*a ε-amines of lysines and to label selectively the N-terminal amino group. The Shiff base formed by the reaction of PEG aldehyde with the protein amino group was reduced by sodium cyanoborohydride, yielding a secondary amine [8, 49]. The obtained conjugate demonstrated an improved pharmacokinetic profile, which is mainly due to reduced kidney clearance. The short half-life of native G-CSF is also due to the internalization of G-CSF-receptor complex in neutrophile cells and it is in some way related to the number of circulating neutrophiles. PEG-G-CSF, which remains in the bloodstream for a prolonged time, stimulates the proliferation of neutrophiles and consequently causes its own clearance when the therapeutic aim is achieved. The PEG-G-CSF conjugate (Pegfilgastrim) has been marketed since 2002.

15.7.3 Thiol PEGylation

Free cysteines are seldom present in native proteins, therefore its thiol residue represents an optimal opportunity to achieve site direct modification. PEG derivatives having specific reactivity towards thiols have been developed, such as maleimide-PEG (MAL-PEG), orthopyridyl disulphide-PEG (OPSS-PEG), iodine acetamide-PEG (IA-PEG) and vinyl sulphone-PEG (VS-PEG). Nowadays, MAL-PEG and OPSS-PEG are the more frequently used. Every PEGylating agent presents specific characteristics, for example with MAL- or VS-PEGs reaction at high alkaline pH must be avoided because some degree of amino coupling might occur. OPSS-PEG is very specific for thiol groups but it forms disulphide bridge with the protein thiol that might be cleaved in the presence of reducing agents such as simple thiols or glutathione. MAL-PEG is slightly unstable due to ring opening degradation of maleimide. IA-PEG tends to form iodine that can react with protein tyrosine residues and VS-PEG presents a lower reactivity with respect to the other reagents.

hGH was extensively studied with this strategy; to overcome the common poly-substitution of amino modification, several cysteine muteins were synthesized by

genetic engineering. A cysteine added at the C-terminus leads to a fully active mutein that was PEGylated by PEG-MAL 8 kDa. To achieve a good yield of conversion, it was necessary to reduce the protein with 1,4-dithio-DL-threitol (DTT) before the coupling step to obtain the free cysteine at the C-terminal. This step might be risky because it can also cause reduction of the natural disulphide bridges of the protein, thus hampering the coupling. In the case of rhGH, after removal of DTT excess by gel-filtration, the conjugation leads to a mono-PEGylated derivative with yield over 80% [39].

Hemoglobin (Hb) is another good example of thiol PEGylation. The limit of the native protein is its vasoactivity as consequence of extravasation. Hb PEGylation was studied to avoid this drawback [50]. After preliminary unsuccessful attempts of amino PEGylation, which yielded highly heterogeneous conjugate mixtures, a thiol coupling approach was performed. Maleimidophenyl-PEG of 5, 10 or 20 kDa was linked to Cys-93(β) obtaining a conjugate with two polymer chains per Hb tetramer [51]. This derivative was more effective than the previously studied polymerized Hb*s* (Hb -octamer or -dodecamer).

An interesting combination of thiol PEGylation and genetic engineering techniques is represented by Certolizumab pegol (CIMZIA®), a new therapeutic agent for the treatment of tumor necrosis factor α (TNFα) related inflammatory diseases. TNFα is a proinflammatory cytokine involved in chronic inflammations such as rheumatoid arthritis, Crohn's disease and psoriasis. Certolizumab pegol is a PEGylated, humanized, antigen-binding fragment (Fab′) of an anti-TNFα mAb. The protein moiety has been designed by genetic engineering. In particular, the short hypervariable complementarity-determining region of the murine mAb HTNF40 was grafted into the framework of a human Ig Fab′ fragment [52]. Furthermore, this fragment was engineered with a single free cysteine at the hinge region, thus allowing a site specific thiol PEGylation at the C-terminus that preserved the recognition ability of the Fab′ fragment to bind and neutralize TNFα [53]. A branched PEG of 40 kDa was coupled to achieve a prolonged half-life of about 2 weeks. This approach generates a conjugate that can be dosed every-other-week or even monthly. Certolizumab pegol, with respect to the other two mAbs in therapy for TNFα related diseases (Infliximab and Adalumimab), is devoid of the Fc region; therefore it does not present complement fixation and cell lysis [54]. Furthermore, Certolizumab pegol possesses only one binding site and it does not form large aggregates with the TNF trimers, as occurs with the full antibodies [52].

15.8 Beyond Protein PEGylation

15.8.1 Aptamer PEGylation

PEG has been studied also for the delivery of small drugs and oligonucleotides, but so far only one non-protein conjugate has reached the market, pegaptanib (Macugen). This ophthalmic drug is used to treat wet age related macular degeneration.

The aptamer specifically binds and blocks the vascular endothelial growth factor (VEGF) that causes the disease. To further improve the stability of the leading aptamer towards nuclease and to prolong the in vivo half-life, it was coupled to a 40 kDa branched PEG. In particular, the 5′ terminus was converted to a primary amino group and then reacted with an N-hydroxysuccinimidyl activated PEG. The conjugate retained great affinity for VEGF, around 200 pM [55], and a half-life of 12 h after subcutaneous injection, or of 94 h when injected into the vitreous humor of the eye [56].

15.8.2 Small Drug PEGylation

Most of the reagents and strategies found useful for protein conjugation can be transferred to the PEGylation of small organic drugs, mainly to prolong their body residence time, but some considerations apply. In these molecules, the biological activity is easily lost following stable conjugation. In this case releasable conjugation strategies, as reported above, are generally mandatory [57]. Furthermore, on the contrary of what is desirable for protein conjugation, polyfunctional PEGs are often needed to overcome the limitation of the low drug loading of linear PEGs. Diol-, multi-arm or dendronized-PEGs were, therefore, investigated. Although no PEG-drug has yet reached the market, many conjugates are in clinical trials [58]. For example PEG conjugates with SN38, irinotecan and docetaxel, are under clinical investigations. PEGylation in these cases is mainly aimed to increase the water solubility of the drugs, to prolong their pharmacokinetic profile, and possibly to favor drug accumulation into the tumor by the known “enhanced permeability and retention” (EPR) effect.

15.9 Conclusions

The data summarized in this review are teaching us on one side, the great success of PEGylation that in a few decades has allowed entry into the clinic more biotech drugs than any other drug delivery system and, on the other, how the technology is still open to original discoveries. In the short term we might foresee and hope that the great number of studies by academic and industry researchers will overcome the problems of PEG-small drugs conjugates, bringing these new derivatives to the market also.

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Chapter 16
Self-Assembling Lipid Formulations

Fredrik Tiberg, Markus Johnsson, Catalin Nistor, and Fredrik Joabsson

Abstract Encapsulation of drugs into lipid-based liquid crystalline (LC) phases offers a broadly applicable approach for the in vivo stabilization and sustained release delivery of peptides and proteins as well as small molecule drug substances. This is exemplified by the FluidCrystal®[1] Injection depot, an adaptive drug delivery system, combining ease of manufacturing, handling, and injection, with long acting release. The system exploits specific liquid mixtures of naturally occurring polar lipids and small amounts of solvents, which upon contact with minute quantities of aqueous tissue fluids self-assemble into reversed LC phases. The resulting encapsulation of dissolved or dispersed active pharmaceutical ingredients provides a release duration from a small volume injection, which is tunable from days to months.

16.1 Lipid Self-Assembly

Lipids typically consist of a polar group chemically linked to one, two, or three hydrocarbon chains. As molecular entities, they are often classified into polar and nonpolar lipids [1]. Nonpolar-lipids, e.g., triacylglycerols (triglycerides), interact only very weakly with water and do not form liquid crystal phases. Polar lipids, on the other hand, are amphiphilic in nature and interact more strongly with water and self-assemble to form a wide range of aqueous phases. These include solid phases characterized by the localization of hydrocarbon chains into weakly interacting layers, with the polar heads forming end-group planes via stronger interactions. On heating, the hydrocarbon chains are the first to enter into the disordered liquid

[1] FluidCrystal® is a registered trademark of Camurus AB, Sweden.

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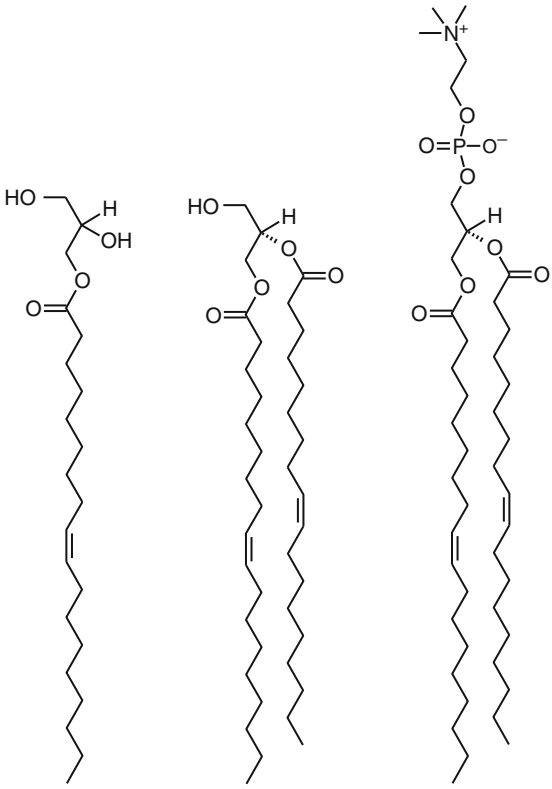

Fig. 16.1 Examples of molecular structures of polar lipids used in LLC delivery systems. *From left*: monoacylglycerol, diacylglycerol, diacyl phosphatidylcholine

state, with the overall bilayer structure remaining intact through the stronger polar head interactions. This combination of long range order and disorder on the submolecular level is a characteristic attribute of lipid-based LC phases. In the present chapter, we will focus exclusively on lipid-based systems forming such LC phases on interaction with water and not on the solid state. Furthermore, the emphasis is on long acting delivery and therefore LC nanoparticle dispersions are not addressed.

Examples of LC-forming lipids that have been used in relation to extended release applications and studies include: unsaturated long-chain phospholipids, monoacylglycerols, and diacylglycerols, Fig. 16.1. The long hydrocarbon chain (−s) ensure low monomer activity (and aqueous solubility) of the lipids, thereby providing important resistance to fragmentation and lipid monomer formation during exposure of the functional LC phase to excess water present at the site of injection.

As noted, the amphiphilic "dual character" of polar lipids gives rise to a strong tendency to self-assemble in water to form different types of supramolecular LC phases, characterized by the separation of polar and nonpolar segments. This occurs through the spontaneous association into phase structures with domain sizes in the

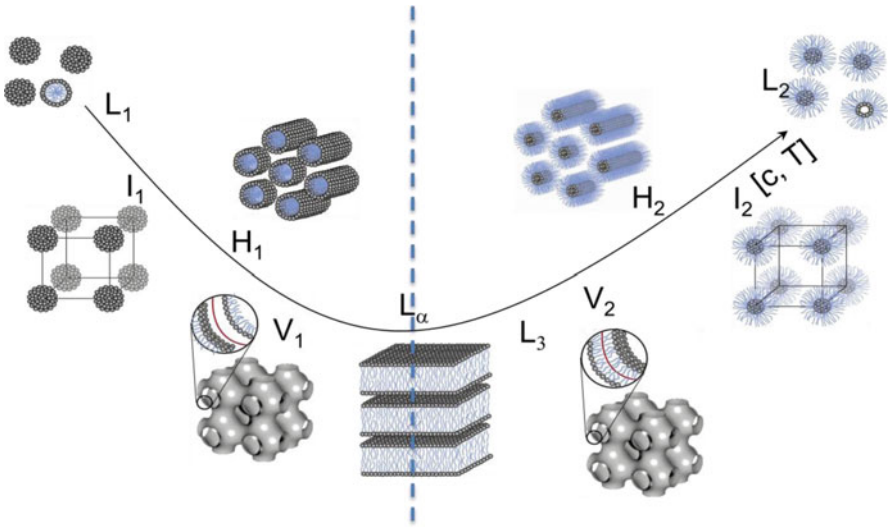

Fig. 16.2 Common self-assembly structures of polar lipids in aqueous media. Phase transitions can be introduced by changes in solvent (water) content, temperature, or introduction of other solution components, including in some cases active pharmaceutical agents. The lamellar liquid crystalline phase (L_α) can be regarded as the mirror plane (*dashed line*) between normal "oil-in-water" ("normal phase") structures and reversed "water-in-oil" ("reverse phase") structures. On both sides, there are possibilities for forming cubic bi-continuous (V_1, V_2), hexagonal (H_1, H_2), cubic micellar (I_1, I_2), and micellar phases (L_1, L_2), as well as other intermediate phases (e.g., sponge, L_3, phase). The phase behavior can be rationalized by considering their effective packing parameter (*v*/*al*), where *v* and *l* are the volume and the length, respectively, of the hydrophobic part of the molecule, and *a* is the effective area per polar head group [2]. *v*/*al* < 1 and >1 favor normal phases (*left side*), and reverse phases (*right side*), respectively, while lamellar phases are favored when *v*/*al* is close to 1

1 and 100 nm (i.e., 10^{-9} m) range, see Fig. 16.2. The rich polymorphism seen in aqueous lipid systems is largely controlled by geometric molecular packing constraints that are further influenced by temperature and concentration [3, 4].

Self-assembly at low amphiphile concentrations typically result in "oil-in-water" structures, such as, spherical and elongated micelles, hexagonal, and normal cubic phase structures, while with increasing concentrations reversed "water-in-oil" phases are formed. The phase sequence in Fig. 16.2 is highly idealized and most lipids do not show the full phase sequence with increasing concentration or temperature, and in some cases additional intermediate phase structures may appear.

From a sustained release perspective, the most interesting phases are the reversed water-in-oil phases, i.e., those on the right side of the mirror plane in Fig. 16.2. Unsaturated monoacylglycerol (e.g., monoolein or glycerol monooleate) is an extensively studied lipid-forming reversed phase, most significantly the bicontinuous cubic phase (V_2) is in equilibrium with excess water, see Figs. 16.2 and 16.3a.

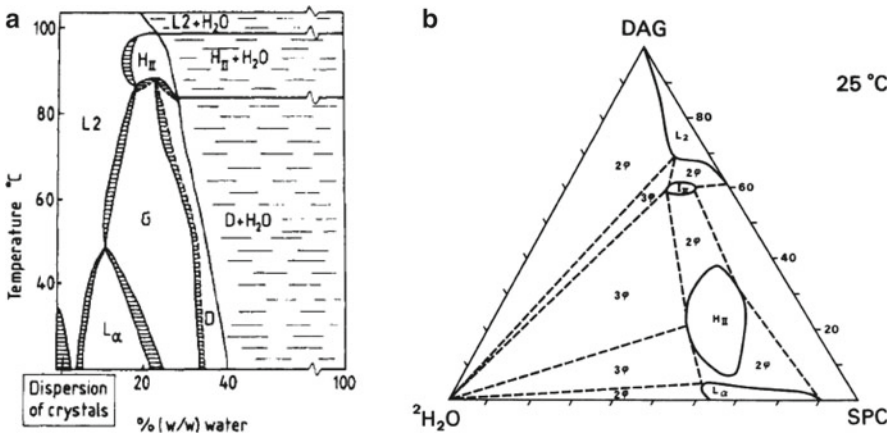

Fig. 16.3 (**a**) Two component phase diagram for glycerol monooleate (GMO) – water. Reproduced with permission from Ref. [5]. The bicontinuous cubic (D or V_2) and reverse hexagonal (H_{II} or H_2) phases are in equilibrium with excess water. (**b**) Three component phase diagram for soy phosphatidylcholine (SPC), DAG (diacyl glycerol, primarily GDO), and water (deuterated), showing the phase sequence: L_a, H_2, I_2, and L_2 with increasing DAG content. In between are two and three phase regions. Reproduced with permission from Ref. [6]

Another example of reversed phase-forming lipids is the mixture of a long-chain phosphatidylcholine and diglyceride lipids, e.g., soy-PC or synthetic dioleoyl-PC and glycerol dioleate (GDO). With increasing GDO content, this mixture undergoes a phase-transition sequence: (1) lamellar (L_α), (2) reversed hexagonal (H_2), (3) ordered reversed cubic micellar (I_2), and (4) reversed micellar (L_2) phase, with the further possibility of intermediate phases, see Fig. 16.3b. The reversed H_2 and I_2 phases and their mixtures are promising LC structures for drug delivery applications due to the anticipated good loading and encapsulation ability in the coexisting lipophilic and discrete hydrophilic (aqueous) nanodomains. The ability of these phases to resist phase changes on exposure to excess aqueous media is a further important property allowing their use as time-persistent reservoirs in drug delivery applications. The viscous PC-GDO LC phase liquid precursor can be tuned into a lower viscosity fluid by the addition of small amounts of solvent, e.g., ethanol and/or propylene glycol, for easier manufacturing, handling, and injection. Limited amounts of water may also be added to the LC precursor to facilitate solubilization of polar drug compounds into the L_2 phase. Importantly, this can be achieved without inducing LC phase formation and associated gelling. On further water uptake, for instance by absorption of interstitial water at the site of injection, the in vivo functional LC sustained release matrix is spontaneously generated; physically materialized as a stiff gel with the active drug evenly enclosed. Due to the fact that the gel-formation process goes from the outside to the inside, see Fig. 16.4, any dissolved drug compound is rapidly encapsulated.

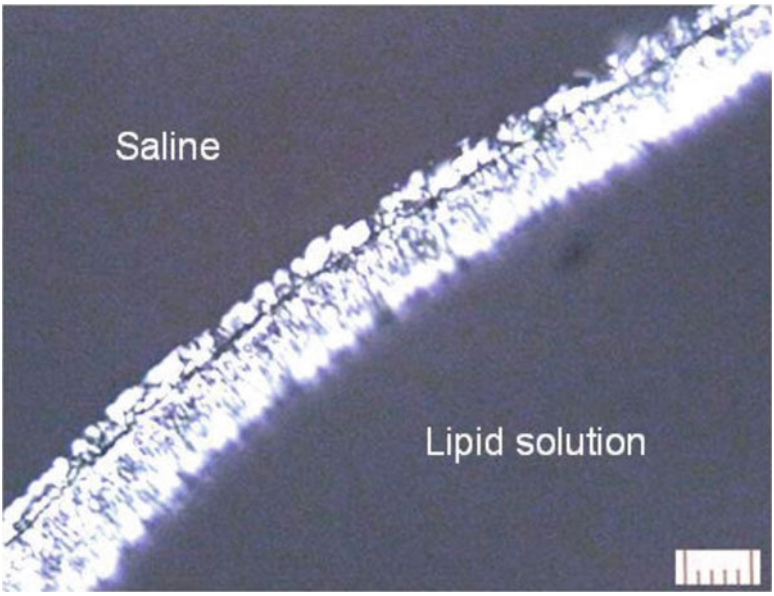

Fig. 16.4 Light microscopy (polarized light) image of the interfacial region between a lipid solution of SPC/GDO (50/50 wt/wt) after contact with saline solution (0.9% w/v NaCl). The image was recorded 2 min after contacting the formulation with the saline solution. A liquid crystalline shell (or surface phase) is clearly observed at the aqueous-lipid formulation boundary as water is being absorbed from the outside in. The magnification was 80 times and the distance between scale bars is 10 μm

16.2 Solubility and Encapsulation of Drugs in Lipid LC Delivery Systems

Important criteria for parenteral products include easy handling and injection through narrow-gauge needles for minimal patient pain and discomfort. The administration volume of subcutaneously administered products should typically be kept small, preferably below 1 ml, and hence the drug-delivery system (DDS) must have a good solubilizing ability. A truly versatile DDS should ideally be capable of handling drugs that may possess very different chemical characteristics and molecular weights, ranging from lipophilic small molecules to amphiphilic or hydrophilic peptides or larger proteins. As lipids are amphiphilic in nature, their solutions and LC phases are typically relatively good solvents by themselves. This property has been demonstrated for a wide range of drug substances such as: acetylsalicylic acid, diclofenac sodium, hydrochlorquine sulfate, and vitamin E [7]; propantheline bromide and oxybutynin hydrochloride [8]; timolol maleate [9]; chlorpheniramine maleate and propanolol hydrochloride [10]; melatonin, pindolol, propanolol and primethamine [11]; haemoglobin [12]; cefazolin [13]; and insulin [14, 15]. The vast majority of these studies feature the GMO water system, forming the bi-continuous

V_2 phase at relevant temperatures (see Fig. 16.3), in equilibrium with excess aqueous solution [5, 16]. Although very interesting, this system has some limitations to its merit as a DDS:

- The relatively high monomer activity of GMO and observable hemolytic activity [17].
- The bicontinuous nature of the cubic phase, which also limits its function as effective barrier to diffusive molecular transport.
- One-component lipid systems such as the GMO matrix do not allow for compositional tuning of the phase structure.

As may be expected from molecular packing considerations, hydrophilic drugs with relatively large head groups may cause transitions from the bicontinuous cubic V_2 phase into the lamellar L_α phase, whereas more lipophilic drugs, for which the volume of the lipophilic chain is large tend to convert the same V_2 phase into the reversed hexagonal H_2 phase. This was nicely illustrated by Engström [18], when solubilizing lidocaine in the GMO water cubic phase, where the water-soluble lidocaine hydrochloride salt induced a transition from V_2 to the sponge and/or the L_α phase, while the corresponding more lipophilic lidocaine-free base converted the same V_2 phase into the H_2 phase. If shown to have unwanted effects on the sustained release characteristics of the system, such transition cannot be compensated for in a one-component lipid system. This illustrates the importance of having a means to tune the phase behavior according to release targets and being able to compensate for curvature effects of dissolved drug, not least to avoid the relatively excessive initial release shown for the GMO and GMO-PC in the insert of Fig. 16.5. On the other hand, having two lipid components where the packing parameter of the two different building blocks favors different sides of the reverse phase sequence in Fig. 16.2, makes it is easy to tune the phase behavior over a wide range, and thereby also optimize the encapsulation and extended release through compositional variation, see Fig. 16.5. This is the principle of the FluidCrystal delivery system, exploiting for instance combinations of lamellar phase forming phospholipids and reversed micellar phase forming diacyl glycerol lipids.

Since the FluidCrystal delivery system typically comprises two lipid-building blocks, potential effects of solubilized drugs on the phase structure formation can easily be compensated for by changing the ratio of the lipid excipients to reverse the effects of the drug substance. Accordingly, release levels, including initial release, can be kept to a minimum. Similarly, the formulation viscosity and injectability may be tuned by the addition of small levels of an appropriate cosolvent, such as ethanol or propylene glycol. By varying the lipid ratio and cosolvent levels of the FluidCrystal DDS, a large number of drug compounds have been loaded to predefined targets for clinical efficacy and duration. A nonexhaustive list of small molecules includes: bupivacaine, benzydamine, testosterone enantate, testosterone undecanoate, granisetron, buprenorphine, and fentanyl, while examples of peptides and proteins include: octreotide, leuprorelin (leuprolide), histerelin, triptorelin, somatostatin, calcitonin, glucagon, glucagon-like peptide-1 and analogues, interferons, different enzymes, and antibody fragments, see Table 16.1 [19–23]. Small molecules have been loaded

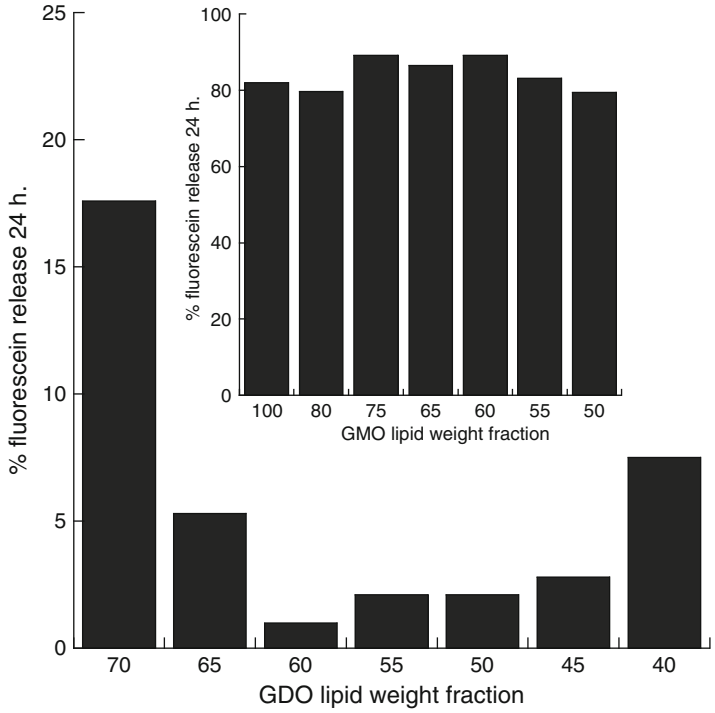

Fig. 16.5 In vitro release of disodium fluorescein from LC formulations based on SPC/GDO and SPC/GMO (inset). All "precursor" formulations comprised 10 wt% ethanol and a fluorescein load of 0.5 wt% (ca 5 mg/ml). The formulations (0.1 g or ca 0.1 ml) were injected into 4 ml of phosphate-buffered saline (PBS, pH 7.4) and kept with gentle rotation at 37°C for 24 h. Sampling of 0.2 ml of the PBS release medium was performed at 24 h and the released fluorescein content was assayed by measuring the optical absorbance at 490 nm. Note the different magnitude of the release when comparing SPC/GDO and SPC/GMO (or pure GMO) formulations

up to about 300 mg/ml, while the upper limits for peptides and proteins typically are of the order 100 and 50 mg/ml, respectively. Buprenorphine, leuprolide acetate, and octreotide chloride represent APIs that currently are in clinical development, (http://www.camurus.com).

16.3 Method of Preparation and Manufacturing

Scale up of manufacturing to commercial scale at reasonable cost is a prerequisite for a successful sustained release DDS technology. Typically parenteral sustained release products are provided as powders for reconstitution, e.g., dispersions of a solid phase in a liquid phase, often water. Examples of this type of product include Lupron Depot® by Abbott (sold as Procren® Depot in parts of Europe), Decapeptyl®

Table 16.1 Examples of "drug" substance levels loaded into the FluidCrystal DDS. These represent in most cases target levels and not maximum drug loads

Substance	Class	Drug loading evaluated at 25°C in FluidCrystal Injection depot (mg/ml)	Molecular weight (g/mol)
Small molecules			
Lidocaine	Local anaesthetic	300	234.3
Bupivacaine	Local anaesthetic	150	288.4
Benzydamine	Local anaesthetic, anti-inflammatory	60	309.4
Granisetron	Anti-emetic	90	312.4
Testosterone enantate	Steroid hormone	300	400.6
Testosterone undecanoate	Steroid hormone	300	456.7
Buprenorphine	Analgesic	100	467.6
Peptides and proteins			
Octreotide	Peptide hormone Somatostatin analog	100	1,019
Leuprolide	Peptide hormone Gonadotropin-releasing hormone analog	60	1,209
Somatostatin	Peptide hormone	40	1,638
Glucagon-like peptide 1	Peptide hormone	50	3,298
Lysozyme	Hydrolyzing enzyme	20	14,300
Interferon alpha-2a	Anti-viral protein	2.5[a]	19,500
Horseradish peroxidase	Enzyme	10	40,000

[a]Higher drug loads were not evaluated

by Ipsen, Risperdal Consta® by Janssen, and Sandostatin® LAR® by Novartis. All of these are based on poly(lactic-*co*-glycolic acid) (PLGA) microspheres that are reconstituted (suspended) in an aqueous medium before injection, either using a syringe and vial (−s) or a two-compartment syringe. This poses challenges in relation to manufacturing, characterization, and process validation and often requires dedicated specialized manufacturing equipment or even dedicated manufacturing plants for the commercial production. In this regard, lipid-based LC systems offer the advantage of being manufactured as simple solutions with the drug substance dissolved within. Only when administered does the "active" controlled release matrix develop in vivo. This means that the highly viscous gel-like release matrix is only present postinjection and is, therefore, not an issue during preparation or administration. Notably, the opportunity of exploiting "nonaqueous" ambient adaptive LC systems, as represented by the FluidCrystal Injection depot technology, was not considered in earlier studies of LLC systems, where the high viscosity and inadequate injectability of lipid-based LC systems was believed to be a major obstacle [24].

Aside from alleviating the problem of handling and injecting a very viscous LLC phase gel, the use of a low-viscous nonaqueous liquid precursor greatly facilitates scale-up and product manufacturing. The FluidCrystal Injection depot uses standard

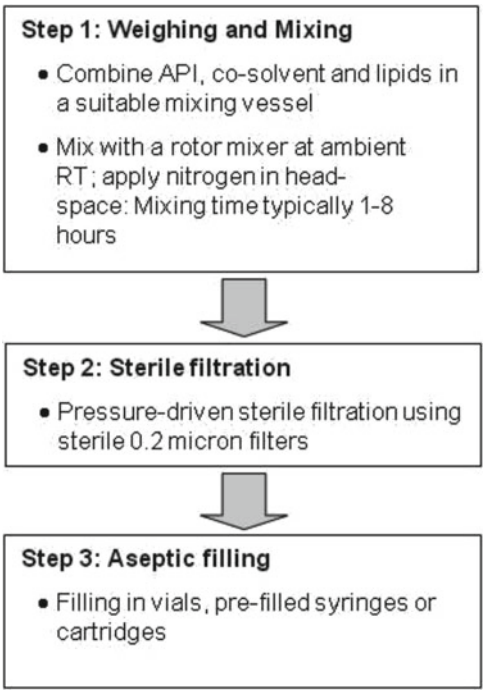

Fig. 16.6 Schematic manufacturing process scheme for an LLC "precursor" product, i.e., based on FluidCrystal Injection depot technology

pharmaceutical processing steps, including standard mixing and sterile filtration steps, followed by filling in vials, prefilled syringes, or cartridges for pen systems (Fig. 16.6). Mixing of lipids, solvents, and API to a homogenous solution is usually achieved in a single step process at room temperature under inert atmosphere. Sterilization is typically accomplished by pressure filtration through a 0.2 μm filter medium and subsequently filling is performed under aseptic conditions, Fig. 16.6.

16.4 Stability in Lipid Delivery Systems

Stability is a key issue in any pharmaceutical product development, both in relation to storage and use. The ideal physical form of long acting LC products is in the form of a ready-to-use liquid with the drug substance dissolved to avoid issues associated with physical colloidal stability. LC liquid precursors are typically nonaqueous, thus presenting a very different chemical environment compared to that of ordinary aqueous parenteral solutions or lyophilizates. As mentioned above, the FluidCrystal system allows entrapment of compounds with a broad range of physicochemical properties, from small molecules to large amphiphilic peptides and proteins, as exemplified in Table 16.1. Importantly, these ready-to-use solutions can also be designed to provide very good chemical stability of dissolved active compounds over time, as demonstrated by the data in Figs. 16.7 and 16.8 below.

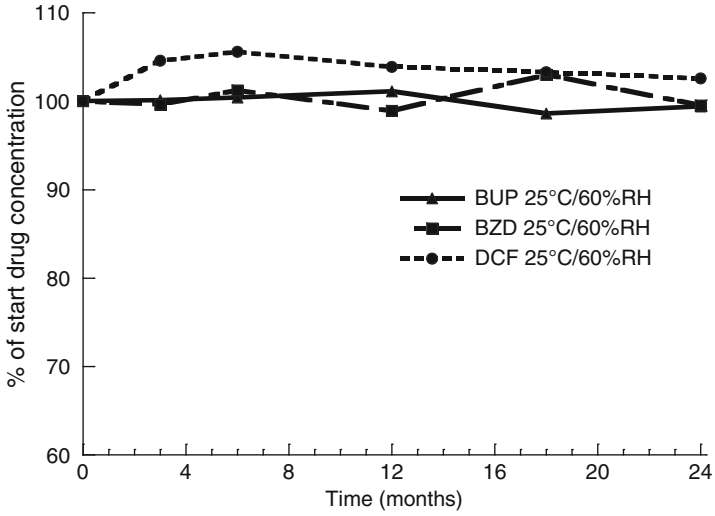

Fig. 16.7 Change in the buprenorphine (BUP), benzydamine (BZD) and diclofenac (DCF) content in their corresponding FluidCrystal Injection depot formulations stored for up to 24 months at 25°C/60% RH

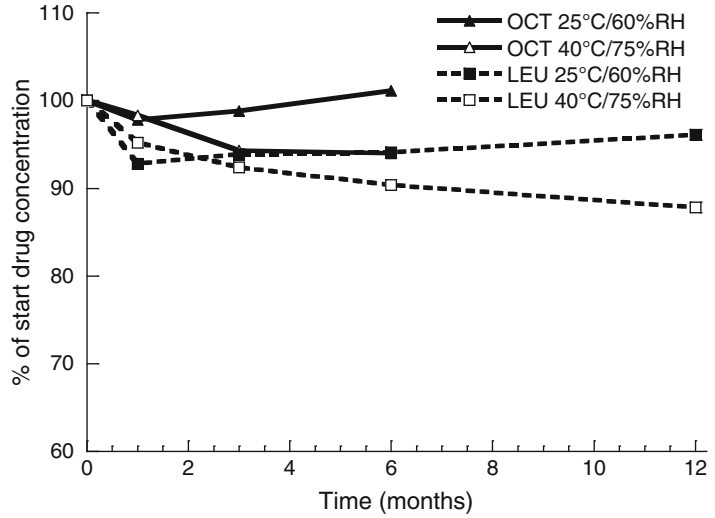

Fig. 16.8 Storage stability of octreotide chloride (OCT) and leuprolide acetate (LEU) in FluidCrystal Injection depot formulations stored at room temperature (25°C/60% RH) and accelerated degradation conditions (40°C/75% RH)

Storage stability is an issue of considerable concern especially for formulations containing peptides and proteins, due to their inherent instability, both chemical (due to hydrolytic processes, isomerization, or oxidation of sensitive amino acid fragments) and physical (e.g., loss of potency due to adsorption to solid surfaces or aggregation). Many of these processes are enhanced in aqueous environments and significantly inhibited in water-free systems, such as lipid-based systems. LLC systems can also be prepared to provide excellent stability of biological compounds and preservation of their secondary and tertiary structures, thus offering good premises for obtaining stable peptide and protein-based drug products.

Figure 16.8 shows the chemical stability of octreotide chloride and leuprolide acetate filled on prefilled syringes; demonstrating that both peptides have excellent stability in the FluidCrystal system over at least 6 months at accelerated degradation conditions (40°C/75% RH). Hence it is possible to develop room temperature stable FluidCrystal peptide products, where corresponding marketed products, i.e., Eligard® (Astellas Pharma), Sandostatin® LAR®, and Lupron® Depot require refrigerated storage.

Another important aspect of a long acting product is the in vivo stability of the active compound. By its entrapment inside the depot structure after in vivo administration, direct enzymatic degradation is avoided. Furthermore, the in vivo physicochemical stability in the depot is of considerable importance; ideally this should be measured directly, but indicative results can be obtained indirectly, by measuring stability of the drug compound in the FluidCrystal Injection depot following storage at simulated in vivo conditions (e.g., in aqueous buffers incubated at 37°C).

Sadhale and Shah showed that the cubic GMO phase protected the small molecules cefazolin and cefuroxime from chemical inactivation by hydrolysis and oxidation [13]. Already in 1983, Ericsson et al. showed for the protein lysozyme that the cubic LC phase can have a favorable impact on protein stability, retaining the native conformation and chemical activity [25]. Also, bovine hemoglobin encapsulated from 1 to 10% by weight in GMO cubic phase retained its native conformation and activity over time and could also be released in a sustained manner. Sadhale and Shah [15] have demonstrated that cubic phase gels can stabilize peptides (insulin) against agitation stress induced inactivation.

Enzymes, such as lactase, glucose, pyranose, and cholesterol oxidase, have also been successfully included into the structure of lipid cubic phases for biosensor development, and their enzymatic activity was shown to be preserved inside the LC phase structures [26]. Proteins as large as immunoglobulin G, albumin, transferrin, and fibrinogen have been successfully stabilized over a broad range of temperature when entrapped into self-assembled mixtures of GMO and maleimide (triethyleneglycol) ether under physiological hydrating conditions [27]. The positive effect of the interaction with lipids for improvement of peptide stability was also demonstrated by inclusion of vasoactive intestinal peptide into liposomes containing egg PC, phosphatidyl glycerol, and cholesterol, where the peptide was stabilized against enzyme catalyzed and uncatalyzed degradation permitting its maintenance of essentially undegraded form at 37°C for 8 days [28].

16.5 Controlled Release Mechanism and Pharmacokinetics

Most early studies of LLC systems as delivery vehicles featured the GMO-water system, c.f. refs. [24, 29]. Notably consistent release results have been obtained by various groups for different substances, showing that release is typically diffusion controlled, with a square-root-time dependent. Considering the fact that GMO spontaneously assembles into a bicontinuous phase with water [16], where both water and lipid channels are interconnected throughout the phase, it is not surprising that the drug release observed is relatively rapid and nondiscriminating between different drug substances. One of the more detailed studies of release kinetics and mechanisms from GMO cubic phases was performed by Chilukuri and Shah featuring the anesthetic bupivacaine in hydrochloride salt and free-base forms, respectively [30]. This study also showed very little discrimination between the product forms in terms of release kinetics, aside from one experiment performed in basic media, pH 9, where lipids are rapidly degraded into fatty acids and glycerol. In their subsequent review of cubic phases as drug delivery systems, Shah, Sadhale, and Chilukuri concluded that "cubic phases may never be able to provide release kinetics longer than 2–3 days." [24]

The FluidCrystal Injection depot distinguishes against earlier investigated lipid based LC systems by being:

1. A low viscous precursor of the "active" sustained release LC phase
2. Forms in vivo a reversed hexagonal (H_2) or a cubic micellar (I_2) phase, or mixtures and intermediates thereof
3. A mixture of two or more lipids, such as a lamellar phase forming phospholipid and a reversed micellar phase forming diacyl glycerol

An illustration of the evolution of the FluidCrystal Injection depot after subcutaneous injection in vivo is shown in Fig. 16.9. The LC phase develops immediately upon contact with aqueous media present in the tissue at the site of injection. As pointed out, the process goes from outside in providing rapid encapsulation of the dissolved substance. Thereafter, the active substance is released by a combination of restricted diffusion and depot biodegradation and erosion.

As is exemplified in Figs. 16.5 and 16.10, the in vitro release of fluorescein and octreotide from the FluidCrystal® formulations comprising PC and GDO is very limited during 24 h. A minimum in vitro release is typically observed at a balanced lipid weight ratio of PC-GDO of about 1 or slightly higher, see Fig. 16.5, where the PC-GDO system forms the H_2 or I_2 phase or mixtures thereof. As shown in Fig. 16.10, only about 1% octreotide is released after 24 h for the PC/GDO 50/50 ratio and almost no further release is observed in vitro over further weeks or months. In contrast, the release from the bicontinuous V_2 phase formed by GMO is much larger, as is the release from PC-GMO mixtures forming the L_α or V_2 phase. This clearly shows that the specific LC phase structure is crucial for its sustained release performance, an observation recently also made by Boyd and coworkers [31].

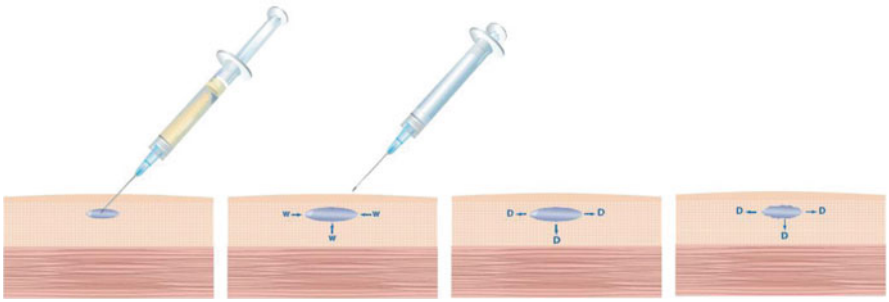

Fig. 16.9 Sketch showing the evolution of a FluidCrystal® Injection depot following subcutaneous injection: absorption of ambient aqueous fluid and lipid self-assembly, followed by releasing the drug by diffusion and lipase-assisted degradation and erosion of the depot. *W* water; *D* drug

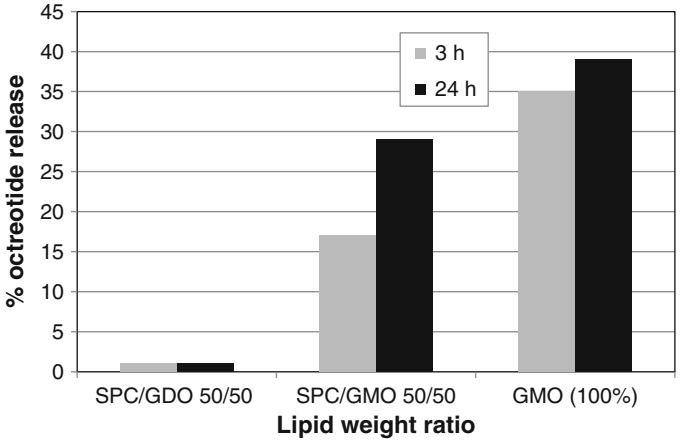

Fig. 16.10 In vitro release of octreotide from LLC formulations based on soy-PC (SPC)/GDO, SPC/GMO and pure GMO (one component system). All "precursor" formulations comprised 15 wt% ethanol and an octreotide drug load of 1 wt% (ca 10 mg/ml). The formulations (0.1 g or ca 0.1 ml) were injected into 4 ml of PBS (pH 7.4) and kept with gentle rotation at 37°C for 24 h. Sampling of 0.2 ml of the PBS release medium was performed at 3 and 24 h and the released octreotide content was assayed by HPLC-UV

Apparently, by carefully choosing the lipid components and phase behavior, it is possible to achieve a very effective in vitro encapsulation and drug release. How does this then translate to the in vivo situation? Relevant in vivo release data for the FluidCrystal octreotide system following single dose injections, intramuscularly and subcutaneously, respectively, are shown in Fig. 16.11. The highest plasma octreotide concentration (C_{max}) was observed after about 30 min. Thereafter, the plasma octreotide levels are only slowly decaying with an apparent half-life of ~15 days, compared to between 1 and 2 h for immediate release octreotide, and with very good consistence between subcutaneous and intramuscular injections.

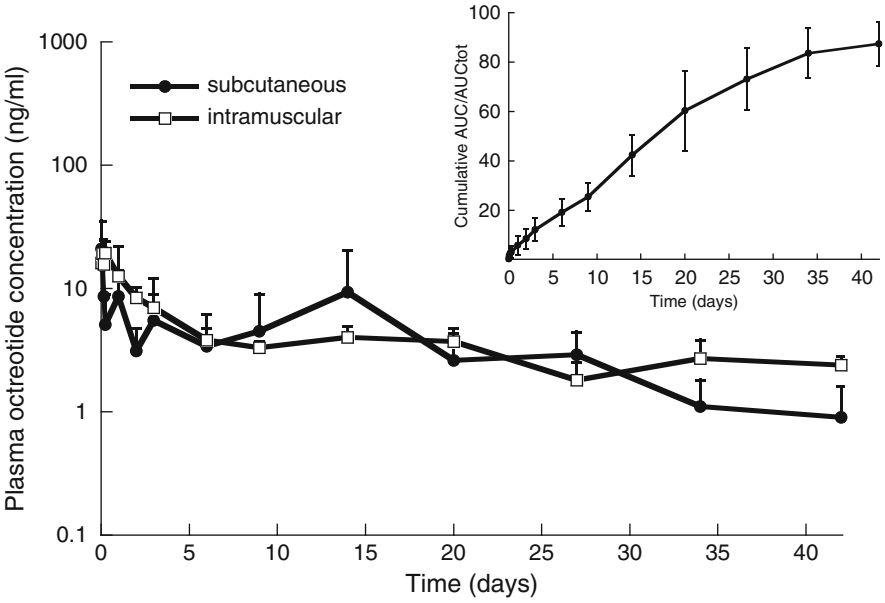

Fig. 16.11 Mean plasma octreotide concentration versus time (semi-log scale) over 42 days after a single dose of a FluidCrystal Injection depot containing 13.5 mg octreotide acetate given subcutaneously (*filled circles*, $n=6$) and intramuscularly (*open squares*, $n=4$), respectively, to beagle dogs. The inset shows the cumulative area under curve (AUC) divided by the total area under curve (AUCtot) [32]

The initial subcutaneous release of octreotide was about 3% after 6 h and 7% after 24 h when calculated as cumulative area under curve ratio AUC(t)/AUCtot. The initial release observed in vivo is despite its very limited extent considerably larger than that seen in vitro for the same SPC/GDO 50/50 system, see Fig. 16.10, suggesting that the main mechanism of octreotide release is biodegradation of the lipid matrix. Typically, the depot matrix is fully degraded and cleared from the tissue at a time corresponding to two times the effective release duration of the depot.

The duration of FluidCrystal Injection depot products can be tuned from less than a day up to 1 month and longer. Figure 16.12 shows a release profile of a FluidCrystal somatostatin (1–14) formulation with approximately 1 week duration.

Somatostatin (1–14) has ordinarily a half-life of only 1–2 min, strongly limiting its therapeutic use. However, due to the encapsulation protection of the LC phase of the FluidCrystal Injection depot, long-term stable plasma levels can be accomplished. Obviously there can be opportunities for using such LC phases as enabling elements of future therapies involving drug substances that normally suffer from limitations of too rapid in vivo degradation.

One advantage of biodegradable depot systems compared with for instance conjugation technologies, such as PEG-ylation or fatty acid conjugation, commonly

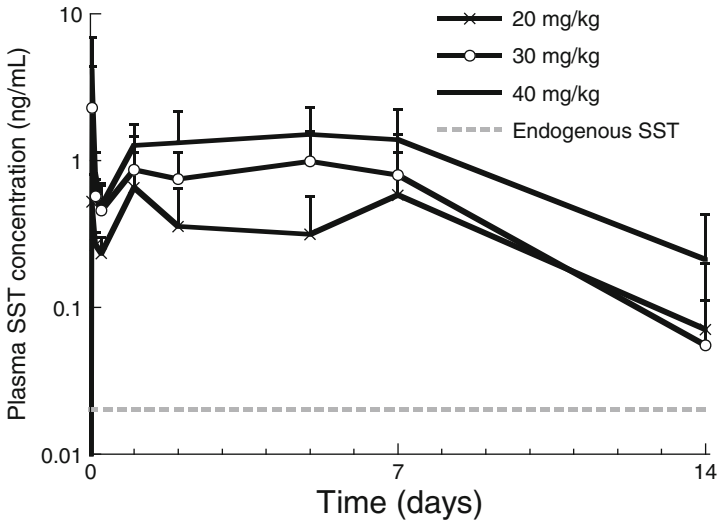

Fig. 16.12 Mean plasma somatostatin (SST) concentration over 28 days (semi-log scale) after a single subcutaneous (*filled circles*) administration of FluidCrystal formulations with different doses of somatostatin (1–14) in a rat model ($n=6$), see [33]

used for increasing half-lives, is that the extensive toxicological, safety, and regulatory documentation associated with the creation of a new chemical entity can be avoided.

When presenting the sustained release characteristics of LC delivery systems, it is useful to make a comparison to established sustained release technologies, such as polyglycolactic acid and polylactic acid (PLGA–PLA) microparticle and gel technologies [34–37], which are currently on the market.

Following injection, the FluidCrystal system provides dose-dependent stable leuprolide plasma values for over 1 month, see Fig. 16.13, indicating zero-order release kinetics in this time frame. Then the release starts to decay more strongly as the depot is emptied and degraded. For the reference Procren® Depot (Lupron® Depot, USA) and Eligard® products, the initial burst release is larger and the subsequent longer term release gives lower and less-stable plasma values over time.

LC delivery systems are also being exploited for development of small molecule therapeutics. Figure 16.14 shows the release profiles obtained for a FluidCrystal Injection depot product of buprenorphine. This product concept is currently being evaluated in a Phase II trial as maintenance treatment for drug addiction and is being further assessed for treatment of acute and chronic pain. As seen, stable buprenorphine plasma levels are accomplished, with only a factor of between two and ten between C_{max} and C_{28days}, depending on the administered dose and dose volume.

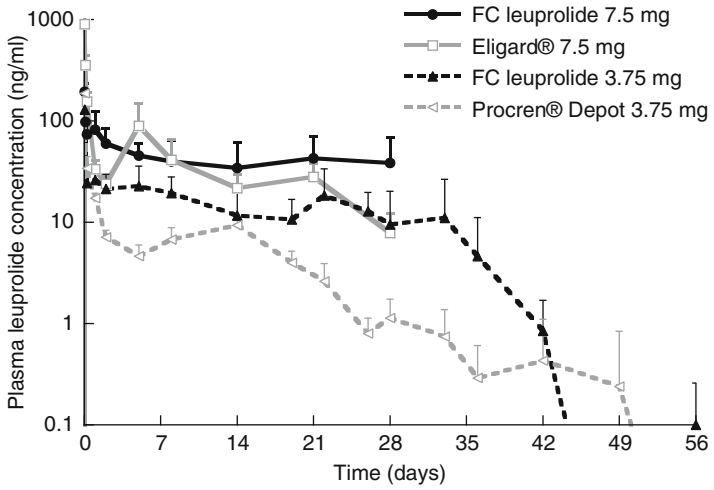

Fig. 16.13 Mean plasma leuprolide concentration versus time (semi-log scale) after single subcutaneous injections of two FluidCrystal® (FC) formulations containing 3.75 and 7.5 mg leuprolide, respectively, and the corresponding marketed Procren® Depot (Abbott) and Eligard® (Astellas Pharma) products in a rat model ($n=6$) (Camurus AB). The in vivo release was followed during 28 or 56 days. All formulations and products are designed for a therapeutic duration of 1 month

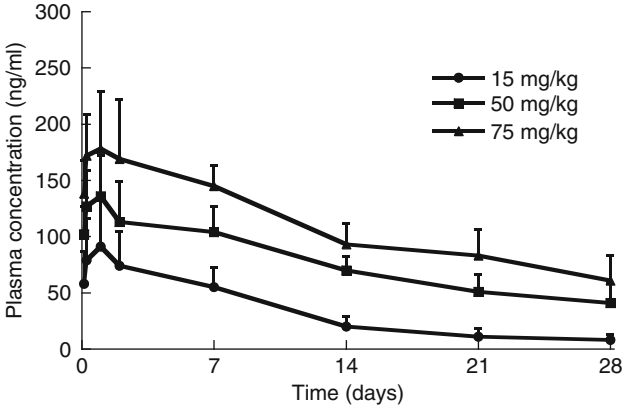

Fig. 16.14 Mean plasma buprenorphine concentration over 28 days after a single subcutaneous administration of different doses of a buprenorphine FluidCrystal formulation in a rat model ($n=6$) [23]

16.6 Current Status of Development of LLC Systems

Approved and marketed products featuring LLC delivery systems are limited to nonparenteral routes of delivery. Significant development advances have, however, taken place in recent years with currently three injectable products being investigated

in clinical trials, ranging from long acting peptide products for acromegaly and prostate cancer, to buprenorphine for treatment opiate addiction (http://www.camurus.com). The so far accumulated preclinical and clinical documentation, with a total exposure time in patients of more than 20 years and in animals of several 100 years, has demonstrated the FluidCrystal Injection depot to be safe and locally tolerable when e.g., administered subcutaneously or intramuscularly. All lipid excipients used, i.e., PC and GDO, are GRAS (generally recognized as safe) and have well-characterized metabolic pathways. PC is also used in other parenteral products, while a bridging toxicology program has been performed in regards to the parenteral use of GDO. With the expanding pool of pharmacokinetic, safety, local tolerability, CMC, and regulatory data, the prospects for bringing forward new drug candidates using LC DDS become increasingly favorable.

Easy manufacturing, using only standard pharmaceutical processing steps and ready-to-use injection devices, distinguishes the FluidCrystal Injection depot from the currently marketed DDS technologies based on polymer microparticles and gels, which typically require specialized processing equipment as well as reconstitution before injection. High compatibility with standard devices, including prefilled syringes and cartridge pen systems, furthermore results in a favorable cost of goods and allows easy handling and injection by patients and healthcare professionals through thin needles, e.g., typically in the range 25–27 G.

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Chapter 17
Implantable Drug Delivery Systems Based on the Principles of Osmosis

John A. Culwell, Jose R. Gadea, Clarisa E. Peer, and Jeremy C. Wright

Abstract Implantable, osmotically driven drug delivery systems are applicable to the delivery of small molecules, peptides, proteins, and other biomolecules, and continue to be used at the forefront of development of new clinical therapies. Sustained, zero-order drug delivery from osmotic implants has been shown to achieve consistent pharmacokinetics in multiple models and therapies. The continuous infusion features of these systems can provide improved economics, convenience, and therapeutic benefit versus bolus dosing. In animal research, ALZET osmotic implantable pumps have demonstrated the benefits of continuous infusion versus bolus dosing in various therapeutic applications, including chemotherapy, angiogenesis, and obesity. Osmotic implantable pumps have been selected for site-specific delivery to enable continuous local site infusion and avoidance of systemic effects. Applications include delivery to the spine (analgesia), tumors (oncology), cochlea (gene expression), and the brain (neurodegenerative applications). Human use applications using DUROS osmotic implantable pumps have provided delivery of agents from 1 month to over 1 year for treatment of pain, cancer, diabetes, and hepatitis.

17.1 Introduction

Implanted drug delivery systems offer a number of advantages, including assurance that the patient or subject is receiving the desired therapy and maintenance of steady drug levels with the avoidance of peaks and valleys of immediate release dosage forms. In addition, implanted drug delivery systems can be utilized for site-directed therapy. For animal research applications, implanted drug delivery systems can

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reduce the frequency of animal handling, potentially require less compound during preclinical research stages, and enable the investigation of novel dosing paradigms. For human health applications, additional advantages of implanted systems include convenience for the patient, cost-savings for the provider and healthcare system as a result of reduced frequency of administration, and the possibility of early termination of therapy if necessary. Balanced against these advantages are the drawbacks of minor surgical procedures for implantation and system removal and possible obstacles in qualifying novel systems for government or insurance reimbursement for human health applications.

Mechanisms/designs for implants include matrix diffusion, membrane diffusion, osmosis, and osmotic pumping. Systems based on osmotic pumping provide steady, zero-order delivery of drugs. Osmosis is the movement of solvent (i.e., water) from a zone of higher thermodynamic activity to a zone of lower thermodynamic activity. Generally, this involves a semipermeable membrane (e.g., a membrane permeable to water but not to ionic species) and aqueous ionic solutions. Osmotic drug delivery systems for oral, animal research, veterinary, and human delivery have been intensively investigated and developed over the past 40 years [1–5]. This chapter describes the ALZET® osmotic pump developed for implantable drug delivery to laboratory animals and the DUROS® osmotic implant for human therapy.

Site-specific delivery can be achieved with osmotic implants either by implanting the system directly at the target site or by use of a catheter attached to the system, with the catheter tip directed to the target site. Achievement of therapeutic concentrations of a drug at a local target site can often require high and sometimes toxic systemic drug concentrations when the drug is delivered systemically. With site-specific delivery, therapeutic concentrations of a drug can be present at the desired target site without exposing the entire body of the patient or subject to a similar dose.

The ALZET pump is designed for subcutaneous or intraperitoneal implantation in unrestrained animals as small as mice and young rats. Although it is primarily used in rodents because of the preference of these species for in vivo research models, the ALZET pump has also been used in a variety of other animal species, including dogs, monkeys, livestock, leopards, tigers, birds, fish, and even reptiles. The ALZET pump has been utilized in experiments performed aboard the space shuttles. The ALZET pump is available in three sizes (100 μL, 200 μL, and 2 mL) and a range of delivery rates (0.11–10 μL/h) and durations (1 day to 6 weeks; Fig. 17.1). The ALZET pump has become a popular research tool for continuous delivery of a wide range of test agents, including small molecules, peptides, proteins, nucleic acids, and lipids, as cited in over 12,000 research publications. It is particularly useful for continuous delivery of compounds with short half-lives, which are notoriously difficult to reliably deliver by conventional dosing regimens. Complete implantation of the infusion device permits free animal movement during the infusion period. In addition, the ALZET pump eliminates the need for repeated animal handling for dosing, thus minimizing unnecessary animal stress and experimental variables which can interfere with study results. The ALZET pump has been used in research applications involving both systemic, and site-specific delivery. Attachment to a catheter enables direct administration of agents to a specific target

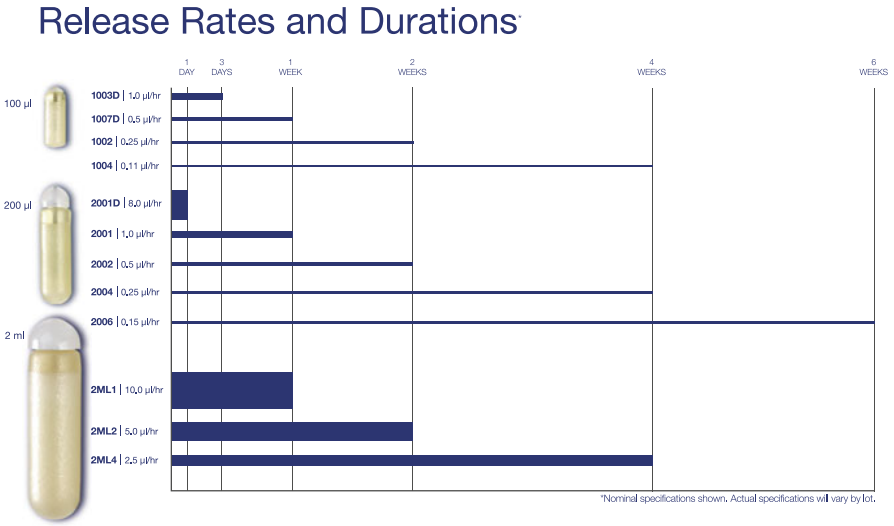

Fig. 17.1 Rates and durations of ALZET Osmotic Pumps (©DURECT Corp.; reprinted with permission)

area, such as blood vessels, the spinal cord, or into the cerebral ventricles and brain parenchyma. Another important capability of the ALZET pump is the possibility of delivering solutions in an intermittent fashion. This is achieved by loading alternating series of drug and placebo solutions within the catheter.

In addition, analysis has indicated that continuous administration of research compounds can produce savings in the amount of compound utilized in research studies. This benefit has greatest potential for compounds that exhibit fast clearance rates in vivo and for which frequent dosing is required to maintain effective steady-state levels or receptor occupancy. The savings can be especially important in the early stages of preclinical investigation when compounds are often expensive to produce and are sometimes only available in minimal quantities [6].

The success of the ALZET pump led to the investigation of osmotic implantable systems for veterinary use (food producing animals). The veterinary implants were further modified for use in human therapy, leading to the design and development of the DUROS osmotic implant system. The DUROS system resembles a miniature syringe and the drug is delivered by the controlled movement of a piston. DUROS implants have been developed to deliver small molecules, peptides, and proteins over periods of several months to a year or longer. The first regulatory approval was granted by the US Food and Drug Administration in 2000 for the Viadur® implant which delivered leuprolide acetate for 1 year. While conceptually the DUROS implant should be applicable to many of the therapies researched utilizing ALZET pumps, DUROS system development has been more narrowly focused, including applications in oncology, pain management, diabetes, and antiviral therapy. DUROS implants have also been investigated for site-specific delivery.

17.2 Design and Principles of Operation

17.2.1 Mathematics of Osmosis

Water transport because of an osmotic gradient is described by the following equation [7]:

$$J = \frac{k(\sigma\Delta\Pi - \Delta p)}{h}, \tag{17.1}$$

where J = water flux across the membrane, k = membrane permeability, h = membrane thickness, $\Delta\Pi$ = osmotic pressure difference (osmotic engine vs. tissue), σ = osmotic reflection coefficient of the membrane, and Δp = hydrostatic pressure difference across the membrane.

Equation (17.1) can be utilized to mathematically describe the delivery rate from an osmotic drug delivery system (with assumptions including $\sigma = 1$ and negligible Δp):

$$\frac{dM}{dt} = k\left(\frac{A}{h}\right)\Delta\Pi c, \tag{17.2}$$

where dM/dt = delivery rate of drug, A = membrane area, and c = drug concentration.

From (17.2), it can be seen that a steady, zero-order delivery rate (dM/dt) over the time course of action of the osmotic implant will result if all of the variables (k, A, h, $\Delta\Pi$ and c) remain constant over time.

17.2.2 ALZET Design and Principles of Operation

The ALZET pump has a cylindrical shape and is composed of three concentric layers: the inner drug reservoir, the osmotic layer, and the outer, rate-controlling membrane (Fig. 17.2). The flexible inner reservoir is molded from a synthetic elastomer which is chemically inert to most drug formulations, and is impermeable to the material in the surrounding osmotic layer. The osmotic layer contains a high concentration of sodium chloride that provides the osmotic driving force. The outermost rate-controlling membrane is formulated with a blend of cellulose esters, and allows selective water permeability. An additional component of the ALZET system is the flow moderator, a 21-gauge stainless steel tube with a plastic end-cap, which prevents random drug diffusion and ensures the delivery profile is osmotically controlled. The component materials of the ALZET pump are tissue compatible, in accordance with USP Class VI Biological Test standards. However, although biocompatible, the ALZET pump employs some materials that have not been qualified for use in human implantable therapy.

The ALZET pump is easily filled by the researcher using a drug-filled syringe connected to a blunt-ended needle. After filling, the flow moderator is inserted

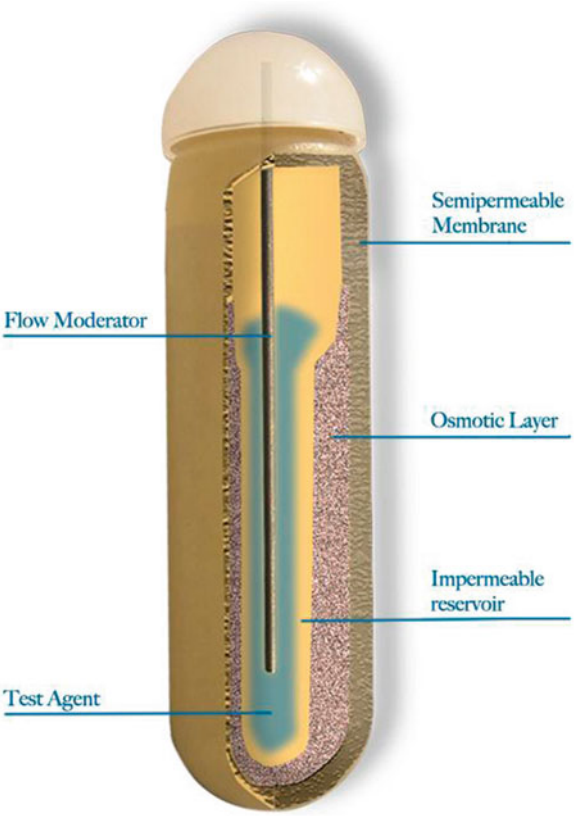

Fig. 17.2 Cross section of an ALZET Osmotic Pump showing its design and components (©DURECT Corp.; reprinted with permission)

through the delivery port. When placed in an aqueous medium (or implanted in the animal), water enters the pump across the semi-permeable membrane into the osmotic layer. This hydration process causes expansion of the osmotic layer and exerts pressure on the flexible reservoir, thus forcing the drug solution through the flow moderator and out via the delivery portal. Since the outer membrane is rigid, the volume of water entering the pump is equal to the volume of drug solution that is released. The delivery rate is dependent on the membrane permeability of the system, and various formulations have been developed to provide researchers with a wide range of delivery rates to choose from.

17.2.3 ALZET Implantation and Explantation

For systemic delivery, the ALZET pump can be implanted subcutaneously or intraperitonealy under general anesthesia. The usual site for subcutaneous implantation in small rodents is on the back, slightly posterior to the scapulae. Once the animal is anesthetized, a mid-scapular incision is made and a subcutaneous pocket

is created by blunt dissection. The filled pump is inserted into the subcutaneous pocket, delivery portal first. The incision is then closed with wound clips or sutures. For intraperitoneal pump implantation in small rodents, a midline incision of the skin and musculoperitoneal wall is made in the lower abdomen under the rib cage. The filled pump is then inserted into the peritoneum. The peritoneal wall is then closed with absorbable sutures and the skin incision is closed with wound clips or sutures.

After its specified infusion duration, the pump becomes an inert object for a period of time lasting half as long as its duration. After that time, because of the continued osmotic attraction of water into the system, it may swell or leak contents of the osmotic layer into the surrounding tissues. For this reason, it is recommended that the pump be removed no later than 1.5 times its duration. Surgical removal of the spent pump is accomplished in the anesthetized animal via a procedure similar to the one described above.

17.2.4 DUROS Design and Principles of Operation

A schematic diagram of the DUROS system is shown in Fig. 17.3. The system consists of a cylindrical drug reservoir, either a biocompatible metal (e.g., titanium alloy) or a biocompatible polymeric material. At one end of the system is the rate-controlling semipermeable membrane, usually constructed from a polyurethane copolymer. The membrane is impermeable to ionic species and other compounds, but is permeable to water. Next to the membrane is the osmotic engine, which consists primarily of NaCl. Following the osmotic engine is the piston, constructed from elastomeric components chosen for compatibility with a specific drug formulation. At the opposite end of the system is the exit port, which can have a catheter attached for site-specific delivery. The drug formulation is filled into the drug reservoir space, which is between the piston and the exit port. Dimensions can range from as small as several millimeters in outside diameter (OD) to 10 mm OD × 60 mm in length. Resulting volumes available for the drug formulation (drug reservoir volume) range from <100 μL to slightly more

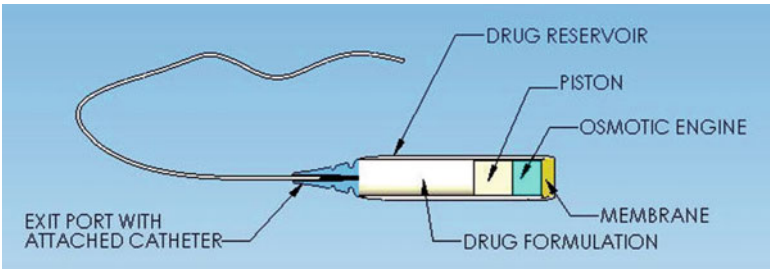

Fig. 17.3 Cross-sectional diagram of DUROS osmotic implant (©DURECT Corp.; reprinted with permission)

than 1 mL. Maximum drug delivery rates are calculated to be on the order of 5 mg/day (40% drug loading, 3-month delivery duration).

When implanted, a gradient in osmotic activity is set up between the water in extracellular fluid on the outside of the semipermeable membrane and the osmotic engine (the osmotic pressure of a saturated solution of NaCl is much greater than the osmotic pressure of extracellular fluid). Water is imbibed into the osmotic engine at a rate controlled by the hydraulic permeability of the semipermeable membrane. The osmotic engine expands at a controlled rate, resulting in the controlled displacement of the piston and delivery of the drug formulation at a controlled rate, as described by (17.2) (above). Sufficient NaCl is usually present in the osmotic engine so that a saturated solution of NaCl is present in the osmotic engine compartment throughout the full delivery stroke of the piston.

DUROS drug formulations can be either solutions or suspensions. Because of the small amounts of formulation delivered per day, nonaqueous formulation excipients (such as dimethyl sulfoxide or alcohols) can be utilized in the drug formulation [4]. Since the DUROS systems deliver over periods of months, the drug formulation must protect the stability of the drug at 37°C for the extended delivery duration (in addition to exhibiting the required shelf stability prior to use). Because of the relatively small drug reservoir volumes (to approximately 1 mL) and extended periods of delivery, drugs must either be very potent or the drug formulations must be fairly concentrated (>5%).

The volumetric delivery rate from the DUROS system depends on the membrane permeability, which can be altered by the choice of the material used for the membrane, or, in the case of a copolymer such as polyurethane, by altering the composition of the copolymer. In addition, the volumetric delivery rate of the pump can be adjusted by the membrane surface area and the thickness of the membrane. This delivery rate is then fixed for the given pump design; however, different doses can be provided from a given pump design by filling it with different formulation concentrations.

The volume in the exit port can be designed to capture any thermal expansion of the formulation or delay startup upon implantation, or can be minimized for a more rapid onset of formulation delivery. In addition, based on the pump delivery rate, the exit port is designed to prevent both diffusional losses and potential back-diffusion into the formulation. The exit port may consist of multiple components, produced by injection molding, extrusion, or machining.

While the specific DUROS systems described below provide zero-order delivery, osmotic technology can be adapted to produce other patterns of drug delivery [8]. These patterns include pulsatile delivery, delivery rates that increase with time and delivery rates that decrease with time.

17.2.5 DUROS Implantation and Explantation

DUROS systems can be implanted in body sites where there is sufficient extracellular water and where there are no biomechanical limitations on implant placement or patient comfort limitations. For systemic delivery, a preferred site is in the subcutaneous

space of the inside of the upper arm. Implantation is accomplished under local anesthesia; a small skin incision is made and a trocar or a custom designed implanter can be used to tent the skin and allow the implant to be inserted [4]. For site-specific delivery, the DUROS system can be implanted directly at the site of action or the system can be implanted at an accessible site and a catheter tunneled to the desired site of action.

Implant removal (explantation) is accomplished under local anesthesia via simple and quick procedures. A small incision is made at one end of the implant site and in the thin capsule surrounding the implant, and the implant is pushed out.

17.3 Osmotic Implant Composition, System Development Program, Nonclinical Evaluations, and Regulatory Aspects

Because ALZET pumps are utilized for animal research applications, they are not regulated products. However, as described above, the pumps have been engineered to be biocompatible. Furthermore, each lot is tested for conformance to labeled specifications.

DUROS implants may be considered combination products by regulatory agencies, as they have device-like characteristics, but the systems deliver pharmacologic agents as their primary mode of action. Hence, they are usually regulated as drugs and a drug division of the regulatory agency (FDA or European agency) will have primary review responsibilities. In the USA, the device division may consult on the regulatory review. The guidances issued by device authorities should also be referenced during product development. These guidances contain useful information that can help in the overall implant system development. Furthermore, the regulatory agencies will expect conformance to the appropriate guidances, either from the device division or the drug division.

Materials selected for the DUROS system must be appropriate for human implantation. Preliminary materials screening for the system components may be performed by examination of the literature for general biocompatibility and chemical compatibility with formulation excipients. Interaction of the system materials with the drug and formulation must be evaluated, and material/formulation compatibility screening is typically conducted prior to design definition.

DUROS implants must be sterile and nonpyrogenic. Either the entire implant can be sterilized by conventional means of terminal sterilization, or if the drug cannot withstand irradiation, steam, or other modalities, an aseptic scheme can be followed. Subassemblies consisting of the reservoir, membrane, osmotic engine, and piston are terminally sterilized as is the exit port. The drug formulation can be filter sterilized and aseptically filled into the subassembly, followed by aseptic assembly of the exit port.

In reference to the guidances described above and in accordance with general product development principles, a typical development program will include

engineering performance studies (e.g., mechanical integrity testing and design robustness studies), in vitro drug release testing under simulated environmental conditions (e.g., temperature conditions, vibration, and/or pressure), stability studies, biocompatibility testing per ISO 10993, packaging and sterilization studies, preclinical/animal safety studies, and ultimately clinical studies. Preclinical/animal studies may include local tolerance studies, in vivo/pharmacokinetic performance studies, pharmacology studies, subchronic toxicity studies, irritation studies, genotoxicity studies (if the API or excipients have not been tested previously), chronic toxicity and irritation studies (in rodents and nonrodent species), and other studies determined on a case-by-case basis (e.g., absorption/distribution/metabolism/excretion studies, pharmacodynamics studies).

17.4 ALZET Applications

17.4.1 Systemic Delivery

Optimizing the schedule of drug delivery can, among other advantages, improve the ratio of therapeutic effects to toxic effects. This is particularly critical in cancer therapy as chemotherapeutics are frequently hampered by their low therapeutic index, wherein side effects occur at relatively low doses while maximal antitumor activity demands high doses. For some agents, continuous infusion is more efficacious and with fewer side effects as compared with administration by immediate release methods, such as injection. The ALZET pump provides a convenient method for this comparison in research animals.

17.4.1.1 Bleomycin for Lung Cancer Chemotherapy

The earliest example of this was a study by Sikic et al. [9], who evaluated the impact of dosing schedules on the anticancer effects and pulmonary toxicity of bleomycin in an animal model of lung cancer. Bleomycin has a short half-life of less than 2 h, undergoing rapid renal excretion. It is also most toxic to cells in certain phases of cell division, so a better antitumor effect would be anticipated from prolonged exposure to tumor cells. Sikic et al. dosed mice with bleomycin via continuous infusion (using 7-day ALZET pumps implanted subcutaneously) compared with injections administered either twice weekly or 10 times weekly. Each regimen was tested for several total doses. Continuous infusion was significantly more effective in reducing tumor size as compared with the injection regimen (Fig. 17.4). In addition, continuous infusion was protective for lung toxicity, defined as collagen deposition as measured by hydroxyproline content (Fig. 17.5).

In the 30 years which have elapsed since the Sikic study was published, more than 1,000 studies have been published in which ALZET pumps were used to examine the schedule dependence of various drugs. Several recent examples follow.

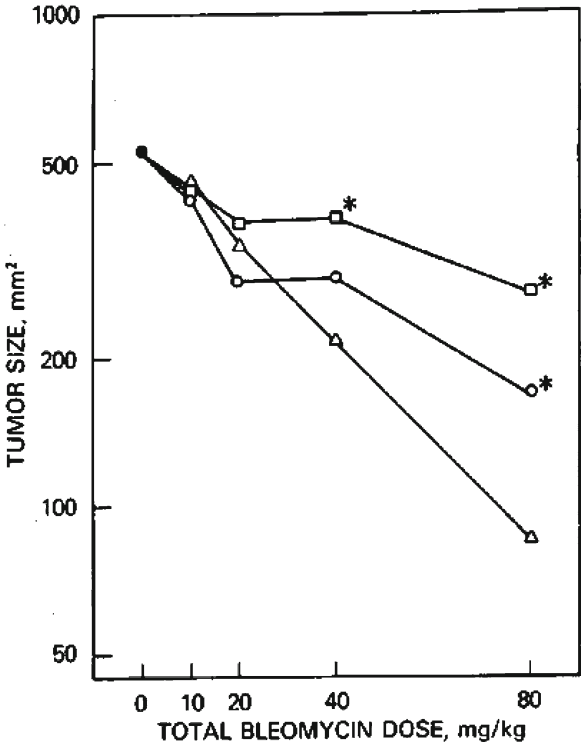

Fig. 17.4 Continuous infusion of bleomycin by ALZET pump (Δ—Δ) achieved greatest reductions in tumor size as compared with twice weekly injections (□—□) or ten times weekly injections (○—○) in a mouse model of Lewis lung carcinoma. *Significantly different from continuous infusion, $p < 0.05$ (reprinted, with permission, from Sikic et al. [9])

17.4.1.2 Antiangiogenesis Therapy

The formation of new blood vessels, or angiogenesis, is essential to sustain tumor growth. Thus methods that inhibit angiogenesis provide a unique therapeutic approach for cancer treatment. Many angiostatic agents have been identified; however, the most promising ones are those that directly inhibit endothelial cell proliferation, such as anginex. Dings et al. evaluated the in vivo efficacy of anginex in mouse xenograft models of ovarian carcinoma [10]. Anginex was administered continuously via ALZET pumps at increasing doses of 5, 10, and 20 mg/kg/day. Anginex treatment resulted in a dose-dependent inhibition of tumor growth in nude mice. Optimum efficacy was achieved with the 10 mg/kg/day dose, which led to up to 80% tumor growth reduction compared with a maximum of 50% in the lower dose group. The therapeutic effects of various treatment regimens were also evaluated on established MA148 tumors. Anginex was administered either by locoregional injections (once- or twice-daily), slow-release alginate beads, or continuous administration by osmotic pumps for 28 days. Continuous administration by osmotic pumps was most effective, leading to up to 80% reduction in tumor growth, compared with 60% and 30% reduction with alginate beads and daily injections, respectively. Anginex was also shown to have a synergistic effect with angiostatin or carboplatin

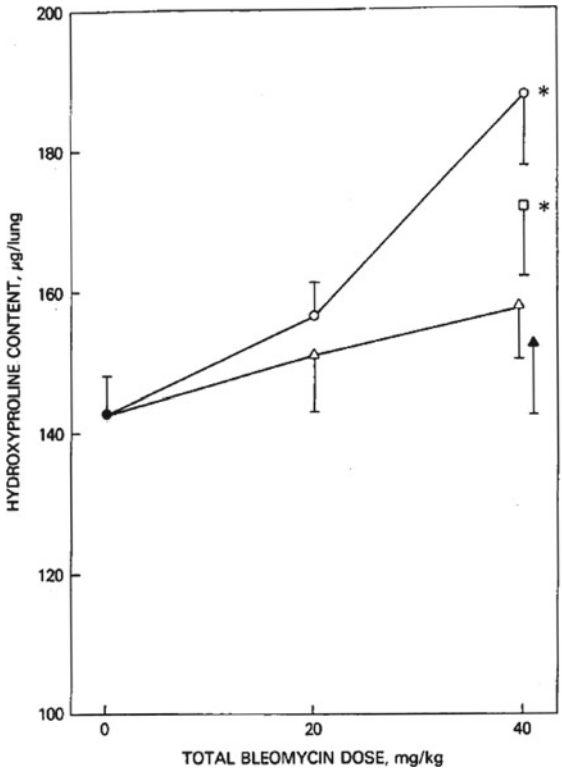

Fig. 17.5 Delivery schedule impacted lung toxicity, as measured by lung hydroxyproline content 10 weeks after bleomycin treatment. Continuous infusion was comparable to saline-treated controls (●—●) even at higher doses and with prolonged treatment: continuous infusion for 1 week (Δ—Δ); continuous infusion for 2 weeks (▲—▲). Injection regimen produced significantly more toxicity: twice weekly (□—□); 10 times weekly (○—○). *Significantly different from controls, $p < 0.05$ (reprinted, with permission, from Sikic et al. [9])

on the inhibition of established ovarian tumors in nude mice [11]. Although continuous, single agent administration was effective, combination therapy of anginex with angiostatin resulted in enhanced tumor inhibition of up to 80%. When anginex was combined with suboptimal doses of carboplatin, animals experienced tumor remission to undetectable levels (Fig. 17.6).

17.4.1.3 Continuous Infusion of Somatostatin Analogs

Somatostatin analogs have been found to induce apoptosis and reduce proliferation of cancerous cells in animal studies. Tejeda et al. found that the therapeutic efficacy of TT-232, a novel somatostatin analog, is significantly dependent upon its mode of administration [12]. Leukemia tumor-bearing mice were treated with various doses of TT-232 either by daily injections for 14 days, or continuous infusion via ALZET pumps for 14–28 days. Although a dose-dependent tumor reduction was evident in both treatment groups, continuous administration was the most efficacious form of administration. In the P-388 tumor cell model, "infusion of TT-232 by ALZET osmotic minipump resulted in 70–80% tumor growth inhibition and 20% tumor-free survival."

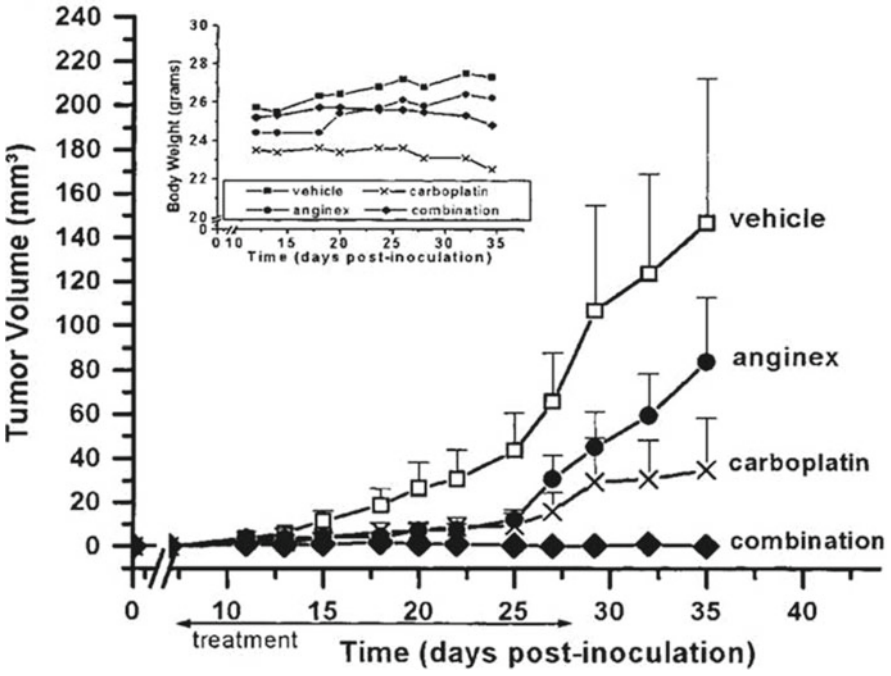

Fig. 17.6 Mean tumor growth curves in a human ovarian carcinoma model. Mice were treated with (square) vehicle, (filled circle) anginex (10 mg/kg/day), (X) carboplatin, or (filled diamond) a combination of both agents. Carboplatin was administered IP once every 3 days at a suboptimal dose of 32.5 mg/kg. Vehicle and anginex were administered continuously via SC pump. Treatments were given for 28 days starting 7 days after tumor cell inoculations. The inset shows animal body weight changes during the treatment period as an indirect assessment of toxicity (reprinted, with permission, from Dings et al. [11], Fig. 2)

Administration by injections resulted in only a modest 26–44% tumor growth inhibition, with no impact on the survival rate. Tejeda et al. also favored osmotic pumps over injections since "serial injections represent significant stress to the animals and require precautions in terms of drug administration." On the other hand, "ALZET minipumps maintained a constant drug level, resulting in a well-defined, consistent pattern of drug exposure throughout the period of drug administration."

Combination therapy with octreotide (a somatostatin analog), galanin, and serotonin has shown promise as a potential treatment for colon cancer, more so than single or double therapy. Studies indicate that the effectiveness of this triple drug therapy is also dependent on the mode and route of administration [13]. Nude mice bearing human colon cancer xenografts were treated with octreotide, galanin, and serotonin, either via daily bolus injections or continuous infusion with osmotic pumps. Drug treatments were maintained for 14 days and were given via the subcutaneous (SC) or intraperitoneal (IP) route. Regardless of the route of administration, triple drug therapy effectively induced apoptosis and reduced tumor volume, weight,

and vascularization. However, continuous IP infusion via osmotic pumps offered the most effective treatment, decreasing tumor weight and volume by 70% compared with only 20% in the IP injection group. Triple drug therapy was well tolerated, as indicated by stable animal weights throughout the study. These agents, like most neuroendocrine gut peptides, have short half-lives. Treatment success was attributed to the low, but prolonged drug concentrations offered by the continuous administration method. On the contrary, intermittent injections provided high drug concentrations, but only during brief periods.

17.4.1.4 Antiobesity and Antidiabetic Effects of FGF21 Infusion

Researchers have found that continuous administration of fibroblast growth factor 21 (FGF21) may offer a safe and efficacious option for the treatment of metabolic disorders [14, 15]. Kharitonenkov et al. used ALZET pumps to administer FGF21 to *db/db* mice for 8 weeks at an efficacious dose of 11 μg/kg/h. Compared with vehicle controls, continuous administration of FGF21 resulted in a significant and prolonged reduction of plasma glucose during the treatment period. FGF21 treatment, even at high doses, was found to be free of the typical adverse effects associated with other therapies. Coskun et al. demonstrated that FGF21 also exerts potent antiobesity effects, and that continuous administration is necessary to maximize its therapeutic action. Diet-induced obese (DIO) mice were treated for 2 weeks with either vehicle, or increasing doses of FGF21 administered by daily SC injections, or continuous infusion by ALZET pump. Although both treatment regimens resulted in a dose-dependent reduction in total body weight, "a 10-fold greater [injection] dose of FGF21 was required to achieve an equivalent weight reduction compared with FGF21 administration via ALZET pumps" ([14]; Fig. 17.7a, b). The researchers speculate that a continuous activation of FGF21 signaling is required to achieve maximal therapeutic effects. Administration via injections leads to a temporary rise

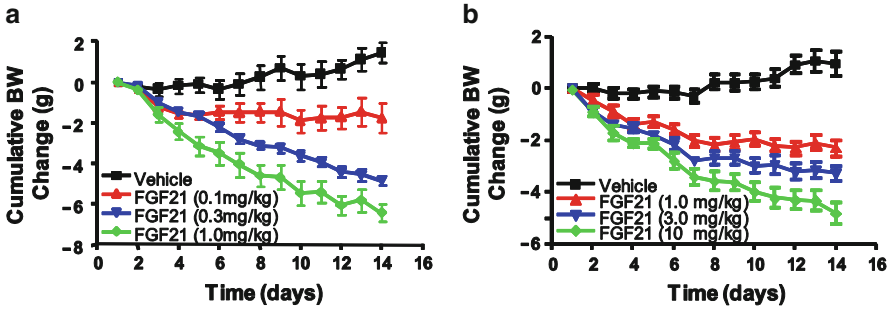

Fig. 17.7 Cumulative change in body weights of DIO mice following continuous FGF21 administration with ALZET pumps (**a**), or daily bolus administration by injections (**b**). Note that a higher FGF21 dose is required with injections to achieve similar antiobesity effects produced following continuous infusion (reprinted, with permission, from [14])

in FGF21 blood levels because of its short half-life. However, "administration via infusion allows for the continuous presence of circulating bioactive FGF21 throughout the course of the study" [14].

17.4.2 Site-Specific Delivery

Site-specific delivery offers many advantages over systemic delivery. High drug concentrations can be achieved and maintained in the target tissue without significant systemic levels, thereby avoiding systemic side effects. In addition, biological barriers to drug entry can be circumvented in order to evaluate a compound's activity in the target tissue. Site-specific delivery using ALZET pumps is typically accomplished using a flexible plastic catheter, which is easily attached to the flow moderator of the pump. In this manner, researchers have delivered agents to numerous tissues, including vessel walls, bone, muscle, wound sites, tumors, and organs such as the brain, ear, eye, ovary, and spleen. Intra-arterial delivery has also been used to target a particular organ, such as the liver and kidney. Whether into the cerebral ventricles, or directly to a specific region or even nucleus, brain delivery is accomplished using a pump implanted subcutaneously on the back of the animal and attached to a catheter leading to a metal or rigid plastic cannula. The cannula is inserted stereotaxically through a drilled hole and affixed to the cranium. The following studies describe the use of ALZET pumps for the delivery of agents to the sciatic nerve, cochlea, and brain.

17.4.2.1 Cannabinoid Analgesia Via Targeted Delivery

The use of cannabinoids for the treatment of neuropathic pain has been hampered by their narrow therapeutic index and association with psychosis risk when delivered systemically. Lever et al. demonstrated that site-specific delivery of systemically ineffective doses of WIN 55,212-2, an aminoalkylindole cannabinoid compound, can effectively induce a peripheral antihyperalgesic effect in Wistar rats [16]. WIN 55,212-2 was administered continuously for 7 days using an ALZET pump, connected to a perineural catheter to enable delivery to the site of sciatic nerve injury. Results show that continuous delivery of WIN 55,212-2 to the injury site significantly reduced hypersensitivity to mechanical and cooling stimuli in a dose-dependent manner. The analgesic effects of WIN 55,212-2 were reversed by co-delivery of the CB_1 receptor antagonist SR141716a, suggesting that localized activity results from overexpression of CB_1 receptors at the injury site. Furthermore, delivery to the contralateral side showed no significant effect on reflex behaviors, thus ruling out the possibility of any systemic analgesic activity. Continuous cannabinoid delivery using an osmotic pump allowed researchers to achieve therapeutic efficacy at doses well below those reported to be effective for analgesia when given by bolus injection. Moreover, localized delivery of WIN 55,212-2 can eliminate the psychoactive side effects normally associated with systemic cannabinoid delivery.

17.4.2.2 Gene Vector Infusion to the Cochlea

An animal model for chronic administration of agents to the inner ear was first developed by Brown et al. to study the effects of tetrodotoxin infusion on auditory nerve responses [17]. The guinea pig was chosen for its ease of surgical manipulation, and ALZET pumps were selected to facilitate the slow, continuous delivery of agents directly into the cochlea. This model was then used as an efficient method for introducing gene vectors to the mammalian cochlea to study and optimize gene expression. A series of studies by Lalwani et al. demonstrated successful in vivo expression of foreign genes in the inner ear of guinea pigs following AAV vector infusion with osmotic pumps [18–20]. Steady-state, intracochlear infusion of AAV vectors containing reporter genes, such as β-galactosidase (β-gal) and recombinant human green fluorescent protein, resulted in transfection and gene expression in a variety of tissues within the cochlea, reflecting the ability of AAV to transfect a broad range of dividing and nondividing cells. Further research showed that AAV was able to establish long-term transgene expression within the cochlea, where β-gal expression was detected for up to 6 months after vector infusion. Luebke et al. reported the use of ALZET pumps for continuous infusion of a replication-defective adenovirus vector carrying the β-gal gene [21]. The modified adenovirus vector was effective at introducing the reporter gene into cochlear hair cells, with reduced toxicity, minimal inflammatory response, and preservation of cochlear function. Wareing et al. demonstrated the feasibility of using cationic liposomes, as a safer alternative to viral vectors, to introduce the β-gal gene into the mammalian cochlea [22]. Gene expression was detected for up to 2 weeks after infusion, and a range of cell types within the cochlea were shown to contain the gene product.

These studies illustrate the feasibility of introducing and expressing foreign genes in the peripheral auditory system of guinea pigs using a variety of vectors. Site-specific delivery with ALZET pumps enables localization of gene vectors to the cochlea while eliminating most side effects commonly associated with systemic administration. In addition, the ability to maintain slow, continuous vector infusion over time helps minimize local tissue trauma and preserve cochlear fluid homeostasis. Furthermore, a slow infusion approach increases the contact time between the gene vector and the cells, thus leading to enhanced transduction efficiency.

17.4.2.3 Treatment of Neurodegenerative Diseases

By providing a means for delivery of agents directly to the brain, ALZET pumps have enabled the evaluation of a vast array of therapeutic agents for various neurological disorders. A review of recent studies of neurodegenerative diseases offers insight into this site-specific use of the ALZET pump across a range of different neurodegenerative models and therapeutic agents (exendin-4, liver growth factor, erythropoietin, and CP2).

Exendin-4. Li et al. [23] used ALZET pumps in a mouse model of Parkinson's disease to demonstrate the neuroprotective effects of exendin-4, a peptide hormone

currently marketed for the treatment of type 2 diabetes, but which also has known neurotrophic activity. The 7-day infusion of exendin-4, or vehicle, was accomplished using a subcutaneously implanted ALZET pump connected to a brain cannula placed in the lateral ventricle (ICV). Following the infusion, mice were subjected to injection of the dopaminergic toxin MPTP, which typically produces a well-characterized model of Parkinson's disease. Assessment of motor function, as well as brain levels of dopamine and its metabolites, revealed that exendin-4 was protective against both motor deficits and damage to dopaminergic neurons.

Prior work with exendin-4 [24] shows the utility of ALZET pumps in a different neurodegenerative model, a rat model of Alzheimer's created by injection of ibotenic acid into the basal nucleus. Rats then received an ICV infusion of exendin-4 for 14 days using ALZET pumps, a treatment which significantly attenuated the loss of cholinergic neurons in the basal forebrain, as measured by choline acetyltransferase immunoreactivity. Exendin-4, also known as exenatide, is also in clinical study in the DUROS implant (see section below).

Liver growth factor (LGF). In a rat model of Parkinson's, established by intracerebral injection of 6-OHDA, Gonzalo-Gobernado et al. [25] used ALZET pumps to evaluate the neurogenic potential of liver growth factor. This agent is a known liver mitogen previously shown to ameliorate certain behavioral and histopathological processes of Parkinson's when infused by ALZET pump [26]. Gonzalo-Gobernado et al. used ALZET pumps to infuse LGF or vehicle into the rat left ventricle for 15 days. Immunohistochemical analysis revealed that LGF increased cell proliferation in the subventricular zone and denervated striatum, as compared with the contralateral side, vehicle-infused animals and the noninfused side of the vehicle controls (Fig. 17.8). In addition, LGF activated microglia and promoted astrogliosis.

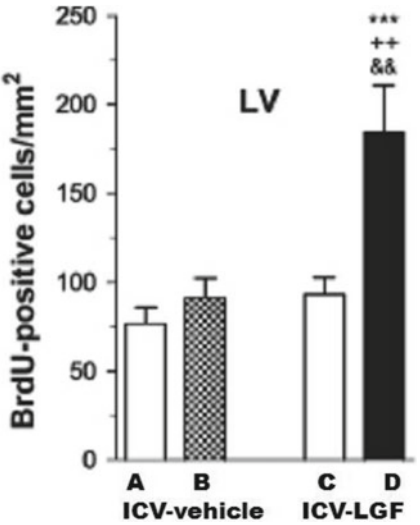

Fig. 17.8 LGF infusion by ALZET pump (D) significantly increased BrdU incorporation in a rat 6-OHDA model of Parkinson's disease, as compared with the contralateral noninfused side (C), and with the vehicle group: vehicle-infused side (B) and contralateral noninfused side (A). Cells in the subventricular zone and striatum were analyzed. ***$p \leq 0.001$ vs. A, ++$p \leq 0.01$ vs. B, &&$p \leq 0.01$ vs. C (reprinted, with permission, from Gonzalo-Gobernado et al. [25])

Erythropoietin (EPO). Also working in a rat 6-OHDA model of Parkinson's, Kadota et al. [27] found that a single bolus of EPO was ineffective. Instead, the group administered EPO by continuous ICV infusion using ALZET pumps to evaluate the regenerative effects of this agent. Delivered in this manner, EPO was neuroprotective, ameliorating behavioral markers of Parkinson's, such as amphetamine-induced rotations, and preserving neurons, without producing systemic effects, such as polycythemia.

CP2. Finally, Hong et al. [28] employed ALZET pumps in a transgenic mouse model of Alzheimer's. The 5X FAD mouse strain is known to manifest the Alzheimer's phenotype unusually early, and with generous deposition of β-amyloid. ALZET pumps provided a 2-week ICV infusion of CP2, a tricyclic pyrone molecule, in the transgenic mice. The group found that ICV infusion of CP2, as compared with vehicle, reduced β-amyloid 1–42 in brain tissue by 40–50%.

17.5 DUROS Applications

17.5.1 Systemic Delivery

DUROS systemic delivery is appropriate for highly potent compounds that (1) can be stabilized for extended periods of time and (2) provide therapeutic benefit via systemic delivery at steady rates for extended periods. Four examples of such DUROS systems are described below.

17.5.1.1 Viadur® (Leuprolide Acetate Implant)

The Viadur implant was developed for the palliative treatment of advanced prostate cancer. Suppression of circulating testosterone is the primary therapeutic approach to the management of advanced prostate cancer [29, 30]. Leuprolide, a synthetic nonapeptide gonadotropin-releasing hormone (GnRH) agonist, acts on the pituitary–testicular axis to produce an initial, transient increase in circulating testosterone, followed by profound long-term suppression to castrate concentrations.

The dimensions of the Viadur system are nominally 4 mm (OD) × 45 mm (length), with a drug reservoir volume of approximately 150 μL. The drug reservoir contains a concentrated solution of leuprolide acetate dissolved in dimethyl sulfoxide (DMSO). The system was designed to deliver leuprolide for 1 year; serum leuprolide levels from patients receiving one implant are shown in Fig. 17.9. In the clinical trials leading to product approval, the expected initial surge in testosterone levels was observed, followed by sustained suppression of testosterone levels [32].

In vivo/in vitro delivery rate correlation was studied for the Viadur implant in rats, dogs, and humans. The in vitro testing was conducted in phosphate-buffered saline (pH 7) at 37°C. In the studies in rats, systems were explanted at 3, 6, 9, and

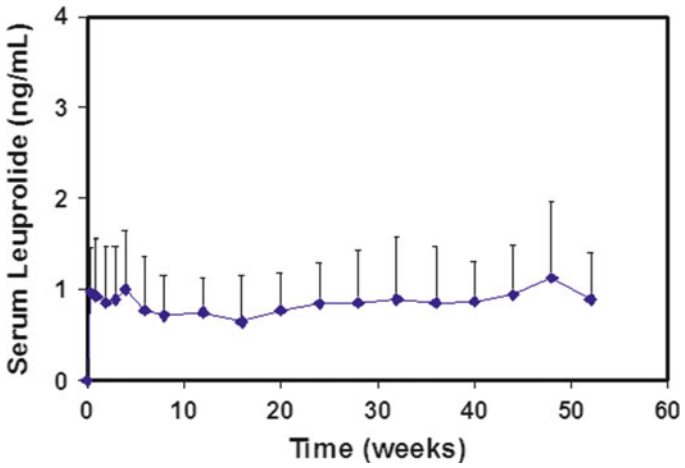

Fig. 17.9 Serum leuprolide concentrations for patients treated with the Viadur® implant (reprinted from [31] with permission from Elsevier)

12 months. There was a good agreement between the drug delivered in vitro and the drug delivered in vivo, as determined by analysis of the drug remaining in the implant at each time point. Similar agreement (to within ±5%) was also observed in canines and in humans for systems explanted at 12 months [31].

17.5.1.2 CHRONOGESIC (Sufentanil) Pain Therapy System

The CHRONOGESIC pain therapy system was developed by DURECT Corporation for continuous systemic delivery of sufentanil for patients requiring around-the-clock opioid treatment for chronic pain. Sufentanil, a potent mu-opioid agonist with a history of use as an analgesic in hospitals, was delivered via the CHRONOGESIC implant for a period of 3 months (Fig. 17.10). A Phase II trial of the CHRONOGESIC System enrolled 66 patients experiencing chronic pain as a result of failed back surgery, cancer, and other malignant and nonmalignant causes. Patients were transitioned from their pre-study opioid medication to a 6-week period of CHRONOGESIC therapy. In a post-study survey, 60% of patients indicated a preference for CHRONOGESIC over their pre-study medication.

17.5.1.3 Omega DUROS for Hepatitis C

Omega DUROS is being developed by Intarcia Therapeutics for delivery of omega-interferon for treatment of hepatitis C. In vitro delivery data for 90 days and stability data for up to 2 years have been reported [33, 34]. The product is in initial clinical

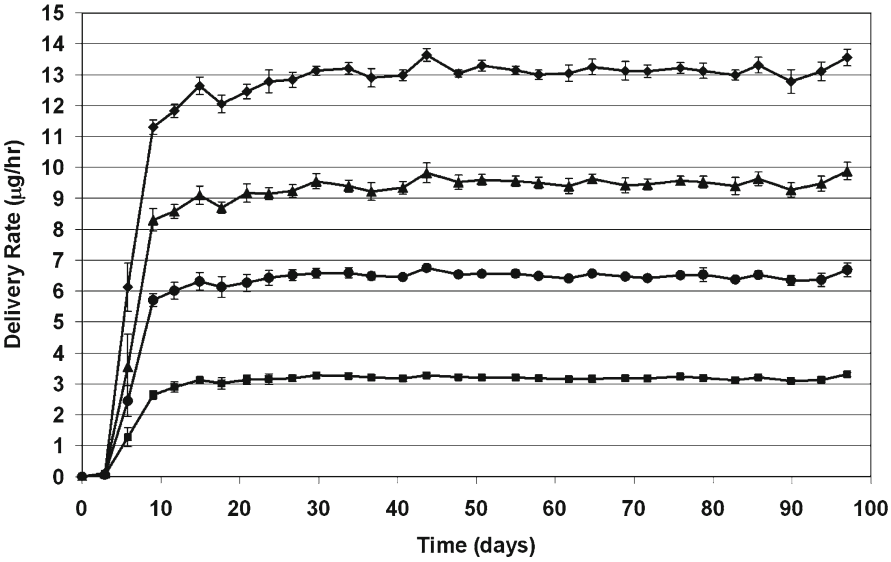

Fig. 17.10 In vitro release rate from four dosage strengths of the CHRONOGESIC System (standard deviation shown by error bars; phosphate-buffered saline, 37°C.) (Reprinted from [4] with permission from Drug Delivery Technology)

trials. Preliminary results reported from this study suggest a highly predictable PK/PD profile and a rapid and profound decrease in HCV RNA [41].

17.5.1.4 Exenatide DUROS for Type 2 Diabetes

Exenatide DUROS for Type 2 Diabetes is being developed by Intarcia Therapeutics. In a Phase 1b study, delivery of exenatide, an approved incretin mimetic, was conducted via the DUROS subcutaneous implant for a 28-day period. Steady-state pharmacokinetics were observed during the implant period [35]. Intarcia has also reported 12-month stability data of exenatide in the DUROS at human body temperature [36]. A Phase 2 study with a 3-month subcutaneous implant has been completed [37].

17.5.2 Site-Specific Delivery

As discussed above, with site-specific delivery, therapeutic concentrations of a drug can be present at the desired target site without exposing the entire body to a similar dose. This section presents two examples of precision drug delivery using DUROS systems with miniature catheters.

17.5.2.1 DUROS Intrathecal Opioid Delivery System

The DUROS intrathecal delivery system was developed for site-specific delivery to the intrathecal space for patients requiring continuous long-term opioid therapy for the treatment of chronic pain. The system consists of a cylindrical titanium alloy reservoir assembled on one end to a rate-controlling membrane and assembled at the other end to an orifice fitted with a strain relief. A specially designed catheter is provided for attachment to the orifice end of the reservoir, through which drug is osmotically pumped to a targeted delivery area (intrathecal space). The implant is nominally 10 mm (in diameter) × 60 mm (in length). The DUROS intrathecal delivery system is currently in the preclinical stage. Nominal formulation delivery rates with the system are 5–15 μL/day, for a pump volume of 1,000 μL, yielding sufficient delivery for 2–6 month delivery applications [4].

17.5.2.2 DUROS Delivery to the Brainstem

Local drug therapy for brain tumors has demonstrated improvements in survival in experimental and clinical studies [38]. Placement of a catheter at the tumor site and connection to a DUROS system placed remotely from the tumor site offer a potential therapy option. In a research study, a catheter was inserted into the pons of cynomologous monkeys and attached to a DUROS system. The catheter consisted of a 30-cm silicone-tubing proximal section and a 2–3 cm Nickel Titanium Alloy (NiTi) distal section. The study demonstrated that the pons of monkeys could be safely accessed via the DUROS catheter system and that saline could be safely delivered via the system for 90 days [39]. Carboplatin was then infused into the brainstem. Analysis of cerebrospinal fluid (CSF) and plasma carboplatin levels showed higher drug levels in CSF [40]. Local neurotoxicity was dose-dependent.

17.6 Conclusions

Osmotically driven, implantable drug delivery systems have demonstrated applicability to both pharmaceutical research and human drug therapies. Sustained, zero-order drug delivery from osmotic implants has been shown to achieve consistent pharmacokinetics in multiple models and therapies.

Systems have been designed with delivery durations from 1 day to 1 year or greater. Osmotic implantable systems are applicable to the delivery of small molecules, peptides, proteins, and other biomolecules. For animal research applications, osmotic systems provide steady delivery, minimize animal handling and enable experimentation that would otherwise be difficult to perform. Zero-order delivery from osmotic implants may reduce overall drug usage over the course of administration. This aspect can be especially important when administering compounds that are expensive to synthesize or compounds with short elimination

half-lives which would otherwise require repeated, frequent injection to maintain near steady-state plasma levels. Research utilizing implantable osmotic delivery systems (i.e., the ALZET) permits investigation of dosing regimes and potential therapies with compounds that are at the frontier of science in fields such as oncology, endocrinology, neurology, cardiology, immunology, and gene therapy. Many of the applications employ the combined benefit of constant dosing at a local delivery site using a catheter attached to the pump. Lastly, research into steady rates of infusion versus repeated or bolus injection may indicate the potential for therapeutic advantages.

For the DUROS system, dedicated drug formulations developed for specific molecules enable long-term stability of the active agent for human therapy. The Viadur implant experience demonstrates that the systems are acceptable to patients. The systems offer the advantage of easy removal should it become necessary to terminate therapy for any reason. Osmotic implantable systems also offer the possibility of site-specific delivery for human applications, allowing better drug utilization and potentially eliminating side effects from systemic levels of drug therapy.

Given their ability to deliver compounds at zero-order, their potential for site-specific delivery, and their established capability to deliver small molecules, peptides, proteins, and other biomolecules, osmotic implantable systems are expected to find continued application in research investigations and human therapy.

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Chapter 18
Microtechnologies for Drug Delivery

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Abstract Microfabrication techniques, originally developed in the microelectronics industry, can be engineered for in vivo drug delivery. Microfabrication uses a variety of techniques including photolithography and micromachining to create devices with features ranging from 0.1 to hundreds of microns with high aspect ratios and precise features. Microfabrication offers a device feature scale that is relevant to the tissues and cells to which they are applied, as well as offering ease of *en masse* fabrication, small device size, and facile incorporation of integrated circuit technology. Utilizing these methods, drug delivery applications have been developed for in vivo use through many delivery routes including intravenous, oral, and transdermal. Overall, microfabricated devices have had an impact over a broad range of therapies and tissues. This review addresses many of these devices and highlights their fabrication as well as discusses materials relevant to microfabrication techniques.

18.1 Introduction

Microfabrication is a collection of methods that creates micro-meter scaled features in a variety of materials. The most common microfabrication method, lithography, was discovered by Nicéphore Niépce in the 1820s where 0.5–1 mm features were

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transferred by photolithography. Since then, photolithography and other microfabrication techniques have been used to produce devices with feature sizes of 0.18 μm or less [1]. One biomedical application of these techniques is for the fabrication of in vivo therapeutic delivery devices. The development of microfabricated drug delivery devices is advantageous in many respects, including the ability to fabricate engineered, miniaturized devices with biologically relevant scaled features, integrate circuit technology and use high-throughput manufacturing practices.

Perhaps one of the most advantageous elements of microfabrication is the ability to control the design of surface topography. For example, traditional light photolithography allows for the development of surface features ranging in size from 1 μm up to the centimeter scale. Electron beam (E-beam) photolithography has resolution on the order of nanometers. The aspect ratio of the fabricated surfaces can be highly manipulated to allow for constrained geometry. Aspect ratios over 25 have been reported for microfabrication of polyester extrusion dyes [1] and aspect ratios of 30 for SU-8 (a photoepoxy resin) features [2]. High aspect ratio microfabricated features can alter cell phenotype, proliferation, and differentiation [3, 4]. Besides precise control over aspect ratio, microfabrication allows management of shape and spacing in order to optimize surface characteristics such as binding molecule functionalization [5, 6], drug release kinetics [7, 8], and surface fouling characteristics [9].

The novelty of microfabrication arises mainly in the miniaturization of features, a design element that was not available until the 1950s with the advent of integrated circuit technology and not applied to biology until the 1990s [10]. The mass fabrication techniques developed for integrated circuit technology carry over to many or all of the features of microfabricated therapeutic delivery devices. Whether on the bench-top or in the factory, the same methods used to generate computer chips can be used to produce microfabricated biotechnological elements *en masse*. One silicon wafer can be processed and contains drug-eluting as well as sensing elements to be implanted in the body [11, 12]. High-throughput, reproducible mass fabrication of these implantable elements allows for low-cost fabrication and precise replication control.

Microfabricated drug delivery devices can be applied to almost every cell type in the body, but have been primarily used for general drug applications, administered through oral, cardiovascular, hepatic, or other tissue routes [13]. Some of these devices release for short periods of time (less than a day) while others release over extended periods of time. This review will focus on the microfabricated elements of both short- and long-term implants. Microfabricated technologies as applied to the delivery of therapeutics as well as a general overview of the techniques employed to fabricate these devices will be highlighted. In addition to the information presented in this review, supplementary information can be gleaned from a variety of other review articles focused on whole implant microsystems [13, 14], medical application of micro- and nano-technologies [15–19], and tissue- or application-specific implants [17, 20–30].

18.2 Microfabrication Techniques

Microfabrication is a process in which micro-scale features are constructed on the surface of a bulk material or patterned into the bulk material itself [1]. The bulk material can be any material but the most prolific materials are silicon, glass, and various polymers [1]. The most commonly applied microfabrication technique is photolithography. The process of photolithography involves the transfer of a pattern from a mask to a thin film via the localization of light. By combining steps of masked exposure and thin film application, multi-layered resists can be formulated with high aspect ratios. A resist is simply a layer, usually thin, of a material that is sensitive to light and thereby altered in some way when exposed to light. It can be made of different materials including a thin layer of a polymer or a chemically modified silicon surface. Higher resolution photolithographic techniques, such as X-ray and E-beam lithography, can lead to smaller feature size as well as more intricate topography [1]. Often photolithography is incorporated in a multi-technique micromachining process for the development of bulk materials. The most common micromachining techniques are chemical etching and surface micromachining [1]. Chemical etching can be either wet (using acids and bases) or dry (typically using plasmas), and removes material from the bulk surface. In contrast, surface micromachining adds to the bulk substrate surface generally by chemical reaction, such as chemical vapor deposition or oxide formation. The location of etching and micromachining is easily manipulated by photolithographic addition of polymer resists. Micromachining and photolithography can also be used in other microfabrication processes, such as micromolding. Micromolding consists of a master with features to be transferred to the polymer or another surface. The initial master substrate can be microfabricated using a variety of methods, including electrochemical microfabrication, lithography, and micromachining. The pattern of the master can be replicated through techniques such as injection molding, hot embossing, and plastics casting [1]. Plastics casting is commonly used for microfabricated implants. By casting a bio-friendly polymer like PDMS, patterns can be transferred to a secondary polymer such as biodegradable PLGA or PCL. These techniques are used in isolation or combination to create implantable biomedical devices. Typically, microfabricated implants are developed in silicon or similar materials, due to ease of manufacturing. After the proof of concept has been established, the implant can then be modified to be cast in additional materials through techniques such as micromolding.

18.2.1 Substrates

18.2.1.1 Silicon

Silicon is the most traditional material for microfabrication due to its use historically in integrated circuit development. Although methods such as wet etching can be performed on other materials such as quartz, silicon is commonly chosen because

of its electrical properties, high yield stress, hardness, and Young's modulus, especially in microelectromechanical systems (MEMS) [1]. The material is available in a variety of single-crystal, thin (0.25–10 mm) wafers of various crystalline orientations [1, 19]. In biomedical applications, many silicon-based devices, such as implantable sensors [31, 32], devices for drug delivery [33–35], and electrical stimulation [36–38] are already under consideration for use within the human body. There are numerous additional examples of MEMS-based biomedical devices [14, 26, 39–46]. Micro- and nano-structured silicon, such as porous and nanowired silicon, have previously been employed as a foundation for sensor technologies [47–55]. It has been shown that topographically modified silicon can elicit specific physiological responses within the body, from bioactivity [56–59] to biodegradation [60–63], displaying the ability to use the material for engineering of cellular response.

With implants, biocompatibility as well as immunogenicity needs to be studied for a surface due to the chronic inflammation foreign body response (cellular encapsulation) and the desire to maintain patient comfort. Biocompatibility is defined as the ability of a material to perform with an appropriate response in a specific application, whereas immunogenicity is defined as the ability of the surface to invoke an immune response, such as inflammation. Micro- and nano-structured silicon has been shown to be biocompatible and non-immunogenic in in vitro studies. A small number of studies have been performed with immune cells on silicon or silane modified surfaces [64, 65]. A study from the Desai group has concluded that micropeaked silicon is more immunogenic than other micro- and nano-structured silicon surfaces based on human monocyte cytokine production, cell viability, and shape factor evaluation. Additionally, the immunogenicity and biocompatibility of flat, nano-channeled and nanoporous silicon are approximately equivalent to tissue culture polystyrene. None of the silicon surfaces were as immunostimulatory as lipopolysaccharide (LPS) in in vitro cell culture studies. Furthermore, in vitro studies indicated that the formation of reactive oxygen species is not a prerequisite for inflammation from silicon-based surfaces [66]. Silicon is not only a user-friendly material, but can also be formulated to be biocompatible and not invoke a significant immune response in vitro or in vivo for extended periods of time.

18.2.1.2 Glass

Like silicon, glass or silicon oxide is available in a variety of sizes and compositions, such as fused silica and borosilicate wafers. Of these two materials, silica offers ease in microscopic imaging [19]. Additional microfabrication techniques are being developed continuously to enhance the role of micro- and nano-structured glass [67, 68]. The use of glass in microfabricated implants is not as universal as silicon, but its use is increasing as new fabrication methods for micro- and nanoscale technology are being developed.

With regard to glass biocompatibility, bioactive silicon oxide based implants have been used since the 1970s, primarily in dentistry, but also in orthopedics [69]. The immunogenicity of glass has been observed with immune cells on both flat and

microstructured surfaces. Increase in size and cell spreading of macrophage cells has been noted, consistent with the high level of cytokine secretion induced by the bioactive glass surface [70]; however, few studies have been performed on micro- or nano-structured glass. Studies have reported that a difference was not observed between the adherence of cells on textured versus flat glass [71]. In addition, glass has been shown to be non-cytotoxic in the particulate form at low concentrations (<1 mg/mL) [72]. We have shown that glass nano- and micro-structure, in the form of nanowires, can reduce the expression of inflammatory cytokine released from human blood derived monocytes, as well as the number of apoptotic and necrotic cells, compared to flat glass surfaces [73]. With the increasing development of micro-structured glass surfaces, as well as the proven biocompatibility in in vitro studies, of such surfaces, the application of these surfaces for implanted devices is inevitable.

18.2.1.3 Polymers

Often the most economical substrate materials for microfabrication are polymer based. Many traditional polymer manufacturing techniques, such as injection molding and embossing, can be carried over into the microfabrication realm at a much smaller scale [19]. Often polymers are introduced through photolithographic techniques. The most common photolithographic polymers are commercially available SU-8 and PMMA. Patterning of additional polymers can be accomplished using micro-molding of SU-8, PMMA, or silicon fabricated masters. The range of polymers available adds an additional design element to the microfabrication process by allowing the developer to tailor specific material properties such as hydrophobicity, biodegradability, and biocompatibility. Furthermore, features such as drug release kinetics, biodegradability, and cell incorporation can be altered with variables such as molecular weight, chemical structure, and/or cross-linking density. A variety of biocompatible polymers are currently used in medical applications, including polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL), poly(glycerol-sebacate) (PGS), and poly(DL-lactide-co-glycolide) (PLGA). Chen et al. have reviewed some of these polymers and have reported average degradation rates and tensile moduli [74].

One concern with polymer-based microfabricated implants is their biocompatibility and in particular their immunogenicity. With respect to biocompatibility, adherence, and viability for many cell types on several different polymer substrates have been reported in literature. Table 18.1 reports studies of cell adherence on polymers that have been patterned using microfabrication techniques. A decrease in cell number was observed with rat epitenon cells on polystyrene (PS) nanometer-sized pillars [82]. In addition, bladder smooth muscle cells (BSMCs) grown on nanostructured PLGA had increased cell adhesion and numbers [77]. With regard to immunogenicity, neutrophils grown on roughened PS surfaces, both in vitro and vivo, have decreased cell viability. Decreased neutrophil viability could be due to a lack of stimulation, rather than due to increased activation [84]. We have shown that

Table 18.1 Immune related and other cell studies with polymers applied in microfabrication techniques

Polymer	Acronym	Cell	Notes
Expanded poly (tetrafluoroethylene)	ePTFE	Macrophages [75, 76]	• Neutrophils mount a substantial burst of reactive oxygen species production, perhaps resulting in inflammation
Poly(dimethyl siloxane)	PDMS	ECs, macrophages, fibroblasts [76]	• ECs, macrophages and fibroblast died rapidly as indicated with trypan blue staining
Poly(caprolactone)	PCL	Bladder smooth muscle (BSMC) [77] Fibroblasts [78–80] Smooth muscle cell (SMC) ECs [80]	• Adhesion of BSMC is enhanced as PCL surface feature dimensions are reduced into the nanometer range • Fibroblasts adhered and grew better on the smooth PCL surfaces with lower surface tensions • Increased oxidative stress was observed in fibroblasts cultured on PCL films • Increase of surface hydrophilicity of PCL before cell culture improves SMC and EC adhesion and proliferation, and reduces oxidative stress
Poly(ethylene terephthalate)	PET	Macrophages ([81], [71])	• Macrophage grown on PET were adhered and/or proliferated more compared to polyacrylamide (PAAm) surfaces • Proinflammatory cytokine release from macrophages on surfaces with different hydrophobicity and surface charge, including PET, was independent of surface chemistry
Polyethylene	PE	ECs, macrophages, fibroblasts [76]	• ECs, macrophages, and fibroblast died rapidly as indicated with trypan blue staining
Poly(lactic-*co*-glycolic acid)	PLGA	BSMC [77]	• BSMC numbers were enhanced on chemically untreated, nano-structured PLGA
Poly-methylmethacrylate	PMMA	ECs, fibroblasts, macrophages [76]	• ECs, macrophages, and fibroblast died rapidly as indicated with trypan blue staining

(continued)

Table 18.1 (continued)

Polymer	Acronym	Cell	Notes
Polystyrene	PS	Neutrophil [76] Epitenon cell [82] Macrophages [75, 76] Mononuclear cells [83]	• Neutrophil adhesion to PS triggers cell spreading, reactive oxygen species production, and changes in intracellular pH • Significant decrease in the number of epitenon cells attached to after 2 h to nanometer-sized PS pillars • Inflammatory cytokines released from macrophages after adhesion to PS surface • Human adherent monocytes revealed apoptotic changes when cultured without any stimuli on uncoated PS (*note: monocytes go through apoptosis at increased rates when unstimulated*) • In vitro mononuclear cell activation on PS surface
Polyurethane	PU	BSMC [77]	• BSMC growth was not supported on chemically treated, nanostructured PU

PCL nanowires with microstructure can elicit a decreased inflammatory response compared to flat PCL [73]. Overall, many of the polymer surfaces, including those that are micro-structured, do not elicit a significant immune response.

18.3 Microfabricated Implant Technology

18.3.1 Delivery of Therapeutic Agents

Microfabricated therapeutic delivery devices can be implanted for short term (up to a few hours) and long term (several days to months) depending on the application. An example of a short-term application is transdermal microneedles which can be applied to the skin to deliver DNA [22] and then removed in a matter of seconds. Devices that are used to treat chronic diseases, such as osmotic pumps to deliver therapeutic proteins [85], are examples of long-term implants, on the order of months or more. Independent of the implant timescale, the ability to control device design as well as drug eluting properties is advantageous in the delivery of therapeutic agents. Microfabrication allows for design control and drug elution. For example, an asymmetric

particle can be microfabricated to deliver drug in a concentrated fashion at the device/intestinal interface (Figs. 18.1 and 18.2) [26, 87–90]. In addition, pore size can be micromachined from silicon to allow for single molecule release of drug, permitting zero-order kinetics (Fig. 18.2) [33, 91]. A representative sample of implantable devices for delivery of therapeutic agents, developed through microfabrication is presented in Table 18.2. These devices are applied in a variety of tissue systems and disease states where several of the obstacles associated with therapeutic delivery can be overcome using a microfabrication design. A review of micro- and nano-electromechanical devices for drug delivery is given by Staples et al. [45].

Although this book is focused on injectable delivery, microfabricated devices for all delivery routes are discussed here due to the considerable activity in this area.

18.3.1.1 Intravenous Delivery

Non-protected, intravenously delivered drugs are diluted upon introduction into the blood stream and are delivered systemically, distributing potential toxicity and harmful side effects in the process. By incorporating the drug into a delivery vehicle, advantages such as targeting, protection, and prevention from dilution are more closely achieved. Currently nanoparticles and liposomes are used ubiquitously for intravenous delivery, but despite phenomenon like the enhanced permeability and retention (EPR) effect, a majority of these particles adversely accumulate in the liver and spleen [115]. Indeed when injected intravenously, microfabricated poly(ethylene glycol) (PEG) hydrogels on the micro- and nano-meter scale, developed in the DeSimone laboratory, accumulated preferentially in the liver (Fig. 18.4) [116]. To make these particles, the DeSimone laboratory creates a silicon master from traditional micromachining techniques (photolithographic application of resist layer with wet etching of the surface) that is then used to create a perfluoropolyether (PFPE) mold to micromold isolated particles on a non-wetting substrate. These particles can be made of a variety of materials including PEG, triacrylate resin, poly(lactic acid), and poly(pyrrole) [96]. Most microfabricated implants, due to their size, cannot be inserted intravenously as there would be a risk of capillary blockage. However, silicon-based microneedles have been implanted into atherosclerotic plaques to deliver DNA [113]. In addition, mesoporous silicon particles have been microfabricated to both image and deliver therapeutics. Q-dots and FITC conjugated single wall nanotubes were added to the silicon particles through surface chemistry. The imaging specificity of these particles was measured on endothelial cells, indicating their potential for intravenous therapy [117]. Most likely, further development for intravenous delivery through microfabricated implants will be enhanced as more microfabricated elements reach the nanometer scales.

18.3.1.2 Oral Delivery

Oral delivery of therapeutics requires drug stability in harsh pH and flow conditions as well as permeation through a thick mucous layer and intestinal epithelial cell tight junctions. These conditions typically prevent the adsorption of most macromolecules,

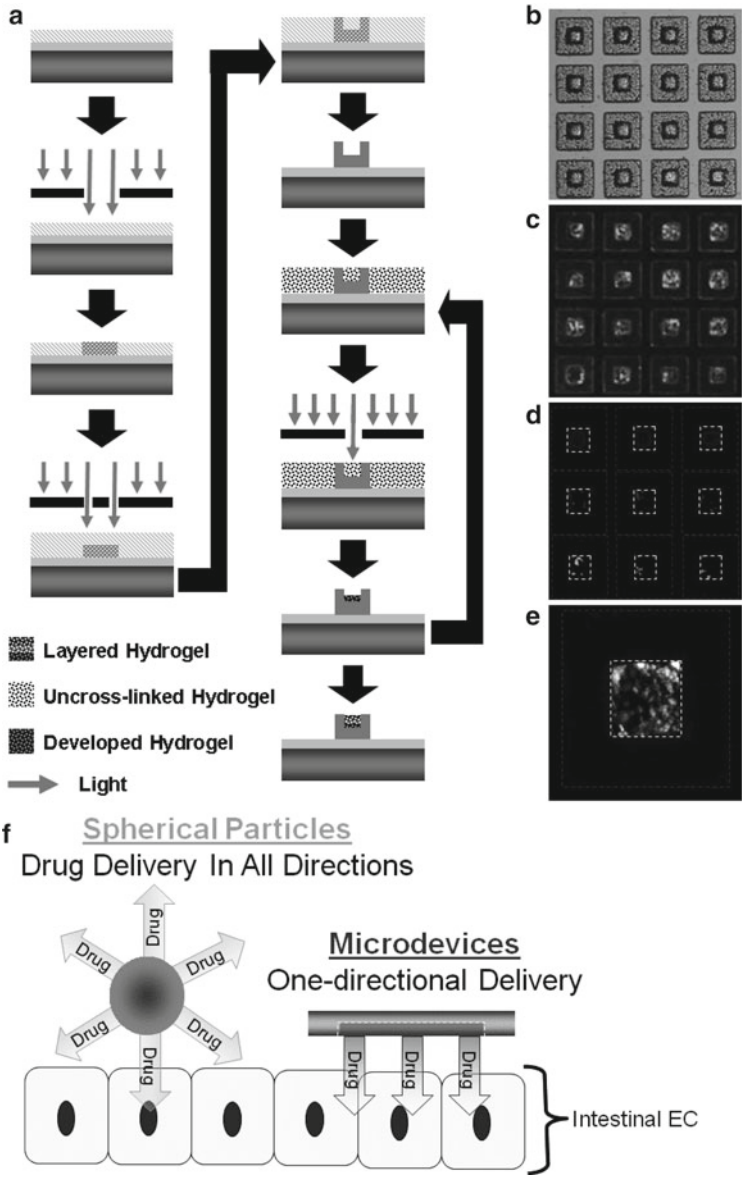

Fig. 18.1 Bi-polymeric particles for oral delivery. (**a**) Process flow diagram for bi-polymeric particles. (**b**) Optical micrograph of hydrogel loaded particles. (**c**) Fluorescent micrograph of FITC-bovine serum albumin loaded hydrogel particles. (**d**) Fluorescent micrograph of multilayered hydrogel prepared with DNP-BSA, FITC-BSA, and Texas-red-BSA (from outmost layer to inmost). The *grey dotted-line* box highlights the reservoir area. (**e**) A fluorescent micrograph of combined filters of hydrogel-filled microdevices. (**f**) Schematic depicting the release of drug from spherical and microdevice particles [86, 87]

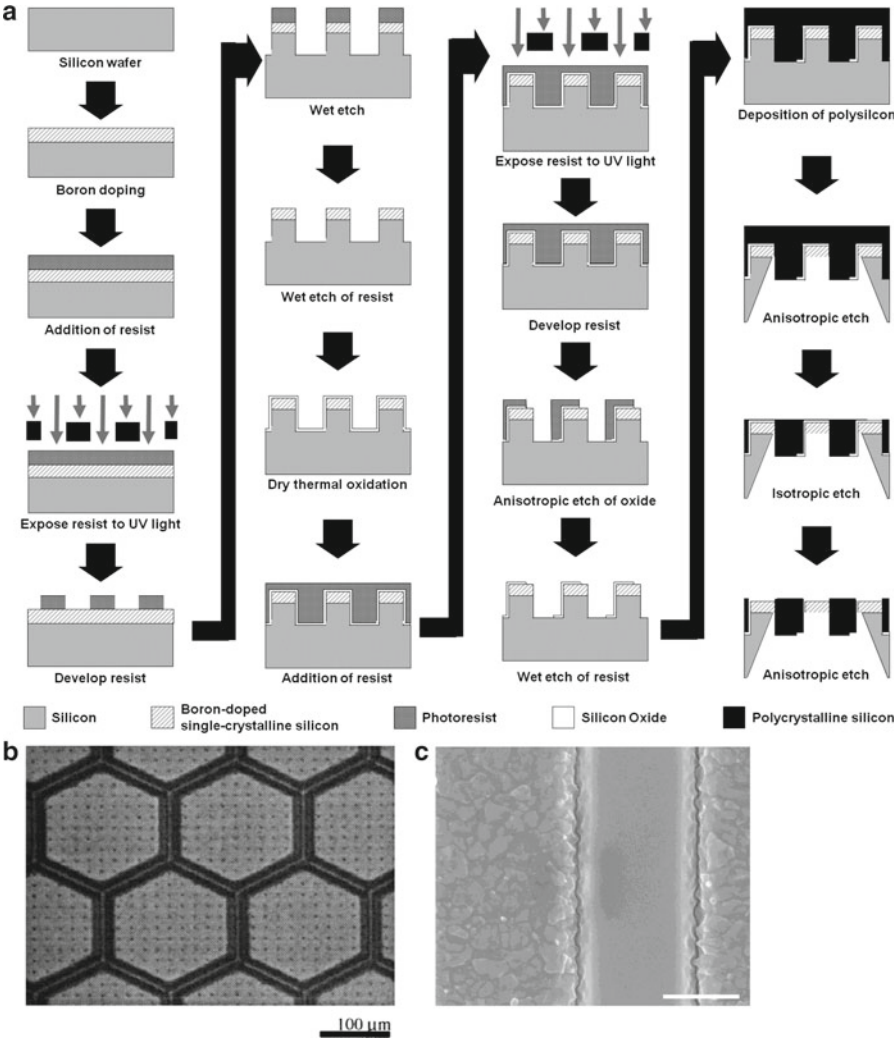

Fig. 18.2 Microfabrication of immunoisolation silicon nano-channeled membrane. (**a**) Process flow diagram adapted from Desai et al. [91]. The diffusion channel is 20–100 nm in thickness. (**b**) Optical micrograph of nano-channeled membrane with pores 78 nm in diameter. (**c**) Scanning electron micrograph of silicon nano-channeled membrane. Scale Bar = 1 µm

and only about 3% of the macromolecules delivered orally reach the blood stream [118]. This can be overcome to a certain extent through administration of copious amounts of drug to the lumen of the intestine. However, this approach is not practical for expensive or toxic drugs, such as those used to treat cancer. Non-microfabricated approaches with nanoparticles and liposomes have had limited success and can be restricted by the hydrophobicity of the therapeutic. Additionally,

Table 18.2 Microfabricated implants used for drug delivery

Tissue System	Description	Microfabrication method(s)	Feature size	Reference
Neural	A microelectrode probe was microfabricated to contain 16 iridium microelectrode sites. This probe is located superior to a drug-delivery catheter and pump-connection adapters for drug delivery	CVD and chemical etching	A surface area of 703 μm^2; spaced 100 μm apart	[92]
Multiple	Nanoporous membranes were fabricated by integrating a block polymer film with a thin (100 μm) silicon substrate and performing multiple etchings	CVD, chemical etching and photolithography	43 nm	[93] [94]
Multiple	A biodegradable PLGA 3-layer device comprised of a diffusion control layer via micro-orifices, diffusion layer, and drug reservoir layers	Micromolding	Orifices on the order of 100 μm	[95]
Orthopedic	bFGF was delivered 40 ng/day on average over 4 weeks from a biodegradable osmotic pump PLGA MEMs device	Micromolding	50×50 μm^2 reservoir, 25 μm deep	[85]
Multiple	Drug eluting microparticles of PEG, PLA and poly-(pyrrole) molded from photocurable perfluoropolyether	Photolithography and Micromolding	>200 nm	[96]
Oral drug delivery	Asymmetric, multilayer microdevices for oral delivery of made of SU-8, PMMA and PLGA with a protected reservoir for therapeutic delivery	Photolithography	150×150×8 μm^3	[26, 27, 87, 89, 90, 97–99], [73]
Oral drug delivery	An asymmetric silicon microdevice for oral delivery of therapeutics with a protected reservoir	Micromachining	50x50x2 μm^3 thick with 25x25x1 μm^3 deep wells	[100]

(continued)

Table 18.2 (continued)

Tissue System	Description	Microfabrication method(s)	Feature size	Reference
Hepatic	A silicon nanochanneled delivery system for zero order release of IFN-α	Micromachining	Nanochannel of 100 nm	[101] [12, 102–104][a] [105] [22, 105–110][a]
Dermal	PLA, PGA, and PLGA microneedles increased dermal permeability almost three orders of magnitude	Micromolding	570 μm high needle with beveled diameter of 10 μm	[35] [45, 111, 112][a] [113] [107, 110][a]
Multiple	A solid-state silicon microchip that provides controlled release of single or multiple chemical substances through the electrochemical dissolution of thin anode membranes covering micro-reservoirs filled with chemicals in solid, liquid, or gel form	Photolithography, CVD, and micromachining	Reservoirs on the order of 70 μm	[114] [45, 111, 112][a]
Cardio-vascular	Silicon-based needles that penetrate into the internal elastic lamina of normal arteries or atherosclerotic plaques to deliver DNA	Micromachining	80 μm high, with a radius of curvature of <0.1 μm	[113] [107, 110][a]
Neural	Silicon-based multi-channel neural probe for therapeutic delivery on the order of 100 pL in 1 s	Micromachining	10 μm features with channel length of 4 mm	[114]

[a] Indicates related references

the therapeutic is often non-specifically delivered to healthy tissue throughout the intestine [119, 120]. An ideally designed oral delivery vehicle would incorporate a protected drug reservoir, asymmetric drug delivery, cyto-adhesion, small device design to resist shear and cell permeability enhancers. The Desai laboratory has designed an oral delivery vehicle that incorporates many of these features (Fig. 18.1) [26, 27, 87–90, 97–100]. These devices were initially developed in silicon through micromachining techniques [100]. Later modifications included multilayer SU-8

devices developed photolithographically, and PMMA and PLGA devices [89]. Building on the multi-layered device, a bi-polymeric device can be formulated with photolithography. Poly (ethylene glycol) dimethacrylate (PEGMA) has been incorporated into SU-8 microdevices for oral delivery of chemotherapeutics [88] (Fig. 18.1). Acrylated PEG polymers were covalently bound to free radicals present on a SU-8 surface [121]. Additionally targeting ligands have been incorporated in to the PEGMA hydrogel for enhanced binding in flow conditions. Indeed, in a developed flow and diffusion chamber, increased drug release was observed with the microfabricated multi-layered device, over the release of free model drug [87]. One advantage of orally delivered vehicles is if the vehicle is larger than several microns, and therefore not endocytosed by the cell, it will be eliminated in a matter of hours by the gastrointestinal tract, avoiding organ accumulation observed with intravenous injected particles. Another potential advantage is that drug release can occur on a relatively short time scale, in the order of hours rather than weeks, months, or years. Certainly, oral delivery of microfabricated devices is a field that holds a lot of promise.

18.3.1.3 Transdermal Delivery

Due to the high density of vascular beds in the dermis, delivering drugs through the epidermis to this region can be advantageous. Currently, non-microfabricated techniques include transdermal patches for birth control and nicotine addiction treatments. Microfabricated silicon- and polymer-based needles have been used to enhance dermal permeability as well as deliver therapeutics [22, 105–110] (Fig. 18.5). The microneedles work like thousands of tiny syringes, on the scale of 10–100 μm in diameter, that permeate the epidermal layer and deliver compounds to the dermis below. Besides the aforementioned application of these microneedles for vascular delivery [113], they have also been used for ocular delivery [108], as well as for vaccines and DNA delivery [105, 107, 110, 113]. In addition to silicon construction, microneedles can be microfabricated through micromolding to form platforms of biodegradable carboxymethylcellulose [109], amylopectin [109], polylactic acid [105], polyglycolic acid [105], and PLGA [105]. Microfabricated microneedles can aid in the *en masse* application of vaccines at relatively low cost.

18.3.1.4 Cell-Based Drug Delivery Therapy

For the treatment of diseases such as diabetes, the therapeutic approach needs to be a dynamic one that incorporates both sensing, e.g., blood glucose, and drug delivery, e.g., insulin. Replacing the cells damaged in such a disease is one way to replicate this dynamic environment. Unfortunately, foreign cells implanted in the body are commonly attacked and destroyed by the immune system, so a protective system needs to be fabricated to facilitate such an implant. Ideally, the system would have a limited diffusion barrier for the analyte and cell nutrients to diffuse in and the

therapeutic to diffuse out. Additionally, this diffusion barrier would need to be impermeable to immune cells and antibodies. Desai et al. have developed a microfabricated membrane that can be incorporated into a device that both limits immune cell signaling and allows for adequate diffusion (Fig. 18.2) ([7, 91, 122, 123, 130]). Nano-channels on the surface of the membrane allow for quick diffusion of glucose and other nutrients, yet inhibit transport of antibodies. Micromachining was used to fabricate the immunoisolating membrane for cellular encapsulation applications. During the microfabrication process, the thickness of the silicon oxidation layer controls the thickness of the diffusion pore and can vary from 10 to 100 nm with a uniform reproducibility of around 5% across a 4 in. wafer [91, 124]. This membrane can be incorporated into a silicon device with a cell reservoir for islet cell transplant [7, 122]. Seigel and co-workers have developed a polymer-based nanoporous membrane fabricated by spin-coating PLA at a thickness of approximately 80 nm on one side of a silicon wafer etched with a silicon nitrate coating (approximately 40 μm thick) on both sides. Using separate chemical etching steps to fabricate pores in the silicon and the PLA, an approximately 43 nm porous structure results in a polymer with approximately 20 μm sized pores in the silicon and silicon nitrate support [93]. Although cells have not yet been incorporated into a polymer membrane based device, the membrane has been incorporated into a hydrogel delivery platform to deliver hormones [94] and insulin, while simultaneously sensing glucose concentrations [125]. For a simultaneous glucose sensor and insulin delivery vehicle, a poly(styrene), poly(isoprene) and PLA co-polymer membrane was self assembled on the surface, as depicted in Fig. 18.3.

18.3.1.5 Other Delivery Routes

Long-term implantation of microfabricated drug delivery devices has been used in a variety of tissue systems and disease states. Through traditional micromachining techniques, a silicon-based multi-channel probe connected to a micropump can be used to deliver therapeutics directly into the neural tissue at a rate of 100 pL/s to stimulate or monitor neural tissue [114]. In a similar application, drug delivery was incorporated with electrochemical sensing, wherein an electrode consisting of 16 iridium microelectrodes was attached to a catheter and a pump [92]. To deliver basic fibroblast growth factor (bFGF), researchers have micromolded a biodegradable PLGA pump for the treatment of bone tissue [85]. Although a considerable amount of microfabricated implants have focused on active delivery through micropumps [126], passive diffusion is also common for therapeutic delivery. A layered system has been developed through micromolding where diffusion is regulated through micro-orifices on the order of 100 μm [95]. The pore size of the device can be controlled through micromolding, which can be advantageous for drug release kinetics. By controlling the pore size of drug delivery devices, traditional concentration-dependent diffusion kinetics can be turned into zero-order kinetics, such as can be seen with silicon membranes [12, 101–104, 127] and is predicted for drug release from titanium nanotubes [128]. A system wherein triggered release of therapeutics has been developed through sili-

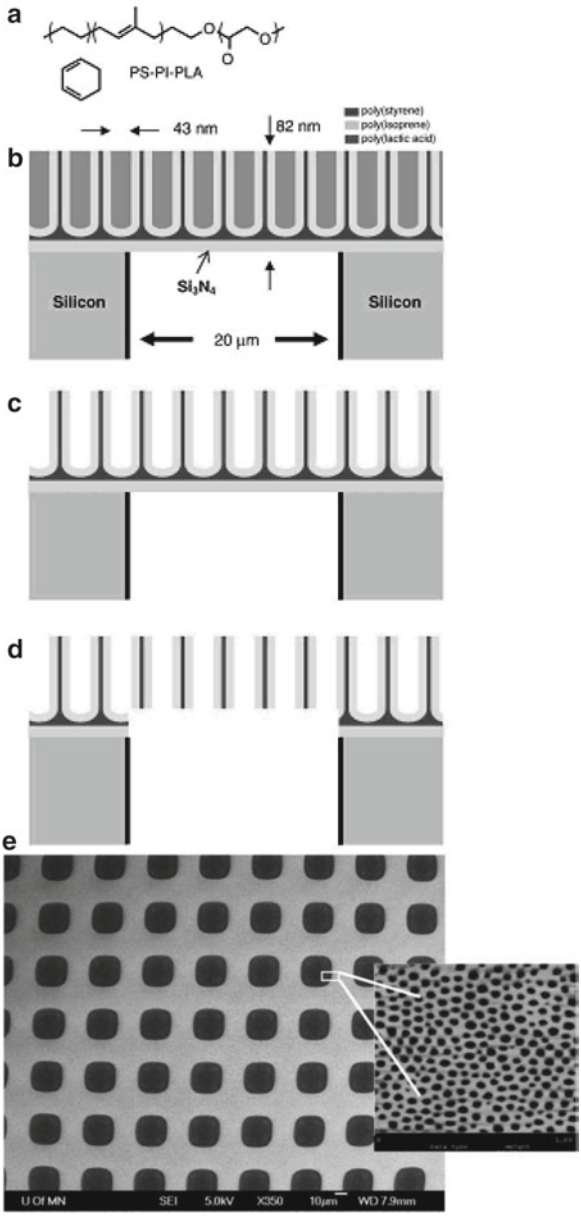

Fig. 18.3 Schematic and micrographs of microfabricated co-polymer membrane. (**a**) Chemical structure of co-polymer. (**b**) Self-assembly of PS–PI–PLA on Si3N4. (**c**) After PLA chemical etching with sodium hydroxide. (**d**) Removal of Si3N3 and wetting PS layer yields continuous nano-microporous membrane. (**e**) An electron micrograph of microporous Si array carpeted with nanoporous block polymer membrane. *Inset*: tapping AFM of nanoporous membrane, area 1 μm^2

con micromachining, photolithographic, and chemical vapor deposition processes was developed by Langer, Cima, and colleagues [35]. Single or multiple reservoirs are incorporated into a silicon device. The reservoirs are gold coated and serve as the anode with a microfabricated cathode also present on the chip. A small electric potential is placed across the chip, dissolving the thin gold membrane and releasing the

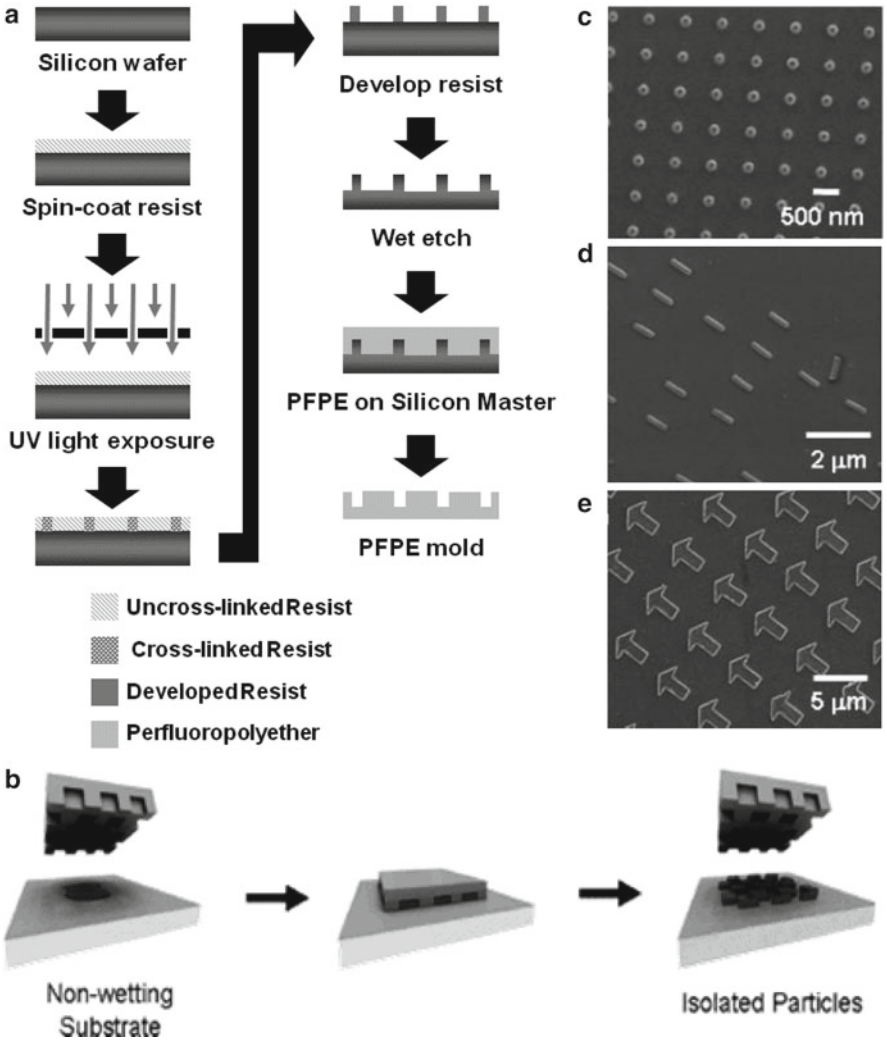

Fig. 18.4 Microfabrication of micro- and nano-scale particles through PRINT method. (**a**) Process flow diagram for fabrication of PFPE mold. Adapted from Rolland et al. [96] (**b**) Schematic of particle formation with PFPE stamp through modified micromolding. (**c**)–(**e**) Scanning electron micrographs of microfabricated PEG particles of various sizes and shapes

therapeutic payload [35]. This technology has been fabricated from PLGA [111], and has been modified to include a sensor aspect for triggered release [112].

Other delivery routes, such as nasal, buccal, and pulmonary, have not yet found suitable microfabricated implant devices, perhaps due to the potential harm incurred with vehicles on the order of 1 μm or larger in some of these systems. Additionally, microfabricated therapeutic delivery systems incorporating vaccines have not been

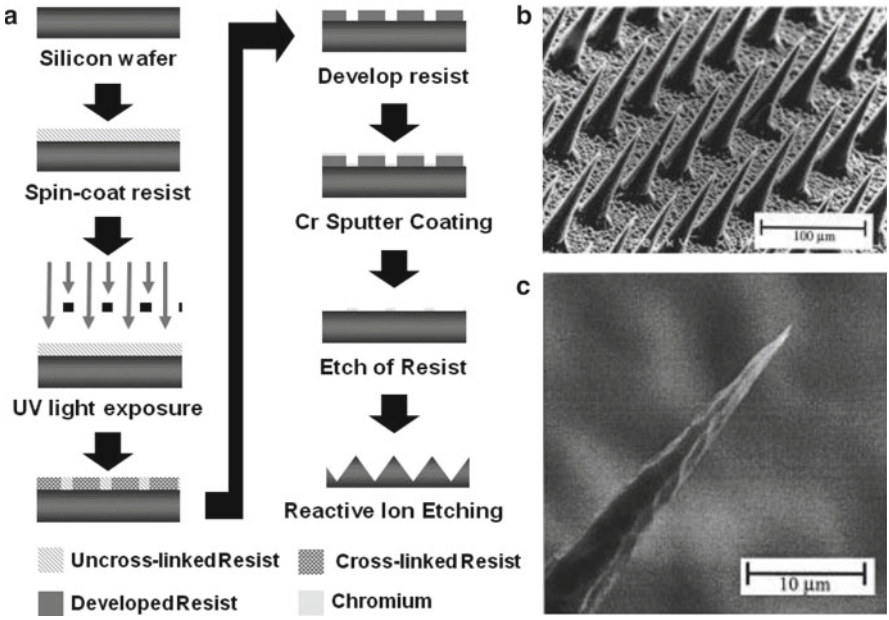

Fig. 18.5 Micromachining of microneedles made of silicon. (**a**) Process flow diagram for microfabrication of silicon microneedles. Adapted from Henry et al. (**b**) and (**c**) scanning electron micrographs of microneedles [107]

explored beyond the application of microneedles [129]. Future applications which reduce the resolution scale of techniques such as photolithography and micromolding can perhaps lead to technologies for alternative delivery routes not yet explored.

18.4 Conclusions

Microfabricated implants have had influence in a variety of fields including drug delivery. Tissue systems throughout the body have been influenced by these devices. The success of these applications has exemplified the ability of microfabrication as an engineering tool to control design of microstructure. Microfabricated elements allow for design at a biologically relevant scale that can easily include integrated circuit technology on a low-cost and high-throughput platform. Although microfabrication might not be relevant for all tissue systems and biotechnological applications, the features of the broad techniques available can produce viable devices, as described here. As time passes and additional microfabrication techniques are developed to produce features at a smaller scale with additional materials, the field of microfabricated implants will no doubt result in more sophisticated and complicated structures.

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Chapter 19
Drug-Eluting Stents

Jonathon Zhao and Lori Alquier

Abstract Coronary stenting is currently the dominant and most cost-effective interventional approach in managing symptomatic artery diseases. Stent restenosis is the phenomenon of arterial vessel re-narrowing following an angioplasty procedure and is the most common complication of the stenting procedure. Drug-eluting stents (DES) nearly eliminate stent restenosis in simple lesions and significantly reduce its incidence in complex lesions. This chapter provides an overview of the pathophysiology of restenosis and the various considerations that go into the design and development of a successful DES including stent platform and materials, drug carriers, selections of various classes of anti-restenotic agents, and the appropriate modulation of drug load and release duration. Detailed examinations of the leading marketed drug-eluting stents are provided, followed by a brief introduction to devices that are in the development stage which may become the next-generation DES.

19.1 Introduction

Heart diseases are the leading cause of death globally, with coronary artery disease being the most common type of heart illness. According to the latest estimates by World Health Organization (WHO), 17.5 million died of cardiovascular diseases in 2005 (WHO Study, 2005, Who Fact Sheet #317). Of these deaths, 7.2 million were as a result of coronary artery diseases. Equally startling is the estimate by the American Heart Association (AHA) that in 2006 about 81 million people in the USA alone suffered from one or more forms of cardiovascular disease. The cost of treating and preventing cardiovascular diseases is staggering and continues to rise

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with each passing year. In addition to the traditional pharmacological treatments, minimally invasive interventions, such as percutaneous coronary angioplasty (PTCA), with and without stent implantation, have been introduced over the years to treat and manage coronary artery diseases more effectively.

Stents are tiny metal wires used to keep arteries patent following balloon angioplasty. Both balloon-expandable (made of stainless steel and other cobalt chromium alloys) and self-expanding (usually made of Nitinol alloy) stent designs have been used for treatment of coronary disease. Stenting of de novo lesions with balloon-expandable stents has been shown to produce a significant reduction in restenosis rates when compared with balloon angioplasty in randomized trials [1, 2]. However, restenosis rates after bare metal stenting still remained at 15–20% in patients with simple lesions and over 30% in patients with complex lesions [3].

Perhaps no other medical device in recent years has been greeted with more scientific enthusiasm and commercial adoption than the introduction of drug-eluting stents (DESs) in 2002. DESs have dramatically increased the use of angioplasty with stent implant in both simple and complete interventions with remarkably low target lesion failure rates. Immediately after the US approval of the CYPHER® Stent (a sirolimus-eluting stent marketed by Cordis Corporation) in 2003, and the TAXUS® Stent (a paclitaxel-eluting stent by Boston Scientific) in 2004, DES quickly displaced bare metal stents (BMS) as the default choice and accounted for more than 90% of all stents implanted. The main appeal of a drug-eluting stent lies in its ability to reduce or even eliminate the restenosis associated with the implantation of a stent, and reduce the need and cost of repeated interventions to reopen occluded or partially occluded arteries.

In addition to the CYPHER® and TAXUS® Stents, two other DESs (Endeavor® from Medtronic and XIENCE™ from Abbott) have been approved so far in the USA. Clinical trials comparing these DES to their respective nondrug-eluting counterparts have demonstrated consistent and significant reduction of stent restenosis and other important clinical endpoints such as target lesion revascularization (TLR) and late loss of the stented vessel [4–8]. The initial enthusiasm toward DES was subsequently tempered by reports and editorials that DES may be associated with increased risk of higher late stent restenosis rate compared to BMS [9, 10]. More recent studies with larger patient populations and longer follow-up times, however, provided a more balanced view, with scientific evidence showing that drug-eluting stents with durable polymer coatings are at least as safe as BMS 3–5 years after implantation [6, 11]. These concerns, as well as the controversy around the widespread usage of DES, among other factors, caused the usage rate of DES to stabilize at the current level of about 70% of all interventional procedures performed in the USA each year. The evolution and development history of DES serve as a good case study for the controlled-release scientific community, as DES is the first blockbuster drug–device combination product and controlled-release technology figures prominently in the success of DES.

A successful DES requires the careful selection of the three components of DES (stent platform, active pharmaceutical ingredient, and a controlled drug delivery system), and meticulous integration of them so that stent restenosis is maximally

suppressed while any unwanted side effects are minimized. This chapter briefly describes the pathophysiology of stent restenosis, followed by a review of the design and engineering of DES and finishing with an overview of the current efforts in the research and development of future generation DES.

19.2 Rationale for DES

The pathophysiology of in-stent restenosis (ISR) is fundamentally a wound-healing process of a coronary artery in response to the trauma caused by balloon angioplasty and stent deployment. This overly aggressive regrowth of arterial tissue, or intimal hyperplasia, at the procedural site is caused by a multi-step event arising from the dilatation of the artery by angioplasty, disruption of the atherosclerotic plaque, physical trauma caused by both the ballooning and stenting, and the foreign body reaction to the stent implant [12].

ISR is reported to occur in 15–20% of patients with a simple lesion, and the occurrence rate can be higher than 30% for complex lesions [3]. Figure 19.1 depicts the three-step process of ISR. It is clear that mechanical stenting will help only with the recoil process immediately following the stenting procedure. The positive remodel of the vessel is beyond the capability of a bare stent and can only be addressed by pharmacological interventions. The key to successful intervention, therefore, is to find the right drug that can cause maximal suppression of intimal hyperplasia and to locally deliver the optimum dose for the required duration.

Sustained local drug delivery from the coating of a stent is a logical and practical approach to treating the positive remodeling of stent restenosis as the stent itself provides mechanical scaffolding of the vessel while its coating serves as a platform or depot for sustained-release local drug delivery. An important requirement for the success of this strategy is to find an effective drug and drug carrier and to successfully integrate these components. A study of the interactions of various polymers with porcine coronary arteries suggests that such efforts would be challenging as a

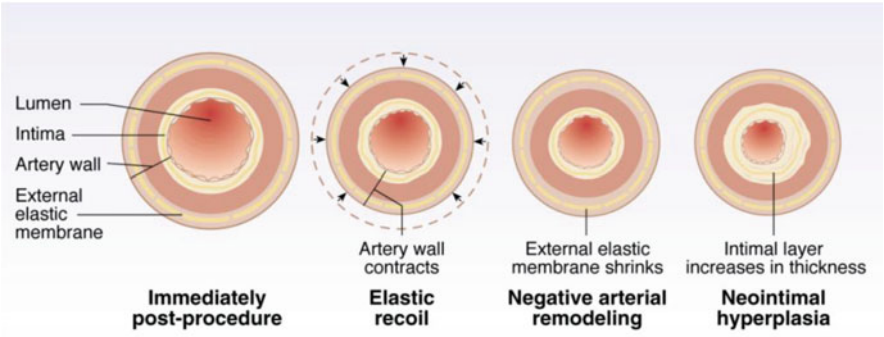

Fig. 19.1 Various processes leading to restenosis

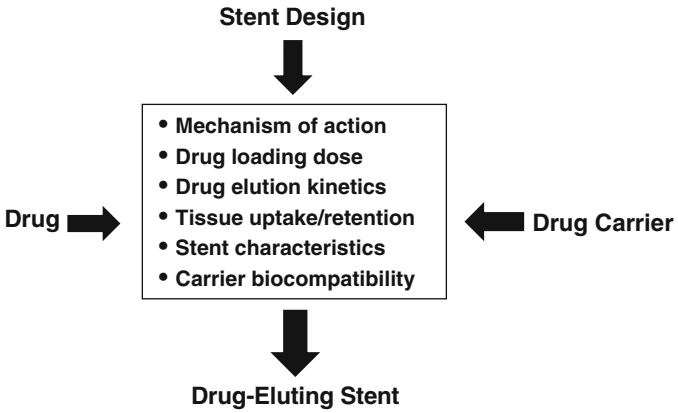

Fig. 19.2 Three critical components of a DES

result of polymer-induced inflammatory reactions [4]; however, persistent efforts that included identification of a potent antirestenotic drug and a biocompatible polymer matrix, as well as an effective coating technology, have proved the contrary.

As shown in Fig. 19.2, the three components of a DES (stent design (platform), drug carrier, and a restenotic drug agent) are all critical to the overall success of a DES. Failure to optimize any of these three aspects will result in a suboptimal DES system.

19.3 Three Major Components of a Drug-Eluting Stent

19.3.1 Antirestenotic Drug

Localized drug delivery from drug-eluting stents provides an efficient and efficacious treatment for restenosis. Applying the drug(s) directly to the target site via the stent drug delivery system provides the maximum dosage of drug delivered while minimizing toxicity and systemic side effects. The physicochemical properties of the drug, duration of action, and the release profile of the drug are all considerations in drug selection.

It has been suggested that the vascular smooth muscle cells start proliferation only a day after the injury resulting from balloon angioplasty/stent deployment and continue for up to 14 days [12]. Consequently, release of drugs from drug-eluting stents usually targets duration in excess of 4–6 weeks to ensure adequate drug release over the timeframe needed.

Multiple drugs have been proposed to prevent restenosis using a stent delivery system. The ideal drug should have a wide therapeutic window with a broad safety profile at the intended dosage. The drug should reduce neointimal hyperplasia by

Table 19.1 Biostable polymers used in approved DES

Stent	Manufacturer	Coating	Drug	Status
CYPHER®	Cordis Corporation	PEVA/PBMA blend	Sirolimus	WW approval
TAXUS®	Boston Scientific	Translute SIBS block	Paclitaxel	WW approval
Endeavor®	Medtronic	PC-copolymer	Zotarolimus	WW approval
Endeavor® Resolute	Medtronic	Biolinx polymer system	Zotarolimus	CE Mark
ZOMAXX	Abbott Laboratories	PC-copolymer	Zotarolimus	IDE trial
XIENCE™ V /Promus	Abbott Laboratories /Boston Scientific	PVDF–HFP copolymer	Everolimus	WW approval

suppressing one or more of the processes of platelet activation, acute inflammation, smooth muscle cell proliferation, extracellular matrix production, angiogenesis, and vascular remodeling [13]. It should also promote vascular healing, allow re-endothelialization of the vessel wall, and should not incite thrombosis or inflammation. Table 19.1 shows the drugs which have been evaluated for use in drug-eluting stents.

Vascular smooth muscle cells and endothelial cells in adult blood vessels are normally quiescent, non-proliferative, and are in G0 phase of the cell cycle. When blood vessels are injured, growth factors stimulate the proliferation of cells in response. Cell proliferation occurs by means of the cell cycle. There are four phases of the cell cycle; G0 or a quiescent phase, G1 or gap 1 phase, S or synthetic phase in which DNA replication occurs, and M or mitosis when the cell divides. As the inflammatory and proliferative responses are the result of a complex series of events, effective drugs targeting different stages of the cell cycle via diverse mechanisms of action may all produce the desired reduction in neointimal proliferation.

In general, drugs that affect cell functions early in the cell cycle (G_1 phase) are considered cytostatic. These agents generally elicit less cellular necrosis and inflammation compared with agents that interact in the later stages of the cell cycle (beyond the S phase) [14]. For this reason, the majority of drugs selected for use on commercial drug-eluting stents inhibit cell functions at the early stages.

On the basis of the mechanism of action of the drug and its target in the restenotic process, drugs can be categorized as immunosuppressive agents, cellular proliferation inhibitors, anti-inflammatory agents, or antithrombotic and prohealing agents.

19.3.1.1 Immunosuppressive Agents

Immunosuppressive drugs, such as sirolimus, tacrolimus, and everolimus, generally bind to FK506-binding protein 12 (FKBP12) in smooth muscle cells. Sirolimus, a potent anti-rejection drug produced by *Streptomyces hygroscopicus,* subsequently binds to the mammalian target of rapamycin (mTOR). This prevents the smooth muscle cell to progress from the G_1 to the S phase of the cell cycle, and thus arrests the growth of smooth muscle cells in the G_1 phase [15]. Sirolimus is strongly lipophilic and has extremely low water solubility.

As everolimus is more hydrophilic than sirolimus or tacrolimus, it exhibits a shorter elimination half-life (approximately 30 h) and demonstrates greater relative bioavailability compared with sirolimus [16]. Like sirolimus, inhibition of mTOR appears to be the mechanism by which everolimus inhibits cell proliferation. Unlike sirolimus, everolimus has increased solubility in organic solvents and has a two- to threefold lower affinity for the receptor FKBP12.

Tacrolimus, produced by *Streptomyces tsukubaensis*, in contrast, binds only to FKBP12 and interferes with cell cycle progression by means of calcineurin inhibition [17]. Despite this slightly different mechanism of action, tacrolimus is thought to prevent restenosis by halting the cell cycle in the G_1 phase also. Tacrolimus is lipophilic and highly bound to plasma proteins [18].

19.3.1.2 Cellular Proliferation Inhibitors

Cellular proliferation inhibitors, such as paclitaxel, actinomycin-D, and methotrexate, inhibit cell proliferation by binding to DNA. Paclitaxel inhibits cellular replication by binding to microtubules and inhibiting cell processes that depend upon microtubule turnover, such as mitosis, migration, and secretion. Paclitaxel acts at multiple points of the cell cycle, inhibiting cell replication at the G0/G1 transition as well as the G2 and mitotic phases. The lipophilic nature of paclitaxel favors transit of the drug through membranes [19] and its insolubility in water and lipophilic character minimize loss in the extracellular fluids (including blood), In addition, the binding of the drug to microtubules results in low, single-dose applications providing a sustained effect [20]. Its narrow safety margin, however, makes it ideally suited for low-level, local applications.

Other drug candidates from this group have been studied, with disappointing results. Actinomycin-D is a chemotherapeutic drug which blocks the cell cycle in the S-phase by blocking the transcription of DNA. In animal studies, actinomycin-D inhibits smooth muscle cell proliferation in a dose-dependent manner. The ACTION trial was prematurely stopped because of increased restenosis rates in the actinomycin-D groups.

19.3.1.3 Anti-inflammatory Agents

Because of the role of inflammatory cells in restenosis, anti-inflammatory agents are commonly used in conjunction with drug-eluting stents. The mechanism of action by which steroids inhibit smooth muscle cell proliferation is multifold. Due to their anti-inflammatory properties, steroids reduce the aggregation of inflammatory cells at the site of vessel wall trauma. The steroid-induced anti-inflammatory actions include inhibition of leukocyte adhesion to endothelial cells, reduction in leukocyte aggregation, suppression of platelet derived growth factor, and reduced production of cytokines, nuclear proteins, fibroblasts, and macrophages [42].

19.3.1.4 Antithrombotic and Pro-Healing Agents

Several antithrombotic agents have been or are undergoing clinical evaluation. Heparin, originally used as a passive fixed coating, was also gaining interest as an active coating on drug-eluting stents [21]. Clinical studies of drug elution with other steroid anti-inflammatory agents are underway, including 17-β estradiol [22] which may have a role as a pro-healing agent. Some researchers have suggested that pro-healing agents may provide a safer approach to restenosis prevention than other types of agents [13].

Estrogens have also shown promise as the active component on drug-eluting stent. Estradiol stents were found to reduce neointimal growth compared with a control stent [23]. This reduction in neointimal growth is attributed to estradiol's potential to reduce smooth muscle cell migration and proliferation, promote vascular healing, and stimulate angiogenesis [24].

19.3.2 Stent Platform

Balloon-expandable coronary stents are fabricated from stainless steel, cobalt chromium, and other metal alloys and composites. Important attributes of a stent platform include high radial strength, low recoil, minimal foreshortening, uniform scaffolding, and radiopacity [25, 26]. As drug delivery platforms, stent surface area and cell pattern (open-cell or closed-cell designs) are important factors in determining the amount of drug that can be loaded and the uniformity of drug delivery to the vessel wall [27]. The stent platform should provide the necessary capacity for, and be compatible with, the drug-loading process such as sufficient surface area and adhesion strength for drug/polymer coatings [28]. In many cases, a primer coating layer is used to improve the adhesion between the stent surface and drug/carrier layers.

19.3.3 Drug Carrier

Other than the careful selection of a drug that is potent enough to inhibit intimal hyperplasia, equal consideration should be given to the drug carrier system. A successful carrier ensures that the drug is slowly released into the diseased tissue in a controlled fashion to match the pathophysiology of the restenosis and, at the same time, minimizes the potential loss to systemic circulation. Various methods have been proposed to deliver drugs from a stent, including non-polymeric drug coatings [29, 30], covalent drug attachment to the stent surface through linkers [31], drug-infused polymer sleeves [46], non-absorbable polymer carriers, and bioabsorbable polymer carriers. Regardless of the nature of the drug carrier, it needs to meet a certain number of requirements. For instance, the carrier needs to have excellent

tissue compatibility, good physical/chemical compatibility with the drug, adequate resistance to cracking and peeling during manufacturing and sterilization processes, conformability during stent expansion and the ability to control the drug elution over the required number of weeks after implantation. Another important design parameter to consider for a drug carrier is the terminal sterilization method, as it is known to affect many carriers. The most commonly used method for DES sterilization is ethylene oxide (EO), which may interact with active functional groups such as amine, sulfhydryl, and unsaturated bonds in a drug molecule. A polymer carrier can help to shield the drug from the effects of EO. Similarly, the polymer material itself may be affected by the sterilization process, causing the rearrangement of the drug in the polymer matrix and ultimately affecting the shelf-life and release kinetics of the drug. Likewise, e-beam and gamma irradiation are not compatible with most drugs because their high energy can disrupt molecular structure. In the following section, the commonly used drug carriers are reviewed, with in-depth discussions given to biostable and bioabsorbable polymers, as they have been successfully used as drug carriers in current and future generations of DES.

19.3.3.1 Biostable Polymers

Biostable polymers have been chosen for use in the majority of DESs that are marketed or in late clinical development. All four approved DESs in the USA use a biostable polymer or polymer blend system as the drug carrier (Table 19.1). Newer generation DESs such as Endeavor® Resolute continue this trend with a three-component polymer blend to replace the original phosphorylcholine (PC) block polymer carrier in order to improve the control of drug elution kinetics.

The prevalent use of biostable polymers as drug carriers for DES is not surprising in that those polymers have been used in previously approved implantable devices and have a good safety record, or their biocompatibility has been established in related studies. Their physical and chemical properties are well studied and characterized, and are familiar to the material scientists involved in DES development.

By design, the polymer carrier remains stable and durable during coating, crimping, sterilization, packaging, storage on the shelf, and inside the artery after implantation. This physical stability is important as it is critical to maintaining the integrity of the stent coating, the inertness of drug matrix toward the drug, the uniform distribution of drug in the coating and a consistent drug elution, and the shelf life of the product. The important physical parameters to be considered are the glass transition temperature (Tg) and the mechanical properties of the polymer such as Young's Modulus, elongation at break, and phase compatibility between the drug and the polymers. Common ways to adjust the stability are through co-polymerization or polymer blending. In addition, the polymeric carrier should not interact with the drug in the matrix, as many drugs have functional groups that may chemically interact with the carrier system, leading to shortened shelf life of the product. In addition, both the drug and the carrier systems should be compatible with the terminal

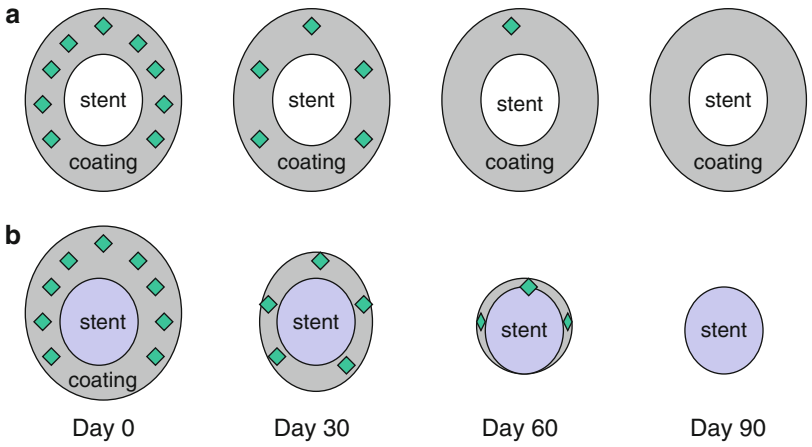

Fig. 19.3 Comparison of drug elution from a DES using a biostable and a bioabsorbable coating matrix. (**a**) biostable coating matrix (**b**) bioabsorbable coating matrix

sterilization methods commonly used for implantable medical devices. The design and development approaches for the biostable polymers listed in Table 19.1 are discussed in full detail in the section on leading DES below.

19.3.3.2 Bioabsorbable Polymers

Bioabsorbable polymers were among the first materials to be used as coatings for DES. The main advantage of a bioabsorbable polymer is the promise of the disappearance of the drug carrier after the complete depletion of the drug payload. Material properties and degradation rates of a bioabsorbable polymer are dependent upon the molecular weight of the polymer and the ratio of monomers in the copolymer. As shown in Fig. 19.3, a biostable coating controls the drug elution mainly by the drug diffusion process, with predominantly first-order release kinetics, in most cases. The bioabsorbable carrier, on the other hand, may have a combination of diffusion control and degradation control over the drug elution. In the beginning, the drug elution is primarily driven by a diffusion mechanism before the onset of drug matrix degradation. As more and more drug is depleted, water ingresses into the drug polymer matrix and causes the bulk erosion and degradation of the matrix. The drug elution is thus influenced by two simultaneous processes. This change makes the design of drug depot system and drug release control more challenging.

The most commonly used bioabsorbable polymers and their key properties are listed in Table 19.2. So far, the majority of the bioabsorbable polymers used in DES come from the family of polylactic acid (PLA) and copolymers of poly(lactic-*co*-glycocolic acid)(PLGA). This is not surprising in that PLA and PLGA polymers have been used in a wide range of implantable devices including resorbable sutures, bone pins, screws, and implantable controlled-release depots.

Table 19.2 Bioabsorbable polymers used in DES

Manufacturer	DES	Drug	Stent material	Coating	Status
Biosensors Inter-national/ Terumo	BioMatrix	Biolimus-A9	stainless steel/ cobalt chromium	PLA	CE Mark
Sahajanand Medical Technologies	Infinnium® Paclitaxel eluting Coronary Stent	Paclitaxel	Stainless steel	PLGA	CE Mark
JIWI	Excel™ Sirolimus-eluting Coronary Stent	Sirolimus	Stainless steel	PLA	CE Mark
Cordis/Conor	NEVO™ Sirolimus-eluting Coronary Stent	Sirolimus	Cobalt chromium	PLGA	Ex US clinical trial
Boston Scientific	JacTax	Paclitaxel	Platinum/Cobalt alloy	PLA	
GDT/biosensor	Champion	Everolimus	Stainless steel	PLA	Stopped
Abbott Labora-tories	Bioabsorbable Vascular Solutions	Everolimus	PLLA	PLA	OUS trial
Reva/Boston Scientific	Polycarbonate	Paclitaxel	Polycarbonate (Bioab-sorbable)		Stopped

19.3.3.3 Natural Biopolymers

Proteins such as albumin, collagen, gelatin, and fibrin, or polysaccharides such as hyaluronic acid and dextran, have been used as natural polymeric materials for DES coatings [32, 33]. Intuitively they are logical choices as a drug carrier because of their natural origins and presumably would have good biocompatibility with vascular tissues. These attempts, however, have not been successful for a variety of reasons. The hydrophilic nature of these materials usually produces a matrix with poor physical integrity and poor ability to control drug elution. In the case of cross-linked gelatin encapsulating paclitaxel, the drug load is completely exhausted in 2 weeks after implantation [34]. Mitigating strategies such as crosslinking often lead to unwanted damage to the drug incorporated. Potential immunogenicity of these biological materials (especially after crosslinking) also raises additional safety concerns.

19.4 Examples of Commercially Available DES and the Applications of Controlled Delivery Technologies

Currently only four drug-eluting stents have been approved by FDA for US markets. They are CYPHER® Sirolimus-eluting Coronary Stent (Cordis Corporation), TAXUS® Paclitaxel-eluting Coronary Stent (Boston Scientific), Endeavor® Zotarolimus-eluting Coronary Stent (Medtronic), and XIENCE™ V/Promus

Everolimus-eluting Coronary Stent (Abbott Laboratories). These four DESs have different stent platforms, balloon catheters, drugs, and coating materials; however, they all share similar principles in their designs of a drug/carrier system and controlled release of drug that results in clinical efficacy.

19.4.1 CYPHER® Sirolimus-Eluting Coronary Stent

The CYPHER® Stent is the first DES approved in CE mark countries in 2002 and in the USA in 2003. The initial clinical trial results of CYPHER® Stents with 0% restenosis [4] caused a big stir in the interventional cardiologist community and ushered in a new chapter of DES development. The development history of the CYPHER® Stent embodied the major principles of a successful DES with a highly potent cytostatic drug, sirolimus, and a biostable polymer blend system as the drug carrier. A closed-cell 316 L stainless steel stent provides uniform coverage of the vessel wall at the lesion site. The bare metal stent platform is the first that was graft-polymerized in situ with a durable thin layer of parylene to promote adhesion of subsequent layers of drug-loaded polymer layers to the metal surface. This thin polymeric layer serves the important functions of enhancing the bonding of subsequent polymeric drug carrier matrix layers to the stent surface to maintain the drug delivery properties.

In the CYPHER® Stent, a blend of poly(ethylene-co-vinyl acetate) and poly(butyl methacrylate (PEVA/PBMA) is used as the drug carrier (CYPHER® Stent Instructions for use). Both PEVA and PBMA have excellent biocompatibility and have individually been used as implants in humans with a proven safety track record with regulatory agencies. The elastomeric nature of both polymers also ensures a durable conformal coating on the stent surface to control drug elution. The selected drug loading level of sirolimus and the choice of the polymer carrier system based upon its excellent compatibility with sirolimus were the result of numerous experiments to ensure a sustained drug release with a first-order kinetics profile. In vivo studies have shown that the majority of the drug is released in a sustained fashion in 30 days with complete drug release in 90 days as shown in Fig.19.4. The majority of the drug release within the first 60 days is important because it is the most critical period for the development of restenosis following stent implantation.

The seemingly small drug load of 160 μg of sirolimus on 3.5 mm by 18 mm CYPHER® Stent, once slowly released into disease lesions, is proved to be effective in suppressing intimal hyperplasia. The durability of the CYPHER® Stent antirestenotic efficacy has been unequivocally proven in large-scale clinical trials after 5 years of clinical follow-up data.

19.4.2 TAXUS® Paclitaxel-Eluting Coronary Stent

TAXUS® Paclitaxel-eluting Coronary Stent was the second DES to gain approval in the USA. In the case of TAXUS®, a tri-block copolymer of poly(styrene–isobutylene–styrene (pSIBS, Translute™ polymer system) is used as the hydrophobic

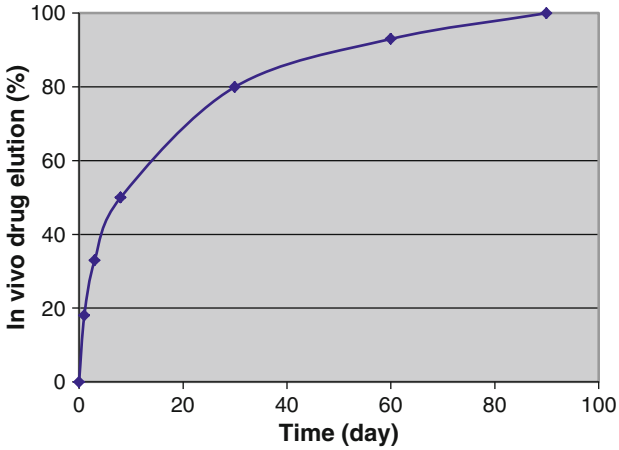

Fig. 19.4 In vivo elution kinetics of Sirolimus from the CYPHER® Stent

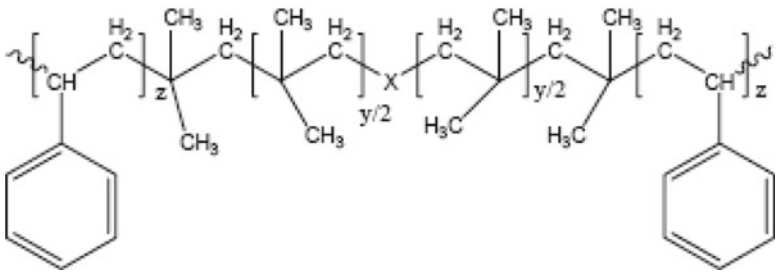

Fig. 19.5 SIBS polymer chemical structure

polymer matrix for controlled release of paclitaxel [48]. PolySIBS (Fig. 19.5) is a soft, flexible, yet very hydrophobic elastomeric polymer system that forms a good conformal coating, but is impermeable to the diffusion of either fluids or gases. This classic elastomeric coating has a continuous polyisobutylene phase and a discontinuous polystyrene phase.

As the polymer is hydrophobic, elution of paclitaxel is slow, and only about 10% of the drug is eluted after implantation, with an additional small amount released afterward (Fig. 19.6). The feasibility of pSIBS as a drug carrier on a stent is made possible by the extreme potency of paclitaxel in treating restenosis. Higher doses or prolonged presence of paclitaxel in vasculature would cause delay in the endothelialization of coronary artery damaged during the angioplasty procedure. The slow release (SR) formulation was chosen for commercialization after clinical trials of fast-, medium-, and slow release of TAXUS® prototypes. The detailed characterization studies later demonstrated that paclitaxel is not compatible with the pSIBS carrier and only the paclitaxel located on or near the coating surface (Fig. 19.7) is eventually released into the vascular tissue [35]. The majority of paclitaxel remained trapped permanently in the polymeric carrier matrix (Fig. 19.5). All subsequent

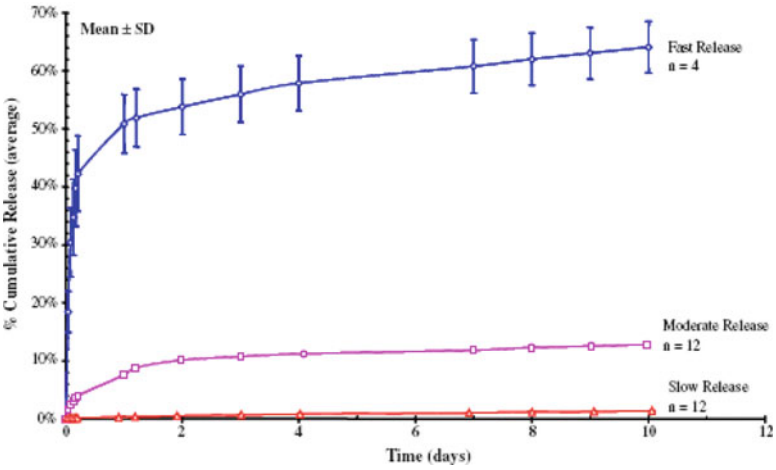

Fig. 19.6 In vivo release of the TAXUS® Stent with various drug levels [35]

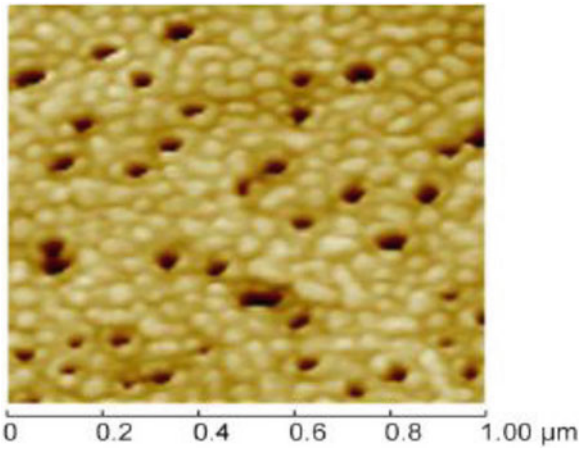

Fig. 19.7 Topography of a Paclitaxel-pSIBS surface after exposure to Tween and PBS [35]

developments of paclitaxel-based DES choose to use drug load of about 10% of TAXUS® and aim at 100% release over their life time [36, 37]. The key to TAXUS® control delivery system lies in the potency of paclitaxel and the impermeability of its carrier pSIBS after sufficient drug release.

19.4.3 Endeavor® Zotarolimus-Eluting Coronary Stent

Endeavor® was the third DES to be approved in the USA. According to its manufacturer, the main advantage of this system is that it incorporates a non-absorbable polymer carrier, phosphorylcholine polymer (PC Polymer, Fig. 19.8). PC polymer is

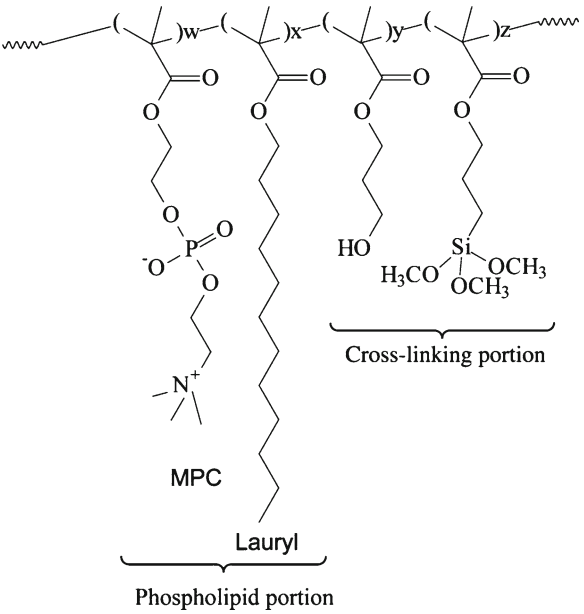

Fig. 19.8 Chemical structure of PC polymer

a synthetic version of phosphorylcholine, which is a constituent of the lipid bilayer of the cell membrane. PC polymer as used in Endeavor® is a biostable methacrylate-based copolymer that contains a phosphorylcholine monomer to mimic the charged phospholipid component of the plasma membrane of cells [47]. The copolymer contains a phospholipid segment (2-methyacryloyloxyethyl PC)(MPC), lauryl methacrylate (LMA), and a crosslinking portion of hydroxypropyl methacrylate (HPMA) and trimethoxysilylpropyl methacrylate (TSMA) [44]. The LMA portion was incorporated to adjust the overall hydrophobicity of the polymer, and the TSMA segment is utilized to effect crosslinking of the polymer network after the coating process to enhance the physical stability of the coating. The rational for use of PC polymer as a drug delivery matrix is to have a biomimetic material that can serve as a biocompatible drug delivery matrix. In practice, the charged moieties in the PC polymer make it relatively hydrophilic and require extensive crosslinking to slow drug efflux.

The anti-restenotic drug used in Endeavor® is zotarolimus. Zotarolimus is a semi-synthetics analog of sirolimus, with a tetrazole ring substituting for the hydroxyl group at the 42 position of rapamycin (Fig. 19.9). The tetrazole group is a hydrophobic group substituted at the C-42 position to reduce its water solubility and increase diffusivity of drug in the local tissue.

Despite the design changes in the both the polymer carrier and the hydrophobicity of the drug, and additional use of a topcoat to further slow down the drug elution,

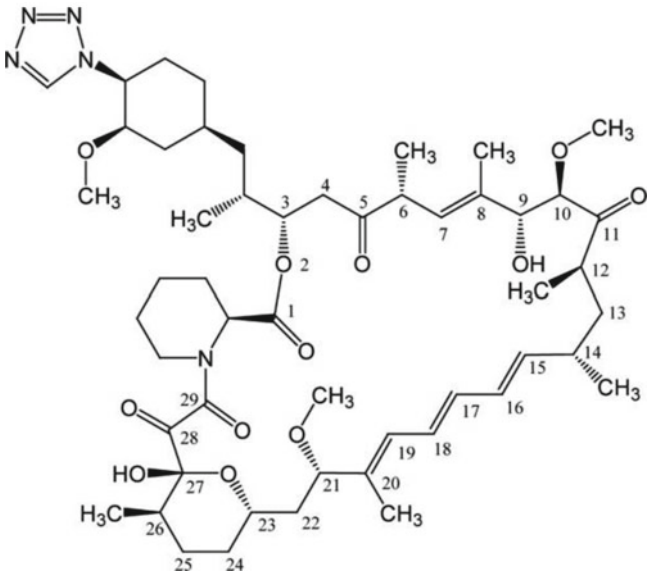

Fig. 19.9 Zotarolimus chemical structure

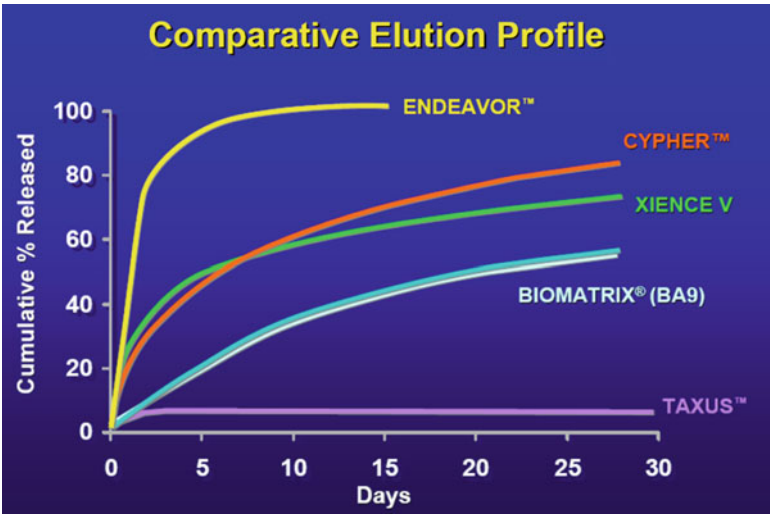

Fig. 19.10 Endeavor® in vivo elution graph [38]

the overall polymer carrier coating results in about 60–70% of zotarolimus released after only 1 day of implantation and almost complete drug elution in the first week. The Endeavor® stent has the fastest release rate of all approved DESs that elute sirolimus or its analogs (Fig. 19.10).

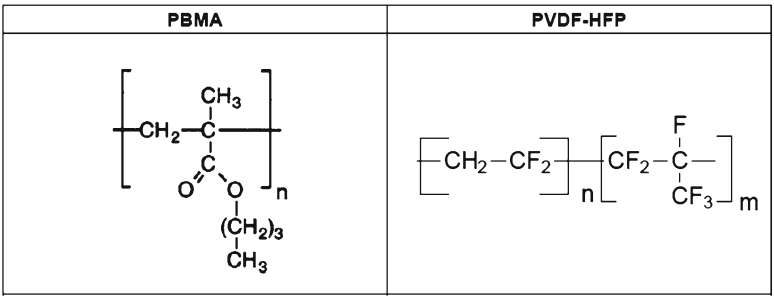

Fig. 19.11 PVDF–HFP chemical structure (from Xience™ Stent IFU)

This fast elution is primarily driven by the comparatively fast water uptake of the PC polymer matrix. As we know from the description of the restenosis pathological process, the local presence of an anti-restenotic drug is needed for at least 2 weeks and preferably 1 month to completely suppress the intimal hyperplasia. The clinical trial results later showed that the late loss (indicator of intimal growth) treated with Endeavor® stent is higher than with competitive products such as CYPHER® and XIENCE™, presumably because of the fast drug elution from Endeavor®. The newer generation from the Endeavor® family, Endeavor® Resolute, incorporates a newly designed polymer system (Biolinx) to reduce the initial burst of drug elution and adjust the duration of drug elution to those shown by CYPHER® and XIENCE™ V.

19.4.4 XIENCE™ V/Promus Everolimus-Eluting Coronary Stent

The latest DES to gain FDA approval in the USA is XIENCE™ V from Abbott Vascular Systems (a subsidiary of Abbott Lab, Inc.). XIENCE™ elutes everolimus (a semi-synthetic analog of sirolimus) from an elastomeric matrix provided by a copolymer of vinylidene fluoride and hexafluoropropylene [49] (PVDF-HFP, Fig. 19.11). The properties of this fluoro-copolymer depend on the ratio between VDF and HFP, and can be stiff and semi-crystalline (PVDF-HFP 80/20) to soft and amorphous (PVDF-HFP 60/40). The copolymer used in XIENCE™ DES is PVDF-HFP 85/15, a semi-crystalline random copolymer with a molecular weight in the range of 254–293 kDa and a melting point of about 160°C. The drug matrix copolymer is mixed with everolimus at 83%/17% ratio and applied onto a PBMA primed stent surface. The structure of the primer and matrix polymer is shown below.

The drug loading of XIENCE™ is about 70% of a comparably sized CYPHER® stent. The release kinetics of everolimus (Fig. 19.10) and the efficacy of the XIENCE™ stent are comparable to previously discussed competitive products.

19.5 Examples of Development-Stage DES and the Applications of Controlled Delivery Technologies

The tremendous clinical and commercial success of the current generation DES has led to intensified efforts in both academia and in the device industry for the design and development of the new DES. The experiences gained from the studies of various components of the current generation DES encourages researchers to produce more innovative stent designs, wider selections of drug carriers, and new ways to control drug elution kinetics and directions. The new DESs being developed promise to be a group with very diverse designs and integration approaches. Not only are people paying more attention to the drug choice (more analogs of sirolimus), loading dose, and release kinetics that are common themes of current DES research, but they are also finding ways to precisely control drug elution direction (luminal and mural directions), and to even do so without a metal stent support. The following leading examples embody one or more of these design trends and provide some contrast to existing products.

19.5.1 NEVO™ Sirolimus-Eluting Coronary Stent and RES TECHNOLOGY™

The NEVO™ sirolimus-eluting Coronary Stent utilizing RES TECHNOLOGY™ is one of many promising advances in DES technology and design. The NEVO™ Stent platform deviates from the traditional conformal coating and is distinctive in that it utilizes a laser-cut cobalt chromium alloy thin strut stent with uniformly spaced reservoirs embedded in the stent struts that serve as drug depots instead of a conformal coating over the entirety of the stent surface (Figs. 19.12 and 19.13). The number of reservoirs on the struts is proportional to the diameter and the length

Fig. 19.12 NEVO™ Stent with in-strut reservoirs as drug elution depots

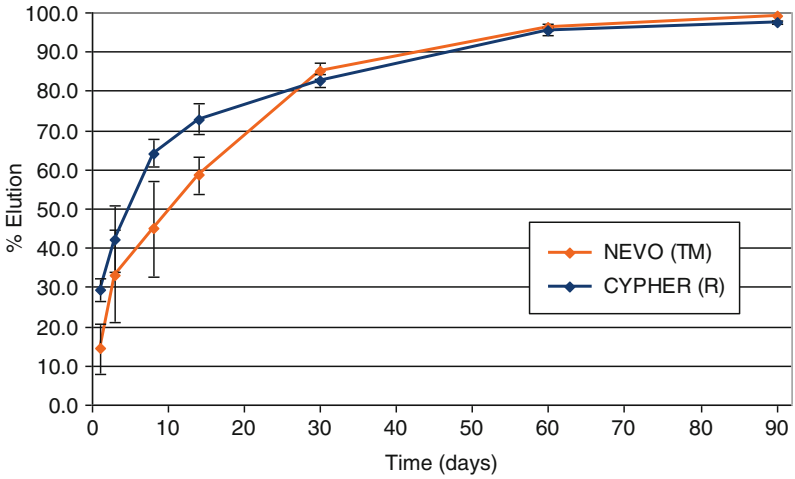

Fig. 19.13 Comparison of in vivo drug elution from the NEVO™ and CYPHER® Stents implanted in a porcine coronary artery

of stent. These tiny reservoirs are filled with a matrix of sirolimus and bioabsorbable PLGA polymer [39].

The carrier polymer, PLGA, has a long history of use in implantable devices such as surgical sutures, orthopedic pins, and screws. In addition, it has been used in other DES, such as Biomatrix and Nobori. A stent designed with reservoirs has a major advantage over traditional conformal coatings in that the drug carrier is mixed with drug and deposited into the reservoirs. These reservoir fillings shield the drug polymer matrix from the mechanical stresses commonly encountered during stent delivery and deployment. This shielding obviates the need for superior mechanical properties, such as high elasticity. This design feature greatly expands the range of absorbable polymers that can be used as the drug carriers rather than being constrained to using only the relatively elastic and mechanically strong polymers used in conformal coating designs. PLGA copolymer, with its relatively weak mechanical strength, affords the desired control of drug elution and provides shorter polymer resorption times to fine tune drug loading, elution kinetics, and matrix resorption. In addition, these reservoirs require comparatively less amount of bioabsorbable polymer to control the drug elution and lessen the burden of the arterial tissue to absorb the polymer degradation. The RES TECHNOLOGY™ design also affords the possibility of two-way drug delivery (Fig. 19.14) or multidrug delivery.

19.5.2 Endeavor® Resolute Zotarolimus-Eluting Coronary Stent (Commercially Available Outside the USA Only)

This DES is the latest iteration of the Endeavor® franchise from Medtronic, Inc. As discussed in previous sections, the chief drawback of the first-generation PC polymer-based

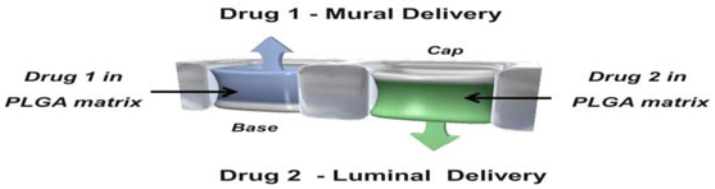

Fig. 19.14 Schematic of reservoir-based dual drug-eluting Stent

Endeavor® Stent is the rapid initial burst of drug elution that results in a complete drug release within the first week. The Resolute Biolink polymer coating was developed with this concern in mind and it consists of a polymer blend of three different polymers and copolymers, C10 copolymer (butyl methacrylate and vinyl acetate), C19 terpolymer (n-hexyl methacrylate, N-vinyl pyrrolidinone, and vinyl acetate); and poly(N-vinyl pyrrolidinone) (PVP) [45].

The three copolymers and polymers are used to serve different functions. As shown in Fig. 19.15, the C1 component mimics the polymer blend used in the CYPHER® Stent (PEVA/PBMA polymer blend) with different molar ratios between PEVA and PBMA. Both C19 and PVP contain a hydrophilic polymer to adjust the overall hydrophobicity and hydrophilicity balance (PVP is a recognized hydrophilic polymer that is widely used in pharmaceutical and medical device applications). When the three polymers are blended at the ratio of 30/70/10 (C10/C19/PVP) as used in the Resolute® system, the resulting zotarolimus elution is more controlled, and the in vivo drug release is much slower compared to the original PC polymer-based Endeavor® Stent.

The main design goal was accomplished successfully in a relatively short time frame. The end result is a redesigned Endeavor® stent with a drastically slowed drug elution profile (Fig. 19.16) that approaches the range of other leading DES that contain a sirolimus analog (CYPHER® and XIENCE™ V Stents). The potential downsides of the newer version with improved drug elution control include a more complicated coating system that requires more attention to the manufacture of these polymers and in the quality assessments used in the manufacturing processes. The long-term biocompatibility of these polymers will also need to carefully assessed.

19.5.3 JacTax Paclitaxel-Eluting Coronary Stent

While no longer in development, JacTax from Boston Scientific is a next-generation DES that incorporates paclitaxel as the drug [40]. In addition to the change in the stent and balloon delivery system that aims at improving the deliverability of the DES system, the drug carrier is changed to a bioabsorbable polylactide-*co*-glycolide coating that has been used in other commercial DES, such as Biomatrix from Biosensor. Even though the polymer/drug matrices are deposited on the abluminal surface to target the delivery to the vessel wall alone, similar to the arrangement of Biomatrix, the drug/polymer matrices (10% loading) are deposited as individual dots

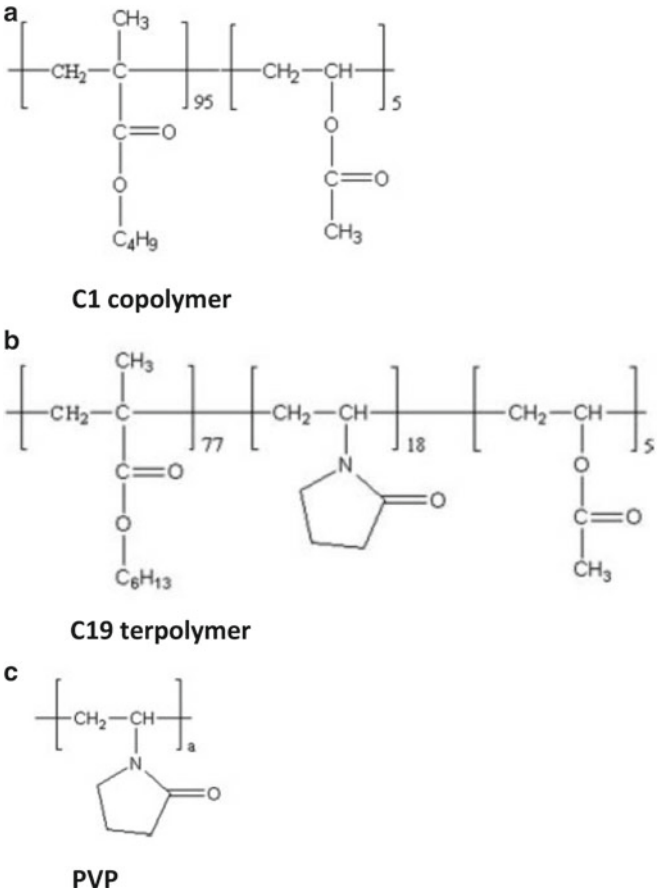

Fig. 19.15 Endeavor® resolute biolinx polymer system

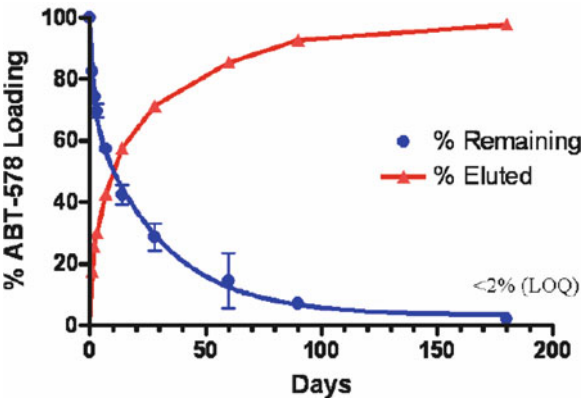

Fig. 19.16 The resolute® Stent elution curve

Fig. 19.17 Abluminal coating of Paclitaxel/D,L-PLA dots on JacTax™ DES [40]

on the outer surface with a thickness of about 10 m (Fig. 19.17). In addition, the drug load is also reduced to about 10% of the original load and targeted for 100% release. This way, the concern of having 90% of drug payload in the durable polymer coating, as is the case with TAXUS®, is removed. Also, the drug carrier, PLGA, is proportionally reduced to lessen the burden of carrier absorption by the local tissue. The advantages of JacTax in using small amounts of poly(dl-lactide) (DLPLA) and paclitaxel are also a potential drawback of the system, for it may be complex to upscale when a larger amount of drug and polymer is needed on the DES.

19.5.4 Totally Bioabsorbable DES

The concept of a totally resorbable drug-eluting stent has been around for a long time. The design is attractive as clinical studies have consistently shown that the restenosis incidence rate is minimal several months after stent implantation and, at that point, the stent scaffolding is no longer needed. However, it is enormously challenging to find a bioabsorbable material that has adequate initial radial strength to keep the artery patent, a good control over drug elution, and satisfactory biocompatibility and reasonable absorption time frame.

The ABSORB Everolimus-eluting Coronary Stent from Abbott (originally from the BVS division of Guidant) is a good example of such a pursuit [41] (Fig. 19.18). A high-molecular-weight poly-L-lactide (PLLA) polymer is used to fabricate the stent scaffold. To meet the required radial strength (normally >10 psi for coronary stent), these laser-cut stents start with a very high-molecular-weight polymer, which is thermally oriented and conditioned to improve strength and reduce elastic recoil. A separate layer consisting of poly(D,L-lactic acid) (PDLA) polymer with lower Mw and everolimus was coated onto the pure PLLA stent scaffold to complete the drug integration.

Despite the appeal of a totally absorbable DES, ABSORB has undergone several iterations of design change to address the practical issues. Being a thermoplastic polymer with a high Mw, the PLLA scaffold will gradually lose its shape memory

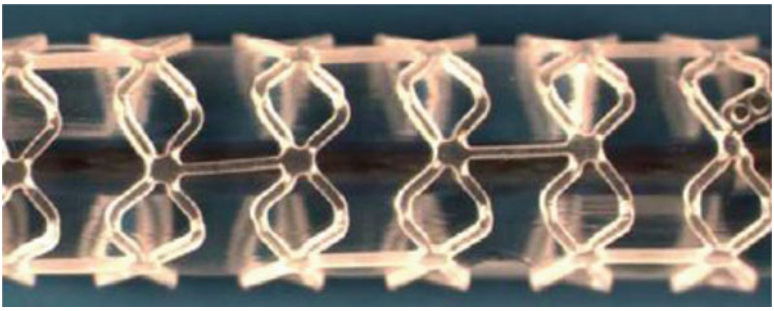

Fig. 19.18 Close-up view of ABSORB stent design from Abbott lab [41]

during storage and, as a result, needs to be refrigerated. After the drug elution and surface PDLA absorption, the core PLLA stent scaffold may gradually lose radical strength and will require a long time (>2 years) for complete stent resorption. Clinical trials with long follow-up time are required to address the long-term biocompatibility aspects of the technology.

19.6 Conclusions

Drug-eluting stents have revolutionized the practices of interventional cardiology and dramatically improved the effectiveness of stent angioplasty and quality of life of the patients. DES has increasingly been used to treat complex coronary lesions in place of more costly cardiovascular bypass surgery. As a prime example of a drug–device combination product, the overall success of a DES is predicated on the collaborative efforts of stent design engineers, material scientists, pharmacologists, and control release technologists. In the coming years, this field will continue to attract active research and development efforts from both academia and medical device industry. DESs currently in clinical trials or late stage development employ a wide array of innovative designs, tailor-made drug carriers and matrices, and precise control over drug elution rate and direction. These DES will continue to provide more treatment options for cardiovascular disease management and improve the quality of life for the patients.

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Chapter 20
Delivery of Peptides and Proteins via Long Acting Injections and Implants

Cynthia L. Stevenson, Christopher A. Rhodes, and Steven J. Prestrelski

Abstract Biomolecules are rarely orally bioavailable and parenteral injections are the industry standard. Development of long acting delivery systems will continue to help biopharmaceuticals reach their full therapeutic potential. Long acting injections have manifested into a range of products designed to target optimal therapeutic dosing requirements (prefilled syringes, long acting injections, sustained release depots, controlled release implants, targeted delivery, and larger payloads). Sustained release implants usually provide a longer term delivery duration than long acting injections and can be categorized into bioerodible systems (rods and cylinders) and implantable devices. This chapter reviews biomolecules currently marketed or in clinical trials, utilizing long acting parenteral technology or sustained/controlled release implants.

20.1 Introduction

Over the past few decades, biotherapeutics (peptides, proteins, oligonucleotides, and antibodies) have emerged as significant tools in the healthcare toolbox. The advent of technologies for large-scale peptide synthesis, recombinant protein production and, most recently, humanized antibody production has lead to significant numbers of these molecules being developed as medicines. Biotherapeutics now represent ~8% of all drugs on the market and account for approximately 10% of the total drug expenditures. Their use is growing at over 20% per year. Further, biotechnology-derived drug

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candidates account for around ~32% of all pipeline research programs. In addition, biological drugs are administered in life-saving or end-stage applications, 74% more than chemically derived pharmaceuticals. The biopharmaceutical market was estimated to be worth over $79 billion in 2007 with antibodies representing around $27 billion sales and protein products generating almost $52 billion. The market is also growing at an annual rate of around 15%, significantly higher than the overall pharmaceutical market (c. 6–7% per annum). By 2020, the market is forecast to be valued over $200 billion, driven by a shift in usage from conventional drugs to biotherapeutics, the relatively high cost of biotherapeutics, the launch of biosimilars, and a new generation of biotherapeutics [1].

Biomolecules are rarely orally bioavailable, and parenteral injections are the industry standard. Additionally, biomolecules usually have short plasma half-lives and require frequent dosing to maintain blood levels in the therapeutic window. Therefore, the driver for developing long acting delivery systems is a less frequent dosing regimen, with increased patient comfort and compliance. These long acting delivery systems will help biopharmaceuticals reach their full therapeutic potential.

Furthermore, sustained release drug delivery systems have been developed to improve the therapeutic response by providing more consistent blood levels than immediate release parenterals. A more controlled therapeutic blood level can result in less adverse reactions, because a high C_{max} after injection is not required to keep the dose in the therapeutic window for the required time period. Additionally, targeted delivery systems (liposomes) offer the possibility that the drug can be delivered to the site, limiting systemic exposure.

As these drug delivery platforms become established, more many companies are exploring prefilled syringes instead of glass vials as the primary container closure. These new long acting injections have become more effective and efficient at delivering biomolecules and have manifested into a wide range of delivery systems to target optimal therapeutic dosing requirements. Further, given the ability to tailor drug delivery to the requirements of the biomolecules, platforms have become more diverse (prefilled syringes, long acting injections, sustained release depots, controlled release implants, targeted delivery, larger payloads, and improved solid-state stability formulations) than ever before. The innovation in new delivery platforms has also resulted in new methods of manufacture and novel carrier materials.

This book chapter reviews the field of pharmaceutically relevant biomolecules currently marketed or in clinical trials, utilizing long acting parenteral technology or sustained/controlled release implants.

20.2 Parenteral Injections

Sustained release strategies for parenteral injections can be grouped into three categories:

1. “Analog design” (generation of drug substance analogs with improved pharmacokinetic characteristics)

2. "Molecular engineering" (the addition of high-molecular-weight "tails" to the drug substance to modify release characteristics and ADME profile)
3. "Formulation strategies" (utilization of excipients to decrease the solubility and/or dissolution rate of the drug substance)

These three approaches are discussed in detail for peptides, proteins, and antibodies, since the approaches to achieving a long acting formulation change as the molecule increases in size and complexity.

20.2.1 Peptides

20.2.1.1 Analog Design

Analog design can be as simple as the substitution of one or more amino acids to improve the proteolytic stability, and thus activity, of the peptide. This strategy is usually used in a site-specific manner, where the substitution does not disrupt the secondary structure or activity of the molecule. For example, the substitution of D-Leu^6 for Gly^6 increases the half-life from 4 min for luteinizing hormone releasing hormone (LHRH) to 3 h (Leuprorelin) [2]. Other examples of successful LHRH analogs for the treatment of prostate cancer and/or endometriosis include Histrelin, Goserelin, and Buserelin (Table 20.1).

A second example is the optimization of somatostatin analogs for the treatment of acromegaly. The half-life of somatostatin ($t_{1/2}$ = 1–3 min) was increased substantially in the generation of the analogs octreotide ($t_{1/2}$ = 1.7 h) and lanreotide ($t_{1/2}$ = 2 h) (Table 20.1) [2]. Both the LHRH and somatostatin analogs have been coupled with additional sustained release formulation strategies, resulting in marketed products discussed below.

A third example is the optimization of the structure/activity relationship for vasopressin analogs, resulting in increased circulating half-lives and the successful treatment of enuresis. These analogs most commonly found in marketed products are Arg^8 Vasopressin (Desmopressin), Lys^8 Vasopressin (Lypressin), and Terlipressin (Table 20.1) [3].

A fourth example is glucagon-like peptide-1 (GLP-1). The native sequence of GLP-1 (7–36) has a short plasma half-life of about 2–5 min because it is cleaved by dipeptidyl peptidase IV (DPP IV), resulting in inactivation [4–6]. DPP IV cleaves the N-terminal dipeptide His-Ala leaving GLP-1 (9–36) [7]. Several companies have created analogs of GLP-1 resistant to DPP IV in order to increase the circulating half-life.

Exenatide (exendin-4; Byetta®) has approximately 50% sequence homology with GLP-1. Exenatide contains a Gly^2 instead of Ala^2 found in GLP-1, is DPP IV resistant, and increases the half-life to ~4 h (Table 20.1) [8].

Beaufour Ipsen and Roche are currently finishing Phase 3 trials with Taspoglutide, an α-aminoisobutyric acid-derivatized GLP-1 analog, and Aib^{8,35} GLP-1(7,37) NH_2 that is delivered once weekly [9]. The once-weekly duration is further helped by a zinc-based formulation, discussed below under Formulation Strategies.

Table 20.1 Peptide sequences

Peptide	Sequence
Luteinizing hormone releasing hormones	
LHRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2
Leuprorelin	pGlu-His-Trp-Ser-Tyr-d-*Leu*-Leu-Arg-Pro-NH2
Histrelin	pGlu-His-Trp-Ser-Tyr-*N-benzyl*-d-*His*-Leu-Arg-Pro-NH2
Goserelin	pGlu-His-Trp-Ser-Tyr-d-*Ser(But)*-Leu-Arg-Pro-Azgly-NH2
Buserelin	pGlu-His-Trp-Ser-Tyr-d-*Ser(tBu)*-Leu-Arg-Pro-*NHEt*
Somatostatins	
Somatostatin	Ala-Gly-Cys*-Lys-Asn-Phe-Phe-Trp-lys-Thr-Phe-Thr-Ser-Cys*-OH, *disulfide bond (3–14)
Octreotide	D-Phe-Cys*-Phe-d-*Trp*-Lys-Thr-Cys*-Thr-ol, *disulfide bond (2–7)
Lanreotide	[cyclo S-S]-3-*(2-naphthyl)*-d-*Ala*-Cys-Tyr-d-*Trp*-Lys-Val-Cys-Thr-NH2
Vasopressins	
Vasopressin	Cys*-Tyr-Phe-Gln-Asn-Cys*-Pro-*Arg*-Gly-NH2, *disulfide bond (1–6)
Desmopressin	1-(3-mercaptopropionic acid)*-Tyr-Phe-Gln-Asn-Cys*-Pro-d-*Arg*-Gly-NH2, *disulfide bond (1–6)
Lypressin	Cys*-Tyr-Phe-Gln-Asn-Cys*-Pro-*Lys*-Gly-NH2, *disulfide bond (1–6)
Terlipressin	Gly-Gly-Gly-Cys*-Tyr-Phe-Gln-Asn-Cys*-Pro-Lys-Gly-NH2, *disulfide bond (4–9)
Glucagon-like peptides	
GLP-1 (7–37)	His-*Ala-Glu*-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-NH2
Liraglutide	His-*Ala-Glu*-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys*-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-NH2, *Glu-C16 fatty acid
ZP10	His-*Gly-Glu*-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Ser-Lys-Lys-Lys-Lys-Lys-Lys-NH2
Taspoglutide	His-*Aib-Glu*-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Aib-Arg-Gly-NH2
CJC-1131	His-d-*Ala-Glu*-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly*-NH2, *N^{ε}-(2-[2-[2-(3-maleimidopropylamido) ethoxy] ethoxy] acetyl) Lys, bioconjugated to Cys^{34} HSA
Exenatide	His-*Gly-Glu*-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala-Pro-Pro-Pro-Ser-NH2
Teduglutide	His-Gly-Asp-Gly-Ser-Phe-Ser-Asp-Glu-Met-Asn-Thr-Ile-Leu-Asp-Asn-Leu-Ala-Ala-Arg-Asp-Phe-Ile-Asn-Trp-Leu-Ile-Gln-Thr-Lys-Ile-Thr-Asp-OH
Insulins	
Insulin A chain	Gly-Ile-Val-Glu-Gln-Cys*-Cys*-Thr-Ser-Ile-Cys*-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys*-Asn-OH
Glargine A chain	Gly-Ile-Val-Glu-Gln-Cys*-Cys*-Thr-Ser-Ile-Cys*-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys*-*Gly*-OH
Insulin B chain	Phe-Val-Asn-Gln-His-Leu-Cys*-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys*-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OH

(continued)

Table 20.1 (continued)

Peptide	Sequence
Glargine B chain	Phe-Val-Asn-Gln-His-Leu-Cys*-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys*-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-*Arg*-*Arg*-OH
LisPro B chain	Phe-Val-Asn-Gln-His-Leu-Cys*-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys*-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-*Lys*-*Pro*-Thr-OH
Aspart B chain	Phe-Val-Asn-Gln-His-Leu-Cys*-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys*-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-*Asp*-Lys-Thr-OH
Glulisine B chain	Phe-Val-*Lys*-Gln-His-Leu-Cys*-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys*-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-*Glu*-Thr-OH
Determir B chain	Phe-Val-Asn-Gln-His-Leu-Cys*-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys*-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-*Lys*$^{\varepsilon}$-OH, $^{\varepsilon}$C-14 fatty acid
Oncology	
Stimuvax	Ser-Thr-Ala-Pro-Pro-Ala-His-Gly-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Leu(palmitoyl)-Gly

A final example is glucagon-like peptide-2 (GLP-2). Native GLP-2 is also susceptible to DPP-IV cleavage, similar to GLP-1. GLP-2 acts in an opposite fashion to GLP-1, enhancing intestinal growth and metabolism with nutrient intake. NPS Pharmaceuticals has created an analog [Gly^2] GLP-2 (Teduglutide) (Gattex™) that is resistant to protease degradation and has completed Phase 3 trials for Short Bowel Syndrome using subcutaneous delivery [10]. Therefore, proteolytic degradation can be mitigated, increasing circulating half-lives by site-specific substitution of amino acids at specific proteolytic cleavage sites.

20.2.1.2 Molecular Engineering

Site-specific mutagenesis usually increases the half-life from minutes to hours; however, other strategies are used, often in conjunction, to further increase the therapeutic duration. These additional alterations to the structure of the biologically active molecule can be described as molecular engineering. Many companies are exploring attaching N- or C-terminal polypeptide tails, fatty acid chains, human serum albumin (HSA), polyethylene glycol (PEG), and fusion proteins (Fc fusion proteins, recombinantly derived PEGylated peptides, and other chimeric entities).

Novo Nordisk recently gained approval for Victoza®, a GLP-1 analog, Liraglutide. Liraglutide contains several sequence alterations: Arg^{28} replaces Lys^{28}, an additional Gly at the C-terminus, and Lys^{20} contains a C16 fatty acid chain (Arg^{28}, Lys^{20}-*N*$^{\varepsilon}$-(γ-Glu (*N*-α-hexadecanoyl)) GLP-1) [11]. The fatty acid portion binds to serum albumin, which extends its plasma half-life to allow a once-daily injection. Furthermore, Novo Nordisk has a once-weekly, extended release product based on a different molecule than Liraglutide (Semaglutide) in Phase 2 trials [12].

Conjuchem, Inc., are using their PC-DAC technology to develop a subcutaneous injection with a novel GLP-1 analog. CJC-1131 has a D-Ala[8] and a C-terminal reactive chemical linker, N^{ε}-(2-[2-[2-(3-maleimidopropylamido)ethoxy]ethoxy]acetyl) Lys, bioconjugated to Cys[34] of HSA [12]. This covalent and irreversible bond to HSA extends the half-life 30-fold relative to native exendin-4 ($t_{1/2}$ 10–12 days), but with a reduction in bioactivity [13]. Conjuchem has also applied their conjugation technology to Exendin (CJC-1134) and completed Phase 2 trials.

Zealand Pharma and Sanofi-Aventis have entered Phase 3 trials with a GLP-1 analog (ZP10), which is modified by attaching six Lys residues to the C-terminus [14]. PolyTherics has also partnered with Zealand Pharma to develop peptide-based therapeutic candidates, which are amenable to PEGylation [15].

GlaxoSmithKline is testing Abliglutide in Phase 3 trials. Abliglutide (Syncria®) was developed by Human Genome Sciences, Inc., and uses a proprietary fusion technology which fuses the gene for HSA to the gene that expresses GLP-1 and produces a protein containing two tandem linked copies of GLP-1 and one copy of HSA [16].

Eli Lilly has developed a novel GLP-1 Fc fusion protein (LY2189265), consisting of a dipeptidyl peptidase-IV (DDP-IV) protected GLP-1 analog linked to a fragment of immunoglobulin G4, which is believed to increase the duration of its pharmacological effect. The molecule is dosed once weekly and is currently in Phase 2 trials [17]. The GLP-1 conjugate made from the Fc region of antibodies increases the size of the active agent and therefore increases the circulating half-life.

Amunix creates a genetic fusion of an unstructured polypeptide to a therapeutic peptide (XTEN technology) to confer a significantly longer plasma half-life. Depending on the molecular size of the construct, dosing intervals of up to once per month are anticipated [18]. Amunix has licensed an exenatide-XTEN program to start up Versartis, who are currently in Phase 1 trials [19].

PhaseBio is developing the ELPylation technology, which is a peptide system based on the elastin-like protein, which is covalently attached to the therapeutic peptide [20]. The elastin-like proteins (ELP) are thermally responsive biopolymers composed of a Val-Pro-Gly-Xaa-Gly pentapeptide repeats and can be recombinantly produced along with the peptide. ELPs are soluble in an aqueous solution at temperatures below their transition temperature, but become insoluble and aggregate at temperatures above their transition temperature and are therefore intended to gel upon injection, increasing the length of the absorption phase and creating a longer plasma half-life.

Affymax has generated a peptidomimetic of erythropoietin (EPO) called Hematide®. Hematide® is a PEGylated peptide (Peginesatide) with no sequence homology to EPO and is anticipated to be dosed monthly. Affymax and Takeda have completed Phase 3 trials for the treatment of anemia associated with chronic renal failure and are in Phase 2 clinical trials for the treatment of anemia in cancer patients [21, 22].

20.2.1.3 Formulation Strategies

Numerous formulation strategies have been tested for their ability to create sustained release formulations of peptides in a biocompatible format. Clearly, the use of poly-D,L-lactic-glycolic acid (PLGA) has been the most successful in creating

commercial drugs. PLGA microspheres are discussed here, as a formulation strategy, while PLGA bioerodible implants are discussed below. The ratio of lactic acid and glycolic acid controls the biodegradation rate, where 50:50 ratios degrade in 50–60 days [23]. In general, increasing the lactic acid increases the half-life. Once injected, the ester bond, linking the lactic and glycolic acids, is hydrolyzed by esterases, releasing the peptide.

As previously outlined, LHRH analogs were successful at increasing the circulating half-life from minutes to hours; however, longer acting injections, lasting months, were required. Several subcutaneous or intramuscular PLGA-based sustained release formulations of LHRH agonists are currently marketed for prostate cancer and endometriosis. The formulations last 1–6 months and include Lupron Depot® 3.75 mg, 7.5 mg, 11.25 mg, and 30 mg (Takeda/Abbott), Eligard® 7.5 mg, 22.5 mg, 30 mg, and 45 mg (Sanofi-Aventis), Trelstar® Depot, Trelstar® LA, Prostap® (Wyeth), Suprefact® (Aventis), and Suprecur® (Aventis) [2].

The Lupron Depot® extended release leuprolide at lower doses (3.75 mg and 7.5 mg) is a mixture of PLGA, while the higher doses (11.25 mg and 30 mg) are polylactic acid. The Eligard® product line of long acting leuprolide acetate formulations is based on the AtriGel® technology (Atrix). Atrigel is a biodegradable formulation that forms a depot gel in situ. The formulation is supplied as a ready-for-use liquid/suspension or powder, which can be reconstituted prior to use. The formulation combines PLGA and poly(D,L-lactide) copolymers in primarily water-miscible solvents (*N*-methyl-2-pyrrolidone), with hydrophobic solvents (triacetin, ethyl acetate, and benzyl benzoate) [24]; also see Chap. 9).

Similarly, the Debio PLGA-2® PLGA microsphere technology has been used to develop long acting delivery systems for several LHRH agonists. These include Decapeptyl®, a formulation of triptorelin pamoate to treat cancer and endometriosis, and Trelstar® Depot (1 month) and Trelstar® LA (3 months for treatment of cancer) [2].

A second class of peptides successfully incorporated into PLGA-based formulations are the somatostatin analogs (octreotide and lanreotide). Sandostatin LAR Depot® (Novartis) is a long acting octreoetide microsphere suspension given as a monthly intragluteal injection [2, 25]. Somatuline® LA (Tercica) is a long acting formulation of lanreotide that employs biodegradable microparticles consisting of PLGA (75:25 lactic:glycolic acid) for injectable, biodegradable, sustained release. Tercica also developed Somatuline® Depot, which provides a 1 month depot as a supersaturated suspension of lanreotide. This depot formulation contains no PLGA, but simply precipitates the active agent at the site of injection and the release becomes limited by the solubility and dissolution rate of the lanreotide.

Finally, Ambrilia/Mallinckrodt is in Phase 3 trials with a novel prolonged release formulation of octreotide using its C2L PLGA formulation technology, dosed every 6 weeks [26].

A third class of peptides benefiting from PLGA formulation are GLP-1 agonists. Amylin has developed a sustained release PLGA microsphere-based depot formulation (Exenatide Once Weekly; Bydureon®) for diabetes that increases the dosing interval from twice daily to once weekly. Phase 3 studies for Bydureon® demonstrate that it has superior efficacy and tolerability relative to Byetta® (BID version) [27].

Taspoglutide (Beaufor Ipsen and Roche) created a DPP IV resistant analog of GLP-1 and formulated it with zinc. This strategy of utilizing a less soluble zinc salt to decrease the solubility and dissolution rate has also been used for insulin and growth hormone [28, 29].

Several companies provide sustained release depot formulation platforms that are not PLGA-based and still amenable to peptide drugs. Camurus' FluidCrystal® injectable depot delivers drug over extended periods of time. The depot is a liquid solution that transforms into a controlled release liquid crystal gel matrix in situ on contact with aqueous fluid at the site of injection [30]. Similarly, Durect offers the SABER® platform, a high-viscosity system, composed of sucrose acetate isobutyrate (SAIB), which provides controlled release of the drug for up to 3 months [31].

Liposomal delivery of peptides has also gained maturity. Pacira offers DepoFoam® which consists of multivesicular liposome particles with numerous internal aqueous chambers contain the encapsulated drug [32, 33]. Merck KGaA and Oncothyreon have been granted Fast Track Status for Stimuvax® and are currently in Phase 3 trials. Stimuvax® (L-BLP 25) is palmitoylated peptide vaccine, formulated as a liposome. The liposomal formulation provides targeted delivery and enhances recognition of the cancer antigen and induces T-cell response to cancer cells expressing MUC1. Stimuvax is indicated for non-small cell lung cancer, where the median survival in Stage IIIB cancer patients was 30.6 months compared to control of 13.3 months in Phase 2b trials [34].

In summary, successful peptide formulation strategies have included PLGA-based systems, depot formulations that change in viscosity upon injection, less soluble zinc salts, and targeted liposomes.

20.2.2 Proteins

20.2.2.1 Analog Design

Extended-action insulin analogs are designed to provide a basal level of insulin. Glargine insulin (Lantus®), marketed by Sanofi Aventis, differs from human insulin by a substitution of Asn for Gly at A21 and the addition of two Arg residues to the C-terminus of the B-chain. These modifications increase the isoelectric point to a more neutral pH, reducing the solubility under physiologic conditions and causing glargine to precipitate at the injection site, thus slowing absorption [35]. Insulin glargine solution is formulated and injected at pH 4.0. Glargine is an extended-action analog that lasts 20–24 h [36, 37].

Rapid-acting insulin analogs are used to control the normal physiological responses to meals when a bolus dose of insulin is required. Lispro (Humalog®), made by Eli Lilly, differs from insulin by switching the Lys at position B28 and the Pro at position B29. The modification of the B-chain C-terminus decreases the nonpolar contacts and β-sheet interactions between insulin monomers, resulting

in less self-association [35]. In the solution formulation, lispro exists as an inherently destabilized hexamer, but when injected, it spontaneously dissociates into a monomeric form.

Aspart insulin (Novolog®), made by Novo Nordisk, acts similarly to lispro. The rapid-acting analog aspart differs from human insulin by a single substitution of Asp for Pro at position B28. This substitution results in charge repulsion between monomers and steric hindrance due to a local conformation change at the carboxyl terminus of the B chain, reducing the formation of both hexamers and dimers and thereby increasing the rate of absorption of monomeric aspart insulin [35, 38]. Lispro and aspart insulin act within 5–15 min of injection, respectively, compared to 30–60 min for regular insulin [39].

Glulisine insulin (Apidra®), made by Sanofi Aventis, is a rapid-acting insulin. It differs from human insulin in that Lys replaces Asn at position B3, and Glu replaces Lys at position B29, destabilizing dimerization and providing a readily available insulin monomer. After injection, Apidra acts more rapidly and lasts longer than regular insulin, so it can be given 15 min before a meal or immediately after a meal.

An example of a larger protein is IL-2. Proleukin®, marketed by Chiron/Novartis, is a des-alanyl-1 analog of human IL-2, where the Cys at position 125 is replaced with Ser [40].

20.2.2.2 Molecular Engineering

Molecular engineering strategies for proteins are similar to peptides, but do include a slightly different set of approaches including fatty acid chains, PEGylation, hyperglycosylation, polysialation, and fusion proteins.

Detemir insulin (Levemir®), made by Novo Nordisk, utilizes conjugation to create a product which maintains basal insulin levels. Detemir differs from insulin by the elimination of Thr at position B30, and acylation of the ε-amino group of Lys B29 with a C14 fatty acid chain ($N^{\varepsilon B29}$-myristoyl des (B30) human insulin). These modifications caused detemir to bind albumin within subcutaneous tissue resulting in slower absorption into plasma, and a subsequently increased plasma half-life [35]. Detemir can be injected once daily and does not exhibit a pronounced C_{max} peak.

PEGylated proteins provide enhanced stability (increased steric hindrance and altered electrostatic binding) and pharmacokinetics (increased size, molecular weight, and hydrophilicity), while still maintaining an inert, amphiphilic nature [41]. Basically, PEG moieties are repeating units of ethylene glycol and can be linear or branched in nature. Branched PEG moieties provide increased size without the need for more attachment sites. The attachment of PEG to the therapeutic protein in usually made at α- or ε-amino groups of the N-terminus, Lys, Cys, His, Arg, Asp, Glu, Ser, Thr, Tyr, or the C-terminus [42]. Attachment of PEG can result in a loss of activity and binding affinity; however, the overall improvement in product performance still warrants the alteration. Nektar's PEGylation technology has provided improvements to seven of the PEGylated proteins currently on the market

Table 20.2 PEGylated products

Product	Drug	Parent drug	Company	Reference
Peptides				
Phase 3; Syncria	PEG-GLP-1	GLP-1	Human Genome Sciences	[16]
Phase 3; Hematide	PEG-Hematide	Hematide	Affymax/Takeda	[21]
Proteins				
Adagen	Pegadamase	Adenosine deaminase	Enzon	[43]
Mircera	Epoetin beta-methoxy polyethylene glycol	EPO	Roche	[55]
Neulasta	Pegfilgrastim	GCSF	Amgen	[44, 45]
Neupeg	PEG-GCSF	GCSF	Intas	[46]
Oncasper	Pegaspargase	Asparaginase	Enzon	[55]
Pegasys	Peginterferon alfa-2a	INF-α2a	Roche, Chugai	[55]
PEG-Intron	Peginterferon alfa-2b	INF-α2b	Schering-Plough, Enzon	[55]
Somavert	Pegvisomant	Growth Hormone antagonist	Pfizer	[47]
Phase 3; PEG-Avonex	PEG-INF-β1a	INF-β1a	Biogen Idec	[48]
Phase 3	PEG-INF-α	INF-α	SunBio	[49]
Phase 3	PEG-EPO	EPO	SunBio	[50]
Phase 3; NN-7008	PEG-Factor VIII	Factor VIII	Neose/Novo Nordisk	[51]
Phase 3; Hemospan	PEG-hemoglobin	Hemoglobin	Sangaart	[52]
Antibodies				
Cimzia	Certolizumab Pegol	Anti-TNF Fab	UCB/Celltech	[55]
Phase 2: CDP791		PEGylated di-Fab fragment inhibiting VEGFR-2	UCB	[53]
Other				
Macugen	Pegaptanib	Anti-VEGF aptamer	Gilead, OSI	[54]

(Table 20.2) [55]. Enzon has been successful with other approved PEGylated products (Adagen® and Oncapser®) (Table 20.2) (also see Chap. 15).

Similar to PEGylation, hyperglycosylation can extend the biological half-life, improve stability, and reduce immunogenicity. Hyperglycosylation may be achieved through N-linked (Asn-Xxx-Ser, where Xxx is not Pro) or O-linked (generally Ser or Thr) oligosaccharides [56, 57]. Hyperglycosylation of Aranesp® (darbepoetin alfa) (Amgen) increased the half-life threefold and extended the dosing from three times per week to weekly [2].

Finally, Lipoxen conjugates naturally occurring polysialic acid (PSA) to proteins, enabling longer circulating half-lives, similar to PEGylation or glycosylation. PSA, an α-(2→8) linked linear homopolymer of *N*-acetyl neuraminic acid, is a sugar found on the cell surface [58]. Polysialation is generally done with two average molecular weights (~22 and ~39 kDa), depending on the requirements of the peptide or protein of interest. The PolyXen technology has been applied to EPO (Phase 1), interferon-α (preclinical), and granulocyte-colony-stimulating factor (GCSF) (preclinical) [59].

Fusion proteins are also designed to slow clearance by increasing the size of the molecule. This is most commonly performed with Fc region of antibodies or HSA [2]. Etanercept (Enbrel®) is a dimeric fusion protein consisting of human soluble tumor necrosis factor (TNF) and the Fc region of IgG1. Human Genome Sciences has fused HSA to INF-α2b (Albainterferon alfa-2b; Zalbin™) and investigated Zalbin™ clinically for the treatment of hepatitis C.

20.2.2.3 Formulation Strategies

Intermediate-acting insulin formulations are subcutaneously administered as suspensions instead of solutions. Humulin® N and Novolin® N provide intermediate-acting insulin by formulating hexameric insulin with protamine (an arginine-rich polypeptide). A precipitate of orthorhombic crystals (3 insulin dimers:2 zinc ions:0.9 protamine) is formed, where the protamine is loosely bound to the crystal [60]. These formulations are referred to as neutral protamine Hagedorn (NPH) or isophane insulin [38, 60]. These insulin formulations have a longer duration of action than regular insulin and lower rates of absorption due to decreased solubility at physiologic pH due to the basic nature of protamine. For example, Novolin® N is effective 1.5 h after injection, lasts 4–12 h, and may last as long as 24 h.

Humulin® L is another intermediate-acting insulin, formulated as a combination of both amorphous and crystalline suspension of human insulin with zinc, also termed a lente formulation [38, 61]. The lente family contains semilente, lente, and ultralente insulins, which are made by complexing insulin with varying ratios of zinc chloride.

Humulin® U Ultralente is a long acting insulin, where crystalline insulin is formulated with an excess of zinc ions, above the two zinc ions per hexamer. When extra zinc ions are added, the insulin changes from an orthorhombic crystal to a rhombohedral crystal form, resulting in a prolonged delivery profile [60]. In addition, the excess zinc can provide a "salting out" effect, decreasing the insulin zinc hexamer solubility and resulting in a slower dissolution rate [29].

Therefore, by changing the dissolution characteristics of insulin (suspension formulation and crystal form), regular insulin peaks at 2–3 h and has a duration of action of 4–8 h; semilente peaks at 5–7 h and lasts 12–16 h; lente and NPH peak at 8–12 h and last 24 h; and protamine/zinc and ultralente peak at 16–18 h and last more than 24 h [61].

While PLGA microsphere systems have proven successful in creating long acting peptide systems, their application to larger and more complex proteins has been

more limited. The Nutropin Depot® product based on the Alkermes ProLease® Technology has proven a technical success [62], but ultimately marketing was discontinued. The ProLease® technology consists of injectable biodegradable PLGA that encapsulates drugs in the absence of water at very low temperatures. Low temperatures are employed to protect sensitive proteins. Attempts have been made to create long acting formulations of EPO and follicle-stimulating hormone using ProLease, but these have not progressed past early clinical trials.

Flamel offers the Medusa® Platform, self-assembling, poly-amino acid nanoparticles, made of a poly-Glu backbone grafted with hydrophobic α-tocopherol molecules, creating a colloidal suspension of nanoparticles (10–50 nm) in water. The polymer is amphiphilic, allows a noncovalent capture of protein, and spontaneously forms stable nanoparticles in water. The sustained drug release is based on reversible drug interactions with hydrophobic nanodomains within the nanoparticles. They are robust over a wide range of pH values and can be stored as either stable liquid or stable dry forms that can be easily reconstituted in water. Flamel is in clinical trials with INF-α (Phase 2), basal insulin (Phase 1), and interleukin-2 (Phase 1) [63, 64].

Successful protein formulation strategies have included less soluble protamine and/or zinc salts, PLGA-based systems, and poly-Glu nanoparticles.

20.2.3 Antibodies

20.2.3.1 Analog Design

Antibodies are distinct from peptides and proteins in that they are much larger biomolecules (typically greater than 100,000 Da in molecular weight) and are administered at much higher therapeutics doses (typically greater than 50 mg). Analog design can be as simple as the substitution of one or more amino acids to improve stability or immunogenicity and sometimes half-life. Modifications are usually site-specific where the substitution occurs at the amino acid level and does not affect secondary structure or activity of the molecule [65].

20.2.3.2 Molecular Engineering

A more profound modification of molecular structure of antibodies involves the selection and rearrangement of subunits or fragments of antibodies for their specific function. A good overview of approved products and molecules in clinical development can be found in these reviews [43, 66, 67]. These fragments have been investigated for targeting to specific receptors to deliver the parent antibodies themselves or other active payloads, used in creative combinations of fragments for multivalent functionality, and designed specifically for enhanced circulation time through their binding affinity to plasma proteins or through the natural recirculation of antibodies by the FcRn system (neonatal Fc receptor system).

In some cases, particularly when smaller antibody fragments are employed, it has been necessary to chemically modify the antibody or antibody fragment to improve stability, immunogenicity, and circulation time. PEG has been the most widely used polymer system for this purpose. Cimzia® (certolizumab pegol) is an antibody fragment that has been optimized for TNF-alpha activity and is PEGylated in a site-specific manner to extend the plasma half-life (Table 20.2). Similarly, UCB is licensing Nektar's technology to PEGylate an anti-VEGF antibody fragment (CDP791) [53]. The molecule is currently in Phase 2 trials for non-small cell carcinoma [66].

20.2.3.3 Formulation Strategies

The vast majority of antibody products are administered by injection and this can produce sufficient exposure over an extended period of time because half-lives can range from days to weeks for most antibodies [68]. Therefore, molecular engineering strategies have been successful, so that less burden is placed on the formulation design.

20.3 Implants

Sustained and/or controlled release implants provide a longer term delivery duration than long acting injections. Controlled release systems can be defined as achieving a slow release of drug over an extended period of time, where the system is capable of providing control at a constant drug level (i.e., zero-order release). Sustained release systems can be defined as prolonging the release of drug over a period of time, but not necessarily at a constant drug level, similar to depot injections and microspheres. In general, these implants can be categorized into bioerodible systems (rods and cylinders) and implantable devices.

20.3.1 Peptides

20.3.1.1 Bioerodible Systems

AstraZeneca provides a subcutaneous depot formulation of another LHRH analog, goserelin, called Zoladex® 3.6 mg and 10.8 mg. The Zoladex® product line consists of implants of bioerodible PLGA rods for 1- and 3-month delivery as hormone ablation therapy for prostatic and breast cancers [69].

20.3.1.2 Implantable Devices

The Viadur leuprolide acetate implant was designed for the palliative treatment of prostate cancer. The implant delivers leuprolide (~400 mg/ml leuprolide in dimethyl sulfoxide) continuously over 1 year at ~120 μg/day (0.4 μl/day) from a 150 μl drug

reservoir [70]. The DUROS® implant is a 4 mm × 45 mm, osmotically driven, zero-order drug delivery system [71]. In operation, water is drawn through the semipermeable membrane in response to an osmotic gradient between the osmotic engine and moisture in the surrounding interstitial fluid. At the end of the 1-year delivery duration, the empty system is explanted. In vitro release rate data from the Viadur leuprolide acetate implant demonstrated zero-order delivery for up to 1 year [72]. Systems were also implanted into the dorsal subcutaneous space of both rats and beagle dogs [72, 73]. Serum testosterone and leuprolide levels were monitored and showed steady release rates for 12 months. Intarcia has also used the DUROS® implant for the delivery of Exenatide and is in Phase 2 studies with a 3 month version [74]. (Also see Chap. 17.)

The Hydron Implant (Valera Pharmaceuticals), a polymer biomaterial, subcutaneous, nondegradable reservoir implant capable of long-term (1 year or longer) continuous zero-order and pseudo-zero-order parenteral delivery has been used to develop Supprelin® LA for precocious puberty and Vantas® for prostate cancer, both containing the LHRH agonist, histrelin acetate, for a 12-month period [75]. Supprelin® LA (Indevus) and Vantas® (Endo) are nonbiodegradable, subcutaneous implant, implanted with a trocar. The implant looks like a small thin, 3 mm × 2.5 cm, cylindrical hydrogel reservoir and contains 50 mg histrelin acetate [2].

The Memryte Implant (Durect) is being developed for the treatment of Alzheimer's disease using the DURIN™ technology to provide sustained release of leuprolide acetate. The implant is approximately 2.5 mm × 3 cm and allows drug loadings of 60–80%. The DURIN™ biodegradable implant technology is based on the use of biodegradable polyesters as excipients for implantable drug formulations and achieves little to no burst. This family of materials, which is used extensively in medical devices and drug delivery applications, includes the polymers and copolymers prepared from glycolide, D,L-lactide, L-lactide, and ε-caprolactone. These thermoplastic materials are stable when dry but degrade by simple hydrolysis of the polymer backbone when exposed to an aqueous environment [76].

MicroChips has developed an implantable multireservoir array. The array contains descrete reservoirs that provide precise dosing control [77]. Both leuprolide and PTH have been formulated and dosed from reservoirs containing ~20 μg peptide (~200 nl fill volume) [77, 78].

20.3.2 Proteins

20.3.2.1 Bioerodible Systems

Bone Morphogenic Proteins (BMP), specifically BMP-2 and BMP-7, are potent promoters of osteoblast differentiation [79]. Medtronic Sofamor Danek (Infuse® Bone Graft) and Stryker (OP-1 Implant and OP-1 Putty) have products incorporating BMP-7 into a collagen scaffold for spinal fusion surgery and fracture repair [80, 81].

20.3.2.2 Implantable Devices

Intarcia has also delivered ω-interferon from the DUROS® device to improve the treatment of Hepatitis C. Delivery of ω-interferon from the DUROS® device provides a continuous and consistent dose for an extended duration without the need for frequent self-injection. In addition, a constant therapeutic level of ω-interferon is delivered without peaks associated with severe side effects, and troughs associated with subtherapeutic drug levels and viral breakthrough. Intarcia is currently conducting a Phase 1b trial [82].

20.3.3 Antibodies

20.3.3.1 Bioerodible Systems

Because of the long circulating half-life for antibodies, sustained release strategies are only required in some special circumstances. In particular, bioerodible systems have been evaluated for site-specific delivery to increase the efficiency of delivery to the site and to maintain a continuous elevated exposure. For example, a PLGA formulation was created for an anti-VEGF antibody and used to treat macular degeneration by injection into the intravitreal humor of the eye [83]. Other attempts have been made to produce sustained release depot systems to treat local inflammation using gel-forming polymer systems such as hyaluronic acid and carboxymethylcellulose [68].

20.3.3.2 Implantable Devices

Genentech has licensed Surmodics biodegradable implant technology to develop an ocular sustained release formulation of Ranibizumab (Lucentis) for the treatment of wet age-related macular degeneration [84].

20.4 Conclusions

As these long acting parenteral and sustained release platforms gain maturity and find their niche in the market place, two factors will impact their success. First, with the advent of biosimilars, the acceptability of the parenteral delivery platform becomes ever increasingly important to the success of the product. The competition will quickly expand from currently successful biopharmaceutical companies to biogeneric companies and drug delivery companies looking to diversify.

Secondly, the effective and timely development of biomolecular sustained release products requires forethought into the regulatory hurdles in order to receive timely

market approval. These include adequate shelf life stability and in vivo use life stability to ensure that the final dosing period was as efficacious as the initial dosing period. Formulation of biomolecules often is more challenging than formulation of small molecules because of their high level of complexity and the interrelationship between chemical and physical (conformational integrity) stability.

Furthermore, the development of a rugged in vitro release rate test that can be used to demonstrate lack of dose dumping, percentage of total formulation released from the delivery system, in vitro stability, and an in vivo/in vitro correlation is critical. Ideally, the final product must provide a minimum of 1–2 years of storage stability (preferably at room temperature) and meets the additional requirements that are unique to its delivery. Any excipients used should preferably be Generally Regarded As Safe (GRAS) and any polymers utilized (implants) should be Medical Grade, with an acceptable leachable/extractable profile. In addition to these challenges, the development of the drug formulation goes hand in hand with development of the delivery device, and the resulting product may require approval as a drug/device combination.

While tremendous progress has been made in the development and commercialization of long acting delivery systems for biomolecules, much additional work is ongoing to develop more advanced and patient-friendly product presentations. Ultimately, for biomolecules to reach their full potential as medicines, advanced delivery systems, including long acting injectables, will be necessary.

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Chapter 21
Injectable PLGA Systems for Delivery of Vaccine Antigens

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Abstract Micro and nanoparticles prepared from poly (lactic-*co*-glycolic acid) (PLGA) polymer represent a unique and promising delivery system for vaccine antigens. Administration of PLGA with adsorbed or encapsulated antigens has been shown to improve immunogenic responses in mammals relative to administration of soluble antigen. PLGA microparticles are capable of delivering a number of agents simultaneously and presenting multiple copies of antigens on the polymer surface, both which lead to a stronger activation signal. PLGA microparticles can also trap and retain the antigens in local lymph nodes and protect them from proteolytic degradation, ensuring longer stimulation by the antigen. Since the interior of PLGA microparticles is not a "friendly" environment for most proteins, each protein antigen vaccine requires careful optimization. Over the last 10 years, significant advances in antigen stabilization have been made. It is expected that the first clinical use of this dosage form for vaccines will be for treatment of diseases such as cancer instead of prophylactic immunization in healthy populations.

21.1 Introduction

21.1.1 Diversity of Vaccine Antigens and Applications

Vaccine introduction into medical practice at the beginning of the twentieth century has had a tremendous impact on overall human health. Vaccines are considered to be one of the most safe and effective medical interventions currently available and

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are second only to the introduction of a clean water supply in having a favorable impact on human health. Each year, vaccines prevent up to three million deaths and protect 750,000 children from serious disabilities [1]. Traditional vaccines mainly consist of live attenuated pathogens, whole inactivated organisms, or inactivated bacterial toxins. The success of these vaccines is ascribed mainly to the induced antibody responses that neutralize viruses and toxins, prevent pathogen binding to cells, and prompt pathogen uptake by phagocytes [2].

Despite their success, there is a need for the development of new vaccines against a number of infectious diseases for which vaccines are still not available (e.g., HIV, hepatitis C virus, and malaria), emerging and reemerging infectious diseases, pandemic strains of influenza, and even cancer. To combat many of these pathogens and potentially cancer cells, much stronger immune responses, that include activation of T helper-type 1 cells (Th1) and cytotoxic T lymphocytes (CTLs), may need to be induced. However, many of the modern vaccines currently in development are based on highly purified yet less immunogenic recombinant proteins or synthetic peptides. Therefore, these new generation vaccines require strong and safe adjuvants or delivery systems to improve their immunogenicity [2, 3].

The most widely used adjuvants in human vaccines are aluminum compounds (mostly aluminum hydroxide and aluminum phosphate), which are commonly referred to as “alum” after potassium aluminum sulfate. However, alum has several limitations (1) alum is not effective for all antigens, (2) it can induce local reactions, and (3) it activates mainly antibody responses and generally fails to activate potent T cell responses [4–6]. An additional adjuvant that has been licensed in Europe for over a decade is MF59, an o/w emulsion of biodegradable and biocompatible squalene oil [7]. MF59 has an acceptable safety profile and is a potent adjuvant in influenza vaccine that can efficiently deliver antigens and stimulate production of strong antibody responses [8]. Although MF59 has been shown to be more potent in inducing antibody and T cell responses than aluminum-based adjuvants and has been licensed in more than 20 countries [7], it has yet to be approved in the United States. Other important adjuvants on the horizon have been recently reviewed [8].

21.1.2 Objectives of Antigen-Delivery Systems

The terms “adjuvant” and “delivery system” are often used interchangeably, yet a distinction can be made between the two. Adjuvants act in combination with antigens to safely maximize the desired immune response relative to that produced by the antigen alone [8, 9]. Antigen-delivery systems can be considered an important class of adjuvants that mimic the carrier properties of various pathogens (e.g., bacteria and viruses). Common objectives when employing antigen delivery systems may include (a) protecting the antigen from degradation before processing by the immune system (e.g., by encapsulation), (b) decreasing the dose of antigen required in the vaccine, thereby reducing costs and/or increasing vaccine coverage based on limited vaccine availability, (c) targeting the antigen to professional

antigen-presenting cells (APCs) (e.g., by controlling size, charge, and hydrophobicity of the carrier), (d) controlling the amount and timing of antigen exposure (e.g., by controlled release), (e) controlling the alignment of antigen (e.g., by adsorption) exposed to immune cells, (f) coincorporating immunostimulatory substances to increase the immunogenicity of weak antigens, (g) recruiting immune cells (e.g., via proinflammatory signals), (h) directing intracellular processing of the antigen once taken up by APCs, and (i) increasing the quantity, quality, and duration of effector T cell and/or antibody responses. By one or more of these mechanisms, the delivery system ensures that a strong, protective immune response is formed against the antigen of interest [2, 8, 10].

For a vaccine to function optimally, it is necessary to first stimulate the innate immunity. The innate immune response is not specific for the antigen but it is required to activate and guide the correct adaptive immune response. The adaptive immune system (cellular or humoral) works in an antigen-specific manner and generates the immunological memory that is critical for vaccine function. APCs act as a link between the innate and adaptive immunity by taking up any antigen they encounter in the periphery and presenting it to naïve T cells in the local lymph nodes, which activates the adaptive T cell response [11]. Delivery systems promote the uptake of antigen by APCs and enhance the concentration of antigen that reaches the lymph nodes, thereby improving the immune response. A number of delivery systems exist, including microparticles, liposomes, iscoms, emulsions, and virus-like particles [12]. Systems that allow for gradual antigen release and prolonged antigen exposure, thereby enhancing the immune response, such as biocompatible and biodegradable particles, are very promising candidates.

21.1.3 Objectives of the Chapter

Micro/nanoparticulates prepared from biodegradable polymers represent a unique delivery system for vaccine antigens owing to their well-established safety, their development and use in clinical controlled-release dosage forms of drugs, and their flexibility and potential to accomplish each of the aforementioned goals of antigen-delivery systems [8, 10, 13–15]. Among those studied, copolymers of lactic and glycolic acids (polylactic-*co*-glycolic acid – PLGA) remain to date the most common synthetic biodegradable polymers for preclinical, clinical, and scientific evaluation. Although actively investigated for vaccine delivery for more than 20 years, initially because of the potential to obviate booster vaccinations, PLGA antigen delivery has yet to fully benefit from significant recent advances in the mechanistic understanding of PLGA drug-delivery systems. This chapter focuses on PLGA vaccine delivery for the more commonly studied parenteral administration route (i.e., via the needle). Although an interesting body of literature is available for PLGA carriers in mucosal vaccination [16–20], clinical data to date have largely fallen short of expectations [21], and this type of vaccine delivery requires several additional considerations, which are beyond the scope of this chapter.

The objective of this minireview was to describe and discuss the following: (a) various elements of injectable PLGA particulates that make them flexible antigen-delivery systems, (b) combinations of various technologies (e.g., other adjuvants) with PLGA carriers, (c) limitations of PLGAs as antigen carriers, (d) different currently developed approaches to overcome these limitations, (e) some of the latest findings with these dosage forms, mainly from our laboratories, and (f) some recent preclinical in vivo data employing PLGA-based systems, particularly those focused on new antigens and applications, which may require strong cellular immune responses.

21.2 PLGA Micro/Nanoparticles as a Flexible Antigen Delivery Platform

21.2.1 Brief Overview of PLGA Particulates

PLGAs are polyesters formed from lactic and glycolic acids and are readily available for preclinical and clinical use from reputable commercial suppliers (e.g., Boehringer Ingelheim Chemicals Inc., Petersburg, VA, USA; Purac Biomaterials, Lincolnshire, IL, USA; SurModics Pharmaceuticals, Birmingham, AL, USA; Wako Chemicals USA Inc., Richmond, VA, USA). The racemic D,L-lactic acid monomer (i.e., often prepared with cyclic D,L-lactide) is generally desired to avoid complications of polymer crystallization, such as increased hydrophobicity and reduced polymer solubility. The amorphous PLGAs are usually synthesized by ring-opening polymerization with a tin-based catalyst and can be end-capped with a variety of functional groups, although free acid and long-chain aliphatic ester terminations are most common. Molecular weights of commonly used polymers range from 10 to 150 kDa with a lactic/glycolic ratio between 50/50 and 100/0 to adjust continuous vs. discontinuous release [22] and degradation time (e.g., weeks to >6 months) of the polymers [23], respectively. The polymer itself can be terminally sterilized by gamma irradiation without antigen, although this sterilization process significantly decreases the molecular weight of the polymer and can influence the release kinetics of the encapsulant [24].

By dissolving in an appropriate carrier organic solvent, e.g., methylene chloride, ethyl acetate, acetone, or *N*-methylpyrrolidone, PLGA can theoretically be processed into virtually any shape and size, forming a matrix for delivery of antigen and/or other immunologically active substances. PLGA microparticulates are formed by a number of various physical–chemical processes such as emulsification, spray drying, and coacervation. Although the polymer itself does not present any known epitopes, mild inflammatory responses are commonly observed after administration of PLGAs, owing to polymer degradation (i.e., nonenzymatic hydrolytic cleavage of the ester bonds by water absorbed in the polymer), which steadily produces proinflammatory acidic products (e.g., glycolic and lactic acids) [25].

Despite this, the safety of PLGA is undisputed as they are used every day in healthy children as resorbable suture materials and form the major excipients in numerous controlled release products, the earliest of which have been marketed for >20 years [e.g., Lupron Depot® and Zoladex® (http://www.lupron.com, http://www.zoladex.com)].

21.2.2 PLGA Polymers as Adjuvant-Active Delivery Systems

After years of study, it is now well established that administration of PLGA particulates with adsorbed or encapsulated antigens commonly improves immunogenic responses in mammalian subjects relative to soluble antigen. Some of the anticipated mechanisms of enhancing the immune response by PLGA particulate thus far include (a) preventing premature degradation of antigen before reaching immune cells, (b) providing slow release of antigen, (c) targeting and uptake by APCs (and the resulting cascade of events) [26], (d) immobilizing antigen epitopes on a surface, (e) inducing mild inflammation, and (f) release of immunostimulators, if added to the polymer.

To accomplish these effects, PLGA particulates have been mostly studied within three different size ranges (1) larger microsphere depots (10–100 μm), (2) smaller microspheres for targeting APCs (0.5–10 μm), and (3) nanospheres (50–500 nm) that have been suggested to improve targeting of APCs relative to small microspheres [27], but are also capable of circulating in blood [28]. Most approaches focus on particulates in the form of smooth and spherical polymer matrices ideal for injection, although particulates of either rough nonspherical [29] and/or other specific shapes [30] have been investigated.

21.2.3 Immunopotentiators for Incorporation in or Co-administration with PLGA

Immunopotentiators represent a category of adjuvants that have a direct immunopotentiating effect on immune cells, mostly through toll-like receptors (TLRs) or other receptor families. These adjuvants are usually derived from pathogens and act mostly by stimulating innate immune cells to produce proinflammatory cytokines that trigger and control the adaptive immune response [31, 32]. Immunopotentiators are formulated into delivery systems to enhance their effect on APCs, maximize their potency, and minimize their effect on nonimmune cells. Considering that for safety reasons new vaccines are often highly purified, and therefore have reduced proinflammatory effects, the use of immunopotentiators may be necessary in certain applications for inducing a strong protective immune response. The concept of simultaneous delivery of antigen and immunopotentiator into the same APC population

is very attractive since it ensures that the potentiating effect focuses on the cells responsible for immune response induction and minimizes systemic distribution from the site of injection.

Codelivery of immunopotentiators in microparticles with adsorbed antigen has been shown to be an effective way to enhance the effect of immunopotentiators. Cationic microparticles might have advantages for certain formulations since they may be optimal for uptake into macrophages and dendritic cells [33], and adsorption to cationic microparticles can activate some otherwise inactive oligonucleotides as immunopotentiators [34]. Some of the immunopotentiators assessed for antigen co-delivery in microparticles include monophosphoryl lipid A (MPLA) [35], CpG oligonucleotides [36–38], muramyl dipeptide (MDP), and QS21 [39–41]. MPLA, a synthetic mimic of lipopolysaccharides (LPS) from the cell wall of Gram-negative bacteria, has been evaluated in clinic as a part of various formulations and is already used in Europe in allergy vaccines [42]. In October 2009, the FDA approved Cervarix, a vaccine for cervical cancer prevention, with the adjuvant called AS04, a combination of aluminum hydroxide and MPLA. Therefore, after more than 80 years since the approval of aluminum adjuvants, AS04 became the second adjuvant licensed for use in vaccines in the US.

Immunopotentiating and safety aspects of several adjuvants yet to be approved for use in vaccines have been evaluated extensively. For example, Singh et al. used CpG oligonucleotides adsorbed to cationic PLGA microparticles to improve both antibody and CTL responses against recombinant HIV-1 p55 gag and gp120 proteins in mice [43]. CpG oligonucleotides adsorbed onto cationic PLGA microparticles were also used to increase the potency of anthrax vaccine against bacterial challenge in mice [38]. Co-administration of CpG-PLGA microparticles with Anthrax Vaccine Adsorbed (AVA, the licensed human anthrax vaccine) induced stronger and faster IgG response than AVA alone [38].

Tabata and Ikada were the first to entrap MDP in microspheres and to show that microencapsulation reduced the pyrogenicity of MDP [44]. The immunopotentiating effect of MDP entrapped in microspheres was also demonstrated by Puri and Sinko [45] who showed that by decreasing MDP release rate from microspheres loaded with MDP and ovalbumin (OVA), a stronger IgG antibody response was induced in mice. Cleland et al. co-entrapped immunopotentiator QS21 with recombinant gp 120 in PLGA microparticles [46] and showed a strong antibody response in immunized guinea pigs. Interestingly, their study also demonstrated that only antigen is required for the booster immunization and that a higher antibody response was induced when antigen was delivered by a discrete autoboost compared to initial continuous release.

The effect of immunopotentiators is strongly affected by the formulation. Although both encapsulation and adsorption of immunopotentiators could provide their sustained release, the duration of release may be different for the entrapped antigen. This should be kept in mind since longer continuous release might be beneficial in some circumstances while in others it might be more desirable to have the immunopotentiator available only in the early phase of response induction. It seems that the optimal delivery formulation for each immunopotentiator needs to

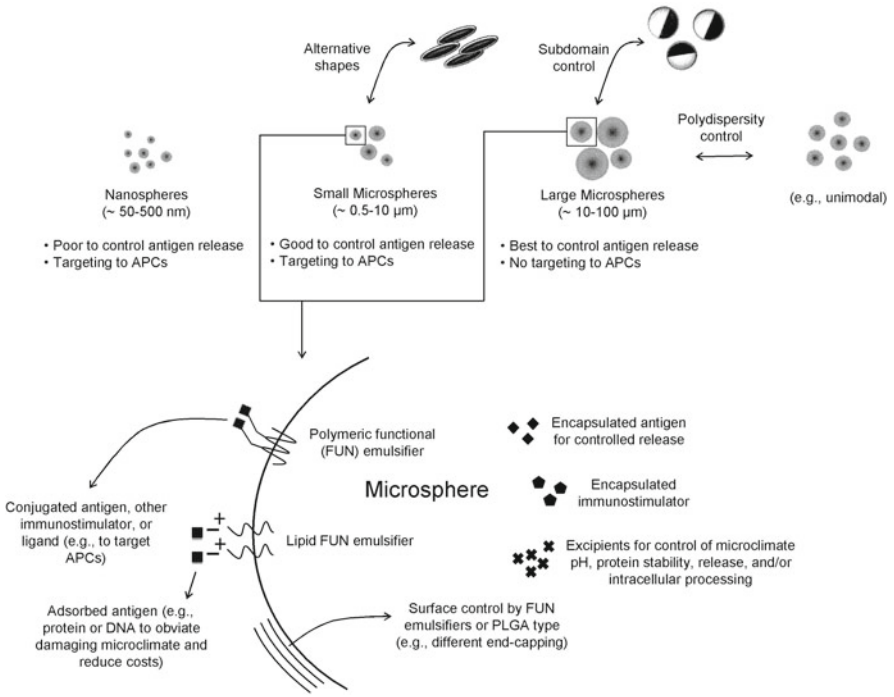

Fig. 21.1 Toolbox of PLGA technology available for antigen delivery

be determined depending on its characteristics, mechanism of action, and the overall goal. For example, immunostimulators may be more acceptable in patient populations with serious illnesses where the risk-to-benefit ratio is more favorable.

21.2.4 Description and Examples of the PLGA Toolbox

Much of the flexibility of PLGA as an antigen delivery system arises from its polymeric properties, which have been intensively studied primarily by material scientists, polymer chemists, engineers, and pharmaceutical and other life scientists over the better half of the last century. As shown in Fig. 21.1, the flexibility and physical–chemical characteristics of the copolymer particulate carrier include:

(a) Its capability to microencapsulate and protect antigens, immunostimulators or other biologically active agents, and excipients (e.g., protein stabilizers, pore-forming agents, plasticizers) for extended periods – upon removal of organic solvent, the polymer forms a dense film through which both the antigen and endogenous proteolytic enzymes cannot penetrate (see below for improvements on antigen stabilization and unexpected adjuvant effect upon encapsulation of antacid excipient).

(b) Facile modification (e.g., charge, hydrophobicity) and functionalization (e.g., antigens or other immunotargeting agents) of the polymer surface – this has been accomplished by numerous physical and/or chemical means with varying levels of complexity, safety, and cost (see below for how to safely simplify surface modification by the surface entrapment of functional emulsifiers).
(c) The ability to tune antigen release rate and duration and whether to be continuous or discontinuous – the ability to accomplish this with peptides has been known for almost 30 years [22], but has been more complicated with protein antigens (see below for methods to make release continuous while minimizing protein damage).
(d) Control of the size, shape, and the subdomain composition of the PLGA particle – the ability to adjust particle size has been known for decades, but additional fine control of particle characteristics has been accomplished only in the last decade (see below for creating monodisperse particles, alternative shape particles, and particles with controlled subdomains).

21.3 Overcoming Limitations of PLGA Polymers for Vaccine Delivery

21.3.1 Limitations of PLGA Delivery

21.3.1.1 Cost and Safety

Even in the scenario where perfectly formulated PLGA antigen delivery systems were prepared and functioned ideally in preclinical in vivo models, advancement into the clinic could be prevented by high production costs. Thus, cost is a significant issue for developing improved vaccine adjuvants for routine childhood immunization, where the required cost of a unit dose must be held to an absolute minimum. Virtually any microencapsulation method to date requires aseptic manufacture, which adds significant costs to development. In addition, whereas new "designer polymer materials" may provide inspiration for what the future might hold, the probability of reducing these fantastic techniques to practice for most antigens within budget may be relatively low in the near future and must be rigorously evaluated before commencing development. Similarly, although safety and biodegradability of PLGA are some of its greatest assets, when performing chemistry (e.g., to the surface for conjugation of antigen) or adding specialized excipients (e.g., immunostimulators) to adjust polymer performance, a keen eye must be maintained on safety to avoid unwanted side reactions upon administration.

When concerned with both practical issues, the vaccine target (e.g., cancer or pertussis) will dictate the allowable cost and safety. For sick patients immunized with a cancer vaccine, the acceptable cost and tolerability of unwanted reactions/toxicity may be orders of magnitude greater than for healthy children immunized with diptheria-tetanus-pertussis (DTP) antigen. It is therefore reasonable to anticipate

that if and when the first advanced PLGA vaccine delivery methods become approved by regulatory agencies, the vaccine therapy most likely will be administered to sick patient populations. Hence, there is clearly a need for new approaches to reduce the cost of PLGA delivery system manufacture while retaining its desirable safety profile.

21.3.1.2 Instability of Antigens

Controlled release of stable vaccine antigens (particularly protein antigens) with intact primary and three-dimensional structures (i.e., in which linear and conformational epitopes are embedded) has proven to be an ambitious goal. By the late 1990s, a long list of proteins reported to have undergone damage when encapsulated in PLGA had accumulated [47]. Extensive analysis of the timeline of physical–chemical events between the time of antigen encapsulation to antigen release in vivo [47, 48] revealed some important facts concerning instability of protein antigens in PLGAs [13, 49]. In general, antigen instability can be triggered by numerous physical–chemical stresses during any of the following processes (1) antigen encapsulation, (2) drying and storage of encapsulated antigen, and (3) antigen release from the particles. During antigen encapsulation, micronization processes (e.g., emulsification, atomization), exposure to organic/water interfaces, and elevated temperature are expected to be most deleterious for the protein. During the drying and storage phase, proteins lose their native structures and are more susceptible to irreversible damage, unless stabilizing excipients are added. During release, proteins are exposed to moisture, variable and typically highly acidic pH, and a moderately hydrophobic and/or charged polymer surface, all of which can damage the protein. Protein unfolding, noncovalent and covalent aggregations, and hydrolysis of the peptide backbone are expected to be the most common molecular mechanisms of instability resulting from the aforementioned deleterious stresses.

Damage to PLGA-encapsulated antigens is not limited to proteins. For example, loss of supercoiled plasmid DNA when exposed to shear associated with microencapsulation in PLGAs has also been recorded [50]. Moreover, PLGA-encapsulated peptides with alpha- and epsilon-amino groups have been found to react directly with the carboxylic acid end group of the polymer to yield the acylated product (i.e., the amide functionality) [51–54].

21.3.1.3 Biological Barriers of the Immune System

Induction of an immune response is an extremely complex process that needs to overcome several major barriers, including low numbers of naïve lymphocytes, different types of responses required to eliminate different pathogens, assistance of various immune cells, restrictions, and tolerance [55]. The complexity of the immune system is necessary to ensure that every foreign antigen in the body gets recognized and eliminated by immune cells.

In addition to the barriers mentioned, successful vaccines need to overcome even more difficult hurdles to induce a strong and efficient immune response that would provide long lasting protection against an antigen of interest. Some of these additional barriers may include (1) antigen degradation by the host enzymes before reaching the immune cells, (2) inefficient recruitment of immune cells by the antigen, (3) poor antigen uptake by the innate immune cells, (4) ineffective antigen processing and presentation to the adaptive immune system, and (5) unsuccessful costimulation of the antigen-specific immune response. To ensure optimal vaccine function, every step from vaccine administration to development of a potent immune response needs to be carefully analyzed and taken into consideration during vaccine development.

Upon vaccine administration, it is very important that the antigen remains intact and exposed in order to recruit and stimulate immune cells. In the best scenario, vaccine antigen should be delivered by a system that protects the antigen from degradation before reaching the immune cells and at the same time increases its immunogenicity. This is particularly important for recruitment of innate immune cells (e.g., APCs) to the site of antigen delivery as activation and assistance of these cells are necessary to generate a strong and long-lasting immune response.

After the antigen is taken up by APCs, it is processed and presented to T cells in complex with major histocompatability complex (MHC) class I and class II molecules. $CD4^+$ Th cells recognize complexes of antigens (from processed extracellular proteins) with MHC class II molecules, while $CD8^+$ CTLs recognize antigens (derived from intracellular proteins) bound to MHC class I molecules. Antigen uptake by these cells is determined by size, composition, and other physical–chemical characteristics of the vaccine particulate. It is worth noting that different vaccine formulations recruit and stimulate diverse types of APCs, thereby altering the repertoire of activated T helper cells [56]. For example, antigens co-delivered with adjuvants that create antigen depots most likely target tissue-derived DCs (e.g., Langerhans' cells) [57], while antigens codelivered with dispersible adjuvants activate inflammatory monocytes and naïve antigen-specific B cells [56].

Considering the different capacities of APC subtypes to uptake, process, and present antigens, the type of recruited APCs will also affect the specificity of the T cell response [58–60]. Although exogenous proteins are processed through an MHC II pathway that stimulates only Th cell populations through still not completely understood mechanisms of antigen cross-presentation, APCs (mainly DCs) are able to process and present antigens derived from extracellular proteins in complex with MHC class I molecules and stimulate CTLs [61–63]. The variety of endolysosomal proteases specific for a type of APC [64, 65] determines the diversity and number of various epitopes presented on the surface of APCs. It is worth noting that antigen presentation could be altered by various posttranslational changes in MHC-bound peptides that occur in APCs after protein antigen uptake (e.g., nitration of tyrosine, tryptophan oxidation, citrullination of arginine) [66–68]. These modifications might stimulate a set of T cells distinct from the one specific for unchanged peptides [66–68] thereby not inducing the desired response.

In addition to stimulatory signals provided by either antigen alone (for B cell activation) or antigen presented in complex with MHC molecules (for T cell

activation), full stimulation of naïve T and B cells requires co-stimulatory signals delivered by activated APCs. Activation of APCs is also crucial for secretion of cytokines that direct T and B cell differentiation into long-lived memory cells that are ready to respond if the same antigen appears again. Therefore, providing full activation of APCs and their migration from the periphery (where they get activated by the antigen) to lymph nodes (where they activate naïve T cells) is one of the most important assignments the vaccine needs to accomplish.

21.3.2 Methods to Overcome Limitations

21.3.2.1 Simplifying Antigen-Loading Methods

As recently reviewed [13], cost and antigen stability considerations have primarily driven investigators to seek simpler methods to associate the antigen with the PLGA carrier. An important alternative to antigen encapsulation, namely surface-immobilized antigen, has been studied for at least 20 years, but probably with increased interest in recent years after cost and antigen stability issues were raised more prominently. Although many antigens will inherently adsorb to the PLGA surface (see Jiang et al. [13] for discussion of several interesting examples), this adsorption may either denature the protein (for the more hydrophobic PLGAs – e.g., crystalline poly(L-lactic acid) with high MW) or result in too rapid antigen release in vivo when sorption is nondenaturing. Early attempts to surface modify PLGA for surface-conjugation of ligands involved changing the polymer backbone [69]. To simplify surface modification, a key finding was to replace standard emulsifiers (e.g., polyvinyl alcohol, PVA) used in the solvent evaporation method with emulsifiers possessing biofunctional groups [41, 43, 70–74]. By using the functional (FUN) emulsifier/PVA switch, the functional group ideally becomes exposed on the surface for associating with antigen (or other bioactive species) by either physical adsorption [43, 73, 74] or chemical conjugation [41, 70–72]. The surface-associated antigen has been shown to exhibit excellent stability when switching PVA for cationic lipid (e.g., DOTAP used in gene delivery for both proteins and DNA) [73, 75]. In addition, with the antigen on the surface of the carrier, e.g., by conjugating antigen to FUN emulsifiers possessing free amino groups [70, 71], the particle may be more readily taken up by APCs [41]. This technique also has advantages relative to copolymerization of functional monomers, since simple processing of existing polymers is much easier than special polymer synthesis to create a new molecular entity, which will also influence the bulk properties of the base polymer.

21.3.2.2 Stabilization of Microencapsulated Antigens

Over the last 10 years, significant advances facilitating encapsulation and slow release of proteins from PLGA with minimal effect on protein stability have been

made [49, 76]. Stabilization has been achieved through both empirical and mechanistic approaches. A useful example involves the vaccine antigen for tetanus, tetanus toxoid (TT), with excellent solution stability. This 150 kDa formalinized antigen is prepared by detoxifying the native tetanus toxin by extensive exposure to formaldehyde. The World Health Organization (WHO) was first interested in this antigen for development of a single dose vaccine in the 1990s to minimize the neonatal tetanus mortality in developing countries [77].

The antigen was found to be extensively damaged during formation of water-in-oil emulsions necessary to micronize the protein [78]. By minimizing molecular motions (and reactivity) in TT via reducing water content during organic solvent exposure, aggregation and immunoreactivity losses during encapsulation were minimized [79, 80]. A similar approach in minimizing TT instability during release from the polymer was used by Sanchez et al. [81] who embedded the antigen in gum Arabic and mineral oil (~1:1,000 antigen to excipient ratio) before its encapsulation in PLGA. This approach showed some promise, but at the expense of impractically low antigen loading.

Mechanistic studies were used to identify, characterize, and inhibit two key instability pathways for TT during its release from PLGA. The first studies identified the mechanism of moisture-induced aggregation of the antigen, as moisture is ubiquitous since the antigen is retained in the PLGA matrix. Two salient features of the denatured state of TT, insoluble covalent nondisulfide aggregates (i.e., not soluble in combined reducing and denaturing agent) and changes in amino acids that strongly interact with formaldehyde (lysine, tyrosine, and histidine), implicated that formaldehyde bound to the antigen initiates a formaldehyde aggregation pathway (FMAP) [82]. The FMAP was later proven by comparing aggregation of bovine serum albumin (BSA) and formalinized BSA [83]. This served as a guide to identify two potent free amino acid stabilizers (lysine and histidine), which could prevent moisture-induced aggregation of f-BSA in the solid-state and in PLGA [84].

A similar mechanistic approach was used to elucidate the pathway of PLGA-induced damage to BSA (i.e., acid-induced noncovalent aggregation and peptide bond hydrolysis [85]) and stabilize the model protein by co-encapsulated antacid excipients (e.g., $Mg(OH)_2$, $MgCO_3$, or $ZnCO_3$). Finally, as shown in Fig. 21.2, by combining stabilizers against FMAP (free lysine), PLGA acidity ($MgCO_3$), and an empiric stabilizer (sorbitol) (whose stabilization mechanism has only been postulated, [86]), TT was slowly released for over 1 month, without significant release-induced damage, from PLGA 85/15 microspheres. The microspheres were prepared by an anhydrous solid-in-oil-in-oil emulsion method at a high antigenically active TT loading (>1% w/w) [76].

In another important study, growth hormone was stabilized by an elaborate anhydrous spray-congealing method (atomizing a polymer solution with suspended solid protein in liquid nitrogen) and zinc-complexation of the protein to maintain the protein in the solid (and immobilized) state [87]. Similarly to proteins, damage to plasmid DNA during encapsulation was minimized by freezing the primary emulsion before forming the second emulsion by homogenization. This processing

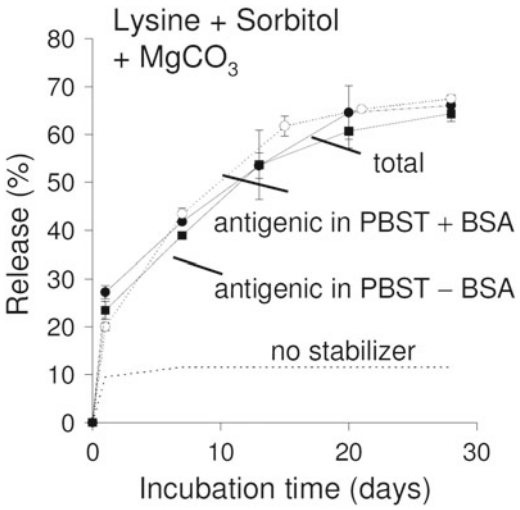

Fig. 21.2 Stabilization of tetanus toxoid (TT) from PLGA 85/15 (i.v. = 0.86 dL/g) microspheres co-encapsulated with stabilizers (lysine + sorbitol + $MgCO_3$) as indicated by overlapping antigenically active and total TT released. Protein content was determined by modified Bradford assay (*filled circle*) and antigenically-active TT (*filled square*) when PBS + 0.02% Tween 80 (PBST) release medium contained no BSA, and by antigenically-active TT when PBST contained 0.2% BSA to minimize TT adsorption to the release tubes (*circle*). *Dashed line* represents release kinetics of TT (Bradford assay) from PLGA microspheres containing no stabilizers. All data are mean ± SD, $n = 2$. Data from Jiang and Schwendeman [76]

immobilized the biomacromolecule during the damaging sheer stress [50]. More recently, soluble multivalent cations (e.g., Mn^{2+} and Ca^{2+} in the form of chloride salts) have been employed to minimize acylation of a model peptide, octreotide acetate, by disrupting interactions between the free-acid end group of PLGA and the protonated primary amine-containing peptide [53, 54]. Finally, in addition to raising microclimate pH with poorly soluble basic additives, the inclusion of >20% PEG as a polymer blend with PLGA has been shown to prevent acid-induced aggregation of BSA [88] and raise the microclimate pH in the polymer [89].

21.3.2.3 Improved Understanding of How to Control PLGA Properties

In addition to forging new frontiers in antigen stabilization, improved understanding of the material science of PLGA may soon be useful in development of polymer delivery of antigens. For example, a combination of pore opening, polymer matrix swelling, and spontaneous pore closing was implicated in control of the high initial burst release of octreotide acetate from PLGA microspheres [90]. The newly identified pore-closing mechanism may be useful to minimize unwanted initial burst release of antigen [91, 92] and shed more light on how antigens are released during

the erosion period. Although acid-stable peptides, such as the luteinizing hormone-releasing hormone analogs, could be continuously released from PLGA by including a significant fraction of low molecular weight PLGA and raising peptide loading [22], this formulation approach is generally not desired for proteins owing to the potential interactions of low molecular weight PLGA with protein (e.g., similarly as found with eroded PLGA and BSA or growth hormone [93]). Hence, it has been conveniently found that inclusion of poorly soluble bases, which are also used to raise microclimate pH [85, 94], in fact creates new pores and accelerates protein release [94, 95]. Similarly, blending partially miscible water-soluble polymers such as PEG with PLGA has also been shown to facilitate continuous protein release [88, 96].

In addition to understanding and adjusting release kinetics, considerable strides have been made in creating and understanding the significance of new polymer morphologies. For example, very precisely controlled size distribution of microspheres (e.g., unimodal, about one desired size) has been achieved by precise control of the emulsion droplet size before polymer hardening [97]. Similarly, composition of specific subdomains within the PLGA particle could be controlled by combining multiple streams of separate polymer solutions in a laminar flow (with very slow lateral mixing) briefly before particle formation [30]. Finally, the shape of the microspheres (e.g., oblong vs. spherical) is now being manipulated to expand on existing knowledge (e.g., effects of charge and hydrophobicity) of how to optimize particle uptake by APCs [98].

21.3.2.4 Improved Stimulation of Complete Adaptive Immune Response

For stimulation of a complete adaptive immune response, vaccines need to overcome various biological barriers to the generation of the desired immune response, as mentioned previously. As will be discussed in this section, particulate delivery systems have several properties that, if carefully formulated, could help to overcome at least some of these barriers.

The size of particulate carriers is similar to the size of pathogens recognized by the immune system and therefore these carriers could be efficiently taken up by APCs [8]. Numerous studies have shown that, by using different preparation methods, the size of polymeric microspheres could be adjusted for optimal uptake [99–101] and that microspheres smaller than 10 μm are preferentially phagocytosed by APCs [2, 102, 103]. The efficiency of PLGA as antigen delivery system has been demonstrated by rapid and efficient phagocytosis by APCs in vitro and in vivo [104–107], by increased antibody titer after single administration of antigen-loaded PLGA microparticles [108] and by induction of the CTL response [109].

Additionally, microparticles in the size range of 0.1–10 μm have 1,000-fold improvement in their ability for cross-presentation by APCs [15, 110]. Since cross-presentation allows for MHC class I complexation of an extracellular antigen, microparticulate delivery systems with size <10 μm may improve the CTL immune

response toward the antigen [111]. At the same time, when encapsulated in the particles, antigen is sequestered from host enzymes and protected from degradation, which prolongs its persistence until it reaches and stimulates immune cells [1]. Therefore, by adjusting particle size and choosing a suitable method for antigen capture, two barriers could be overcome: premature antigen degradation outside APCs and poor antigen uptake by APCs.

Another important barrier that needs to be overcome by a delivered vaccine is the recruitment of immune cells. Although vaccines are formulated to closely resemble the pathogen, for safety reasons they usually lack some of the important features that efficiently recruit innate immune cells. To overcome this barrier, vaccine antigens have been either delivered by PLGA particles or co-encapsulated in PLGA with various immunopotentiators. For example, by loading hepatitis B core antigen (HBcAg) into PLGA nanoparticles with MPLA, Chong et al. increased Th1 immune response with a predominant IFN-γ profile against HBV [112]. Moreover, Ali et al. have recently demonstrated that a macroporous PLGA matrix could be designed to recruit, host, and activate DCs, which enhances their migration to lymph nodes and significantly improves the immune response [113].

Coencapsulation with appropriate immunopotentiator could also help target specific APC populations that produces a desirable panel of cytokines. For example, it has been shown that GM-CSF (granulocyte-macrophage colony-stimulating factor) recruited myeloid DCs, while CpG-ODN (cytosine-guanosine oligonucleotide) increased the number of plasmacytoid DCs, which were known to secrete mostly Th1-type cytokines and promote cytotoxic T cell immunity [113]. Also, uptake of particulate adjuvants, such as PLGA and polystyrene, by DCs activates NALP3 inflammasome-enhancing secretion of IL-1β by DCs and promotes antigen-specific IL-6 production by T cells [26]. This is particularly important for antigen processing and presentation, since some cytokines (e.g., IL-6, TNF-α, IL-1β, and IL-10) can modulate the activity of lysosomal enzymes involved in protein antigen degradation and affect their presentation [114, 115].

In addition to overcoming these immunological barriers, stability of vaccine antigen plays an important role in the induction of a strong and long lasting immune response. To improve its stability, vaccine antigen has been coencapsulated with antacid excipients and stabilizers [10, 13, 88] or surface immobilized [75]. Since T cell receptors recognize antigens in the form of short linear amino acid sequences associated with MHC molecules on the surface of APCs, antigen stability may not be as high of a concern when inducing cell-mediated immunity. However, since B cell receptors recognize antigens through their three dimensional conformation, maintenance of the stable native conformation of an antigen is imperative for an antibody response against discontinuous epitopes [10]. This is important when delivering a toxoid vaccine (e.g., tetanus toxoid) which requires the effects of neutralizing antibodies to induce protection. Encapsulation of toxoid within polymeric microspheres has been found to preserve the immunogenicity of the antigen for production of a stronger antibody response [10, 78, 116, 117].

21.4 Immunogenicity to PLGA-Microencapsulated/Associated Antigens

21.4.1 PLGA-Antigen Vaccines Induce Humoral Immunity

PLGA microparticles are good candidates for vaccine delivery systems since they have the advantage of being able to present multiple copies of antigens on their surface [118]. These organized arrays of antigens are able to efficiently cross-link B cell receptors and constitute a strong activation signal [119–121]. Additionally, PLGA microparticles can trap and retain the antigens in local lymph nodes and protect them from systemic degradation, ensuring longer stimulation by the antigen.

The adjuvant effect of microparticles with entrapped antigens has been known for many years [122]. O'Hagan et al. demonstrated that microparticles with entrapped ovalbumin (OVA) had comparable immunogenicity to OVA dispersed in the most potent adjuvant available – Freund's adjuvant [123]. Similarly, when the 38-kDa recombinant antigen from *Mycobacterium tuberculosis* was microencapsulated and used to immunize mice, the induced antibody level was comparable to the response induced by Freund's adjuvant [124]. The adjuvant effect of PLGA microparticles was also confirmed by Eldridge et al. with staphylococcal B enterotoxoid as an antigen [116].

One of the important parameters that can affect the quality of antigen delivery by PLGA particles is microparticle preparation. During antigen encapsulation in PLGA microparticles, protein frequently gets degraded and denaturated [87]. However, when recombinant antigens from HIV envelope or *Neisseria meningitides* type B were adsorbed onto PLGA microparticles, the antigen structure was maintained, promoted optimal epitope interaction with B cells, and induced potent antibody responses in mice [75]. Moreover, we have shown (Fig. 21.3) that PLGA-adsorption of pCMVgag and pSINCPgag vectors led to a substantial increase in anti-gag antibody titer in rhesus macaques [125].

Another important parameter is size: smaller particles (<10 μm) are often shown to be more immunogenic compared to larger particles, most likely due to better uptake by APCs [116, 126]. Although antigen adsorbed to the surface of microparticles has similar immunogenicity as entrapped antigen [126], antigen adsorption restricts the potential of microparticles for controlled-release delivery. This should be considered while designing microparticle vaccine delivery systems.

Frangione-Beebe et al. have also shown that MVFMF2 peptide construct (designed to mimic the native envelope from HTLV-1) encapsulated in PLGA produced a sustained antibody response in mice and rabbits that lasted for 23 weeks without requiring a booster immunization [127]. However, inoculated rabbits were not protected from cell-associated viral challenge, emphasizing a need for eliciting both antibody and cell-mediated immune responses for a full protection against cell-associated viral infection.

A recent example of the unexpected enhancement of immune response to a poorly immunogenic human chorionic gonadotropin (hCG)-based peptide was observed

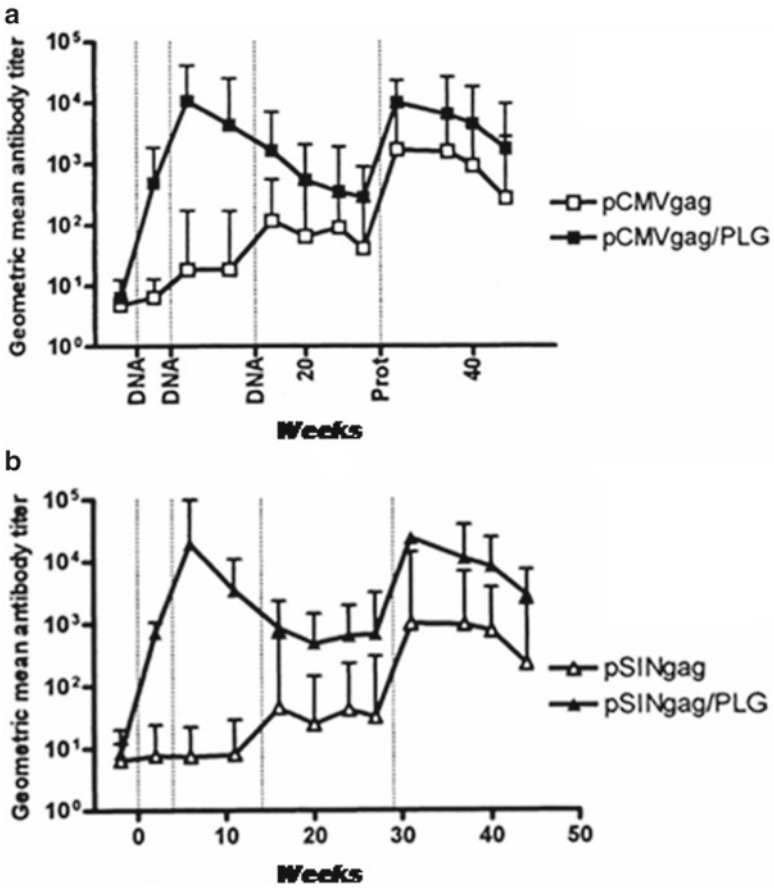

Fig. 21.3 Gag-specific antibodies induced by DNA or DNA/PLG priming and protein boosting. Groups of five rhesus macaques were vaccinated at weeks 0, 4, and 14 with pCMVgag or DNA/PLG (**a**) or pSINCPgag DNA/PLG (**b**). Rhesus were boosted at week 29 with recombinant Gag protein adsorbed onto anionic PLG microparticles. Anti-Gag antibodies are plotted as group geometric mean ELISA titers for DNA/saline (*open symbols*) and DNA/PLG (*closed symbols*), and error bars extend to 95% confidence upper limits. Reproduced with permission from Otten et al. [125]

when it was coencapsulated with an antacid ($MgCO_3$) to stabilize PLGA microclimate pH. Surprisingly, the anti-hCG antibody titer in rabbits was increased from 21 nM for soluble antigen, to 730 nM for the antigen coencapsulated with $MgCO_3$ in PLGA (Table 21.1). This was similar to three doses of antigen in water-in-squalene (positive control) [41]. The effect of $MgCO_3$ on immunogenicity of other peptide antigens and the mechanism responsible for this effect are currently under investigation. This study was particularly noteworthy as it showed that PLGA-microencapsulated peptide could elicit a strong antibody response with acceptable safety profile without oily vehicles.

Table 21.1 Peak serum anti-hCG response to PLGA/C-TT2-CTP35 microspheres[a]

Peptide formulation[b]	Peak serum anti-hCG level (nM)
Soluble antigen (negative control)	21 ± 6
Antigen in squalene (positive control)	730 ± 290
Encapsulated antigen + $MgCO_3$	720 ± 190
Encapsulated antigen – $MgCO_3$	15 ± 3

[a]Data from [41]; experimental values represent mean ± SEM ($n = 5$)
[b]Antigen dose was 1 mg; soluble and emulsified antigen groups were boosted at 4 and 10 weeks; positive control group also contained 25 μg nor-MDP

21.4.2 PLGA-Antigen Vaccines Induce Cell-Mediated Immunity

The first studies of PLGA microparticles as antigen-delivery systems were conducted in the early 1990s and showed that these microparticles were able to induce not only antibody but also cell-mediated immunity in rodents [118]. CTL responses were induced in mice following systemic and mucosal immunization [61, 128] with protein and peptide antigens [61, 128, 129]. Based on cytokine responses measured in animals immunized with encapsulated antigens, microparticles preferentially induced a Th1-type response [61, 124], including delayed-type hypersensitivity (DTH) [128]. It seems that particulate antigens are phagocytosed and presented mainly by macrophages through an MHC class I restricted pathway [130], although DCs also contribute to the induction of cytokines that promote the Th1-type response [131].

As mentioned earlier, to avoid problems associated with microencapsulation of vaccine antigens, O'Hagan et al. adopted a new approach of antigen adsorption onto the surface of PLGA microparticles. This resulted not only in a stronger antibody response but also in significantly increased T cell response against recombinant HIV antigens in mice [132] and in nonhuman primates [133]. Although PLGA microparticles with adsorbed p55 gag induced potent antibody and T cell proliferative responses in rhesus macaques, they were not able to induce effective CTL response [133].

CpG adjuvants appear to be very helpful for the induction of Th1 responses [36]. They target toll-like receptor 9 (TLR9), which activates maturation of DCs and facilitates cross presentation of antigens. By adsorbing HIV antigens, p55 gag and gp120, to PLGA and mixing it with CpG also adsorbed to PLGA, Singh et al. have significantly increased both antibody and CTL responses in mice [43]. Importantly, in mice TLR9 is broadly expressed on both the major DC subtypes, plasmacytoid and myeloid DCs, as well as in B cells, macrophages, and monocytes (Ketloy et al. 2008). In humans, B cells and plasmacytoid DCs are the only immune cells that are known to express TLR9 and to be activated by CpG, making it challenging to extrapolate mouse data to humans [134].

21.4.3 Recent Application to Cancer Vaccines

Several lines of evidence suggest that human cancers are subject to immunosurveillance and that many cancer patients harbor preexisting immunity that could potentially be reactivated and used to prevent or treat cancer [135–140]. However, the main obstacle in eliminating cancer seems to be its poor immunogenicity and the immunosuppressive cancer microenvironment.

The principal mechanism for killing cancer cells by the immune system involves CTLs that recognize tumor peptide antigens in complex with MHC class I molecules. Presentation of these antigens is amplified by APCs, the cells that not only provide primary signals to activate T cells (MHC–peptide complex) but also costimulatory signals necessary for their full activation. Unfortunately, cancer cells have developed several mechanisms to escape the host's immune response, including (1) low expression of antigen, (2) downregulation of MHC class I molecules, and (3) production of immunosuppressive cytokines. Therefore, a major challenge in the field of cancer immunology is developing a strategy that will enhance tumor antigen presentation and reverse the immunosuppression of the tumor microenvironment.

Two commonly used approaches in the cancer vaccine field are (1) antigen-based vaccines and (2) cell-based vaccines [141, 142]. Antigen-based vaccines usually contain peptides derived from proteins mutated or overexpressed in cancer. This approach had some success in colorectal carcinoma [143], metastatic melanoma [144, 145], follicular lymphoma [146], acute myeloid leukemia, and multiple myeloma [147].

Cell-based vaccines use either (1) autologous or allogeneic cancer cells combined with adjuvants (e.g., GM-CSF), (2) cancer cells fused with DCs, or (3) DCs loaded with specific tumor antigen [148]. One of the major limitations of DC vaccination is considered to be the inability of DCs to migrate from the injection site to the lymph nodes and their insufficient activation suppressed by the host regulatory T cells [149]. In April 2010, FDA approved Provenge (Dendreon Corporation), the first autologous cellular immunotherapy for patients with prostate cancer. This therapeutic vaccine is based on APCs that are collected from the patient, exposed to a prostate cancer antigen – prostatic acid phosphatase (PAP) – and infused back into the patient. Provenge extended median survival of asymptomatic or minimally symptomatic metastatic prostate cancer patients beyond 2 years, showing a median improvement of 4.1 months and reduced death risk by 22.5% compared to the control group (http://www.provenge.com).

Another approach in cancer vaccine development is to use long synthetic peptides with multiple epitopes, which can potentially overcome MHC restriction [150] and lead to more efficient antigen presentation [151], thereby activating both antibody and T cell immune responses. PLGA particles could be used to improve antigen delivery to APCs, which then process PLGA-encapsulated antigens by two independent pathways, leading to presentation by both MHC class I and class II molecules for simultaneous activation of both $CD8^+$ and $CD4^+$ T cell immune responses, respectively [15]. In addition, PLGA-based cancer vaccines would be ideal for

coencapsulation of immunostimulators or molecules that can reverse the immunosuppression of the tumor microenvironment.

Several investigators have already evaluated PLGA for cancer vaccine delivery. In a recently conducted study by Heit et al., a single injection of PLGA microparticles with coencapsulated chicken OVA and CpG oligonucleotides efficiently activated OVA-specific T cells and caused complete tumor regression in 80% of mice bearing OVA-expressing B16 melanoma (B16-OVA) [152]. However, such a strong T cell activation can be partially attributed to the fact that OVA is a foreign antigen in mice, which is different from the situation found in cancer.

Another study with mice bearing melanoma B16 tumors evaluated the efficacy of PLGA-based vaccines with poorly immunogenic melanoma antigen, tyrosinase-related protein 2 (TRP2), and 7-acyl lipid A adjuvant [153]. This formulation was shown to be very effective in inducing a TRP2-specific CTL response and, more importantly, reversed the immunosuppressive milieu of the tumor microenvironment. Luo et al. showed that carcinoembryonic antigen (CEA) – based DNA vaccine adsorbed onto cationic PLGA microparticles was more potent in CEA – transgenic mice than corresponding naked DNA vaccine [154]. Protective immunity was amplified by boosts with a plasmid encoding murine GM-CSF, leading to a complete rejection of murine colon carcinoma cells in 50% of mice. Protection was provided by activated DCs (shown by increased expression of costimulatory and MHC class II molecules), T cell activation (shown by a dramatic increase in proinflammatory cytokines), and by production of a strong CTL response [154].

Recently, Ali et al. demonstrated that PLGA polymers could be used not only to deliver antigens and immunopotentiators, but also to activate and control trafficking of immune cells in vivo [113]. They designed a PLGA polymer matrix capable of releasing GM-CSF cytokine, housing recruited DCs, and activating them by presenting melanoma antigens and immunopotentiators. This formulation was able to efficiently recruit and activate DCs and promote their migration to lymph nodes, thereby increasing survival in a mouse model up to 90% [113]. This study further supported the concept of using PLGA polymers for successful vaccine development.

21.5 Real World Perspectives of PLGA Development Relative to Alternative Adjuvants

Whereas PLGA microparticles have a number of potentially useful characteristics for vaccine delivery, they also have significant limitations that have impeded their clinical use thus far. Probably the main disadvantage of microparticles is the overall cost resulting from a number of key factors described above, particularly the need for aseptic manufacturing with organic solvents. Hence, currently available PLGA microparticle formulations are unlikely to be the best choice for a simple adjuvant, since alum and various emulsions, including MF59, are already established and manufactured by simpler and less-expensive processes. A potential caveat to this

statement is their use in developing countries that, despite clear progress, continue to have difficulties with reaching entire populations with full immunization schedules and reducing neonatal mortality. PLGA has been shown to compete extremely well with alum for childhood vaccines in preclinical studies after a single dose relative to the required 2–3 doses of conventional adjuvant. However, a sponsor to fund further development for this humanitarian cause has been elusive. By contrast, in head-to-head comparisons undertaken so far, the less expensive antigen-adsorbed PLGA has been comparable to alum at equivalent dosing, and generally less potent than emulsions [74]. Thus, in essentially all vaccine applications, the performance of PLGA would need to significantly exceed the currently available alternative adjuvants to justify inclusion in a vaccine. The future PLGA-adjuvanted vaccine would likely be a "premium" product to justify the associated costs.

In addition to these significant challenges, it also has become abundantly clear that the PLGA interior of the microparticle is not a "friendly" environment for most proteins when the pH is left uncontrolled. Moreover, formulation approaches that must deal with the number of additional factors (e.g., organic solvents, large interfaces, shear, and moisture) that often result in instability of protein antigens during encapsulation and long-term release certainly appear overwhelming when compared with simple mixing of antigen with standard adjuvants. This is particularly true if specific alteration in the PLGA formulation is needed for each antigen of interest. Stabilization of PLGA-encapsulated protein antigen is not trivial and requires careful optimization of the microencapsulation method and selection of PLGA type and excipients. However, it must be emphasized that unlike protein drugs, the bar for stabilization is not nearly as high, since an immune response to a fraction of antigen, which is denatured in a vaccine preparation, would be expected to be much less of a regulatory concern relative to protein pharmaceuticals.

Another independent area of investigation that has inspired much interest for the use of PLGA microparticles is the potential to develop orally administered vaccines through encapsulation in PLG microparticles. However, after two published clinical trials [21, 155], of which the authors are aware, and several others that have not been reported, it is clear that this concept has thus far been unsuccessful. Of course, the challenges highlighted above with regards to antigen instability and costs are relevant factors, but most likely the main reason for failure of PLGA microparticles for oral vaccination is the lack of potency.

Many reports describing the extensive uptake of microparticles across the gut appear fundamentally flawed in their methodology, which have encouraged unreasonable optimism about what might be achieved. If the uptake of particles occurs in humans under normal circumstances, the rate of efficiency is too low to be readily exploitable. Hence, the vast majority of the microparticles and entrapped vaccines must simply be lost to excretion by the normal path following oral administration and not taken up across the intestine. This example serves as another lesson in caution about the interpretation of findings in small animal models, particularly when conditions are manipulated unreasonably, perhaps with unreasonably high doses, or even the use of ligated intestinal loops, which block the normal clearance processes.

Given these various challenges and limitations, a legitimate question to ask is "do PLGA microparticles have real potential for vaccine delivery?" We believe that the answer is an unequivocal yes, but they must be used optimally if they are to succeed and we must focus on their strengths. Fundamentally, microparticles are a flexible system able to deliver a number of agents simultaneously. This can be exploited to ensure the co-delivery of antigens and immunopotentiators to immune cells. This principle has already been demonstrated on a number of occasions, particularly involving the co-delivery of vaccine adjuvants, e.g., CpG or MPL, and an antigen of interest. It has been shown that immunopotentiators are most effective when they are delivered in combination with the relevant antigen, and the flexibility of PLGA allows this to be achieved relatively simply. Antigen adsorption, and perhaps new microencapsulation approaches on the horizon, may be used to obviate aseptic processing to reduce costs and simplify dosage form preparation.

21.6 Conclusions

PLGA microparticles for parenteral vaccine delivery have been studied for a high number of antigens, demonstrating several important preclinical benchmarks. This platform is unique considering (1) the biomaterial's safety record and flexibility to meet a more sophisticated set of desired adjuvant/carrier criteria, and (2) the information available from intensive scientific examination of PLGA mostly for delivery of drugs. However, these advantages are tempered by significant issues of cost, simplicity of encapsulation, and antigen stability. Recent approaches to antigen adsorption and antigen stabilization when encapsulated provide important avenues to overcome these concerns. Thus far, parenteral administration has received more positive results than mucosal vaccination. The first clinical use of this dosage form for vaccines is expected for treatment of diseases such as cancer instead of prophylactic immunization in healthy patient populations.

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Chapter 22
Methods of Sterilization for Controlled Release Injectable and Implantable Preparations

Alpaslan Yaman

Abstract The typical understanding within the pharmaceutical and biologic industries is that heat labile products cannot be terminally sterilized and there is an automatic presumption that aseptic manufacturing will be required. This presumption rests solely on the inherent assumption that the method of terminal sterilization would be the autoclave process (steam over pressure). The concern for heat instability of large molecules or polymeric matrixes is valid; however, terminal sterilization by an autoclave is not the only process available to the pharmaceutical and biologic industries. Other nonthermal terminal sterilization processes are available and may be used to render a biologic or polymeric matrix system sterile. These processes will be reviewed in this chapter. Practical considerations will be given for how these processes can be used to effectively render the product sterile and successfully fulfill the regulatory expectations for these types of products.

22.1 Introduction to Sterilization

22.1.1 Definition and Specifications

Sterilization is defined as "the process by which all living cells, viable spores, viri, and viroids are either destroyed or removed from an object or habitat" [1]. Once an item or object has been sterilized, it is rendered sterile, an object that is "totally free of viable microorganisms, spores, and other infectious agents" [1]. This is the objective for the preparation of all injectable or implantable pharmaceutical, biologic, and

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medical device products. Sterilization can be rendered either through the use of a passive process, an aseptic sterile filtration and manufacturing process (nonterminal process), or through an aggressive process, as is employed in terminal sterilization unit operations. Typical methods used for terminal sterilization are steam (autoclave), ethylene oxide, gamma irradiation, and e-Beam (beta irradiation). The sterility assurance level (SAL) is dependent on which method of sterilization is employed for a product. The SAL is a statistical probability of a nonsterile product unit emerging from the sterilization process; in other words, the probability of a failure in the unit operation to effectively sterilize the finished product. For example, the SAL for an aseptically manufactured unit is 3 (the unit value is based on a logarithmic determination). This means that there is a statistical probability that 1 in every 1,000 units from this process may be contaminated or nonsterile. To get a true appreciation for what this implies, if one were to assume an average batch size for an aseptic product to be on the order of 50,000–80,000 units, there would be a statistical probability of 50–80 potentially unsterile units in that batch. Considering that the sterility test for such a batch could be as few as 20 units, the probability of testing or locating those unsterile units is very unlikely. Therefore, great concern or attention is given to the complete approach or practice of a manufacturing facility that purports to manufacture by this approach. It is for this reason that the regulatory agencies throughout the world have a preference for products that have been sterilized by the terminal sterilization unit operations approach. Thus, it is incumbent on pharmaceutical companies to demonstrate that a product cannot be terminally sterilized before allowance is granted for the aseptic manufacturing process. This is because a product that has been terminally sterilized has an SAL of 6, indicating a statistical probability of one in every one million units processed as being unsterile. For the typical batch of injectable or implantable products, it is rare for the batch size to exceed 150,000. Thus the statistical probability of occurrence of an unsterile unit in a sterile product or implantable batch is effectively zero.

From the considerations detailed above, aseptic manufacture is viewed as high-risk processing within the regulatory agencies; terminal sterilization processes are perceived as low risk. Much work has been done over the years to mitigate the risk associated with aseptic manufacturing processes. This work has resulted in a presumption by some that the risk has been mitigated to some extent, but the risk has not reached the level of mitigation as that afforded by terminal sterilization processes. In the ensuing chapter, processes will be reviewed and practical considerations will be given for how these processes can be used to effectively render the product sterile, through a terminal sterilization process and successfully fulfill the regulatory expectations for these types of products.

22.1.2 Aseptic Sterile Manufacturing Process

Aseptic processing is the processing of drug product components in a manner that precludes microbiological contamination of the final sealed product. Typically, the

drug product or components, the container, and the closure are first subjected to separate sterilization methods and then brought together in a final aseptic step (or steps). For a product manufactured by an aseptic process great concern is given to the entire process and the qualification of each component or aspect of that process, since the breach of any one aspect of this type of process would greatly increase the probability of an unsterile unit making it to the end user. As noted above, all components of the final product, the container, the stopper (if a vial), and the seal (required in Europe) are all sterilized prior to the final assembly with the processed drug dosage form material. Further, the entire process, starting with the incoming raw materials, the personnel and the entirety of the facility is highly controlled. This control transcends the specific batch manufacture, but rather begins with the actual fabrication of the facility, selection and control of the components (all ingredients, materials used to process and equipment) and the training and qualification of the personnel. The details of aseptic manufacture and qualification are not the point of this chapter as there are numerous publications available that discuss in detail how an aseptic facility is designed, built and qualified and how an aseptic process is qualified, including personnel training and media fills [2–4]. It should be understood that although media fills are used to qualify a manufacturing process (including the aseptic training of the operators), media fills do not absolutely validate the sterilization of the process. The manufacturing process involves manipulations and other activities downstream from the sterilization step, the filtration process; thus, there is no absolute sterilization process that can be validated. The operators can only be qualified, as is the case with any other personnel-dependent process activity. It has been stated by experts in the sterile manufacturing field and by individuals from the FDA at various meetings that during the manufacture of a media fill operation, personnel are more "careful" than during a standard manufacturing operation. Further, in order for a failed vial to be evident in a media filled lot or run, a gross contamination must have been present; that is why the SAL of 3 is a well established and recognized characterization of the capability of this type of process. The assumption that with some types of facility manipulations this SAL can be improved to a level of 4 or 5 or even equivalent to that of terminal sterilization (SAL of 6) is unsubstantiated and is not justifiable based on the current level of data and aseptic experience. It should be noted that even if the process is such that human intervention and interaction is minimized downstream to the sterilization process, the assembly of that equipment train, especially the assembly of the sterilizing filter is in most cases performed manually in an aseptic environment. Aseptic processes are not absolute and thus can never be equivalent to a terminal sterilization process, which by design is absolute.

22.1.3 Traditional Terminal Sterilization Process

A traditional terminal sterilization process, regardless of the means for sterilization, is the same overall process. This means that none of the components are sterilized prior to the sterilization unit operation which occurs after the assembly of the final

and complete product into the final primary package. Each of the unit operations are conducted in a controlled environment for low bioburden and control against contamination of the product under manufacture. For products that are drug/device combinations or complex drug formulations, a filtration step involving the drug may still occur, but this filtration is not claimed nor substantiated to be a sterile filtration step, even if the filter is a 0.2 μm filter. The maximum filter size that is used in a typical traditional terminal sterilization process is a 0.45 μm nominal filter; however, it is not uncommon to use a 0.2 μm absolute filter. This filter is typically not integrity tested after or before use since it is not claimed to be a critical filtration step.

The traditional terminal sterilization process is an all or nothing process; it is designed and validated to be an overkill 12 log reduction process for the entire product load that is being sterilized (assuming that the sterilization run is a batch process). It is possible to have a traditional terminal sterilization process that is a continuous process where a lot can be defined by a process time frame such as those units manufactured in a day as receiving the lot designation for that sterilization run. What makes this type of sterilization a "Traditional Terminal Sterilization" is not the means which is used to sterilize the product, but the overall process for the manufacture of the product and where in the point of the process the unit operation of sterilization occurs. What is critical for this type of process is that no other operation occurs to the sterile product (other than labeling and final secondary packaging which occurs to the exterior of the finished product). If a breach of the sterilized product occurs, then the product is no longer deemed sterile and should be destroyed.

As noted above, typical methods used for terminal sterilization include steam (autoclave), superheated water, dry heat, ethylene oxide (ETO, EO, or EtO), gamma irradiation, and e-Beam (beta irradiation). In the later sections of this chapter, details will be given on the governing principles by which sterilization occurs and how these methods of terminal sterilization can be used for sterilization of controlled release and drug/device implantable products.

One of the significant advantages to terminal sterilization over aseptic manufacture is that a terminally sterilized product can achieve parametric release capability, the ability to be able to release material for sale without having to wait for the completion of sterility testing. In parametric release, products are not sterility tested. The validated process controls are monitored and documented. If the parameters are achieved during the specific execution of that unit operation, then the sterilization load of the product is released as sterile, the sterilization process having achieved the validated process conditions. For product that is sterilized in accordance with the validated sterilization parameters, without breach of conditions, an approved parametric release capability affords the manufacturing company a time savings and provides for further capitalization of the expiration dating without spending time in sterility testing, review of test results, and the chance of spending further time in OOS investigations resulting from lab testing failures.

The traditional method of terminal sterilization that is most known within the pharmaceutical industry is the use of steam over pressure, the autoclave. The mechanism of kill or sterilization is contact with steam at a temperature in excess of 121°C.

To achieve this temperature conditions for steam, the pressure of the chamber needs to be in excess of 1.5 bar, where the boiling point of water is raised from 100 to 121°C and thus results in the thermal death of even the most virulent microorganism, e.g. *Bacillus stearothermophilus*. To achieve kill, the direct contact of the steam to the product or surface being sterilized must be achieved for the required duration to achieve the sterilization endpoint of an SAL of 6 process (overkill process sometimes referred to as a 12 log kill cycle). The overkill process is referred to as a 12 log kill process since the validated cycle is typically designed for a 6 log kill. The validation is performed by the inoculation of the test article with a 6 log population of the test spore-forming organism. The cycle is designed to achieve absolute kill, meaning that the test article is returned from the sterilizer with a result of zero recoverable microorganisms, indicating that all six logs of microorganisms were killed. Mathematically, this would be reported as 10° (or 0 Log), which equates to the numeric value of 1. Thus to truly achieve an absolute zero, sterile state, the cycle is doubled yielding a 12 log kill cycle, and thus achieving a true zero microorganism endpoint rendering the product, by definition, sterile. This concept has been established for the pharmaceutical industry, largely through the work done by the food industry, but has been established for the manufacture of sterile pharmaceutical products and the approach and understanding to sterilization cycle development and validation as defined and determined for the autoclave process is applied in principle to the other methods of sterilization, which will be discussed later in this chapter. The test organisms and test articles are different for the different methods of terminal sterilization; however, the thought process and the technical approach to the understanding and establishment of the cycle definitions and ultimately the validation of that unit operation remains consistent with that defined above for the steam sterilization process.

22.2 Alternative Methods of Sterilization

22.2.1 Gamma (γ) Irradiation

Gamma irradiation has been used for sterilization for decades. The industries where this type of sterilization has been the most prevalent have been the medical device and food industries. Use within the food industry is perhaps more applicable to pharmaceuticals especially where macromolecular entities are the intended target of sterilization. It has been the subject of many studies which had been published as early as the 1960s that examined and discussed the dosing requirements to sterilize meat without causing the negative impact to the produce, thus resulting in the changing of the taste of the product. One such publication showed that meat could be sterilized at a dose up to 100 Mrad without any negative impact to the product [5].

The typical source of the gamma particle is Cobalt 60. There are two types of terminal sterilizers; the most common is the chamber where bins containing packages are loaded and then pulled into the sterilizer chamber on a track. Once inside the

chamber the bins are typically moved around in a form of a moving cube to allow each bin equal exposure and time in each of the various positions allowed by the design. The second type of sterilizer is a continuous belt that feeds into the chamber and then out the other end. The belt passes directly through the middle of the sterilizer chamber without the bins being rotated or formed into a moving cube array as is in the case stated preceding.

The Cobalt 60 serves as the source for the gamma (γ) particle. The γ-particle is a high-energy electromagnetic wave. The "gamma-rays have the smallest wavelengths and the most energy of any other wave in the electromagnetic spectrum" [6]. The mechanism of sterilization (of microbial kill) is by ionization of the chemical bonds within the microorganism. The primary target for the ionization-induced chemical breakdown is that of the nucleic acid of the microorganism. Another molecule within the microorganism that is susceptible to ionization is water, thereby creating more free radicals within the organism for perpetuation of the chemical breakdown within the organism.

The ionization of chemical bonds that occur within the bacteria is the same mechanism by which this method of sterilization will attack the bioactive macromolecule of the drug product. Thus, one needs to balance the dose of γ-radiation necessary to achieve the desired level of sterility assurance with an acceptable level of product degradation. The accepted overkill dose that had been established by the Association for the Advancement of Medical Instrumentation (AAMI) is 2.5 Mrad (25 kGy). At this dose level, it would be very difficult to sterilize a pharmaceutical product and not have some level of impact to the product that would need to be addressed. For products where the drug would survive but the polymeric matrix is used to illicit the controlled release effect, the polymers may be degraded such that the release rate would be faster. Thus, the company would have to consider one of two options, either formulate with a higher molecular weight polymer fraction or manufacture in an aseptic environment and use a lower irradiation dose. The latter option would most likely result in a lower level of degradants in the final product. Thus if the facility used to manufacture the filled containers of product is in fact an aseptic manufacturing facility, then one can establish the use of a lower dose (as low as 18 kGy) or potentially even lower doses are possible and still have an overdose validated cycle.

Recently, the VD_{max} method of establishing or substantiating the required parameters for a validated sterilization cycle has been developed [7] and is achieving acceptability within the regulatory community. A USP monograph is being drafted that will establish the parameters and ranges for specifications and thus qualification of terminal sterilization of products using the VD_{max} approach. What is the VD_{max} approach and how can it work for a pharmaceutical product that is otherwise incapable of being terminally sterilized by the traditional thermal process? The VD_{max} approach is based on knowing the normal or facility flora specifically in the environment of production. It requires knowing and controlling the environment of production. This flora is therefore the most likely source of microbial contamination to a product. The use of more resistant spore-forming bacterial is not required when this method is employed. There is now a recognition by the

regulatory bodies that since terminal sterilization is more desirable than manufacturing by aseptic processing and since spore formers that are typically used for the traditional cycle validation are not common within the flora of a production and environment and thus very unlikely to be a contaminate in the product (a very low probability or risk of contamination), then the more applicable approach to sterilization cycle development and validation is the establishment of a kill cycle based on the most resistant or virulent organism of the flora of the process and facility. This would thus be a risk-based approach, a practical approach for a product manufacturing outcome. To employ this approach for a product or facility requires an active monitoring program and constant assessment of the manufacturing environment flora, especially with respect to the virulent isolates (including a comparison of these isolates to those used to validate the sterilization cycle). It is thus prudent to have the cycle on a re-validation or at least a re-assessment program to determine the current state of acceptability of the defined validated cycle with respect to the current state of virulent organisms within the current flora of the manufacturing environment.

With the VD_{max} approach for cycle development and validation, it is conceivable to have a terminal sterilization cycle for an aseptically manufactured controlled release injectable product where the target sterilization dose for a gamma irradiation cycle could be as low as 5–10 kGy.

22.2.2 e-Beam (β Irradiation)

Beta irradiation, also known as e-Beam sterilization, has gained in popularity over the past 5 years or so due to the improvement of the technology used to deliver the energy source required for this type of sterilization. The particle that is used for this type of radiation is the electron and is emitted from an electromagnetic source and then accelerated toward the target. The potential for penetration is much less than that of gamma irradiation and that, until recently, was the main reason for the limited use of this technology for terminal sterilization of proteins and polymeric controlled release and implantable preparations. The item being sterilized had to be directly in front of the beta particle accelerator source. This is still the case; however, the energy capability of the current machines and the energy source bulbs are such that these devices can now be placed directly on a packing line. In addition, the energy source with accelerator can now produce e-beam sources in the 10 MeV range, thus allowing for the penetration of small packaged products, not requiring the units being sterilized to be single units only. There are many significant advantages of this technology over gamma radiation not the least of which is the ease of use and an increase in production efficiency. An e-Beam sterilizer can be fitted directly onto the production line of a vial or syringe operation either just after capping or at any point downstream until final packaging, resulting in an one line operation for a terminally sterilized controlled release product containing either a small molecule or a biologic.

22.2.3 Thermal Methods: Microwave

Microwave is a form of radiation; however, the mechanism of sterilization is a thermal sterilization process. Therefore, the validation of the process is similar to that of an autoclave in that the test organism is *B. stearothermophilus*. The significant difference between microwave sterilization and an autoclave is the time in which the product is exposed to the latent heat used for sterilization. For an autoclave, the minimal time at thermal kill temperature is at least 15 min for a traditional overkill cycle. For microwave sterilization, the time at the kill temperature is on the order of seconds to milliseconds depending on the temperature used for kill. Due to this extremely short time frame, despite the high temperatures used to acquire microorganism kill, the time and temperature is typically insufficient to cause damage to the controlled release formulation for either small molecules or protein formulations. A case study is presented below where this type of technology is used for the fluid stream sterilization of a liposomal protein formulation.

There are presently two types of production capabilities for this technology. One is a flow-through system where the fluid stream is passed through a microwave sterilizer unit prior to the filling operation. Note that this is not a true terminal sterilization process and still requires aseptic filling and capping. The other process is a true terminal sterilization process and is referred to as RIMM. This type of microwave sterilization technology will be discussed in the next section.

The flow-through sterilization process has a particular use for those manufacturing processes where the sterilizing filtration operation occurs at the beginning of the process and then is followed by several manipulations prior to the aseptic filling of the finished product. In these cases, the finished product that is ready for aseptic filling is not capable of sterilization by filtration just prior to the filling machine. This may be the case for liposomal formulations where the liposome is greater than 0.2 μm in size or for suspension formulations where final filtration would result in the breakdown of the intended product formulation. The product fluid stream is then sterilized by microwave just prior to filling and stopper sealing of the container. The device that is used to sterilize the fluid stream fits into the fluid flow processing stream and has within it a heat exchanger and means to pre-heat the fluid stream prior to the sterilization step. The fluid stream is microwave sterilized in a matter of seconds (or as little as 14 ms at 156°C) and then instantly cooled. All of this is controlled using a predetermined and set flow rate through the device. One such device is available through Charm Science, Inc. of Malden, MA (Dr. Stanley E. Charm, http://www.utherm.com).

22.2.4 Resonance, Interference, and Microwave Method (RIMM)

This method uses the same basic technology as described in the previous section; the principles of sterilization and the mechanism of microorganism kill are the same. What differs is how the microwave energy is channeled and focused upon the

target. This particular device is used such that the final outcome is truly terminal sterilization. This device is placed in the product manufacturing equipment train after the final product container has been filled and sealed. This technology uses a high intensity of microwave energy by having two microwave energy waves traveling from opposite directions focused on the target; this produces more intensity of energy per unit surface area resulting in a high temperature for sterilization. This higher temperature is produced in a smaller footprint then would have been possible for the same level of intensity if a more standard approach were employed, nonresonance interference wave intensification. Because of this intense temperature produced from this intensification of the microwave energy, the residence time required under these latent temperature conditions is shorter than that for the microwave sterilization process described above. The drawback to this particular technology is that currently it is only available for a controlled release product that is packaged into an ampoule. Because of this type of requirement, the use of this technology is limited; this limitation is due to the means by which the contents are sterilized. In steam sterilization, kill is achieved through the induced heat resulting in necessary temperature conditions that sterilize the contents of the container. The actual temperature exterior to the container is used solely to transfer heat through the container to the contents thus inducing the required temperature for thermal kill. The kill conditions within the liquid sterilize the actual liquid and any direct contact surface within the container. This is the reason why the product needs to be in an ampoule for this particular technology. The tip of the ampoule is not in direct contact with the liquid being sterilized; it is actually sterilized by being passed through a dry heat sterilization tunnel built into the microwave sterilization machine. (This machine is only available through Eisai Co. Ltd., who developed the process.)

22.2.5 Gas: Ethylene Oxide

Use of gases to induce sterilization is available through many different possibilities: nitrogen oxides, chloride dioxide, and also ethylene oxide. The most prevalent use of these gases has been in the medical device and food industries. Of the available gases, ethylene oxide (EO or ETO) has seen the most use; it has been used for many years, with the highest use being in the medical device industry. The use of EO in the pharmaceutical and biotech industry has not been significant due to the perceived potential impact to product stability. With the advent of newer technologies for design and, most importantly, the out-gassing of the EO to acceptable levels in the final product, the impact of EO to product instability can be abated. The use of EO has become more prevalent in the last 5 years with the advent of drug/medical device combination products.

These products have a primary use as a medical device that is augmented by the use of a drug that is predominately released from a controlled release formulation which is incorporated into or onto the medical device. If the primary method of sterilization for the medical device is the use of a gas such as EO, then typically the

medical device manufacturer will opt to use EO as a method of sterilization for the medical device/controlled release drug combination product.

For sterilization by EO to be effective, there has to be direct contact of the gas (the sterilant) and the intended area being sterilized. Therefore, in medical device product sterilization, the device is packaged in a final pack that permits the penetration of the sterilant and then the poststerilization cycle allows for the dissipation of that gas to a level that is acceptable to the end user and the product. This method of sterilization has proven to be effective not only for the medical device industry but also effective in the terminal sterilization of pharmaceutical and biotech products formulated as controlled release and implantable preparations. The key is the ability of the gas to penetrate to the product through the packaging of the finished product. Although this method of sterilization is acceptable for controlled release products containing small molecules or biologics, it is dependent on how the formulation is packaged in the primary container.

Finally, as with any type of unit operation that is used in the course of manufacture, potential impact to the product needs to be assessed prior to the full implementation or technology transfer of the product into the commercial manufacturing facility. This is especially true for sterilization processes, be they thermal, irradiation, gas or passive sterilization.

22.3 Aseptic/Terminal Sterilization Combination Processes

Aseptic manufacturing, as described in the earlier section of this chapter, involves the sterilization of each of the components of the product prior to the final assembly of the product. The components referred to here are not only the packaging components but also include the drug product formulation. Thus, the product formulation is aseptically sterile filtered through a 0.2 μm sterilizing grade absolute filter. The filter must be selected for compatibility with the product, then sized based upon the surface area requirements for the intended batch size and then qualified for microbial retention. Additionally, the sterilization of the filter using either a sterilize-out-of-place (SOP) process (autoclave or irradiation) or a sterilize-in-place (SIP) process must be validated. Once the sterilizing filter has been assembled into place – double filtration is required by the European Authorities – the product fluid stream is encompassed between the filtrate holding vessel, the filtration fluid path and the final sterile bulk holding vessel. Once the product is sterilized by filtration, the product is filled into prewashed and depyrogenated containers. At this point, the container can either be closed hermetically as in the case of glass ampoules or stoppered as in the case of vials. If stoppered, the stopper can be inserted completely or partially in the case of the lyophilization process requirement. Stoppers that are fully seated are not considered completely sealed since the over seal is not yet in place. The over seal is placed onto the vial at the capping station which may or may not be within an aseptic environment. Once the over seal is crimped into place over the stopper and vial, then the vial is considered sealed and the process for aseptic manufacture

is complete. For those vials that are to be lyophilized (also known as freeze drying), the vial and the contents remain open until after the lyophilization process is complete; the stoppers are seated into the vials prior to the opening of the lyophilizer chamber door. (The stoppers are seated into the vials by the use of the collapse of the shelves to seat the stoppers.) However, as with the case of the stoppered vials above, even if the stoppers are seated, the vials are not considered sealed until after the over seal has been placed and crimped. This entire process of filling into the vials or ampoules until the stopper seating process must take place in an aseptic environment.

Once the product has been manufactured by this aseptic process, the product, prior to individual unit labeling is sent to an autoclave for terminal sterilization using steam. The requirement by the European Authority is that these product vials need to be sterilized within 48 h of the first draw of water for the compounding of the product. For aseptically only manufactured product, the EU requires that the product must be filled and sealed in the final container within 24 h of draw of water for the compounding of the drug solution.

The aseptic environment in the United States is typically referred to as a Class 100 dynamic environment. With the European Authority this environment is referred to as a Class A/B zone. Whatever it is called, the requirement is that filling and processing environment be monitored for viable and nonviable particulates. The classification requires that the airborne particulate level is controlled and validated to be below 100 particulates per cubic foot per hour and is actively monitored using both an air probes for nonviable particulates and settling plates for viable particulates (microbiologically active particulates). Class A/B per the European Union is specified such that Class A is equivalent to Class 100 dynamic conditions and Class B is equivalent to Class 100 static conditions. The Class A environment has to be within the Class B environment which thus makes up the background for the Class A region, which is typically confined to those areas housing the open container (discharge from the depyrogenation tunnel), the filling line up to and including the stoppering line until the container passes into the capper zone.

There needs to be documented proof of the ability to render the product sterile prior to the aseptic manufacture of a product in addition to the continual monitoring and documentation of the aseptic state of the production environment. Especially important is the aseptic control of the product through each unit operation that occurs after the aseptic sterilization of the product stream. Any breach of the controlled state after the sterilization point for the product will render the product as a failed lot, incapable of release to the consumer.

Many new facilities that are constructed to manufacture sterile small volume injectable products are designed as aseptic manufacturing facilities because for many professionals this is the only means of sterilizing many products currently being developed, especially lyophilized and/or large molecular compounds such as proteins. It is rare to see a facility designed solely for the manufacture of a product by terminal sterilization only. Generally, this makes good sense if a company wants to have one facility that can manufacture both aseptic and terminally sterilized products rather than a facility for each type of process. This is why if a product is to be

terminally sterilized, it is not unusual for it to be aseptically manufactured and then terminally sterilized. This is an added benefit since the product is manufactured under a strict control from potential environmental contamination and then finalized with the added assurance of terminal sterilization. This last step renders a level of absolutism that is afforded from the terminal process unit operation. It should be understood that if aseptic manufacturing is stated to be the means or method of rending the product sterile, then the sterile filter must be integrity tested per production activity. For the EU, this requires a pre-use integrity test as well as redundant sterile filtration of the fluid stream. For those that are only manufacturing for the U.S. market, a post-use integrity test is the only qualification proof that is required for each lot of sterile product sterilized by this method of manufacture. Although, some may argue that an integrity test is critical, this is more a carryover of the historical use of 293 flat stock filters. Most filters used today are preassembled by the filter manufacturer and integrity tested prior to being shipped, so the need to integrity test is not as critical as it once was and for products that are in a nonaqueous formulation, this test is definitely not value-added.

22.4 Case Studies

22.4.1 Case Study 1: Terminal Sterilization of Microspheres Using Gamma Irradiation

In this example, a pharmaceutical controlled release product was developed for the delivery of an analgesic by formulation into an intramuscular depot consisting of co-polymers poly-lacto-glycolic acid (pLGA) and poly-lactic acid (pLA). The manufacturing process involved the sterile filtration of each of the components of the formulations into their respective solution tanks; each of the polymers were dissolved into ethyl acetate and the drug into the same tank and the water for injection sterile filtered into a separate tank. From this point on, the product was processed in a closed presterilized in-place equipment train and then dried using a vibratory vacuum dryer. The dried microsphere encapsulated drug powder was then removed from the drying unit and filled into powder filling canisters. Although the process was controlled as strictly as possible, including clean-in-place and sterilize-in-place for the equipment train, the process was not considered or documented as aseptic manufacture. The dry powder-drug-encapsulated microspheres were then filled and stoppered into vials using a drug powder-filling machine. The filled, stoppered, and then crimped sealed vials were tray-packaged into packs of 25 vials. The packs were then placed into a specially designed master shipper which contained four trays of vials. The master shippers were transported to a gamma irradiation facility where they were terminally sterilized using gamma irradiation at a target dose of 25 kGy. The maximum exposure for the run for the entire load was 40 kGy. These values were determined using dosimeters that were placed at the worst case location

for penetration (to establish minimum kill dose) and the best case location (to monitor maximum exposure dose for the product).

The minimum kill dose was based on the presumption that the current standard set by AAMI as 25 kGy had to be achieved as per the requirements for terminal sterilization (by overkill). Today, this approach would not be necessary (as was discussed earlier in this chapter). The VD_{max} approach is a method that may be used to substantiate a lower kill dose for this terminal sterilization cycle and thus a lower gamma irradiation dose could most likely be validated and approved. Regardless, this example shows that terminal sterilization is a viable approach for polymeric controlled release product formulations and should be considered during product development rather than directly assuming that aseptic processing is the only means of manufacture for these types of preparations.

22.4.2 Case Study 2: Thermal Sterilization of a Liposomal Product Fluid Stream Using Microwave Sterilization Technology

This example involves a multi-laminate liposomal formulation with a protein entrapped with the lipid layers. This product was manufactured using aseptic process technique. During development and initial scale-up, the process involved over 99 aseptic connections. In an effort to minimize this potential product liability, many of the connections were eliminated by transferring into a dedicated equipment train where many of the aseptic connections were converted to welded connections. This still left approximately 20 plus aseptic connections that could not be converted. This equipment train was sterilized-in-place using steam (SIP) and then the major components of the train, which were separately sterilized either by SIP or by use of a parts autoclave, were transferred to a sterile filling suite and aseptically assembled.

The product was manufactured by aseptic processing. Each of the components were dissolved in the appropriate formulation solvent and then sterile-filtered into the central mixing tank where the liposomal product was formed. The entire process of pumping, extruding, and other further processes all continued aseptically in the closed sterilized equipment train within the Class 100 clean room.

The microwave sterilization process that was employed for this product was of the flow-through type. It was added just prior to the filling machine. The fluid stream of the product was pumped from the holding tank through the microwave sterilizer, through the filling manifold, then through the filling needles and finally into the presterilized glass product containers, which were stoppered and crimp sealed as is typical for a sterile product. As stated above, this would not be considered true terminal sterilization since the sterilization step occurs prior to the filling and sealing of the final product, but it did constitute an enhancement to the sterility assurance of the product. Much like adding an additional sterilizing filter to a fluid stream prior to filling can enhance the sterility assurance for an aseptic product, this thermal operation afforded an additional level of sterility assurance for a product

whose sterilization step occurred at the beginning of the labor and manipulation intensive process. Unlike filtration, this thermal process would be accepted as providing greater sterility assurance, since the qualification and validation of this unit operation would be similar to that of an autoclave validation (where the process of fluid stream sterilization cycle is validated using a thermally resistant spore-forming microorganism).

22.4.3 Case Study 3: Terminal Sterilization by Ethylene Oxide Gas of a Drug, Polymer, and Medical Device Combination Product Implant

This example involves a medical device that is co-extruded with a drug/polymer matrix formulation to yield a medical device implant containing a controlled release drug formulation. The entire product was manufactured in a nonaseptic manufacturing operation and thus it was required the final primary packaged product be terminally sterilized. The method that had been used for the medical device had always been ethylene oxide gas (EO). It had been shown that EO did not negatively impact the drug potency or long-term stability or the release rate of the drug/polymer controlled release formulation. The final product was packaged in a Tyvek pouch with a breather flap that allowed for the EO gas to penetrate into the package and directly come in contact with the product surfaces of the device and the controlled release formulation. The EO gas was then removed to an acceptable residual level through cycles of vacuum and nitrogen purge. At this point, the final package was sealed and the Tyvek flap was removed. This process proved to be effective in the terminal sterilization of a controlled release formulation containing a biologic protein and a co-polymer matrix contained within the medical device. Additionally, the process allowed the long-term functionality of the medical device drug combination implant to be retained.

22.5 Conclusion

The purpose of this chapter has been to show that there exist options for the sterilization of controlled release or implantable sterile pharmaceutical preparations and that the typical initial assumption of aseptic processing is not the only possible manufacturing method. This chapter has presented a representation of what is involved with each method or process approach used in the production of a sterile controlled release or implantable pharmaceutical product. As with any method of sterilization, thermal, irradiation, gas or passive (e.g., filtration), the impact to the product must be addressed during development and then confirmed during process transfer and scale-up. This impact assessment requires a formal study design protocol and report and should be included in the development report for the final product.

Finally, the case studies have provided examples of how these principles can be applied for three different types of controlled release formulations. The first was a microsphere formulation that was sterilized using gamma irradiation, the second was a liposomal product containing a biologic protein interwoven within the layers of the multilaminate liposomal preparation that was sterilized using a microwave sterilization process, and the third was a drug/device implantable combination product that was terminally sterilized using a sterilant gas.

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Chapter 23
In Vitro Drug Release Testing and In Vivo/In Vitro Correlation for Long Acting Implants and Injections

Michail Kastellorizios and Diane J. Burgess

Abstract An advantage of long acting implants and injections is their ability to maintain a relatively constant drug concentration at the site of interest. In vitro drug release testing, particularly where an in vivo/in vitro relationship has been established, can help in ensuring in vivo performance. There are various in vitro release testing methods, which are chosen, based on availability, dosage form specifications and drug properties. These are categorized into three groups: sample and separate, flow-through and dialysis. Although there are currently no standard methods for in vitro release testing of controlled release parenterals, standard dissolution apparatus specified by the United States Pharmacopeia have been adapted in some cases and the development of compendial monographs is anticipated. An in vivo/in vitro correlation can predict the bioavailability characteristics of a product based on release in vitro.

23.1 Introduction

The controlled and sustained delivery of therapeutics has attracted a lot of attention over the past decades due to the advantages that such formulations exhibit over conventional dosage forms. One of the main reasons is the ability of such formulations to maintain therapeutic drug concentrations for a long time [1–10]. On the contrary, maintaining even an approximately constant concentration of a drug after repeated administration is very challenging [4, 11–14]. Figure 23.1 shows a schematic of the concentration pattern that follows repeated drug administration.

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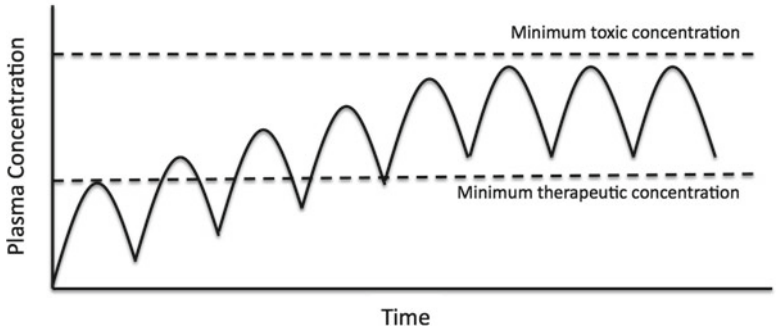

Fig. 23.1 Plasma concentration versus time after repeated administrations

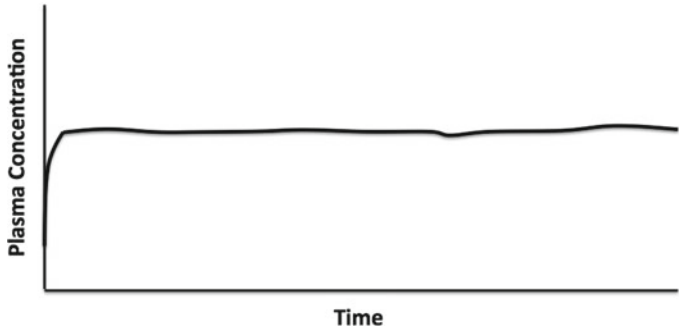

Fig. 23.2 A typical in vitro release profile obtained from push–pull osmotic pump systems where the plasma concentration remains approximately constant for the duration of the treatment

Ideally, the concentration of a drug at the site of interest should remain not only constant, but also well within the boundaries defined by the minimum therapeutic and toxic concentrations. Long acting implants and injections have the ability to release drugs in a sustained fashion [4, 15]. This prolonged release in combination with drug elimination from the site (which usually follows first-order kinetics) results in an approximately constant concentration of the therapeutic at the site of interest.

The drug delivery system that offers the most effective constant in vivo release profile is based on an osmotic pump principle [16–21]. The first and most popular of these systems is the OROS® system, developed by Alza. These systems release drug continuously due to an osmotic pressure difference effect. Figure 23.2 demonstrates a typical in vivo release profile from such a delivery system. (The osmotic pump principle has been adapted to parenteral implants; refer to the chapter on Implantable Systems based on Osmosis.)

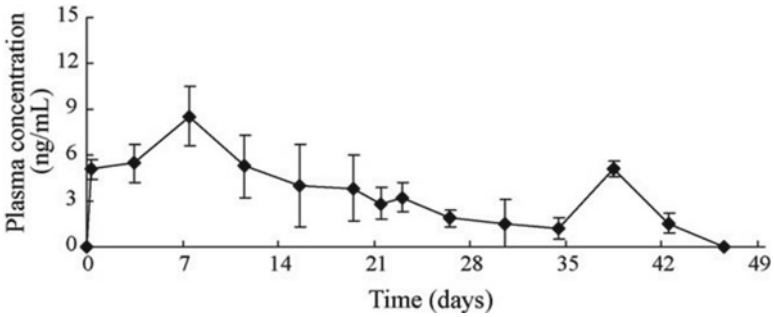

Fig. 23.3 Plasma levels of HupA in rats after subcutaneous injection of drug loaded microspheres [22]

However, other than the osmotic pump system, most parenteral controlled release systems produce only an approximately constant release profile, typically beginning with an initial burst release (such as the HupA microsphere system shown in Fig. 23.3) [22].

The product release profile will affect the pharmacodynamic response. For example, in some cases an initial burst release can be useful in providing a bolus dose to help control a specific condition or disease [23]. Parameters such as burst release, release rate and mean release time directly affect the maximum concentration that is achieved as well as the overall duration of treatment.

When developing a controlled release formulation, it is crucial to have a good understanding of the drug release kinetics in vivo in order to ensure effective and predictable product performance. However, in the early formulation design stages, it is not practical to test each formulation in vivo due to the time, expense and animal life that this would involve. Accordingly, there has been a great deal of research effort around the development of appropriate in vitro release models that can provide a predictive tool to ensure product performance and batch-to-batch reproducibility [2, 24–28].

In vitro release methods should be relatively easy to perform and ideally should be predictive of in vivo release. If an in vivo/in vitro correlation (IVIVC) can be established, then the in vitro release profile obtained for different batches can be used to ensure product behavior in vivo, for both quality control purposes and bioequivalence studies [29]. Accordingly, the FDA may grant a biowaiver for cases such as manufacturing site changes, raw material supply and instrument changes. It should be noted that it is not possible to develop an IVIVC for all drug products. However, an in vitro release test can be developed which satisfies the requirements for batch release of product. Such in vitro release tests must be able to discriminate between in specification and out of specification batches and should demonstrate a complete or greater than 80% release of drug [30, 31].

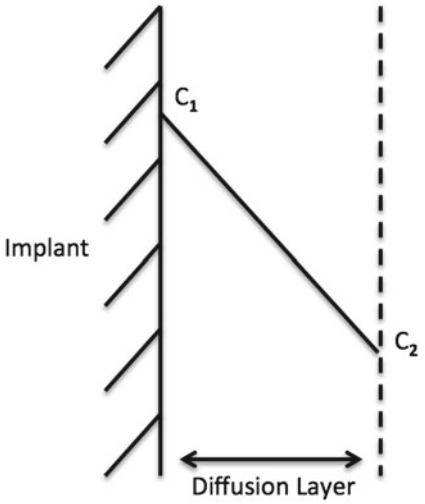

Fig. 23.4 Diffusion layer and concentration gradient surrounding the implant. C_1: Drug concentration near the implant (*high*); C_2: bulk concentration in the receptor solution (*low*)

23.2 Parameters to Consider When Developing In Vitro Release Tests

23.2.1 Sink Conditions

In the case of sustained release parenterals, sink conditions are defined as the conditions where if 100% of the labeled drug amount were released into the media, the concentration would not exceed one-third of the saturation concentration [32]. It is common practice to maintain the drug concentration in the media less than 10% of the saturation concentration at any given time [33]. The physicochemical significance of maintaining sink conditions is to ensure that the rate and extent of dissolution will not be affected by any disturbance of the concentration gradient [34]. The concentration gradient is the driving force when the drug escapes the implant/injection. Figure 23.4 demonstrates the diffusion layer that forms around the implant and the concentration gradient through it.

Extra care needs to be taken in order to avoid any breach in sink conditions throughout the study. There are several steps that may be taken in order to achieve this. First of all, the saturation concentration of the therapeutic molecule needs to be estimated and the total as well as the sampling volume should be adjusted so that sink conditions are not breached. In some cases, when a drug has very low saturation concentration, total media removal during sampling is necessary. Flow-through dissolution methods are able to maintain sink conditions rather easily, due to the ease of media replacement without sample loss, and the lack of a saturated layer around the implant as a result of the laminar flow conditions (this will be discussed later).

23.2.2 Burst Release

There is no official definition of burst release, a fact that has allowed the creation of multiple gray zones in this subject. Usually, burst release is considered the amount of drug released from a delivery system up to 24 h after administration. Some researchers do not use specific time points to define the burst release phase; instead they base their definition on the observation of a change in the release rate [from first-order release to zero-order release or no release at all (lag phase)].

A convenient approach to defining burst release is to calculate the theoretical amount of drug released due only to diffusion from the surface of the delivery system. This might prove challenging in the case of short-term delivery systems. One example is release from polymeric systems with low molecular weight. In this case, the amount of drug released in 24 h may include both drug release as a result of diffusion of surface associated drug (burst release) and drug released from the polymer matrix due to polymer degradation.

In order to avoid confusion and allow comparison of data between laboratories, a standard definition of burst release needs to be established.

23.2.3 Drug Stability

A problem associated with in vitro release testing of slow releasing products is that released drug is susceptible to degradation in the aqueous media and considerable degradation may occur prior to the end of the study (which may take days or even months). This leads to inaccuracies in the determination of the release profile. Small molecules are usually subject to chemical degradation (e.g., hydrolysis), while large molecules such as proteins are also subject to physical denaturation and subsequent aggregation [35].

Since the primary role of the media is to successfully mimic the in vivo conditions, it is better not to make extensive adjustments to the media to prevent drug degradation. Rather, changes can be made in terms of sampling (such as increased frequency and total media removal if necessary) and sample storage conditions (such as pH and temperature adjustment). Depending on protein stability, samples may be either frozen or lyophilized prior to analysis. In the case of drugs that are susceptible to hydrolysis, the pH of the media can be adjusted postsampling.

A drug stability study is often carried out in parallel with dissolution testing, and the stability profile can be used to adjust the drug release data to account for drug degradation. Kim and Burgess have reported this type of analysis for an antibiotic microsphere system [36].

An interesting approach to tackle the issue of insufficient drug stability in the dissolution media was suggested by Viosine et al. They calculated the isosbestic point (the wavelength at which the drug and its degradation products have the same absorbance) for the released drug, and compared the cumulative release calculated at the isosbestic point and at the UV max. An incomplete release profile was

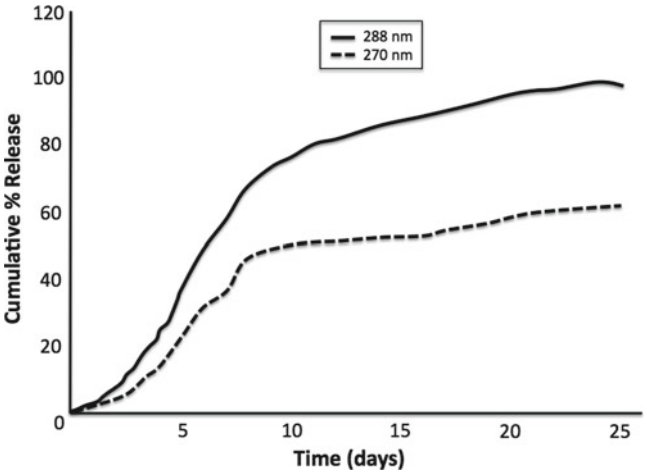

Fig. 23.5 Comparison of cumulative release profiles from cefazolin sodium microspheres obtained by UV max (*continuous line*) and isosbestic point (*dashed line*) (redrawn from [37])

observed when the calculation was made using the UV max data. However, a complete cumulative release profile was obtained using the isosbestic point data (Fig. 23.5) [37].

Another factor that needs to be considered is drug stability within the dosage form during the release testing period. As well as degradation in the media, it is possible that the unreleased drug may degrade. When degradation of unreleased drug within the dosage form is of a significant level, this must be taken into consideration. This is of particular concern, as it would imply that the drug could degrade prior to release in vivo. This would decrease the total amount of drug available and also there is a possibility that the degradation product(s) could have toxic effects (e.g., in the case of protein therapeutics this could lead to immune response).

23.2.4 Extent of In Vitro Release

It is generally accepted that at least 80% of the label claim amount of drug in the dosage form should be released from a long-term drug delivery system [38, 39]. It is important to understand the reason for incomplete release (e.g., due to drug instability in the media). As mentioned above, proteins are susceptible to denaturation and aggregation after release into the media and care needs to be taken in order to prevent such aggregation. Otherwise, a falsely incomplete release profile will be observed that will not be truly representative of the release in vivo.

At the end of the in vitro release test, the amount of drug remaining in the formulation can be analyzed to determine if this together with the cumulative percent release is consistent with the theoretical drug loading. This is further complicated if significant drug degradation has occurred and in this case the analysis must include determination of the degradation products.

23.2.5 In Vivo Relevance

When designing an in vitro release method, it is pivotal to select appropriate conditions and method design that allow for good in vivo predictability. Conditions such as temperature (physiological or elevated), agitation, sampling method, as well as pH and buffer capacity of the media have to be fine tuned in order to achieve a method that mirrors the in vivo release [40]. In addition, product and drug stability in the media during the study as well as a logical time frame for the study must be taken into consideration (refer to section below on accelerated release testing).

23.2.6 Accelerated Release

"Real-time" in vitro release testing is crucial in the development of new drug delivery systems, as it can offer a complete view of the release kinetics and some insight into the mechanistic factors governing release. Nevertheless, in the case of formulations that are designed to release long term (i.e., weeks to years), an accelerated release test should be developed for batch release of product [41–44].

Acceleration in release may be obtained by altering the temperature, level of agitation, solvent, presence of surfactants or enzymes, etc. As discussed earlier in this chapter, all these parameters may affect drug stability and therefore care needs to be taken while altering these parameters.

The accelerated release profile should be predictive of the real-time release profile. Moreover, it should be biorelevant. It is accepted that the two release profiles should exhibit specific correlation in three time points: one early on, one in the middle of the release testing and one near the end (>80% cumulative release). Model-dependent approaches have also been suggested, where the real-time and accelerated data are compared following mathematical modeling [42].

23.2.7 Dissolution Vessel Selection

Other than for specific USP apparatus, dissolution vessel specifications such as dimensions and the type of material can vary significantly. Such variation can lead to considerable differences in the release profiles obtained making it difficult to compare data between different analytical groups. For example, variations in vessel size and shape can affect hydrodynamics and hence drug release [45, 46]. Gao et al. have reported some differences in the dissolution of prednisone using four different commercially available vessels [47].

Vessels are usually made of either glass or styrene acrylonitrile. Generally, high-quality glass is preferred, although plastic vessels do exhibit several advantages. Plastic containers show less variation in size, are easier to work with and are economic. More importantly, plastic demonstrates less protein adsorption as compared to glass.

On the other hand, plastic tends to deform and lose its transparency over time, is susceptible to scratches that may lead to extended particle adsorption and any leachables may interfere with sample analysis. Glass or appropriate plastic vessels should be selected to avoid any significant drug adsorption, which would affect the release profile.

For all these reasons, when setting up a dissolution test it is important to clarify the vessel specifications instead of merely the media volume, as is commonly practiced. This will allow for ease of method transfer enabling researchers to accurately recreate the dissolution testing and acquire comparable results.

23.3 Methodology

Currently there is no standard in vitro release testing method for parenteral products. Therefore, researchers have used a variety of methods including USP apparatus designed for other routes of administration as well as custom-designed methods.

The various in vitro release methods that have been utilized for parenteral products may be categorized into three general groups: sample and separate, continuous flow and dialysis. Even though all demonstrate certain advantages, they also have limitations. In addition, hybrid methods have been designed, such as a dialysis/flow-through.

It is important to have a clear view of the drug release mechanism in order to select/design an appropriate dissolution apparatus. Choosing the right apparatus has proven to be pivotal since literature has reported significant variations in release profiles between different apparatus [48–50]. Figure 23.6 shows an example where two different flow-through apparatus were used for the same formulation and the resultant in vitro release profiles were very different.

23.3.1 Principles of In Vitro Release Testing

23.3.1.1 Sample and Separate

Sample and separate is the most popular method for particulate parenteral products, mainly due to its simplicity and practicability [50–62]. In general, micro- or nanoparticles are suspended in a certain amount of release media and the system is subjected to agitation. Sampling occurs at different time intervals, the samples are then filtered or centrifuged to separate the particles from the media and this is followed by media replacement to the dissolution vessel. Figure 23.7 shows a schematic of the sample and separate method. This technique is also used for implants, although the separation part simply consists of transfer of the implant to fresh dissolution media.

There are several parameters that may be adjusted in order to facilitate release, such as the level and type of agitation, media volume, sample separation techniques and sampling volume.

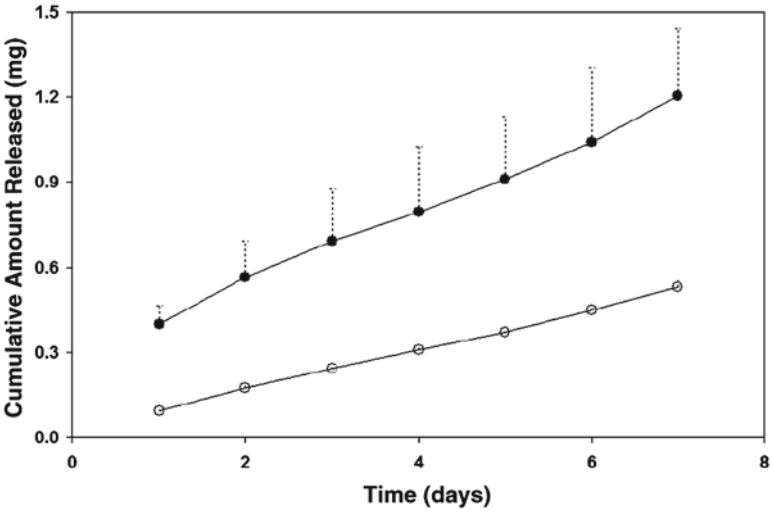

Fig. 23.6 Comparison of two flow-through dissolution apparatus using a capillary device (*void circles*) and a flow-through cell (*full circles*) for release from naltrexone implants [47]

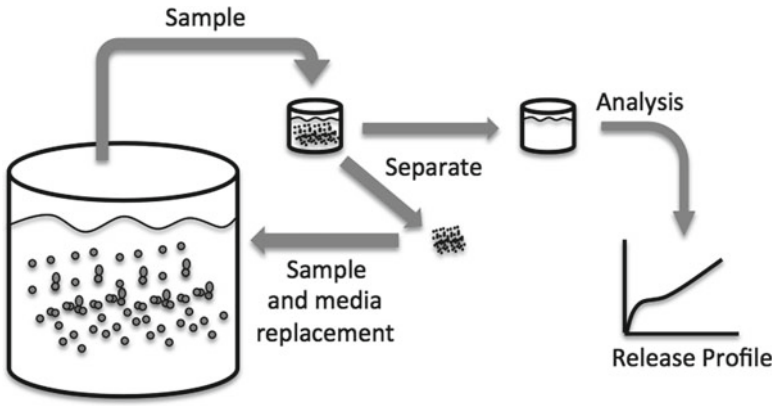

Fig. 23.7 Schematic representation of the sample and separate method

Agitation: Typically, an agitation level that is representative of physiological flow rates is the goal and this is achieved at approximately 100 rpm. In the case of accelerated release studies, more vigorous agitation may be used. There are various means to integrate agitation into an in vitro release study: magnetic stirrer, shaking water bath, shaking incubator, as well as bottle rotation [51, 63, 64]. All these methods are acceptable and they are chosen based on availability and compatibility with the overall dissolution testing apparatus. There have also been reports where no agitation was employed [65].

The volume of media used depends mainly on drug solubility but is also affected by the apparatus used. In vitro release testing methods have been reported using

vials with volumes less than 500 μl, as well as larger containers with volumes up to 500 ml. Obviously, the maintenance of sink conditions throughout the release study is a driving force for selecting media volume.

Sample separation: Sample separation may be achieved either by filtration or by centrifugation [4, 36, 61, 62, 65, 66]. In the first case, the sample is pumped through a filter with appropriate pore size that allows no particulates to pass through, and the analysis takes place either in the supernatant or by retroanalyzing the filtered particulates. This technique, although it offers total separation, is tedious and there is a possibility of either the polymer or the drug interacting with the filter material. In the second case, the only difference is that the sample is centrifuged instead of being filtered.

Sample separation by means of filtration or centrifugation, apart from being labor intensive, leads to volume and/or sample loss. Alternatively, total particulate removal and analysis at different time intervals minimizes this deficiency. However, large amounts of particulates are needed, deeming this approach impractical especially when high manufacturing costs are involved.

There have been reports of particulates being simply allowed to settle on their own and then sampling from the top of the media [67]. In some other cases, sample separation was not necessary and analysis was performed in situ [68]. For example, in the case of liposomes encapsulating a fluoroscent dye, a very small fraction of the total sample volume was needed for analysis and the liposomes did not interfere with the analysis of the released fluoroscent molecule; thus there was no need for sample separation.

Sampling volume: Sink conditions play an important role in determining the sample volume. For poorly water soluble drugs, complete media removal at each sampling point is necessary in order to maintain sink conditions. Another critical factor that accounts for complete media removal is inefficient drug stability in the release media. If the maintenance of sink conditions and the stability of the drug are not a concern, the sampling volume depends on the sensitivity of the quantification technique used for sample analysis and the separation method requirements.

In the standardized sample and separate method (USP Apparatus 2, as outlined below) large media volume is required, which makes this apparatus impractical for small-volume injectable dosage forms. In addition, the use of surfactants is often necessary in order to prevent particulate aggregation [69].

Overall, sample and separate techniques are easy to use and do not necessarily require expensive instrumentation. This technique is used for a wide range of release testing, including real time and accelerated. Shortcomings include particulate aggregation, large media volume, labor intensiveness and inaccuracies due to sample loss.

23.3.1.2 Continuous Flow

Continuous flow release methods have been used in in vitro release testing in order to assist in simulating in vivo conditions [48–50, 70–77]. A continuous flow apparatus consists of a flow-through cell that holds the sample, a filter at the top of the

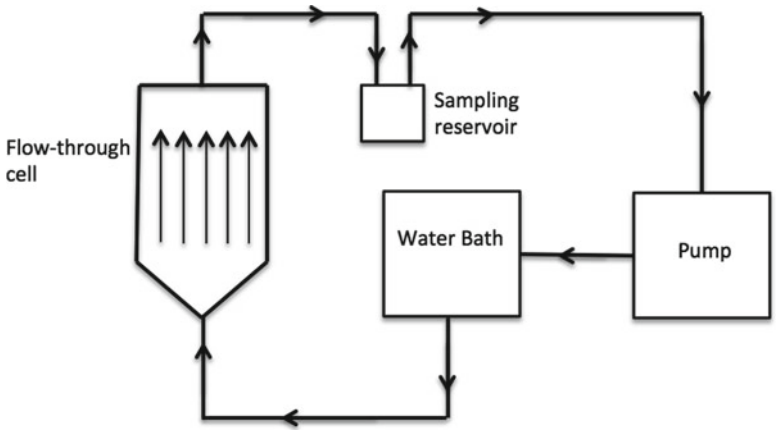

Fig. 23.8 Schematic representation of a *closed loop* flow-through apparatus

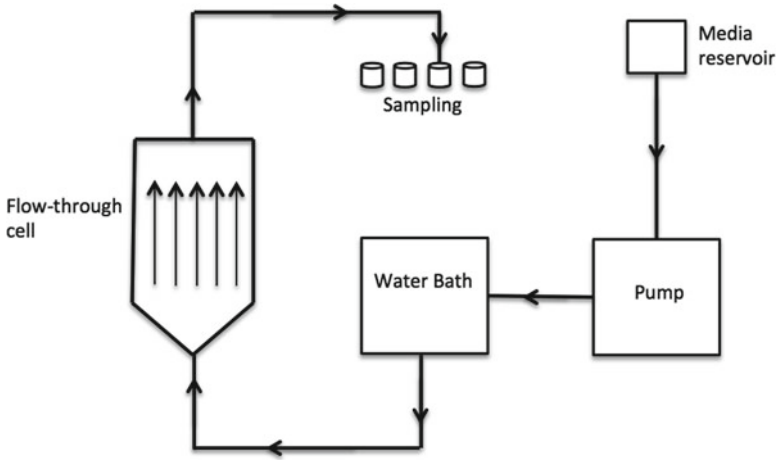

Fig. 23.9 Schematic representation of an *open loop* flow-through apparatus

flow-through cell to prevent particulates from passing out of the sample cell, a pump that drives the dissolution media through the flow-through cell, a water bath that maintains the media at a desired temperature and a media reservoir [75]. The apparatus may be set up in a closed (Fig. 23.8) or an open loop (Fig. 23.9) configuration.

In the closed continuation the media is continuously circulated, whereas in the open configuration a sample collector is attached and media only passes through the cell one time. In the case of the open loop configuration, the flow rate is representative of drug clearance from the release site (either subcutaneous or intramuscular in the case of long-term implants and injections) and has been shown to affect the extent as well as the kinetics of drug release for diffusion-controlled release.

Although commercial apparatus based on continuous flow are available, some researchers use in-house fabricated equipment [74].

There are certain parameters that may be adjusted in the continuous flow method such as sample immobilization, flow rate and media recycling.

Sample immobilization: Glass beads can be used to assist in positioning formulations such as tablets, implants and microparticulates [78]. The glass beads also serve to maintain laminar flow conditions. Glass beads have been used to prevent microsphere aggregation and incomplete release profiles. This modification of the flow-through cell with glass beads is under investigation to be adopted as a standard in vitro release testing method for microspheres (refer below).

Flow rate: The continuous flow in these systems is established with the aid of a pump. Three types of pumps have been employed: peristaltic [37, 48, 50, 78], syringe [79] and high-performance liquid chromatography (HPLC) [80]. These pumps cover a wide range of flow rates, from 0.4 μl/ml (HPLC pump) to 200 l/h (peristaltic pump).

The flow rate greatly affects the release profile observed for dissolution-controlled systems. In principle, the flow rate will affect the hydration of the material, and thus drug diffusion. Low flow rates can result in slow and incomplete release due to insufficient media transfer.

Media recycling: In the case of an apparatus with a peristaltic pump, the media may be recycled back to the sample chamber through a closed circuit set up. Recirculation of the buffer is more practical in terms of sampling and total volume of media required, given that sink conditions are maintained. For this, the solubility of the drug needs to be taken into consideration, just as in other release testing methods. Alternatively, fresh buffer may be pumped through the flow-through cell (open circuit).

The continuous flow method, which was originally developed for controlled release oral products [75], has been suggested as the most appropriate method for controlled release parenterals [38–40, 50, 81–83]. Disadvantages of this method include possible filter clogging by nanomaterials and polymer degradation products, which can result in back pressure. Moreover, the high surface due to the tubings and glass beads may lead to significant drug adsorption. However, this can be overcome by appropriate selection of tubings and beads and/or the use of surfactants. Additionally, although there is no limit to the maximum amount of media volume that can be used, the minimum value is not as flexible. A decrease in media volume (i.e., 10–20 ml) disproportionally increases backpressure and the flow rate should be adjusted accordingly.

23.3.1.3 Dialysis Methods

In this method, the sample is isolated from the bulk media by placement in a dialysis sac. This sac consists of a porous cellulose membrane with various molecular weight cut offs (MWCO) that allow the released drug to diffuse out of the sac and into the bulk media for sampling [2, 48, 84–92].

It is common practice to use membranes with MWCO ten times greater than the released compound. This ensures unobstructed drug diffusion through the membrane and equal drug concentration inside and outside the dialysis sac.

This technique has been extensively used in the past for controlled release lipophilic solutions [93], suppositories [94] and for nanoparticles such as liposomes [48]. This technique has also been used for emulsions, micelles, oral suspensions and implants. In order to maintain a driving force for drug diffusion from the dialysis sacs, the inner dialysis volume is maintained significantly lower than the bulk volume (from 10 to 100 times less based on drug solubility in the media). In addition, the bulk media should be agitated. There should be no breach in sink conditions either inside or outside the dialysis sac.

Agitation may be achieved by use of a magnetic stirrer, a paddle and a horizontal shaker. There are commercially available dialyzers (specially made vials with dialysis sacs inside) that are easy to assemble and convenient to use [2].

Dialysis techniques fall in two general categories: dialysis where the drug is placed inside the dialysis sac and sampling takes place outside; and reverse dialysis where the drug is placed outside and sampling takes place inside the sac.

Regular Dialysis

Samples may be placed in a dialysis sac where drug diffusion takes place all along the dialysis sac wall (Fig. 23.10) or samples may be placed in a tube where only one end bears a dialysis membrane (smaller drug diffusion surface area). There has been a lot of controversy on the appropriateness of the dialysis technique for in vivo relevant release testing. It has been shown that in vitro release profiles obtained with a dialysis sac do not correlate well with in vivo data for both oral and intravenous delivery systems [95, 96]. This is thought to be due to lack of agitation and consequent aggregation inside the dialysis sacs. This is particularly problematic for microparticulate systems. Aggregation may lead to slow and incomplete release and cause variations in the release profiles. Lack of sufficient stirring inside the dialysis sac creates static media layers around microparticles that can slow down polymer hydration and drug diffusion. In the case of subcutaneous and intramuscular injections, where the sample is practically immobilized and can be surrounded by a stagnant membrane, it is speculated that dialysis may offer good in vivo predictability [97]. Another problem with the dialysis sac technique is that the drug may be released from the formulation (such as emulsions or liposomes) relatively rapidly. The rate-controlling step in drug release from the formulation can then become transfer of the drug across the dialysis membrane and although sink conditions may be maintained in the media outside the dialysis sac, there may be violation of sink conditions within the dialysis sac. This was reported by Chidambaram and Burgess for emulsion systems and to overcome this problem, the reverse dialysis sac technique (refer below) was proposed [84].

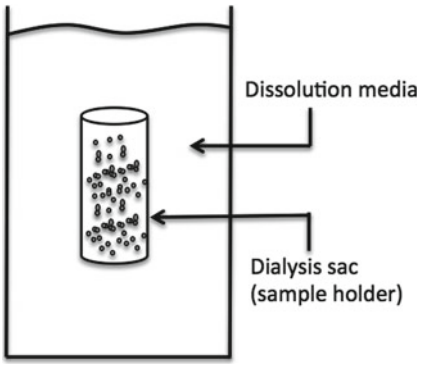

Fig. 23.10 Schematic representation of dialysis technique

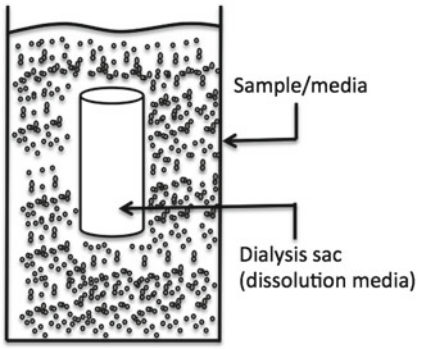

Fig. 23.11 Schematic representation of a reverse dialysis technique

Reverse Dialysis

Reverse dialysis follows the same principle as dialysis, with the exception that the sample is located in the bulk media instead of the dialysis sac (Fig. 23.11) [84]. Dialysis sacs containing only media are placed in a bulk container and sampling takes place either by opening the dialysis sac and removing certain amount of media, or simply by removing the whole dialysis sac and replacing it with a fresh one.

This method has the advantage that agitation takes place in the suspension of microparticles instead of the bulk media, thus preventing aggregation and facilitating polymer hydration and drug diffusion. Accordingly, results are more reproducible. This method also avoids the problem of violation of sink conditions previously observed for the dialysis technique.

A shortcoming is sample loss while handling the dialysis membrane especially in the case of viscous materials such as emulsions or injections.

23.3.2 Standardized Methods

USP offers several standard methods to test dissolution both immediate and controlled release formulations [75]. Seven apparatus are available (USP Apparatus 1–7) and standard procedures for each apparatus are supplied. Out of the seven, only four have been used for testing parenteral products (USP Apparatus 1, 2, 4, and 7). A brief description of these standardized apparatus is given below.

23.3.2.1 USP Apparatus 1 (Basket)

Apparatus 1 was developed for oral solid dosage forms. In this apparatus, the sample is located inside a cylindrical basket. The basket is submerged in dissolution media and rotated with the aid of a motor. Sampling occurs outside the basket where certain amounts of media are removed and replaced with fresh media.

This apparatus has been used in conjunction with the dialysis method, where the dialysis sac is placed in the basket or a modified basket that bears a dialysis membrane is used [98].

Other than its use with the dialysis method, this apparatus is not suitable for release testing of micro- or nanoparticulates, suspensions and viscous gels due to its inability to hold such samples within the basket. Moreover, the large volumes of media required deem it rather impractical [69].

23.3.2.2 USP Apparatus 2 (Paddle)

The USP Apparatus 2 was developed for solid oral dosage forms and has been applied to controlled release parenterals [75]. In the case of parenteral microparticulates, the samples are dispersed in large volumes of media (500 ml) and stirring occurs with the aid of a motor-driven paddle as represented in Fig. 23.12. Sample volumes are removed at certain time points, separated from the microparticles (see Sect. 23.3.1.1 for separation methods) and the microparticulates are dispersed in fresh media and transferred back to the dissolution vessel.

Due to the extensive availability of this apparatus, it is often used as a reference point when developing a new dissolution test or when release kinetics of a new formulation are required for comparison with older formulations [48].

This apparatus has certain limitations. As in USP Apparatus 1, large volumes of media are required, thus it is impractical to use when materials are scarce (usually due to either cost or preparation limitations). Furthermore, as in all sample and separate methods, sample loss during separation is inevitable. Finally, hydrodynamic issues have been reported, where a cone of static water under the paddle is created. This limitation can be dealt with via the use of a specially made “peak” vessel where there is a raised cone shape in the center of the base [99].

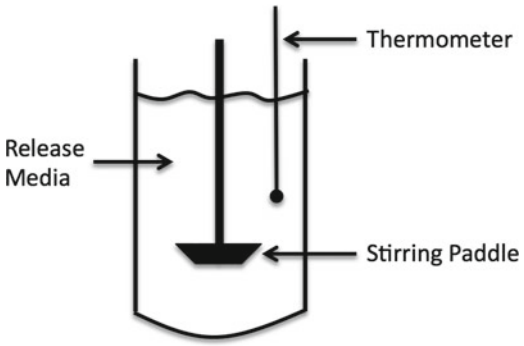

Fig. 23.12 Schematic representation of the USP Apparatus 2

23.3.2.3 USP Apparatus 3 (Reciprocating Cylinder)

The USP Apparatus 3 consists of a glass cylinder containing a glass reciprocating cylindrical vessel [75]. The sample is placed in the inner vessel and sampling takes place from the outer vessel. This apparatus has not been used for in vitro release testing of parenterals. Problems associated with extensive media evaporation have been reported.

23.3.2.4 USP Apparatus 4 (Flow-Through Cell)

The USP Apparatus 4 was originally developed for controlled release oral dosage forms [75] and has been applied extensively to controlled release parenteral formulations. Several workshops have been held where regulatory, industrial and academic experts have considered the issues surrounding in vitro release testing of controlled release parenterals [38–40, 81, 82]. One conclusion of these workshops has been that USP Apparatus 4 appears to be the most appropriate apparatus for in vitro release testing of controlled release parenterals. In particular, a body of work has concluded that Apparatus 4 is the preferred method for in vitro release testing of microsphere products [39, 40, 81, 95]. Apparatus 4 uses the flow-through principle, where the dissolution media is pumped through a specially designed cell that holds the sample as described above under Sect. 23.3.1.2.

Although designed for controlled release oral products, this apparatus may be used for immediate, sustained or delayed release formulations. Currently, the USP does not describe methodologies for using Apparatus 4 for in vitro release testing of parenteral formulations such as microspheres, nanosuspensions, emulsions, liposomes and drug eluting stents. Nevertheless, adaptations of this apparatus that can accommodate such formulations have been developed in the past several years [48]. New flow-through cells as well as adaptations of flow-through cells have been developed.

There are a number of commercially available flow-through cells for use with USP Apparatus 4. These cells are designed to accommodate tablets, capsules, powders, granules, suppositories and implants.

One notable modification is the design of a dialysis sac adapter that can fit into a standard USP Apparatus 4 flow-through cell [48]. This dialysis sac has been reported to successfully accommodate liposomal suspensions and is currently under testing for nanoemulsions and nanosuspensions. This combination of dialysis sac with USP 4 Apparatus was able to discriminate between different liposome formulations and the data were highly reproducible compared to conventional dialysis sac methods.

Another significant modification concerns release testing of microspheres in a flow-through cell. The microspheres are dispersed throughout a bed of glass beads. The incorporation of the beads leads to significantly less microsphere aggregation during the release testing and ensures laminar flow [76–78].

Another focus of USP Apparatus 4 related research is the development of standardized protocols for the release testing of parenterals. Standardized methods suitable for various types of formulations are currently lacking. As mentioned in Sect. 23.3.1.2, it is very common for researchers to use self-made flow-through apparatus, a fact that leads to inevitable variations in release profiles. In recent years, however, researchers have been working with the USP toward the establishment of standardized protocols for the USP Apparatus 4 [83].

23.3.2.5 USP Apparatus 5 (Paddle Over Disk) and 6 (Cylinder)

The USP Apparatus 5 and 6 are modifications of USP Apparatus 2 and 1, respectively. They are both used for transdermal delivery systems and do not bear any significance for long active implants and injections [75, 100].

In the USP Apparatus 5 a disk assembly (usually made of stainless steel) is placed at the bottom of the dissolution vessel and the transdermal delivery system is immobilized underneath it (referred to as a Transdermal Sandwich). In several modifications of this apparatus, a metallic mesh and a transparent glass cover have been used.

The USP Apparatus 6 has a setup very similar to that of Apparatus 1, where a special metallic cylinder capable of containing the transdermal patch has replaced the basket.

23.3.2.6 USP Apparatus 7 (Reciprocating Holder)

The USP Apparatus 7 has been used for transdermals, osmotic devices and stents [75, 100]. It is a modification of USP Apparatus 3 where the reciprocating cylinder is adjusted to contain a holder that carries the implant. There are various types of holders available to choose from, depending on the nature of the delivery system. However, fixing the sample in the holder has proven problematic.

23.4 Special Case: In Vitro Release from Lipophilic Solutions

Long active lipophilic solutions have been in use for over three decades in schizophrenia and enzyme replacement therapy. Lipophilic vehicles may deliver the drug systemically (by intramuscular administration) or locally. The appeal of such delivery systems is understandable given that new chemical entities tend to be hydrophobic in nature. Various dissolution apparatus for lipophilic solutions have been described in the literature, some of which are presented in Table 23.1 [84, 93, 101–113].

In general, diffusion of the drug from the oil to the water phase is considered to be the limiting factor for the release kinetics [102, 103, 108, 109]. Thus, the interphase area, the stirring conditions and the maintenance of sink conditions need to be chosen carefully when designing in vitro release tests for such lipophilic solutions.

As in any liquid delivery system, the interfacial area between the vehicle and the release media is a critical factor affecting the rate and extent of drug release [112]. For lipophilic solutions, the oil–water interphase largely determines the release kinetics and should remain constant throughout the release study. Apparatus where

Table 23.1 Selected in vitro release apparatus for lipophilic solutions

Principle of model	Apparatus description
Lipophilic solution floating on the top of the release media	Lipophilic formulation floating on the release media. Constant stirring in both phases
	Inverted cup: lipophilic solution held in the inverted cup, which is placed in the release media. Constant stirring
	The lipophilic formulation is placed in the eccentrically open tube on the top of the release media. Constant stirring
	Single drop technique: the lipophilic formulation is dispersed as a single drop in the release media. The drop is held in a continuously rotating downward flow of solution inside the tube
Dialysis techniques	Rotating dialysis cell. The dialysis cell containing the lipophilic solution and the media is placed in a large acceptor phase. Constant stirring (rotation) of the dialysis cell
	Float A Lyzer®: lipophilic solution installed in the donor phase inside the dialysis tube, which is placed in the acceptor phase. Constant stirring
	Reverse dialysis bag method: lipophilic solution placed in large donor compartment where numerous dialysis bags containing release media are immersed. Dialysis bags withdrawn at appropriate time intervals. Constant stirring
Continuous flow methods	Lipophilic solution floats on release media. Media circulated through the cell with the aid of tubing

Adapted from [113]

the oil is either floating on top of or suspended in the release media are able to maintain the oil–water interfacial area constant. Dialysis is another method whereby the oil–water interfacial area can be maintained constant, as long as the dialysis membrane is completely filled with the lipophilic solution prior to submerging in the aqueous release media [93].

In the case of oil floating on top of the release media, appropriate stirring may be problematic without disturbing the oil phase and affecting the oil–water interphase. Moreover, stirring inside the lipophilic solution is usually left optional based on the simulated in vivo conditions.

Due to the importance of the interfacial area and the drug diffusion through the interphase, the drug partition coefficient appears linked to the release profile. In future, it may be possible that partition coefficient can replace, at least to some extent, dissolution testing.

23.5 Comparison of In Vitro Release Profiles

As discussed above, in vitro release testing is used as a quality control test for batch release of product. Accordingly, the in vitro release test should be able to discriminate between in specification and out of specification batches.

In vitro release profiles are compared as necessary to support changes in formulation, manufacturing site, scale-up or instrumentation changes. Although there are no specific FDA guidance documents for parenteral products, the principles of the FDA guidance documents for immediate release and extended release oral dosage forms have been utilized to assist in comparing release profiles of parenteral products. The following section summarizes the principles of dissolution profile comparison as taken from the FDA guidance for industry on Dissolution Release Testing of Immediate Release Oral Dosage Forms [114].

A comparison of a few single points in the release profile is usually sufficient when dealing with only minor changes. In the case of major changes, however, complete release profile comparison is necessary. There are two methods that are being followed to compare release profiles: model-independent and model-dependent methods.

The model-independent method uses two statistical factors, one expressing the difference (difference factor, $f1$) and one expressing the similarity (similarity factor, $f2$) of the two curves, based on a point-to-point comparison [115].

These factors may take values from 0 to 100. In order for two release profiles to be declared as equivalent, $f1$ should be less than or equal to 15 and $f2$ should be greater than or equal to 50.

In the model-dependent method, the most appropriate model is selected for the release profile. Subsequently, all the release data (pre- and postchange) are fitted into this model and tested for similarity by mapping out a similarity and a confidence region and making sure that the latter falls completely into the former [116].

23.6 In Vivo/In Vitro Correlations

The FDA IVIVC guidance [31] has defined IVIVC as "a predictive mathematical model describing the relationship between an in vitro property (usually the rate or extend of a drug dissolution or release) … and a relevant in vivo response, e.g., plasma drug concentration or amount of drug absorbed." An IVIVC is intended to predict accurately and precisely the expected bioavailability characteristics of a product from its dissolution profile. An IVIVC may used to apply for a biowaiver, where bioequivalence studies are waived and in vitro release is used instead. However, the FDA will usually not grant a biowaiver if significant changes in the formulation have been made. For example, when changing the dosage strength of a formulation, IVIVCs for multiple dosage strengths must be established and meet certain criteria before a biowaiver can be granted [117].

Although there is currently no FDA IVIVC guidance document for parenteral products, the same principles as detailed in the FDA IVIVC guidance document for extended release oral dosage forms have been applied to parenteral drug products. There are five types of IVIVC, as explained below: level A, level B, level C, multiple level C and level D [29, 118]. However, as level D is merely a rank-order comparison, only levels A, B, C and multiple level C are detailed in the FDA guidance document.

23.6.1 In Vivo Release

In order to obtain an IVIVC, an in vivo release profile is necessary. In systemically delivered drugs this is a simple matter of measuring drug concentration in blood samples [119]. These values can then be compared to the in vitro release profile and tested for possible correlation.

For locally active implants, drug blood levels may never reach any detectable levels and, in any case, are irrelevant since it is the concentration of drug at local site that is the important parameter for product performance. However, it is difficult to measure drug concentrations at the local site without an extremely invasive procedure. Accordingly, it has been very difficult to achieve an IVIVC for a locally active controlled release parenteral formulation. Animal models can be used where tissue samples are taken in a serial sacrifice experiment. However, due to species differences, an in vitro–in vivo relationship based on an animal model may not be appropriate or acceptable to the FDA. According to the European Agency for the Evaluation of Medicinal Products, biowaivers based on an IVIVC obtained from animal studies may be possible depending on the route of administration. No specific guidance for parenterals exists [120].

Much research effort has been expended in investigating in vitro–in vivo relationships using animal data and successful correlations have been made between in vitro release profiles and in vivo animal data [119, 121–123].

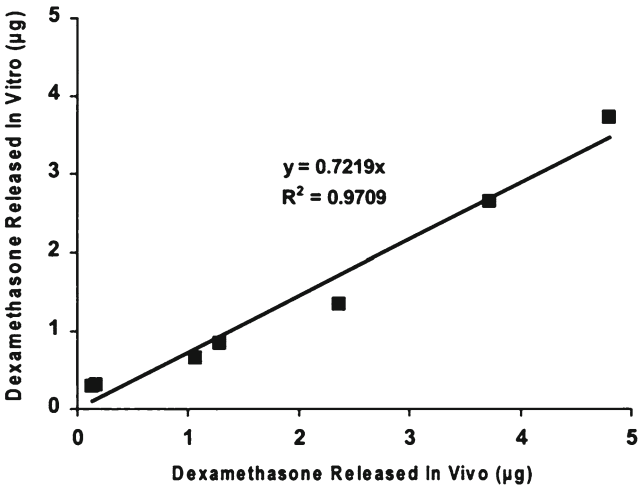

Fig. 23.13 An in vivo/in vitro relationship for dexamethasone release from PLGA microspheres: amount released in vivo plotted against amount released in vitro [15]

Microdialysis is a minimally invasive approach that can be used to obtain drug concentration data at the local site. The microdialysis probe is placed in the immediate vicinity of the implant and the perfusate is tested for drug content. Certain issues, however, need to be taken into consideration. The concentration of drug in the perfusate is typically much lower than that in the tissue; therefore, preliminary experiments must be conducted to understand the relationship between the concentration of drug in the perfusate and that in the tissue. In addition, the presence of the microdialysis probe is likely to cause tissue inflammation and irritation. Inflammation may affect the drug recovery in the perfusate and this effect may become particularly significant during long-term testing.

23.6.2 Types of IVIVC

23.6.2.1 Level A IVIVC

Level A correlations are the most informative and the most complete of all and are recommended by the FDA. These are point-to-point correlations between in vitro and in vivo release profiles. These correlations can be either linear or nonlinear. In the case of nonlinear relationships, appropriate modeling is required. An example [15] of a linear level A IVIVC is shown in Fig. 23.13.

23.6.2.2 Level B IVIVC

Level B IVIVCs are the least informative or predictive. These are based on statistical moment analysis where the mean in vitro dissolution time is compared to the mean in vivo dissolution time or mean residence time. The lack of sufficient predictability of such correlations lies in the fact that various in vivo release profiles may result in the same mean in vivo dissolution time or mean residence time. However, a level B IVIVC is able to predict the overall period of release in vivo, a parameter that is important in long active implants and injections.

23.6.2.3 Level C IVIVC

In level C IVIVCs, a single point correlation between an in vitro release parameter and a pharmacokinetic parameter is achieved. In vitro parameters that are often used are: disintegration time, time for a certain percentage of drug dissolved, dissolution rate, etc. The pharmacokinetic parameters that are most commonly used are C_{max}, T_{max}, K_a, time for a certain percentage of drug release, AUC, etc.

Since this type of IVIVC is based on a single point analysis, it is not as informative as a level A IVIVC. However, multiple level C IVIVCs are sometimes obtained where multiple in vitro dissolution parameters are correlated with multiple pharmacokinetic parameters. Multiple level C IVIVCs can be useful. However, if a multiple level C IVIVC is obtainable, a level A IVIVC should also be possible and is more desirable.

23.6.2.4 Level D IVIVC

Level D IVIVCs (referred to as rank-order correlations) compare release profile rates in vivo and in vitro. A level D correlation is qualitative and as such is not considered useful for regulatory purposes.

23.7 Conclusions

In vitro and in vivo release testing is important in formulation development and in product quality assurance. There are seven USP specified apparatus for dissolution testing, four of which have been used for in vitro release from controlled dosage forms (USP Apparatus 1, 2, 4 and 7). USP Apparatus 4 is speculated to be the most appropriate for release testing of controlled release parenterals and methods using apparatus 4 have been developed for microsphere and liposome formulations. This apparatus is based on a flow-through cell method, and has several advantages over

other apparatus for controlled release formulations. However, there are many factors (drug solubility and stability in the media, delivery system type, etc.) that contribute in the selection of a release testing apparatus.

An IVIVC serves as a predictive platform where a direct relationship between in vitro and in vivo data is established. When formulation changes occur, an established IVIVC can result in waiver of bioequivalence studies and in vitro release studies can be used instead (biowaiver).

Based on the information presented in this chapter, it is clear that there are no standardized methods for in vitro release testing for controlled release parenteral products. Currently, many different methods are being used which makes comparison of data very difficult. Standardized methods need to be established and considerable research is underway to achieve this.

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Chapter 24
Regulatory Issues and Challenges Associated with the Development of Performance Specifications for Modified Release Parenteral Products[1]

Marilyn N. Martinez and Mansoor A. Khan

Abstract Once a parenteral product has been shown to be safe and effective, specifications need to be developed to ensure consistent product performance across batches and throughout the shelf life of that product. This in turn necessitates an appreciation of the physiological variables and critical quality attributes that influence product performance. The assessment of the critical quality attributes and manufacturing processes of new drugs provides the basis for establishing these important quality standards. This chapter provides an overview of the questions and background information that regulators of human or veterinary parenteral dosage forms may consider when establishing the criteria that will ensure repeatable product quality and performance.

24.1 Introduction

The evolution of parenteral extended release platforms pushes the bounds of our understanding of host physiology, pharmaceutical technology, and the regulatory sciences. In human medicine, these ingenious formulations ensure patient compliance, minimize undesirable fluctuations in systemic drug concentrations, and can minimize the generalized systemic side effects associated with the administration of highly

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potent anticancer therapies. In veterinary medicine, these formulations serve a similar function. In addition, when administered to food-producing animals (e.g., cattle and swine), they can reduce both the economic burden associated with repeated visits by the veterinary practitioner and the animal stress associated with repeated handling.

Once the parenteral product has been shown to be safe and effective, specifications need to be developed to ensure consistent product performance across batches and throughout the shelf life of that product. This in turn necessitates an appreciation of the physiological variables and critical quality attributes that influence product performance.

Considering the diversity of products, this chapter does not attempt to provide a description of the critical quality attribute(s) associated with each possible parenteral modified release technology. Rather, it provides an overview of the questions and background information that regulators may consider when establishing criteria to ensure repeatable product quality and performance.

24.2 Statutory Considerations

24.2.1 Human Pharmaceuticals

Under current regulations in the United States, use of a human drug product not previously authorized for marketing in the United States requires the submission of an Investigational New Drug application (IND) to the Agency before administering the drug for clinical studies. The U.S. Food and Drug Administration's (FDA's) regulations 21 CFR 312.22 and 312.23, respectively, contain the general principles underlying the IND submission and the general requirements for content and format. Section 312.23(a)(7)(i) requires that an IND for each phase of investigation include sufficient chemistry and manufacturing controls (CMC) information to ensure the proper identity, strength or potency, quality and purity of the drug substance, and drug product. The type of information submitted will depend on the phase of the investigation, the extent of the human study, the duration of the investigation, the nature and source of the drug substance, and the drug product dosage form.

A pre-IND meeting between the drug sponsor and the FDA takes place before the IND is submitted for review. This IND review is intended primarily to determine if the product will be safe for administration to healthy human volunteers, and to determine if the drug will present with an efficacy profile that justifies its further development. INDs are not approved but rather are called "open INDs" once they are in effect. With an "open IND" a pharmaceutical firm can initiate clinical trials. IND development is depicted in Fig. 24.1.

If the drug demonstrates adequate safety during the initial human studies, termed Phase 1, progressive human clinical trials through Phases 2 and 3 are undertaken to assess safety and efficacy. Review is mainly performed to assess whether the proposed drug formulation is safe and effective and whether the benefits associated with product use will outweigh the risk of adverse (toxic) reactions. The number of

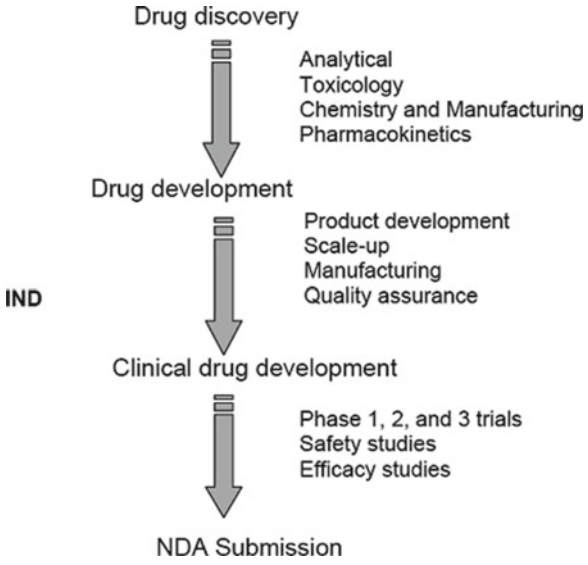

Fig. 24.1 Progressing a product from an IND to a New Drug Application (NDA)

Table 24.1 Number of subjects in clinical trial phases

Phase	Approximate number of human subjects	Approximate length	Purpose
1	20–200	Months	Safety
2	100 s	Months to 2 years	Short-term safety, dosage, efficacy
3	100–1,000 s	1–4 years	Safety, dosage, efficacy

subjects in clinical trials varies, and Table 24.1 provides an idea of the numbers of subjects included in various phases.

As the clinical trials progress, product design, development, scale-up, process controls, labeling, and packaging are established by the pharmaceutical firms. Ultimately, the sponsor files a NDA with the FDA, seeking approval to market the new drug product. The NDA is a registration document submitted by a sponsor to the FDA and serves as an official request for FDA review and determination of the appropriateness for approval for marketing a new drug. The NDA contains CMC data, pharmacology, toxicology, metabolism, clinical safety and efficacy data, as well as proposed labeling. The NDA review process is schematically depicted in Fig. 24.2.

These various components of the NDA can be described as follows:

(a) CMC: The CMC review provides an assessment of the critical quality attributes and manufacturing processes of new drugs and it establishes quality standards to assure safety and efficacy. Impurities profile and stability data are also reviewed under this section. For generic applications (ANDA-Abbreviated New Drug Applications), the CMC review process has recently been modified to include Question-Based-Reviews (QBR), with a vision to transform the review into a modern, science and risk-based quality assessment.

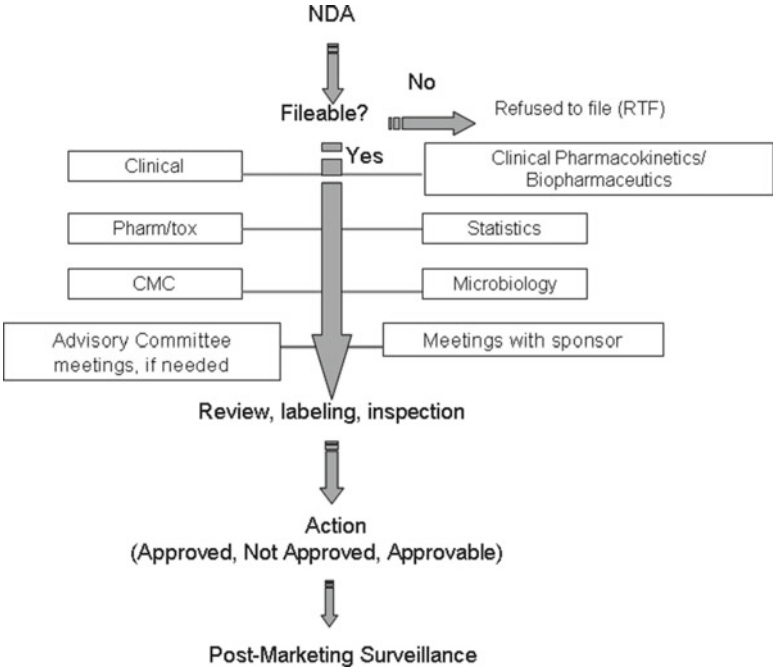

Fig. 24.2 The NDA review process with components

The components of all CMC reviews include:

1. Drug Substance: For drug substance the information submitted includes general information (chemical structure, name, IUPAC name, solubility, etc.), manufacture (manufacturer, manufacturing process and process controls, structure of the proposed starting materials, control of critical steps and intermediates), characterization of the drug's chemical structure, and its physical properties (i.e., polymorphic or solid state form). Some laboratory controls include identification, melting range, specific rotation, water content, impurities (total and individual), assay, appearance, residual solvents, and microbial limits.
2. Drug Product: For drug products, the following information is included in the NDA:

- Description of the composition of the drug product: quantitative composition of all the ingredients
  - Manufacture (manufacturers, batch formula, description of manufacturing process and controls), physicochemical tests (e.g., identity, assay, content uniformity, degradants, impurities, dissolution, viscosity, particle size), biological, pharmacological, toxicological, and microbiological data

- Data on the particle size distribution and/or polymorphic form of the drug substance used is included for a modified release acting parenteral dosage forms (if applicable) so that relevant correlations can be established between data generated during early and late drug development and in vivo product performance
- A stability indicating analytical procedure to track degradation process and impurities, container closure system, and actual stability data generated on a biobatch of the drug products
- Product quality attributes for modified release parenteral products, which may include residual solvents, particle size, viscosity, in vitro dissolution, osmolarity, sterility, stability, biomaterial characteristics, impurities, aggregation, syringability, and endotoxins

(b) Pharmacology and toxicology review: This component provides an evaluation of the nonclinical data to aid in selecting safe doses for "first in man" clinical trials and points to potential toxicities that should be monitored in the clinic. It also assesses toxicities that cannot be addressed in clinical trials such as carcinogenicity, teratogenicity, mutagenicity, and chronic toxicity. Various screening test methods are used to identify whether DNA damage is a possible outcome from exposure to the proposed drug substance. Tests are also conducted to ascertain the drug distribution characteristics within the body. If the molecule does not cause DNA damage during in vitro testing, if it is rapidly metabolized, if it does not reach sensitive organs, or if it is not systemically absorbed, then the drug is considered to be less likely to present certain kinds of health hazards

(c) Clinical safety and efficacy review: These reviews serve to evaluate if the proposed drug is safe and effective in humans based on clinical study data

(d) Statistics: The statistical significance of the clinical trial data is evaluated under the "Statistics" component of the NDA

(e) Clinical pharmacokinetics/biopharmaceutics and microbiology: Drug release profiles for modified release parenteral dosage forms are a key quality attribute and are reviewed in conjunction with the CMC review. In vitro in vivo correlation (IVIVC) if developed for the long acting parenteral formulations is also reviewed and is briefly outlined in the chapter later. The microbiology reviews establish susceptibility criteria for anti-infective drug products. These reviews are no less important to the NDA than are the clinical trial data because they provide pivotal information with respect to dosing regimens/conditions of administration

During FDA's review of emerging technologies, such as Modified Release parenteral dosage forms, the Agency may request additional data from the sponsor to support the applications if such data were not supplied in the original application. Such data are requested when the Agency considers such information relevant for the determination of product safety and effectiveness. If FDA determines such data are needed for a specific class of compounds, FDA may issue guidance to applicants, recommending that such data be submitted in the original application.

Emulsion: A dosage form consisting of a two-phase system comprised of at least two immiscible liquids[1], one of which is dispersed as droplets (internal or dispersed phase) within the other liquid (external or continuous phase), generally stabilized with one or more emulsifying agents. (Note: Emulsion is used as a dosage form term unless a more specific term is applicable, e.g. cream, lotion, ointment.)
Solution: A product, usually a solid, intended for solution prior to administration.
Suspension: A product, usually a solid, intended for suspension prior to administration.
Suspensions, Extended Release: A product, usually a solid, intended for suspension prior to administration; once the suspension is administered, the drug will be released at a constant rate over a specified period.
Gel: A semisolid dosage form that contains a gelling agent to provide stiffness to a solution or a colloidal dispersion. A gel may contain suspended particles.
Graft: A slip of skin or of other tissue for implantation.
Implant: A material containing drug intended to be inserted securely or deeply in a living site for growth, slow release, or formation of an organic union.
Injection: A sterile preparation intended for parenteral use; five distinct classes of injections exist as defined by the USP.
Injectable, Liposomal: An injection, which either consists of or forms liposomes (a lipid bilayer vesicle usually composed of phospholipids which is used to encapsulate an active drug substance).
Injection, Emulsion: An emulsion consisting of a sterile, pyrogen-free preparation intended to be administered parenterally.
Injection, Lipid Complex: Definition pending.
Injection, powder for solution: A sterile preparation intended reconstitution to form a solution for parenteral use.
Injection, Powder for Suspension: A sterile preparation intend for reconstitution to form a suspension for parenteral use.
Injection, Powder for Suspension, Extended Release: A dried preparation intended for reconstitution to form a suspension fo parenteral use which has been formulated in a manner to allow least a reduction in dosing frequency as compared to that drug presented as a conventional dosage form (e.g., as a solution).
Injection, Powder, Lyophilized for Liposomal Suspension: A sterile freeze dried preparation intended for reconstitution for parenteral use which has been formulated in a manner that wo allow liposomes (a lipid bilayer vesicle usually composed of phospholipids which is used to encapsulate an active drug substance, either within a lipid bilayer or in an aqueous space) be formed upon reconstitution.
Injection, Powder, Lyophilized for Liposomal Solution: A dosage form intended for the solution prepared by lyophilizati ("freeze drying"), a process which involves the removal of wat from products in the frozen state at extremely low pressures; tl is intended for subsequent addition of liquid to create a solutio that conforms in all respects to the requirements for Injections.
Injection, Powder, Lyophilized for Suspension: A liquid preparation, intended for parenteral use that contains solids suspended in a suitable fluid medium and conforms in all respects to the requirements for Sterile Suspensions; the medicinal agents intended for the suspension are prepared by lyophilization ("freeze drying"), a process which involves the removal of water from products in the frozen state at extremely low pressures.
Injection, Powder, Lyophilized for Suspension, Extended Release: A sterile freeze dried preparation intended for reconstitution for parenteral use which has been formulated in a manner to allow at least a reduction in dosing frequency as compared to that drug presented as a conventional dosage form (e.g., as a solution).
Injection, Solution: A liquid preparation containing one or more drug substances dissolved in a suitable solvent or mixture of mutually miscible solvents that is suitable for injection.
Injection, Solution, Concentrate: A sterile preparation for parenteral use which, upon the addition of suitable solvents, yields a solution conforming in all respects to the requirements for Injections.
Injection, Suspension: A liquid preparation, suitable for injection, which consists of solid particles dispersed throughout a liquid phase in which the particles are not soluble. It can also consist of an oil phase dispersed throughout an aqueous phase, or vice-versa.
Injection, Suspension, Extended Release: A sterile preparation intended for parenteral use which has been formulated in a manner to allow at least a reduction in dosing frequency as compared to that drug presented as a conventional dosage form (e.g., as a solution or a prompt drug-releasing, conventional solid dosage form).
Injection, Suspension, Liposomal: A liquid preparation, suitable for injection, which consists of an oil phase dispersed throughout an aqueous phase in such a manner that liposomes (a lipid bilayer vesicle usually composed of phospholipids which is used to encapsulate an active drug substance, either within a lipid bilaye or in an aqueous space) are formed.
Injection, Suspension, Sonicated: A liquid preparation, suitable for injection, which consists of solid particles dispersed throughout a liquid phase in which the particles are not soluble. In addition, the product is sonicated while a gas is bubbled through the suspension, and this results in the formation of microspheres by the solid particles.
Insert: A specially formulated and shaped non-encapsulated solid preparation intended to be placed into a non-rectal orifice of the body, where drug is released, generally for localized effects.
Insert, Extended Release: A specially formulated and shaped non-encapsulated solid preparation intended to be placed into a non-rectal orifice of the body, where the medication is released, generally for localized effects; the extended release preparation is designed to allow for a reduction in dosing frequency.
Liquid, Extended Release: A liquid that delivers a drug in such manner to allow a reduction in dosing frequency as compared to that drug (or drugs) presented as a conventional dosage form.
Pellet, Implantable: A small sterile solid mass consisting of a highly purified drug (with or without excipients) made by the formation of granules, or by compression and molding; they are intended for implantation in the body (usually subcutaneously) for the purpose of providing continuous release of the drug over long periods of time.

Fig. 24.3 Dosage form definitions

Parenteral formulations are defined in the Center for Drug Evaluation and Research (CDER) data standards manual (http://www.fda.gov/Drugs/DevelopmentApprovalProcess/FormsSubmissionRequirements/ElectronicSubmissions/DataStandardsManualmonographs). A list of these definitions is provided in Fig. 24.3. The importance of defining specific dosage forms is that these definitions are incorporated into determinations of pharmaceutical equivalence. Products need to be pharmaceutically equivalent and bioequivalent in order to be considered therapeutically equivalent (a prerequisite for the approval of generic products).

For example, if a firm markets an implant, then, a generic firm also needs to develop an implant as an equivalent product for a 505(j) application.

The CDER standard provides for all drug dosage forms (FDA, CDER, 2006). The granularity of data often requires that more specific dosage form terms be stored in automated databases than are represented in publications. These dosage form terms are available not only for use in databases that track approved drug products, but also for drug products such as investigational drug products, homeopathic drug products, biologic products, veterinary drug products, and bulk drug products.

If a product of emerging technologies yields a dosage form other than one of those listed in the manual, appropriate review divisions need to be consulted for defining this new dosage form. It should be noted that several dosage forms or delivery systems such as microspheres do not as yet appear in the CDER Data Manual. This may be partly because the FDA does not approve technologies; rather the products that are safe and effective and produced by several different technologies are approved.

24.2.2 Veterinary Pharmaceuticals

To be legally marketed, new animal drugs must be the subject of an approved new animal drug application (NADA) or abbreviated new animal drug application (ANADA) under section 512 of the Federal Food, Drug, and Cosmetic Act (the Act) [21 U.S.C. 360b], a conditional approval under section 571 of the Act [21 USC 360 cc], or an index listing under section 572 of the Act [21 USC 360ccc-1]. While statutory requirements for safety and effectiveness are similar for veterinary and human pharmaceuticals (section 512(b) of the Act), the pertinent sections of the CFR differ.

Animal drug sponsors may submit data under an investigational new animal drug file (INAD) for what is termed the "phased review" process. This involves the submission of data or information in support of a technical section to the Division responsible for its review. The reviewing Division will notify the sponsor in writing of its conclusions on acceptance or nonacceptance of the data submitted relevant to a technical section. If the reviewing Division finds the data for the technical section to be complete, it will issue a technical section complete letter. A final decision on the approval of an application will be made when the Administrative NADA is submitted and CVM evaluates whether all of the data for all technical sections viewed as a whole support approval (CVM Guidance #132). Alternatively, the sponsor can submit all data for all technical sections at one time. This is referred to as a traditional NADA. The same requirements under 21 CFR 514.1 apply to all NADAs whether for phased review or traditional.

There are potentially seven technical sections: Chemistry, Manufacturing, and Controls; Effectiveness; Target Animal Safety; Human Food Safety; Environmental Impact; Labeling; and All Other Information. These technical sections are described in 21 CFR § 514.1. Additionally, the Freedom of Information (FOI) summary is prepared by the FDA and is described in greater detail in 21 CFR § 514.11(e) (2). Additional information regarding contents and regulatory requirements for safety,

Table 24.2 Technical sections in a new animal drug application as described in CVM Guidance #132 (see citations)

Technical section	Contents	Statutory basis for requirement
Effectiveness	Requirements to show that the drug is effective for its intended use	21CFR § 514.1(b)(8)(i)
Effectiveness	CVM must refuse to approve an NADA unless the sponsor demonstrates by substantial evidence that the drug will have the effect it purports or is represented to have under the conditions of use prescribed, recommended, or suggested in the proposed labeling	Section 512(d)(1)(E) of the Act, 21 USC § 360b(d)(1)(E)
Target animal safety	Requirement for the full report of all studies that shows whether or not the new animal drug is safe to the target species	21 CFR § 514.1(b)(8)(i)[a]
Human Food Safety	This section must, by regulation 21 CFR § 514.1(b)(7), include a description of practicable methods for determining the quantity, if any, of the new animal drug in or on food, and any substance formed in or on food because of its use, and the proposed tolerance or withdrawal period or other use restrictions to ensure that the proposed use of the drug will be safe. This section should also contain any data relating to residue toxicology (including the impact of residues on human intestinal microflora), residue chemistry, and, if the new animal drug has anti-infective properties, microbial food safety[b]	21 CFR § 514.1(b)(7)*
Environmental impact	This section must contain either an environmental assessment (EA) under 21 CFR § 25.40, or a request for categorical exclusion under 21 CFR § 25.30 or 25.33. Under 21 CFR§ 25.15(a), a claim of categorical exclusion must include a statement of compliance with the categorical exclusion criteria and must state that to the sponsor's knowledge, no extraordinary circumstances exist. "Environmental Impact Considerations" and directions for preparing an EA can be found in 21 CFR Part 25	21 CFR § 514.1(b)(14)

[a]Also see CVM Guidance for Industry #185: VICH GL43 – Target Animal Safety for Veterinary Pharmaceutical Products, April 2009

[b]Also see CVM Guidance #3: General Principles for Evaluating the Safety of Compounds Used in Food Producing Animals (July, 2006)

*CVM Guidance #149: VICH GL33 – Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Testing (March 2009), and CVM Guidance #159: VICH GL36 – Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Establish a Microbiological ADI (August, 2006)

effectiveness, human food safety, and environmental impact is provided in Table 24.2. Sponsors are generally advised to generate all in-life data using the final product formulation. If significant CMC changes occur subsequent to these studies, a re-opening of the previously reviewed and accepted technical sections can occur.

As with our human drug counterparts, the Center for Veterinary Medicine (CVM) seeks to understand the critical quality attributes that influence product performance and to determine if a proposed in vitro test is adequate for assuring lot-to-lot quality and performance. A unique challenge to CVM in this regard is that when the product is intended for use in food-producing animals, very small changes in the rate and extent of drug release (which may have negligible therapeutic impact) can lead to the presence of drug levels that exceed the FDA's maximum residue limit (tolerance). Furthermore, oftentimes one in vitro test and set of product specifications will need to assure in vivo product performance across multiple target animal species. All of this is accomplished on a research budget for most drug sponsors that is only a fraction of that available to the development of human pharmaceutical products. In the United States, animal health companies spend approximately $704 million on research and development per year (http://www.ahi.org/content.asp?contentid=692). In contrast, for human drug research and development, U.S. companies spent approximately $63 billion dollars on the development of medicines and vaccines.

24.3 Physiological Variables That Can Influence Parenteral Drug Absorption

Whether considering the human or the veterinary patient, the basic physiological considerations that govern in vivo product performance are identical. What differs is the relationship between these attributes and their interaction with the formulation. Appreciating these relationships is fundamental to the development of clinically relevant product specifications and can influence FDA's decisions regarding product specifications and the evaluation of biowaiver requests that may accompany modifications in product CMC.

24.3.1 The Interstitium

The extracellular matrix (ECM) is a complex mixture of ions, fluid, lipids, proteins, and carbohydrates, including [1]:

1. Collagen: accounts for more than 2/3 of the ECM protein content of many tissues
2. Fibrin: fibrillar matrix found at wounds and in tumor stroma
3. Elastin: gives tissues the property of elastic recoil
4. Proteoglycans: large, brush shaped molecules that consist of carbohydrates attached to a core protein with one or more covalently attached glycosaminoglycan

(GAG) chain(s). Proteoglycans can have a mass of several hundred thousand Daltons and are present in connective tissues

5. GAGs: long unbranched polysaccharides consisting of a repeating disaccharide unit. The repeating unit consists of a hexose (six-carbon sugar) or a hexuronic acid, linked to a hexosamine (six-carbon sugar containing nitrogen). These polyanionic carbohydrate polymers are negatively charged under physiological conditions (usually due to the occurrence of sulfate and uronic acid groups). The negative charge of the GAG attracts cations, including sodium, which promotes tissue hydration. With the exception of hyaluronan (which is a mobile proteoglycan), GAGs are covalently bound to a protein backbone to form proteoglycans which are immobilized in the interstitium. The fixed charges associated with various matrix proteins lead to a surrounding aqueous layer that effectively increases the surface area of these charged molecules. Examples of GAGs include:

- Heparin
  - Heparan sulfate
  - Chondroitin sulfate
  - Dermatan sulfate
  - Hyaluronan (hyaluronic acid): this is the only GAG that is exclusively non-sulfated. Hyaluronan is not immobilized and may be removed from the interstitium via the lymph in a flow-dependent manner

In all tissues, the interstitial space consists of fluids and structural molecules that support a gel phase consisting of negatively charged GAGs, salts, and plasma-derived proteins. Along with collagen, the GAGs play a fundamental role in molecular size exclusion within the interstitium [2–4]. For this reason, the drug diffusion through the interstitium is influenced by drug size, charge, hydrophilicity, and its interactions with the endogenous components of the interstitial fluids. Accordingly, simple formulation changes in drug concentration, injection volume, ionic strength, viscosity, and pH can influence the rate of diffusion from the subcutaneous (*s.c.*) injection site (http://www.drugdeliverytechonline.com/drugdelivery/200906/?pg=38#pg38). In addition, the larger the molecule and the more negative its charge, the greater the amount of space that it will occupy within the interstitium (due to hydration) [3].

By acting as a molecular filter, the ground substance results in a difference in the diffusion rate of solvent versus solute molecules. This leads to a concentration gradient whereby the concentration of drug in the fluid immediately adjacent to the injection site is elevated, thereby retarding its dissolution. Disruption of this sieving property can lead to a significant increase in the rate of drug absorption [5, 6]. This sieving property is also responsible for the negative relationship between particle size and the absorption of aqueous suspensions. In other words, the relationship between rate constant for absorption and particle size appears to follow a negative Emax model whereby the absorption rate constant decreases as particle size increases, with a maximum slowing effect occurring at particle sizes of about 3 μm in diameter [7, 8].

In contrast to that observed with aqueous suspensions, the initial drug concentration does not appear to influence the absorption rate constant of drug in lipophilic solutions [9]. Rather, the rate of absorption depends upon the relationship between the affinities of the drug for the solvent versus the interstitial fluids. When the solute rapidly diffuses through the solvent, the rate-limiting step is the partitioning of drug between the vehicle and the interstitial tissue fluids. Clearly, one can see how the composition of the tissue fluids, as influenced by disease or animal species, could alter this partitioning and therefore, in vivo absorption characteristics. Furthermore, given the importance of surface area and spread, it is not surprising that for any given concentration, the amount of drug remaining at the injection site (rodent model) is inversely proportional to the volume of the injection [9] and is highly dependent upon vehicle viscosity. A similar relationship was observed in people [10].

24.3.2 How the Interstitium Can Influence Drug Delivery to Tumors

In normal tissues, the interstitial fluid pressure has a value of −2 to 0 mm Hg. However, the pressure measured in solid human or experimental tumors can reach values ranging from 3 to 40 mm Hg [11]. Therefore, while the altered pore capillary structure of tumors may allow liposomes to enter the tumor, the distribution of the extravasated liposomes within the tumor interstitium is not uniform but rather appears to form perivascular clusters. Accordingly, while the drug may have entered the tumor, it would not be effective because it would fail to reach the site of action [12, 13]. Even more importantly, this uneven distribution of drug within the tumor tissue can lead to experimental error in efforts to relate product formulation to target tissue delivery if these assessments are based upon tissue homogenate concentrations. These total tissue concentrations may poorly reflect the actual concentrations of drug that interact with the tumor core [12].

In addition to size, negative charges hamper the drug's ability to extravasate into and accumulate within the tumor. In fact, in contrast to the belief that the restricted uptake is due solely to an elevated interstitial fluid pressure within solid tumors, there is now evidence to suggest that the challenge may also relate to size and charge exclusion [3]. For example, negatively charged IgG is excluded from a volume almost twice that of the positively charged IgG, suggesting that the negatively charged hyaluronan had a more important role than the positively charged collagen as an excluding agent [4]. Using a human osterosarcoma model (xenographed into mice), both collagenase [14] and hyaluronidase [15] demonstrated a small but significant increase in the concentrations of osteosarcoma-associated monoclonal antibody found at distances proximal to the blood vessels.

Based upon this information, both size and charge of the nanoparticles (e.g., liposomes) used for tumor targeted uptake need to be very carefully regulated by the development of narrowly defined product specifications. Whether or not there may be differences among tumor types with regard to their "forgiveness" in size and

charge variation remains to be determined. However, a potential drug-by-tumor type interaction is likely to exist [16] and may need to be considered when one product is being targeted for use against cancers of multiple etiologies.

24.3.3 Lymphatics

Failure to consider potential lymphatic uptake when evaluating product bioavailability can lead to prediction errors and suboptimal delivery platforms. For example, Yoshikawa showed that the relative proportion of drug absorbed into the lymphatics versus the blood depended upon the route of administration [17]. Since their work involved a molecule that targeted the immune system (the antineoplastic effect of human fibroblast interferon is dependent upon lymph and not serum drug concentrations), the route providing the higher blood levels may have been incorrectly assumed to be preferable. Similarly, when the lipophilic drug, fluphenazine deconoate, was administered in sesame oil into the femoral muscle of rats, drug concentrations in the lymph nodes were 216 times higher than that observed in plasma by day 1 postdose, but rose to 1,560 times above that seen in the blood by day 21 postdose [18].

The capillary endothelial barrier is relatively impermeable to large hydrophilic macromolecules, and therefore endogenous proteins are primarily cleared from the interstitium via lymph. The majority of capillary pores are small (~4 nm) with a few nonsize selective pathways permitting the movement of particles within the range of 25–30 nm. Exceptions include inflamed tissues, tumor tissues, and sinusoidal tissues (e.g., liver, spleen and bone marrow). Drugs that can be cleared either by blood or lymph (<2 kDa) will preferentially enter the blood because the blood flow is approximately tenfold greater than that of lymph fluid filtration and re-absorption. However, due to size exclusion, larger molecules must be absorbed via the lymphatics [2, 19]. Greater than 50% of a SC dose is expected to drain into lymph when MW > 16,000 [2, 20]. The rate at which proteins are removed from the interstitium is largely sensitive to both physical and pathophysiological changes, with increases of three to fourfold having been reported during muscle activity or edema [3].

24.3.4 Blood as a Carrier: Factors Influencing the Safety and Effectiveness Characteristics of Nanoparticles and Other Large Molecules

Macromolecular complexes of lipids and proteins serve as transport vehicles for moving compounds through the vascular and extravascular body compartments. The lipoproteins consist of a nonpolar core containing triacylglycerols and cholesterol esters, and a surrounding surface monolayer of amphipathetic lipids such as phospholipids and unesterified cholesterol. The surface also contains apolipoproteins (proteins containing both polar and nonpolar amino acid residues that help to

solubilize and stabilize the insoluble lipid of the lipoprotein molecule). The greater the relative proportion of lipid to protein, the lower the density of the molecule. The diameters of these particles range from 75 to 1,200 nm (the lowest density chylomicrons) to 5–12 nm (the high density lipoproteins (HDL)) [21].

Marked differences in the relative proportion of cholesterol, triglycerides, and proteins are observed across the various lipoprotein fractions of dogs, rats, and people [22]. The prandial state of the subjects prior to harvesting the plasma was not indicated. The resulting comparison of the various lipoprotein fractions in dogs, rats, and people is shown in Fig. 24.4a–c. The same analytical method was employed in a study using fasted subjects [23]. Despite differences in the absolute values of the various components, similar interspecies differences in the composition of the three lipoprotein fractions were observed.

These interspecies differences can impact the effect of formulation on the pharmacokinetics of liposomal preparations [22, 24]. For example, with negatively and positively charged liposomes, there was an increase in relative proportion of particles associated with the HDL fraction [25]. These interspecies differences also provide insights into the variations in drug response that can occur when disease alters lipoprotein content of humans. For example, the renal toxicity of amphotericin B increases in patients presenting with elevated cholesterol levels [26]. Similarly, Gardier observed greater cyclosporine toxicity in heart transplant patients presenting with high plasma LDL [27]. Therefore, these potential differences should be considered when using preclinical species during product development and when setting product specifications.

In their reviews, authors discuss how formulation factors such as lipid composition, particle size, liposomal membrane fluidity, vesicle morphology and curvature, and net phospholipid charge can determine the plasma distribution, pharmacokinetics, and pharmacological effects of lipid-based drug formulations [28–31]. In fact, formulation can influence drug partitioning within and the formation of complexes with the HDL fraction of the blood. Interaction between the vesicles and HDL can lead to the removal of certain phospholipids from the liposomes, thereby releasing encapsulated drug at a rate dependent upon the extent of liposomal damage. In addition, formulation can influence vesicle opsonization, which promotes its uptake by the reticulo-endothelial cells. These serum opsonins cause the liposomes to interact with receptors on the surface of macrophages and hepatocytes, thereby enhancing liposome uptake by these cells. The specific proteins that associate with liposomes determine the extent to which a particular liposome formulation interacts with a particular cell population.

Whether the classical or alternative complement pathway will be activated is a function of species-specific formulation effects. For example, the classical complement pathway, which leads to vesicle opsonization, appears to be activated by the presence of negative charges, cholesterol, and saturated acyl chains in the liposomal surface. This relationship has been observed across all mammalian species. In contrast, while positive charges tend to activate the alternative complement pathway in human serum, it still allows for the activation of the classical pathway in rats. Particle size is also important in determining the extent to which there is activation

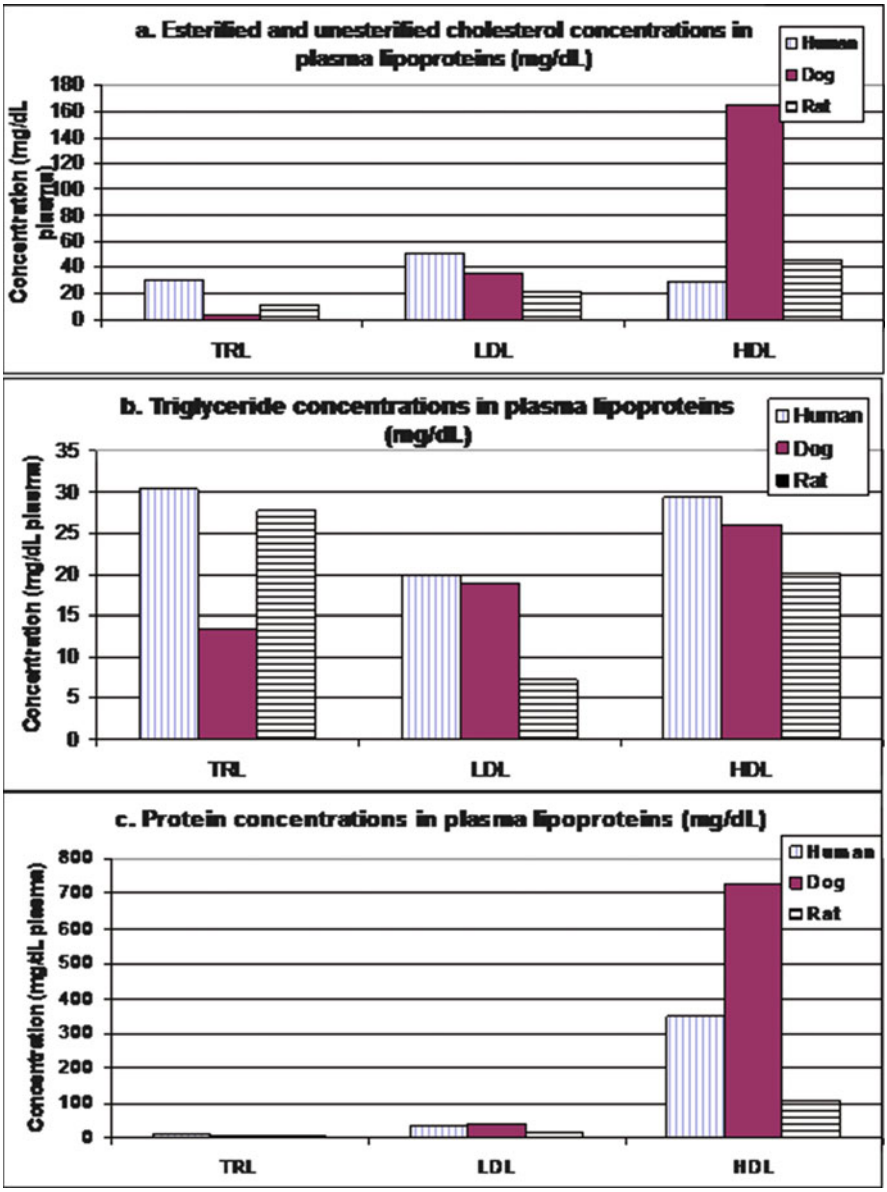

Fig. 24.4 Interspecies differences in lipoprotein composition. Based upon data from Wasan et al. [22]

of the complement system. Liposomal elimination associated with complement fixation may also be associated with dose-dependent kinetics as the available complement is depleted. Lastly, it was noted that nonopsonic blood proteins may play a role in particle clearance, and the phagocytic removal of these vesicles appears to be influenced both by opsonization-dependent and opsonin-independent pathways [32, 33].

Citation	Finding	Insights	Species
Ramaswamy et al., 1999 (61)	Interspecies difference in drug and liposome distribution across the various lipoprotein fractions of the plasma.	In all three species the majority of nystatin as free or liposomal encapsulated drug (Nyotran) is recovered in the LPDP fraction in humans, rats and dogs. However, the relative amounts of drug across the various fractions differed as a function of lipoprotein composition. Proportionally, a greater percentage of the drug was in the HDL fraction of dogs as compared to that in humans or rats. The difference in proportional distribution across the various lipoprotein fractions varied as a function of the lipid composition of that fraction. It would appear that the dimyristoyl phosphatidylglycerol (DMPG) component of the liposome results in its predominant distribution into the HDL lipoprotein fraction of the plasma	In vitro study of plasma from human, dog, rat
Wasan et al., 1993 (25)	When neutrally charged liposomes are incubated in human serum, 57-79% of the encapsulated amphotericin B (AmB) is found in the HDL fraction. In contrast, with positively charged lipid composition, 75-80% of the encapsulated drug is found in the HDL. With negatively charged liposomes, 87-92% of the AmB is recovered in the HDL.	Partitioning of liposomal amphotericin B varies as a function of liposomal composition. With an increase in the positive or negative charge, there is an increase in the partitioning of drug into the HDL fraction.	Human serum
Wasan et al., 1998 (22)	Marked interspecies differences in the relative amount of tretinoin observed across the various lipoprotein fractions.	Differences have been attributed to the species-specific lipoprotein compositions of the various fractions. The higher the level of cholesterol in any given lipoprotein fraction, the greater the concentration of tretinoin in that fraction. Differences in the distribution across lipoprotein fraction (as a function of species or disease) may influence the toxicity and pharmacokinetic properties of both the drug and the liposomal delivery system	In vitro plasma from human, rat and dog
Liu et al., 1995a,b (32,33)	Interspecies difference in liposomal clearance from plasma is due to species-specific differences in formulation-related stimulation of phagocytic uptake. This in turn reflects species-specific components of serum (the complement system) that binds to the liposome	Mouse Kupffer cells appear to recognize surface characteristics of the liposomes without the need for complement fixation. Mice seen to be lacking opsinization activity for certain liposomal formulations. The stimulation of opsonizing activity varied as a function of formulation and species	Mouse and rat in vivo, human, rat, mouse and bovine in vitro serum binding an in situ mouse liver perfusion study
Harashima et al., 1996 (28)	Clearance varied with an allometric exponent of 0.5, but this difference did not reflect density of Kupffer cells. It was suggested that differences in uptake was based upon differences in the uptake capability of Kupffer cells among the three rodent species.	At least in part, differences in clearance of liposomes from rats and mice reflect a difference in the mechanism of hepatic uptake. Rats: uptake primarily involves an opsonin-dependent pathway. In mice, an opsonin-independent pathway also seems to exist	Mouse, rat, rabbit in vivo
Huong et al., 2001a,b (62,63)	Pre-treatment with the anti-complement agent (K-76) decreases liver uptake of liposomes in guinea pigs. In rats, the in vivo binding of the C3 component (the third component of the complement system) was not inhibited by K76-pretreatement. This may reflect that some in vivo factor can compete for the binding of C3. Liposome opsonization by the C3 fragments leads to enhanced liposome uptake by liver phagocytic cells.	In vitro, complement-dependent liposomal destabilization was observed in rat but not in guinea pig serum. These findings suggested that the liposomes activated the complement system of rats and not of guinea pigs. In contrast, in vivo, both rats and guinea pigs exhibited a very large activation of the complement system and binding of C3 fragments were inhibited by K76. The reason for the in vivo and in vitro disparity for the guinea pigs was not determined.	Rat and guinea pig serum, in vivo and in vitro
Moghimi and Hunter, 2001 (29)		Complement opsonized vesicles interact with receptors located on different blood cells across animal species	

Fig. 24.5 Interaction between serum components and liposomal carriers: insight into interspecies differences and potential differences in liposomal pharmacokinetics with disease

Examples of how these interactions, which can vary across animal species or as a function of the altered physiology and liposome formulation, are shown in Fig. 24.5.

24.3.5 The Importance of Host Defense System Characteristics

The in vivo relevance of product specifications may differ when evaluated from the perspective of normal healthy subjects versus the patient population. For example, variability in the opsonization activity of serum for monosialoganglioside-containing

liposomes has been observed among human subjects [32]. The opsonization process is a pivotal factor in determining the phagocytosis of these particles. Similarly, the relationship between liposome composition and its clearance from the circulation can be species dependent [32, 33]. Differences have been observed in the formulation-dependent opsonization activity of serum from mice, rats, humans, and bovine, which in turn affects the formulation-related stimulation of particle uptake by phagocytes.

Not only can there be marked interspecies differences in the relationship between formulation and liposome clearance but also in the mechanism of the clearance process. Unlike that observed in rats, mouse Kupffer cells appear to recognize the surface characteristics of liposomes without the help of serum components. In fact, mouse serum may be unique in lacking opsonization activity for certain liposomal formulations. Using a perfused liver system, it was observed that in rats, hepatic uptake of liposomes is primarily explained by an opsonin-dependent pathway while in mice, the uptake relates to an opsonin-independent pathway [34].

Microspheres are also eliminated primarily by phagocytosis. Therefore, understanding the properties contributing to this uptake is integral to in the development of product design specifications. Microsphere uptake into phagocytic cells includes both a linear and a nonlinear (saturable) component. The degree to which each of these two mechanisms contributes to the total amount of cellular uptake is dictated by the nature of the ligand covalently bound to the surface of the microsphere. The cellular uptake of microspheres is also influenced by such particle properties as its diameter, polymer hydrophobicity, surface charge, and roughness [35, 36]. When intentional, the microspheres can provide the benefit of sustained drug release within the targeted cell. However, when unintentional, this uptake can lead to premature drug loss.

24.4 Excipients in Long Acting Parenteral Dosage Forms

Excipients are ingredients that are added intentionally to therapeutic and diagnostic products to provide a function and are not intended to exert a therapeutic effect. According to 21 CFR 210.3(b)(8), an inactive ingredient is any component of a drug product other than the active ingredient. Excipients are used to formulate the active drug substance into a pharmaceutical dosage form. 21 CFR 314.94 (a)(9)ii states that "an applicant shall identify and characterize the inactive ingredients in the proposed drug products and provide information demonstrating that such inactive ingredients do not affect the safety or efficacy of the proposed drug product."

Novel technologies for modified release parenteral formulations may necessitate the use of "new" excipients. New excipient means any inactive ingredients that are intentionally added to therapeutic and diagnostic products, but that: (1) we believe are not intended to exert therapeutic effects at the intended dosage, although they may act to improve product delivery (e.g., enhance absorption or control release of the drug substance); (2) are not fully qualified by existing safety data with respect to the currently proposed level of exposure, duration of exposure, or route of administration. New excipients should be evaluated for safety.

For veterinary drug products, drug sponsors are advised to contact CVM for recommendations on the assessment of the target animal safety (or human food safety) of these new excipients. With regard to human drug products, a Guidance for Industry is available for performing nonclinical safety studies for pharmaceutical excipients (FDA, May 2005). There are numerous other guidances for determining safety which include potential pharmacological actions (ICH S7A, ICH guidance for industry S7A, 2001), genotoxicity (ICH S2B, ICH guidance for industry S2B, 1997), and reproductive toxicology (ICH S5A, ICH guidance for industry S5A, 1994; ICH S5B, ICH guidance for industry S5B, 1996). Acute toxicology studies should be performed in both a rodent species and a mammalian nonrodent species by the route of administration intended for clinical use (FDA, 1996). In some cases, a dose–escalation study is considered as an acceptable alternative to a single-dose design (ICH M3, ICH guidance for industry M3, 1997).

With respect to human drug products, it is recommended that the absorption, distribution, metabolism, and excretion of the excipient be studied following administration by the clinically relevant routes to the same species that are used in the nonclinical safety studies (ICH S3A, ICH guidance for industry S5A, 1995; ICH S3B, ICH guidance for industry S3B, 1995). Novel excipients intended for short-term, intermediate, or long-term use as well as those intended for nonoral routes of administration need to qualify for safety as outlined in the safety evaluation guidance published by FDA (FDA Guidance, May 2005).

For noncompendial excipients, sponsors are asked to provide a specification sheet that identifies the tests, the acceptance criteria, and indicates the types of analytical procedure to be used (e.g., HPLC). A complete description of the analytical procedures and a brief description of the manufacture and control of these components should be provided. Alternatively, sponsors can provide an appropriate reference such as a Drug Master file (DMF) or a NDA. Information for excipients not used in previously approved drug products within the United States should be equivalent to that submitted for drug substances.

Regardless of the dosage forms and delivery systems, excipients must meet the requirements established in 21 CFR 330.1(e) for over-the-counter drug products and 21 CFR 314.94(a) (9) for generic drug products. For generic drug products, the excipients for parenteral, ophthalmic, or otic routes should be same as the reference listed drug products. For oral, dermal, or topical drug products, selection of excipients is flexible, with the only criteria being that it should be safe.

The excipients that have been previously included in FDA-approved drug products for use in humans are usually listed in the Inactive Ingredient Guide (IIG) (http://www.fda.gov/Drugs/InformationOnDrugs/ucm080123.htm). This guide is published on the FDA website. IIG lists the maximum amount of excipient used per dosage unit. For compendial excipients, sponsors are asked to provide references to quality standards (e.g., USP, NF). There should be documented human use in the proposed level or they should be known excipients that should generally be regarded as safe (GRAS). For multiple units, chronicity of dosing, maximum daily dose, etc., the sponsors are asked to justify the proposed amount of excipient to be used.

Due to potential stability issues, drug–excipient compatibility studies are warranted. Examples of adverse drug–excipient interactions include: (1) degradation caused by an interaction between functional groups of drug and excipients; (2) a physical interaction between a drug and an excipient that compromises product quality; and (3) the unintentional adsorption of drug to an excipient, thereby affecting drug release.

24.5 The Challenge of Defining Product Specifications

Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval. A "specification" is defined as a list of tests, reference to analytical procedures, and appropriate acceptance criteria that are numerical limits, ranges, or other criteria (ICH guidance for industry Q6A on Specifications, 2000). Specifications should focus on those characteristics found to be important for ensuring the safety and efficacy of the drug substance and the drug product. Accordingly, specifications should be established on the basis of the experience and data obtained during development of a new drug substance or product.

Conformance to specifications means that the drug substance and/or drug product, when tested in accordance with the listed analytical procedure, meets the listed acceptance criteria. It may be possible to propose excluding or replacing certain tests on the basis of progressive experiences with the product and/or drug substance.

The in vitro tests and product specifications will be relevant only if it accurately reflects those factors governing in vivo drug release. For parenteral delivery platforms, potential challenges include [37]:

1. Microsphere

- Loss of protein conformational integrity during manufacturing process
  - Adverse injection site reactions

2. Nanoparticles: problems in obtaining accurate particle size measurements in the formulated product and challenges of aggregation and agglomeration
3. Liposomes: problems in obtaining adequate drug loading
4. Biodegradable polymer implants: loss of protein conformational integrity during manufacturing process
5. Nonbiodegradable implant: provides excellent and predictable controlled release profile but minor surgery is needed at the time of administration and for implant removal
6. In situ forming gels: may provide a very promising mechanism for achieving well-controlled delivery of peptides and proteins. Gel formation and drug release are also affected by multiple interacting variables such as temperature, drug loading, pH, and adhesiveness to host tissues [38]. Challenges in these systems include the control of burst release and surface-to-volume ratio of the in vivo gel formation. Some polymers are also designed to be sensitive to biochemical

changes within the patient (e.g., blood glucose levels for insulin delivery or folate receptors for the delivery of drugs directly to tumor cells). These "smart" polymers release drug in response to the altered metabolic environment

24.5.1 Understanding Design Product Space: Quality by Design

QBD refers to a systematic approach to product development, beginning with predefined objectives and emphasizing product and process understanding and process control, based on sound science and quality risk management. Quality itself refers to the suitability of either a drug substance or a drug product for its intended use. This term quality includes such attributes as the identity, strength, and purity (ICH Q6A).

Some of the elements of the QBD approach include:

1. Quality Target Product profile (QTPP). QTPP refers to a prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure the desired quality, taking into account safety and efficacy of the drug product
2. Quality Risk Management (QRM) of critical quality attributes (CQA)
3. Formal experiments to evaluate the main effects and interactions of compositional and process variables that affect the CQAs
4. The establishment of a design space and a control strategy that allows for real time release (RTR)

The CQA is a physical, chemical, biological, or microbiological property or characteristics that should be maintained within an appropriate limit, range, or distribution to ensure the desired product quality. As examples, when considering modified release parenteral formulations, the CQAs could include particle size distribution, drug loading, entrapment efficiency, zeta potential, drug release, drug permeability, physical and chemical stability, etc. A formal experimental design or Design of Experiment (DOE) is defined as a structured, organized method for determining the relationship between factors affecting a process (critical process parameters) or material attributes and the output of that process (CQA).

Figure 24.6 provides an example of main variables of a polylactic and glycolic acid (PLGA)-based nanoparticle formulation obtained in-house at FDA. The Pareto Chart obtained after a designed set of experiments indicates that variables such as drug loading and stirring rate have significant effect on the CQA PSD, whereas the other studied variables do not. Statistical significance is defined by an alpha level of 0.05.

This kind of DOEs is invaluable for defining a design space based upon the effect of the main variables and their corresponding interactions. A design space is defined in the ICH Q8 as the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality. When sponsors work within the design space, any adjustments in formulation or manufacturing method is not regarded as a clinically

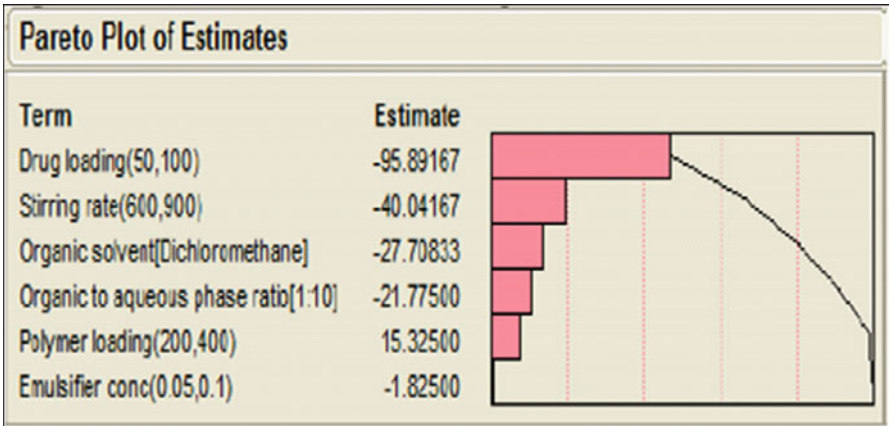

Fig. 24.6 Main variables in PLGA–nanoparticle formulation

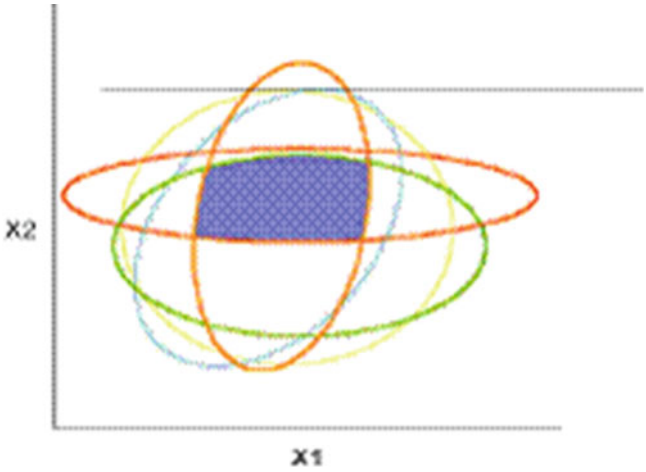

Fig. 24.7 A schematic diagram of response variables of several CQAs

relevant change. However, movement outside of the design space is considered to be a change and would normally initiate a regulatory postapproval change process.

The design space is proposed by the applicant and is subject to regulatory assessment and approval (ICH Q8). Optimally, the design space will ensure consistent product performance, even when certain material attributes and critical process parameters are changed within a certain range. It helps in understanding the potential impact of scale-up on CQAs. The design space allows an opportunity for continuous improvement of process if appropriate information is submitted and approved for this purpose.

Figure 24.7 represents a schematic diagram of a design space where the factors X1 and *X*2 yield response variables of several CQAs indicated by ellipses. The

shaded area inside shows the regions of X1 and *X*2 where all the desired CQAs are within the desired ranges – also known as the optimized range. Assuming that the design space is established on the basis of a well understood and validated set of DOE, there is no reason to believe that the same optimized responses of CQAs will not be achieved when the variables are changed within the optimized zone for continuous improvement.

The design space is often confused with the concept of proven acceptable range (PAR). PAR itself is not a designed space and simply means a characterized range of a process parameter for which operation within this range, while keeping other parameters constant, will result in producing a material meeting relevant quality criteria.

The controlled strategy refers to planned set of controls, derived from current product and process understanding that ensures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in-process controls, finished product specifications, and the associated methods and frequency of monitoring and control (ICH Q10). ICH Q8R and the PAT Guidance also provide an opportunity for RTR. The RTR is the ability to evaluate and ensure the acceptable quality of in-process and/or final product based on process data. Typically, the PAT components of RTR include a valid combination of assessed material attributes and process control (ICH-Q8R). In RTR, material attributes are measured and controlled along with process parameters. Material attributes can be assessed using direct and/or indirect process analytical methods (PAT Guidance, 2004). RTR as defined in the guidance builds on parametric release of heat terminally sterilized drug products. The RTR is comparable to alternative analytical procedures for final product release.

The RTR of a microsphere preparation is illustrated in Fig. 24.8. As the microspheres are formed, the near-IR can actually confirm the nature of the material of microspheres. The focused beam reflectance measurement (fbrm) can measure the dynamic microsphere size measurements. Details of the principles of on-line monitoring can be found elsewhere [39, 40].

It is the experience of these authors that while the QBD has been embraced for immediate release and extended release oral products, as well as conventional injectable dosage forms, there is a considerable opportunity to learn and advance the science of QBD for modified release parenteral formulations. There is a critical need to advance the science of product and process understanding and real-time control strategies for these complex parenteral formulations. Readers are advised to refer to ICH guidelines, Q8, Q8R, Q9, and Q10, as well as the FDA's PAT Guidance.

24.5.2 Targeted Drug Delivery

Using either passive or active targeting mechanisms, nanoparticles can be designed to overcome both anatomical and physiological barriers that challenge the delivery

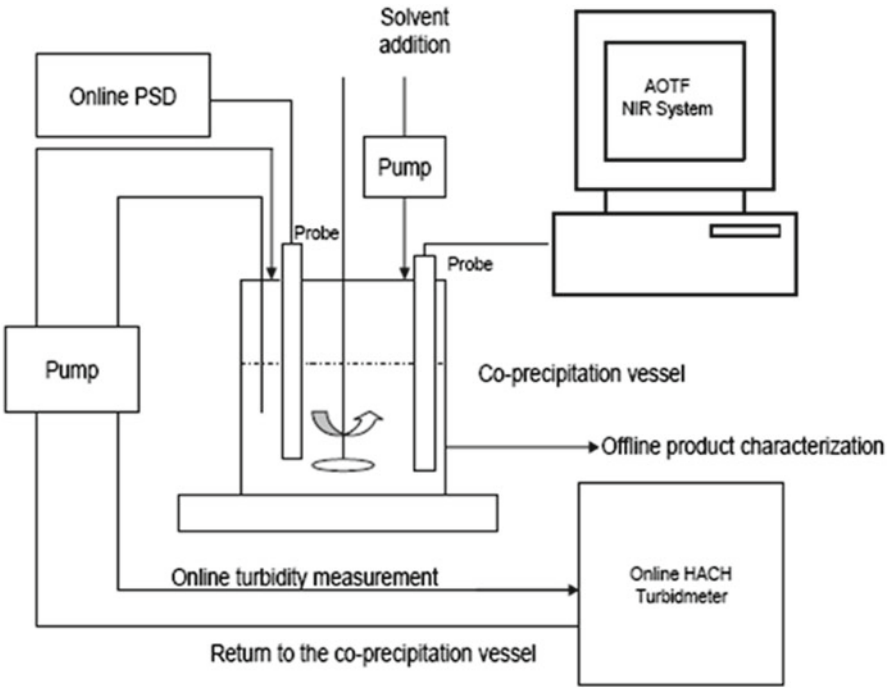

Fig. 24.8 A potential assembly to prepare LA microspheres to monitor particle size by fbrm and near IR in a real time

of therapeutic moieties to the site of action. This targeted delivery can be achieved by addressing specific cellular uptake mechanisms (e.g., receptors on tumor cells) via the attachment of ligands such as lectins, invasins, and peptides [35, 41].

To evade immune system surveillance and allow for a prolonged residence time within the circulatory system, stealth mechanisms need to be established. This is often achieved by particle PEGylation. While both the stealth and targeting features are important for effective and selective drug delivery (e.g., to tumors), it is often difficult to achieve both features simultaneously. Recent targeting strategies utilize the unique extracellular environment of tumors to change the long-circulating nanocarriers to release the drug or interact with cells in a tumor-specific manner [42].

Due to the complexities of this system, the need to identify in vitro test conditions and product specifications that provide batch-to-batch assurance of consistent product performance poses a significant regulatory challenge [43].

24.5.3 In Vivo/In Vitro Correlations and Biowaivers

There are generally four types of correlations that are described (CDER Guidance for Industry, Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In vitro/In vivo Correlations (September 1997)). These are as follows:

LEVEL A: A Level A correlation is usually estimated by a two-stage procedure: deconvolution followed by comparison of the fraction of drug absorbed to the fraction of drug dissolved. A correlation of this type is generally linear and represents a point-to-point relationship between in vitro dissolution and the in vivo input rate (e.g., the in vivo dissolution of the drug from the dosage form). In a linear correlation, the in vitro dissolution and in vivo input curves may be directly superimposable or may be made to be superimposable by the use of a scaling factor. Nonlinear correlations, while uncommon, may also be appropriate.

Whatever the method used to establish a Level A IVIVC, the model should predict the entire in vivo time course from the in vitro data. In this context, the model refers to the relationship between in vitro dissolution of an ER dosage form and an in vivo response such as plasma drug concentration or amount of drug absorbed.

Level B: A Level B IVIVC uses the principles of statistical moment analysis. The mean in vitro dissolution time is compared either to the mean residence time or to the mean in vivo dissolution time. A Level B correlation, like a Level A, uses all of the in vitro and in vivo data, but is not considered to be a point-to-point correlation. A Level B correlation does not uniquely reflect the actual in vivo plasma level curve, because a number of different in vivo curves will produce similar mean residence time values.

Level C: A Level C IVIVC establishes a single point relationship between a dissolution parameter, for example, the percent dissolved in 4 h, and a pharmacokinetic parameter (e.g., time to 50% AUC). A Level C correlation does not reflect the complete shape of the plasma concentration time curve.

Multiple Level C: A multiple Level C correlation relates one or several pharmacokinetic parameters of interest to the amount of drug dissolved at several time points of the dissolution profile.

From a regulatory perspective, it is the Level A correlation that provides the most meaningful information and the highest level of confidence for supporting requests for waiver of in vivo bioequivalence study requirements. However, it is important to recognize that use of this correlation to predict the absorption kinetics of a modified formulation is appropriate only under the condition that the variations share the identical rate-limiting step in drug release. Should a CMC change occur which alters the rate-limiting step, that in vitro test procedure may no longer be adequate to ensure consistent in vivo product performance. Use of an IVIVC to support a biowaiver request relies upon an understanding of the product design space. It is

essentially specific for a product. Therefore, each generic product of a brand is required to have a different IVIVC when biowaivers are sought for CMC changes.

A helpful description of how these relationships can be established and support product development throughout the product development process has been described elsewhere [44]. The IVIVC should be established on the basis of both internal and external validation. The internal validation examines the ability of the model to predict the in vivo performance using the in vitro data that originally went into the model development. Also needed is an external validation, where a formulation with known bioavailability that was not used in the development of the model is used to confirm the adequacy of the overall prediction error.

Within the framework of veterinary medicine or whenever an animal model is used, it may be feasible to establish the IVIVC by doing an explant study (Where the amount of drug remaining as a function of time is evaluated). Such explant studies can provide information on the relationship between product formulation, in vitro performance, and in vivo drug release [45]. From a human food safety perspective, explant studies can also provide important information on the potential variability in residual drug remaining at the injection site. While this residual drug content may have negligible impact on the therapeutic profile of the drug, it can be slowly released over time, thereby influencing product withdrawal time (i.e., the duration of time between drug administration and when the animal-derived edible tissues are safe for human consumption). Explant approaches can also be used when plasma drug concentrations are too low to be adequately quantified [46]. Therefore, these studies can be invaluable in designing an in vitro release test method that supports batch release and, potentially, in vivo biowaivers.

24.6 The Challenge of Developing Clinically Relevant Product Specifications

24.6.1 Physiology-Formulation Considerations

There are several reasons why it may be difficult to develop an in vitro test method that shares the same rate-limiting step as that associated with in vivo drug release. For example, for some injectable products, the limitations in drug absorption are due to the presence of nonsink conditions. This may be particularly important in the case of large therapeutic molecules where convection (i.e., solvent-driven flow of materials) and diffusion (random molecular agitation) will have an important role in in vivo drug release [47]. In other cases, the rate-limiting factor may reflect the ability of the dosage form to successfully enter a specific target site. It will certainly be a challenge to establish an in vitro test method that could mimic the physiological variables that will dictate the in vivo performance of these formulations.

In vitro sink condition can bias release evaluation for polymers whose degradation is catalyzed by a drop in the pH of the microenvironment (e.g., poly(lactic-*co*-

glycolic acid) (PLGA)]. This degradation leads to a drop in pH. The microenvironment may buffer the drop if buffers can diffuse into microsphere or if H^+ can diffuse out. Therefore, in slow-diffusion (in vivo) conditions, there can be an acceleration of polymer autocatalysis [48, 49]. This may not be adequately captured in vitro.

Unlike the small molecule paradigm where the implication of formulation effects is expressed as altered drug absorption, the formulation effects of nanoparticles can result in altered clearance and volume of distribution. For the IV-administered liposome, it is not unusual to see physiological volumes of distribution that increase over time (e.g., days) as an increasing proportion of the administered particles is sequestered by the tissues. Clearance of these particles can reflect phagocytosis or it may reflect a variable drug leakage over time. The challenge of developing an in vitro test that can accurately reflect these physiological variables can be daunting [50].

Another challenging situation is the assessment of in situ forming delivery systems, where the drug is administered as a liquid but forms an implant upon contact with body fluids. These systems reflect complex interactions between product ingredients and interstitial fluid components, and changes in any of these variables can significantly influence product performance [51]. Since the rate-liming step in drug absorption is the drug diffusion through the gel matrix, the in vitro drug release will need to adequately reflect the variation in surface-to-volume ratio that will affect in vivo product performance.

The role of the host immune system can be an important reason why traditional in vitro test methods provide an inaccurate prognosis of factors impacting in vivo bioavailability [52]. An excellent example of this is the relationship between microsphere characteristics and the uptake of microspheres by host phagocytic cells. Particle size, shape, surface characteristics, and mechanical properties can all affect the extent to which host immune cells clear microspheres from the injection site [36]. However, it is unlikely that these properties would impact the in vitro rate of drug release.

The porosity of the implants and microspheres can also influence product biodegradation. Pore size can increase the likelihood of low molecular weight degradation products entering into the microsphere which, in turn facilitates polymer autocatalysis. The surface structure can also increase the likelihood of cellular infiltration into the administration site. This infiltration may take days or weeks to occur, leading to a fibrous encapsulation about the product which results in product fibrous encapsulation and an inability to maintain the intended rate and extent of drug release into the systemic circulation [53]. Given this inherent time delay for altered product release, it is unlikely that an in vitro test will capture these changes, both because it requires the in vivo host response and because most in vitro release tests utilize accelerated test conditions.

24.6.2 Accelerated Test Conditions

To be an effective quality control (QC) tool (particularly when used to support batch release), the in vitro drug release test will need to reach completion within 24–48 h.

A longer timeframe may be prohibitive from a manufacturing perspective for many reasons, not the least of which may be changes to the in vitro test condition that can occur over time (e.g., evaporation of the release test buffer system). However, compressing the duration of drug release from an in vivo timeframe of weeks or months to hours or days could compromise the investigator's ability to monitor the rate-limiting factors governing in vivo drug performance.

Accelerated release for implants and microspheres may be accomplished via modifications of temperature, solvent, ionic strength, pH, agitation rate, or the addition of surfactants or enzymes [54]. However, in each case, such changes could potentially alter the mechanism of drug release. Should that occur, the relevance of these test conditions within a regulatory environment becomes a potential point of concern [55]. Recently, methods for noninvasive, magnetic resonance imaging (BT-MRI) for noninvasive and continuous in vivo studies of in situ forming poly(lactide-*co*-glycolide) (PLGA) implants have been proposed. Although this method did not provide any information on drug kinetics, it did allow for a continuous monitoring of the formation and degradation of an in situ forming gel [56]. In the future, this kind of novel application of existing technologies could provide valuable approaches to enhance our understanding and, therefore regulation, of these novel parenteral products.

The presence of residual solvents or dissolved solutes in a polymeric systemic can lower the glass transition temperature [57]. This is a very important consideration because the transition from a glassy to a rubbery state dictates the ease with which the drug can migrate through the implant or particle. In the glassy state, the macromolecules are immobile, leading to poor diffusivity of the drug. In contrast, in the rubbery state, the mobility of the polymer and the drug are substantially greater, leading to higher rates of drug release. However, the impact of these contaminants on drug release can be obscured by the conditions associated with accelerated release testing. For example, the nature of the buffer system can significantly influence the glass transition temperature of polymeric microparticles [48]. In this regard, the buffer system employed or the use of elevated temperatures can alter the polymer crystalline state, thereby altering the rate of drug release. Accordingly, these "accelerated" test conditions may fail to adequately detect factors that may influence the rate of drug release in vivo.

In other cases, accelerated test conditions may obscure time-dependent changes in product physicochemical properties. Klose [58] observed that high in vitro microsphere concentrations could lead to a pronounced decrease in the pH of the release medium after 10–14 days. This time-dependent drop in pH lead to a decrease in the glass transition temperature, microparticle agglomeration, and an increase in the internal and external microparticle porosity. While this microparticle concentration-dependent effect did not influence drug release of some formulations (e.g., porous, ibuprofen- or lidocaine-loaded microparticles), it had a tremendous impact on others (e.g., nonporous, lidocaine-loaded microparticles and initially porous,

propranolol HCl-loaded systems). Thus, again, we see situations whereby the use of "accelerated" in vitro test conditions may have failed to adequately capture in vivo product performance.

Implants and microspheres are often associated with three phases of drug release: an initial burst release of surface available drug, a secondary slow release controlled by hydration and erosion of the polymer matrix, and a final relatively rapid drug release due to polymer erosion and drug diffusion [59, 60]. Causes of the initial burst phenomenon vary with formulations and include:

1. Flaw in surface coating
2. Changes in capsule coating and saturation of the membrane with drug upon storage product during storage

- Drug diffuses to surface of reservoir membrane
  - Drug saturates the reservoir membrane
  - Drug is released immediately upon injection

3. Sol–gel transition: burst can occur if some drug is not successfully encapsulated
4. Polymer/drug or polymer/solvent ratio: a less compact polymer phase may not adequately suspend the drug, allowing movement of drug towards surface during microsphere production

D'Souza and DeLuca [59] demonstrated that accelerated in vitro test conditions can be developed that adequately describes this triphasic release pattern.

Finally, one of the important questions that have yet to be resolved is whether expiry, as established on the basis of in vitro stability test procedures, is adequate to ensure product performance over the lifetime of its in vivo release. Unlike oral dosage forms, where extended release formulations provide continuous drug exposure for a duration of hours, parenteral extended release formulations may be developed to continue drug release over a duration of months or even years. The question is then whether the expiry date needs to be adjusted so that it encompasses not only shelf life but also the duration of time over which the product is expected to perform in vivo. Clearly, more research is needed in order to resolve this very challenging and important question.

24.7 Concluding Thoughts

Iyer [54] provided a list of considerations for the development of product specifications and in vitro rest methods. Based upon the discussions provided in this chapter, we can add to that list. Figure 24.9 provides a general synopsis of the many points discussed in this chapter that should be considered when developing biorelevant in vitro tests.

Formulation related		*In vivo*		
Type of polymer				
CMC variables	Formulation Method of manufacture Residual solvent Sterility test Pyrogen testing Drug content and content uniformity Drug related substances	Product pharmacokinetics	Absorption kinetics Clearance mechanisms and kinetics Anticipated duration of effect Long term in vivo stability	
			Host-by-formulation interactions	Inflammatory reactions "Stealth" effects Tissue targeting ligands
Dosage form-specific	Size Surface characteristics Charge Ligands	Mechanism of drug release	Mechanism of particle uptake	Lymphatic permeability Macrophage Capillary permeability
Intended duration of action			Intended changes in product physico-chemical characteristics Unintended changes in product physic-chemical characteristics	
Stability	Drug Dosage form			
In Vitro test considerations	Suitability of the selected apparatus (including repeatability and ability to standardize the test conditions). Appropriateness of the flow-rate. Appropriateness of agitation rate Physiological relevance of the medium Rate-limiting factor in *in vivo* vs *in vitro* drug release Informative sampling intervals Methods for accelerated release testing.	Method of administration	Site of administration Volume of administration Surface area Concentration effects	

Fig. 24.9 General considerations in developing product specifications and in vitro release test methods

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