4

Formulation of Biotech Products, Including Biopharmaceutical Considerations

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INTRODUCTION

This chapter deals with formulation aspects of pharmaceutical proteins. Both technological questions and biopharmaceutical issues such as the choice of the delivery systems, the route of administration, and possibilities for target site-specific delivery of proteins are considered.

MICROBIOLOGICAL CONSIDERATIONS

■ Sterility

Most proteins are administered parenterally and have to be sterile. In general, proteins are sensitive to heat and other regularly used sterilization treatments; they cannot withstand autoclaving, gas sterilization, or sterilization by ionizing radiation. Consequently, sterilization of the end product is not possible. Therefore, protein pharmaceuticals have to be assembled under aseptic conditions, following the established and evolving rules in the pharmaceutical industry for aseptic manufacture. The reader is referred to standard textbooks for details (Halls 1994; Groves 1988; Klegerman and Groves 1992; Roy 2011).

Equipment and excipients are treated separately and autoclaved or sterilized by dry heat (>160 °C), chemical treatment, or gamma radiation to minimize the bioburden. Filtration techniques are used for removal of microbacterial contaminants. Prefilters remove the bulk of the bioburden and other particulate materials. The final "sterilizing" step before filling the vials is filtration through 0.2 or 0.22 μm membrane filters. Assembly of the product is done in class 100 (maximum 100 particles > 0.5 μm per cubic foot) rooms with laminar airflow that is filtered through HEPA (high-efficiency particulate air) filters. Last but not least, the "human factor" is a major source of contamination. Well-trained operators wearing protective cloths (face masks, hats, gowns, gloves, or head-to-toe overall garments) should operate the facility. Regular exchange of filters, regular validation of HEPA equipment, and thorough cleaning of the room plus equipment are critical factors for success.

■ Viral Decontamination

As recombinant DNA products are grown in microorganisms, these organisms should be tested for viral contaminants, and appropriate measures should be taken if viral contamination occurs. In the rest of the manufacturing process, no (unwanted) viral material should be introduced. Excipients with a certain risk factor such as blood-derived human serum albumin should be carefully tested before use, and their presence in the formulation process should be minimized (see Chap. 3).

■ Pyrogen Removal

Pyrogens are compounds that induce fever. Exogenous pyrogens (pyrogens introduced into the body, not generated by the body itself) can be derived from bacterial, viral, or fungal sources. Bacterial pyrogens are mainly endotoxins shed from gram-negative bacteria. They are lipopolysaccharides. Figure 4.1 shows the basic structure. This conserved structure in the full array of thousands of different endotoxins is the lipid-A moiety. Another general property shared by endotoxins is their high, negative electrical charge. Their tendency to aggregate and to form large units with M_W of over 10^6 in water and their tendency to adsorb to surfaces indicate that these compounds are amphipathic in nature. They are stable under standard autoclaving conditions but break down when heated in the dry state. For this reason equipment and container are treated at temperatures above 160 °C for prolonged periods (e.g., 30 min dry heat at 250 °C).

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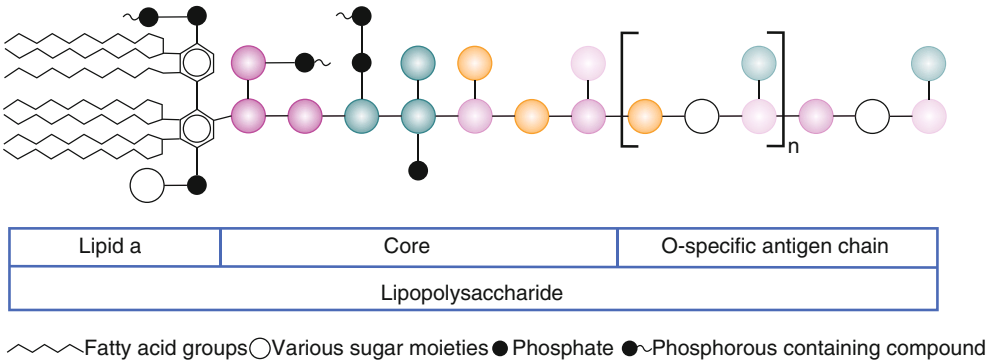


Figure 4.1 ■ Generalized structure of endotoxins. Most properties of endotoxins are accounted for by the active, insoluble "lipid A" fraction being solubilized by the various sugar moieties (*circles* with different colors). Although the general structure is similar, individual endotoxins vary according to their source and are characterized by the O-specific antigenic chain (Adapted from Groves (1988)).

Pyrogen removal of recombinant products derived from bacterial sources should be an integral part of the preparation process. Ion exchange chromatographic procedures (utilizing its negative charge) can effectively reduce endotoxin levels in solution (see also Chap. 3).

Excipients used in the protein formulation should be essentially endotoxin-free. For solutions "water for injection" (compendial standards) is (freshly) distilled or produced by reverse osmosis. The aggregated endotoxins cannot pass through the reverse osmosis membrane. Removal of endotoxins immediately before filling the final container can be accomplished by using activated charcoal or other materials with large surfaces offering hydrophobic interactions. Endotoxins can also be inactivated on utensil surfaces by oxidation (e.g., peroxide) or dry heating (e.g., 30 min dry heat at 250 °C).

EXCIPIENTS USED IN PARENTERAL FORMULATIONS OF BIOTECH PRODUCTS

In a protein formulation one finds, apart from the active substance, a number of excipients selected to serve different purposes. This process of formulation design should be carried out with great care to ensure therapeutically effective and safe products. The nature of the protein (e.g., lability) and its therapeutic use (e.g., multiple injection systems) can make these formulations quite complex in terms of excipient profile and technology (freeze-drying, aseptic preparation). Table 4.1 lists components that can be found in the presently marketed formulations. In the following sections this list is discussed in more detail. A classical review on peptide and protein excipients was published by Wang and Watson (1988).

Active ingredient
Solubility enhancers
Anti-adsorption and anti-aggregation agents
Buffer components
Preservatives and antioxidants
Lyoprotectants/cake formers
Osmotic agents
Carrier system (seen later on in this section)
Not necessarily all of the above are present in one particular protein formulation

Table 4.1 ■ Components found in parenteral formulations of biotech products.

■ Solubility Enhancers

Proteins, in particular those that are non-glycosylated, may have a tendency to aggregate and precipitate. Approaches that can be used to enhance solubility include selection of the proper pH and ionic strength conditions. Addition of amino acids such as lysine or arginine (used to solubilize tissue plasminogen activator, t-PA) or surfactants such as sodium dodecyl sulfate to solubilize non-glycosylated IL-2 can also help to increase the solubility. The mechanism of action of these solubility enhancers depends on the type of enhancer and the protein involved and is not always fully understood.

Figure 4.2 shows the effect of arginine concentration on the solubility of t-PA (alteplase) at pH 7.2 and 25 °C. This figure clearly indicates the dramatic effect of this basic amino acid on the apparent solubility of t-PA.

In the above examples, aggregation is physical in nature, i.e., based on hydrophobic and/or electrostatic interactions between molecules. However, aggregation based on the formation of covalent bridges between molecules through disulfide bonds and ester or amide linkages has been described as well (see also Table 2.4). In those cases, proper conditions should be found to avoid these chemical reactions.

■ Anti-adsorption and Anti-aggregation Agents

Anti-adsorption agents are added to reduce adsorption of the active protein to interfaces. Some proteins tend to

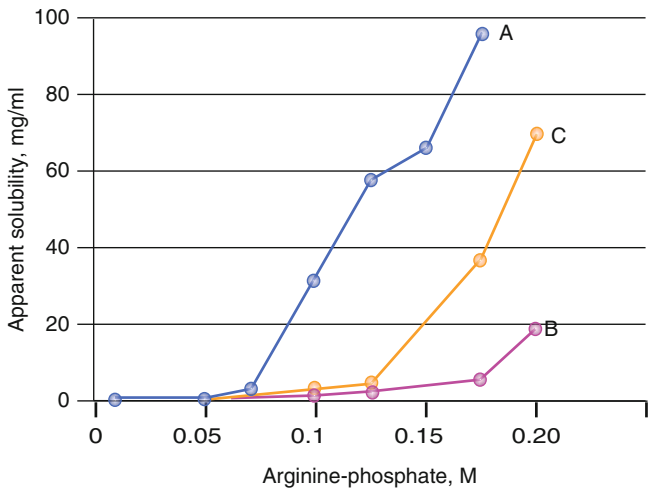


Figure 4.2 ■ Effect of arginine on type I and type II alteplase at pH 7.2 and 25 °C. *A* type I alteplase, *B* type II alteplase, *C* 50:50 mixture of type I and type II alteplase (From Nguyen and Ward (1993)).

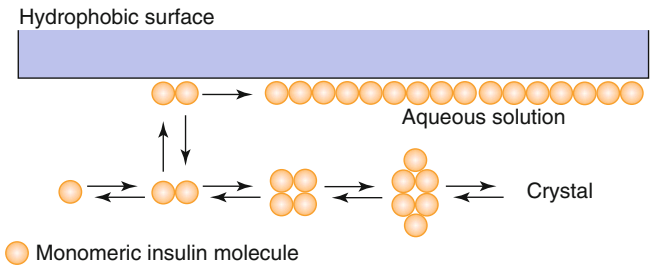


Figure 4.3 ■ Reversible self-association of insulin, its adsorption to the hydrophobic interface, and irreversible aggregation in the adsorbed protein film: ● represents a monomeric insulin molecule (Adapted from Thurow and Geisen (1984)).

expose hydrophobic sites, normally present in the core of the native protein structure when an interface is present. These interfaces can be water–air, water–container wall, or interfaces formed between the aqueous phase and utensils used to administer the drug (e.g., catheter, needle). These adsorbed, partially unfolded protein molecules form aggregates, leave the surface, return to the aqueous phase, form larger aggregates, and precipitate. As an example, the proposed mechanism for aggregation of insulin in aqueous media through contact with a hydrophobic surface (or water–air interface) is presented in Fig. 4.3 (Thurow and Geisen 1984).

Native insulin in solution is in an equilibrium state between monomeric, dimeric, tetrameric, and hexameric forms (see Chap. 12). The relative abundance of the different aggregation states depends on the pH, insulin concentration, ionic strength, and specific excipients (e.g., Zn^{2+} and phenol). It has been suggested that the dimeric form of insulin adsorbs to hydrophobic interfaces and subsequently forms larger aggregates at the interface. This explains why anti-adhesion agents can also act as anti-aggregation agents. Albumin has a strong tendency to adsorb to surfaces and is therefore added in relatively high concentrations (e.g., 1 %) to protein formulations as an anti-adhesion agent. Albumin competes with the therapeutic protein for binding sites and supposedly prevents adhesion of the therapeutically active agent by a combination of its binding tendency and abundant presence.

Insulin is one of the many proteins that can form fibrillar precipitates (long rod-shaped structures with diameters in the 0.1 μm range). Low concentrations of phospholipids and surfactants have been shown to exert a fibrillation-inhibitory effect. The selection of the proper pH can also help to prevent this unwanted phenomenon (Brange and Langkjaer 1993).

Apart from albumin, surfactants can also prevent adhesion to interfaces and precipitation. These molecules readily adsorb to hydrophobic interfaces with their own hydrophobic groups and render this interface hydrophilic by exposing their hydrophilic groups to the aqueous phase.

■ Buffer Components

Buffer selection is an important part of the formulation process, because of the pH dependence of protein solubility and physical and chemical stability. Buffer systems regularly encountered in biotech formulations are phosphate, citrate, and acetate. A good example of the importance of the isoelectric point (its negative logarithm = pI) is the solubility profile of human growth hormone (hGH, pI around 5) as presented in Fig. 4.4.

Even short, temporary pH changes can cause aggregation. These conditions can occur, for example, during the freezing step in a freeze-drying process, when one of the buffer components is crystallizing and the other is not. In a phosphate buffer, Na_2HPO_4 crystallizes faster than NaH_2PO_4. This causes a pronounced drop in pH during the freezing step. Other buffer components do not crystallize but form amorphous systems, and then pH changes are minimized.

■ Preservatives and Antioxidants

Methionine, cysteine, tryptophan, tyrosine, and histidine are amino acids that are readily oxidized (see Table 2.4). Proteins rich in these amino acids are liable to oxidative degradation. Replacement of oxygen by inert gases in the vials helps to reduce oxidative stress. Moreover, the addition of antioxidants such as ascorbic acid or acetylcysteine can be considered. Interestingly, destabilizing effects on proteins have been described for antioxidants as well (Vemuri et al. 1993). Ascorbic acid, for example, can act as an oxidant in the presence of a number of heavy metals.

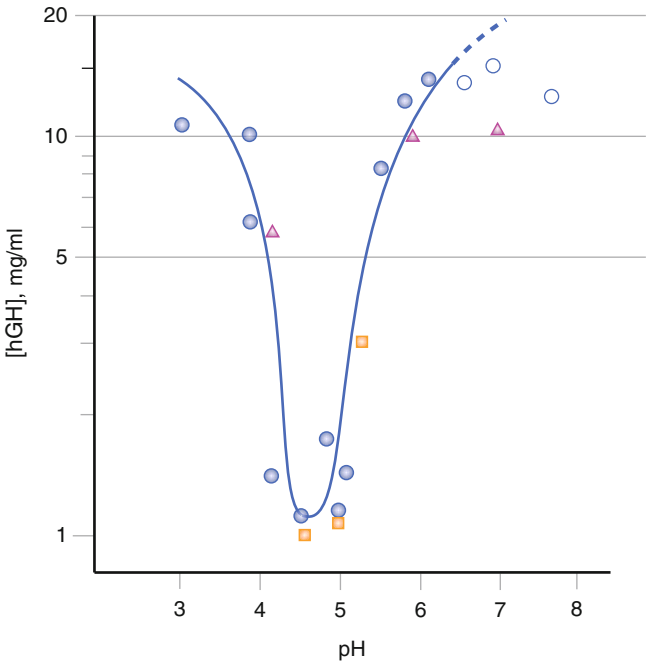


Figure 4.4 ■ A plot of the solubility of various forms of hGH as a function of pH. Samples of hGH were either recombinant hGH (*circles*), Met-hGH (*triangles*), or pituitary hGH (*squares*). Solubility was determined by dialyzing an approximately 11 mg/ml solution of each protein into an appropriate buffer for each pH. Buffers were citrate, pH 3–7, and borate, pH 8–9, all at 10 mM buffer concentrations. Concentrations of hGH were measured by UV absorbance as well as by RP-HPLC, relative to an external standard. The closed symbols indicate that precipitate was present in the dialysis tube after equilibration, whereas open symbols mean that no solid material was present, and thus the solubility is at least this amount (From Pearlman and Bewley (1993)).

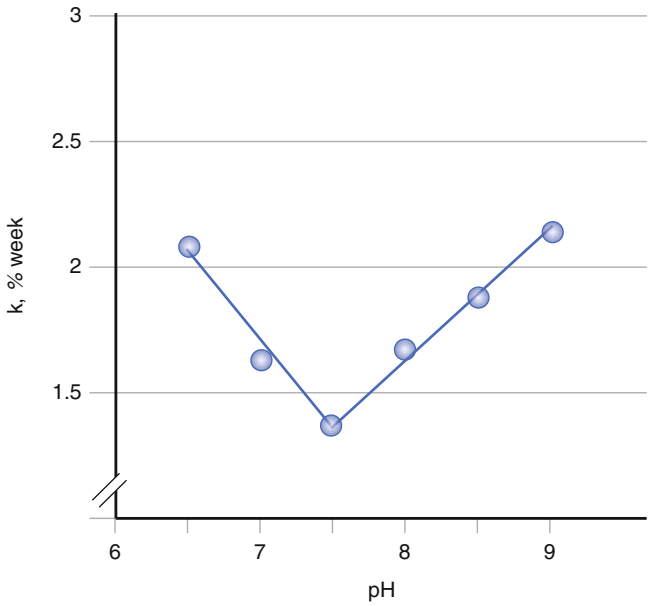


Figure 4.5 ■ pH stability profile (at 25 °C) of monomeric recombinant α_1-antitrypsin (rAAT) by size exclusion-HPLC assay, *k* degradation rate constant. Monomeric rAAT decreased rapidly in concentration both under acidic and basic conditions. Optimal stability occurred at pH 7.5 (Adjusted from Vemuri et al. (1993)).

Certain proteins are formulated in containers designed for multiple injection schemes. After administering the first dose, contamination with microorganisms may occur, and preservatives are needed to minimize growth. Usually, these preservatives are present in concentrations that are bacteriostatic rather than bactericidal in nature. Antimicrobial agents mentioned in the USP 29 are the mercury-containing phenylmercuric nitrate and thimerosal and p-hydroxybenzoic acids, phenol, benzyl alcohol, and chlorobutanol (USP 29 2006; Groves 1988; Pearlman and Bewley 1993). The use of mercury-containing preservatives is under discussion (FDA 2010).

■ Osmotic Agents

For proteins the regular rules apply for adjusting the tonicity of parenteral products. Saline and mono- or disaccharide solutions are commonly used. These excipients may not be inert; they may influence protein structural stability. For example, sugars and polyhydric alcohols can stabilize the protein structure through the principle of "preferential exclusion" (Arakawa et al. 1991). These additives (water structure promoters) enhance the interaction of the solvent with the protein and are themselves excluded from the protein surface layer; the protein is preferentially hydrated. This phenomenon can be monitored through an increased thermal stability of the protein. Unfortunately, a strong "preferential exclusion" effect enhances the tendency of proteins to self-associate.

SHELF LIFE OF PROTEIN-BASED PHARMACEUTICALS

Proteins can be stored (1) as an aqueous solution, (2) in freeze-dried form, and (3) in dried form in a compacted state (tablet). Some mechanisms behind chemical and physical degradation processes have been briefly discussed in Chap. 2.

Stability of protein solutions strongly depends on factors such as pH, ionic strength, temperature, and the presence of stabilizers. For example, Fig. 4.5 shows the pH dependence of α_1-antitrypsin and clearly demonstrates the critical importance of pH for the shelf life of proteins.

■ Freeze-Drying of Proteins

Proteins in solution often do not meet the preferred stability requirements for industrially produced pharmaceutical products (>2 years), even when kept permanently under refrigerator conditions (cold chain).

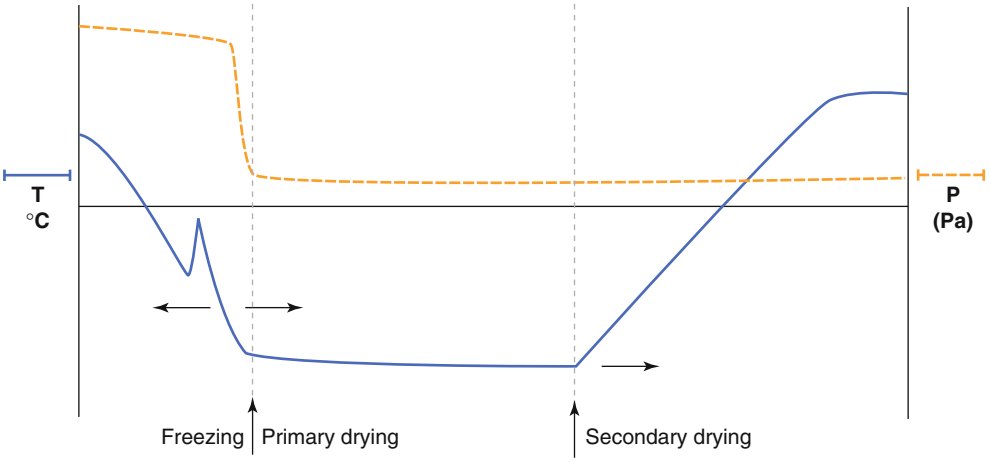


Figure 4.6 ■ Example of freeze-drying protocol for systems with crystallizing water. *T* temperature, *P* pressure.

Freezing
The temperature of the product is reduced from ambient temperature to a temperature below the eutectic temperature (T_e) or below the glass transition temperature (T_g) of the system. A T_g is encountered if amorphous phases are present
Primary drying
Crystallized and water not bound to protein/excipient is removed by sublimation. The temperature is below the T_e or T_g; the temperature is, for example, –40 °C and reduced pressures are used
Secondary drying
Removal of water interacting with the protein and excipients. The temperature in the chamber is kept below T_g and rises gradually, e.g., from –40 to 20 °C

Table 4.2 ■ Three stages in the freeze-drying process of protein formulations.

Bulking agents: mannitol/ glycine	Reason: elegance/blowout prevention[a]
Collapse temperature modifier: dextran, albumin/ gelatine	Reason: increase collapse temperature
Lyoprotectant: sugars, albumin	Reason: protection of the physical structure of the protein[b]

[a]Blowout is the loss of material taken away by the water vapor that leaves the vial. It occurs when little solid material is present in the vial

[b]Mechanism of action of lyoprotectants is not fully understood. Factors that might play a role are: (1) Lyoprotectants replace water as stabilizing agent (water replacement theory). (2) Lyoprotectants increase the Tg of the cake/frozen system. (3) Lyoprotectants will absorb moisture from the stoppers. Lyoprotectants slow down the secondary drying process and minimize the chances for overdrying of the protein. Overdrying might occur when residual water levels after secondary drying become too low. The chance for overdrying "in real life" is small

Table 4.3 ■ Typical excipients in a freeze-dried protein formulation.

The abundant presence of water promotes chemical and physical degradation processes.

Freeze-drying may provide the requested stability (Constantino and Pikal 2004). During freeze-drying water is removed through sublimation and not by evaporation. Three stages can be discerned in the freeze-drying process: (1) a freezing step, (2) the primary drying step, and (3) the secondary drying step (Fig. 4.6). Table 4.2 explains what happens during these stages.

Freeze-drying of a protein solution without the proper excipients causes, as a rule, irreversible damage to the protein. Table 4.3 lists excipients typically encountered in successfully freeze-dried protein products.

■ Freezing

In the freezing step (see Fig. 4.6) the temperature of the aqueous system in the vials is lowered. Ice crystal formation does not start right at the thermodynamic or equilibrium freezing point, but supercooling occurs. That means that crystallization often only occurs when temperatures of –15°C or lower have been reached. During the crystallization step the temperature may temporarily rise in the vial, because of the generation of crystallization heat. During the cooling stage, concentration of the protein and excipients occurs because of the growing ice crystal mass at the expense of the aqueous water phase. This can cause precipitation of one or more of the excipients, which may consequently result in pH shifts (see above and Fig. 4.7) or ionic strength changes. It may also induce protein denaturation. Cooling of the vials is done through lowering the temperature of the shelf. Selecting the proper cooling scheme for the shelf – and consequently vial – is important as it dictates the degree of supercooling and ice crystal size. Small crystals are formed during fast cooling; large crystals form at lower cooling rates. Small ice crystals are required for porous solids and fast sublimation rates (Pikal 1990).

If the system does not (fully) crystallize but forms an amorphous mass upon cooling, the temperature in the "freezing stage" should drop below Tg, the glass

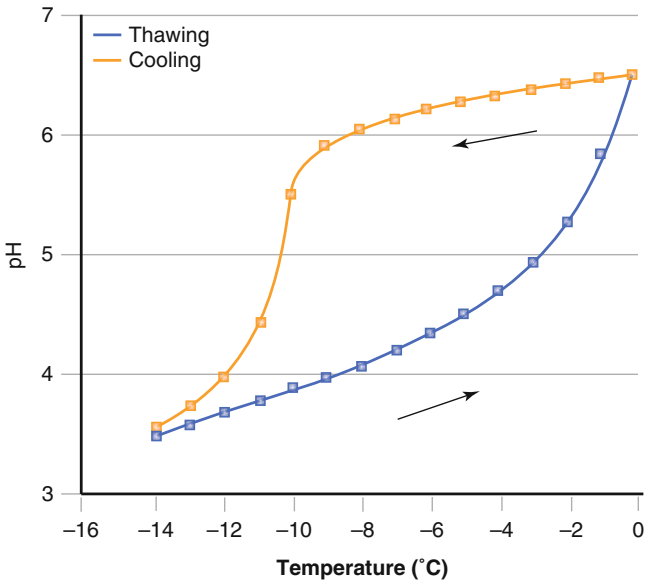


Figure 4.7 ■ Thawing/cooling; ■ thawing, ■ cooling. The effect of freezing on the pH of a citric acid–disodium phosphate buffer system (Cited in Pikal 1990).

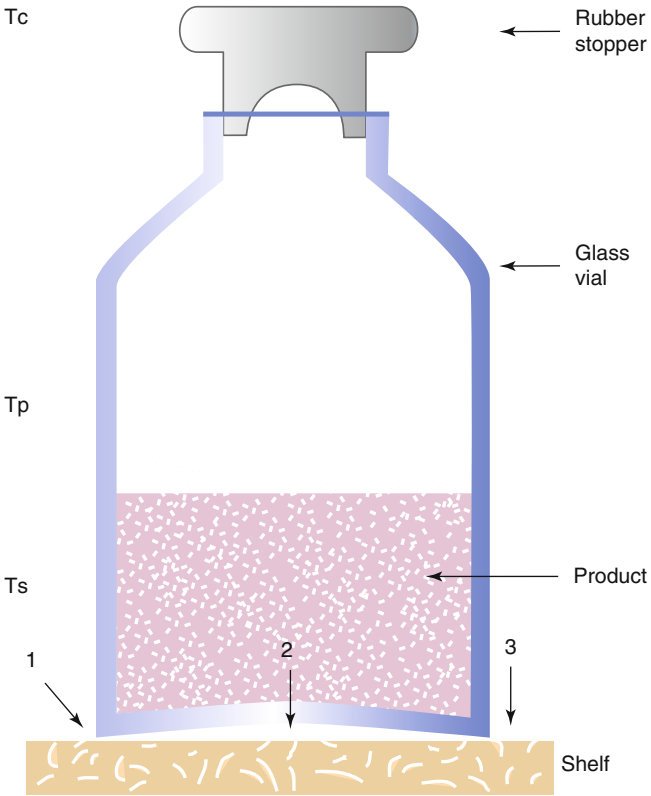


Figure 4.8 ■ Heat transfer mechanisms during the freeze-drying process: (*1*) Direct conduction via shelf and glass at points of actual contact. (*2*) Gas conduction: contribution heat transfer via conduction through gas between shelf and vial bottom. (*3*) Radiation heat transfer. *Ts* shelf temperature, *Tp* temperature sublimating product, *Tc* temperature condensor. Ts > Tp > Tc.

transition temperature. In amorphous systems the viscosity changes dramatically in the temperature range around the Tg: A "rubbery" state exists above and a glass state below the Tg.

At the start of the primary drying stage, no "free and fluid" water should be present in the vials. Minus forty degrees Celsius is a typical freezing temperature before sublimation is initiated through pressure reduction.

■ Primary Drying

In the primary drying stage (see Fig. 4.6.), sublimation of the water mass in the vial is initiated by lowering the pressure. The water vapor is collected on a condenser, with a (substantially) lower temperature than the shelf with the vials. Sublimation costs energy (about 2,500 kJ/g ice). Temperature drops are avoided by the supply of heat from the shelf to the vial, so the shelf is heated during this stage.

Heat is transferred to the vial through (1) direct shelf–vial contact (conductance), (2) radiation, and (3) gas conduction (Fig. 4.8). Gas conduction depends on the pressure: If one selects relatively high gas pressures, heat transport is promoted because of a high conductivity. But it reduces mass transfer, because of a low driving force: the pressure between equilibrium vapor pressures at the interface between the frozen mass/dried cake and the chamber pressure (Pikal 1990).

During the primary drying stage, one transfers heat from the shelf through the vial bottom and the frozen mass to the interface frozen mass/dry powder, to keep the sublimation process going. During this drying stage the vial content should never reach or exceed the eutectic temperature or glass transition temperature range. Typically a safety margin of 2–5°C is used; otherwise, the cake will collapse. Collapse causes a strong reduction in sublimation rate and poor cake formation. Heat transfer resistance decreases during the drying process as the transport distance is reduced by the retreating interface. With the mass transfer resistance (transport of water vapor), however, the opposite occurs. Mass transfer resistance increases during the drying process as the dry cake becomes thicker.

This situation makes it clear that parameters such as chamber pressure and shelf heating are not necessarily constant during the primary drying process. They should be carefully chosen and adjusted as the drying process proceeds.

The eutectic temperature and glass transition temperature are parameters of great importance to develop a rationally designed freeze-drying protocol. Information about these parameters can be obtained by microscopic observation of the freeze-drying process, differential scanning calorimetry (DSC), or electrical resistance measurements.

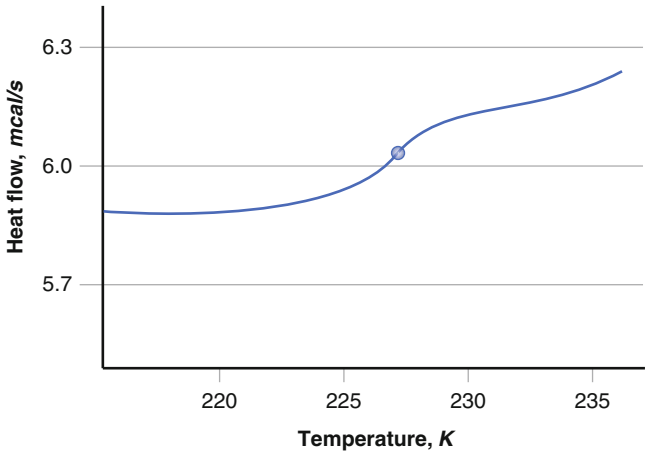


Figure 4.9 ■ Differential scanning calorimetry heating trace for a frozen solution of sucrose and sodium chloride, showing the glass transition temperature of the freeze concentrate at 227 K. For pure freeze-concentrated sucrose, T_g=241 K (1 cal=4.2 J) (From Franks et al. (1991)).

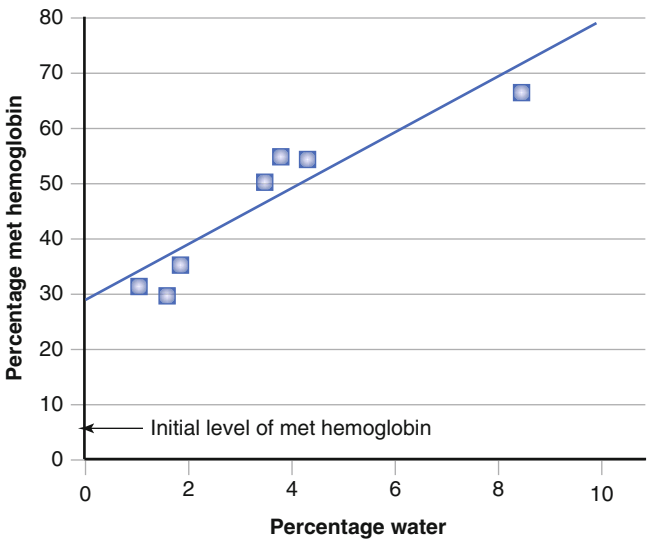


Figure 4.10 ■ The effect of residual moisture on the stability of freeze-dried hemoglobin (–6 %) formulated with 0.2 M sucrose; decomposition to methemoglobin during storage at 23 °C for 4 years (From Pikal (1990). Data reported by Pritoupil et al. (1985)).

An example of a DSC scan providing information on the Tg is presented in Fig. 4.9 (Franks et al. 1991). The Tg heavily depends on the composition of the system: excipients and water content. Lowering the water content of an amorphous system causes the Tg to shift to higher temperatures.

■ Secondary Drying

When all frozen or amorphous water that is nonprotein and non-excipient bound is removed, the secondary drying step starts (Fig. 4.6). The end of the primary drying stage is reached when product temperature and shelf temperature become equal or when the partial water pressure drops (Pikal 1990). As long as the "non-bound" water is being removed, the partial water pressure almost equals the total pressure. In the secondary drying stage, the temperature is slowly increased to remove "bound" water; the chamber pressure is still reduced. The temperature should stay all the time below the collapse/eutectic temperature, which continues to rise when residual water contents drop. Typically, the secondary drying step ends when the product has been kept at 20°C for some time. The residual water content is a critical, end point-indicating parameter. Values as low as 1 % residual water in the cake have been recommended. Figure 4.10 (Pristoupil 1985; Pikal 1990) exemplifies the decreasing stability of freeze-dried hemoglobin with increasing residual water content.

When stored in the presence of reducing lyoprotectants such as glucose and lactose, the Maillard reaction may occur: Amino groups of the proteins react with the lyoprotectant in the dry state, and the cake color turns yellow brown. The use of nonreducing sugars such as sucrose or trehalose may avoid this problem.

■ Other Approaches to Stabilize Proteins

Compacted forms of proteins are being used for certain veterinary applications, such as sustained-release formulations of growth hormones. The pellets should contain as few additives as possible. They can be applied subdermally or intramuscularly when the compact pellets are introduced by compressed air-powered rifles into the animals (Klegerman and Groves 1992).

DELIVERY OF PROTEINS: ROUTES OF ADMINISTRATION AND ABSORPTION ENHANCEMENT

■ The Parenteral Route of Administration

Parenteral administration is here defined as administration via those routes where a needle is used, including intravenous (IV), intramuscular (IM), subcutaneous (SC), and intraperitoneal (IP) injections. More information on the pharmacokinetic behavior of recombinant proteins is provided in Chap. 5. It suffices here to state that the blood half-life of biotech products can vary over a wide range. For example, the circulation half-life of tissue plasminogen activator (t-PA) is a few minutes, while monoclonal antibodies reportedly have half-lives of a few days. Obviously, one reason to develop modified proteins through site-directed mutagenesis is to enhance circulation half-life. A simple way to expand the mean residence time for short half-life proteins is to switch from IV to IM or SC administration. One should realize that by doing that, changes in disposition may occur, with a significant impact on the

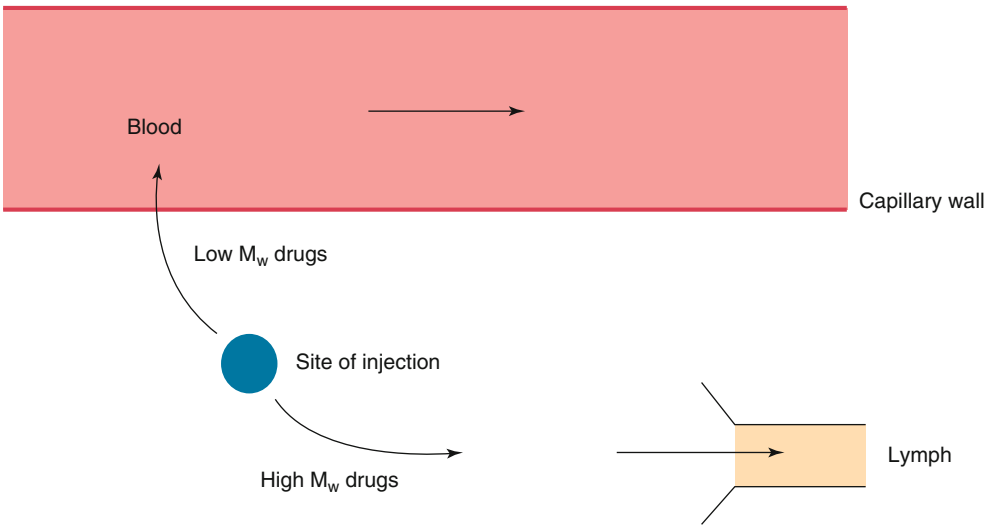


Figure 4.11 ■ Routes of uptake of SC- or IM-injected drugs.

therapeutic performance of the drug. These changes are related to (1) the prolonged residence time at the IM or SC site of injection compared to IV administration and the enhanced exposure to degradation reactions (peptidases) and (2) differences in disposition.

Regarding point 1: Prolonged residence time at the IM or SC site of injection and the enhanced exposure to degradation reactions. For instance, diabetics can become "insulin resistant" through high tissue peptidase activity (Maberly et al. 1982). Other factors that can contribute to absorption variation are related to differences in exercise level of the muscle at the injection site and also massage and heat at the injection site. The state of the tissue, for instance, the occurrence of pathological conditions, may be important as well.

Regarding point 2: Differences in disposition. Upon administration, the protein may be transported to the blood through the lymphatics or may enter the blood circulation through the capillary wall at the site of injection (Figs. 4.11 and 4.12). The fraction of the administered dose taking this lymphatic route is molecular weight dependent (Supersaxo et al. 1990). Lymphatic transport takes time (hours), and uptake in the blood circulation is highly dependent on the injection site. On its way to the blood, the lymph passes through draining lymph nodes, and contact is possible between lymph contents and cells of the immune system such as macrophages and B and T lymphocytes residing in the lymph nodes.

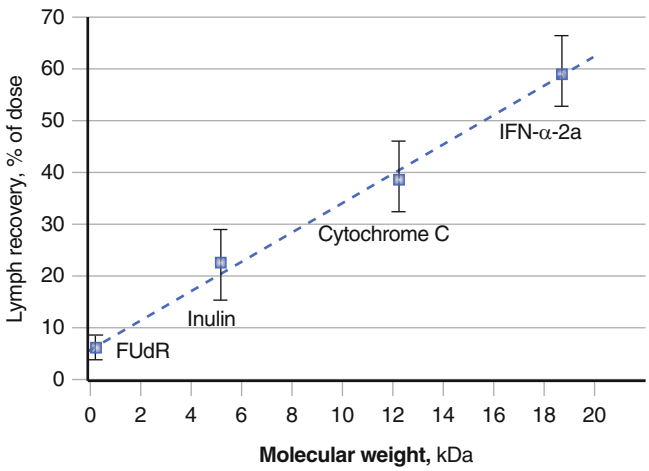


Figure 4.12 ■ Correlation between the molecular weight and the cumulative recovery of rIFN alpha-2a (M_w 19 kDa), cytochrome c (M_W 12.3 kDa), insulin (M_W 5.2 kDa), and FUDR (M_W 256.2 Da) in the efferent lymph from the right popliteal lymph node following SC administration into the lower part of the right hind leg of sheep. Each point and bar shows the mean and standard deviation of three experiments performed in separate sheep. The line drawn is the best fit by linear regression analysis calculated with the four mean values. The points have a correlation coefficient r of 0.998 ($p<0.01$) (From Supersaxo et al. (1990)).

■ The Oral Route of Administration

Oral delivery of protein drugs would be preferable, because it is patient friendly and no intervention by a healthcare professional is necessary to administer the drug. Oral bioavailability, however, is usually very low. The two main reasons for this failure of uptake are (1) protein degradation in the gastrointestinal (GI) tract and (2) poor permeability of the wall of the GI tract in case of a passive transport process.

Regarding point 1: Protein degradation in the GI tract. The human body has developed a very efficient system to break down proteins in our food to amino acids or di- or tripeptides. These building stones for body proteins are actively absorbed for use wherever necessary in the body. In the stomach, pepsins, a family of aspartic proteases, are secreted. They are particularly active between pH 3 and 5 and lose activity at higher pH values. Pepsins are endopeptidases capable of cleaving

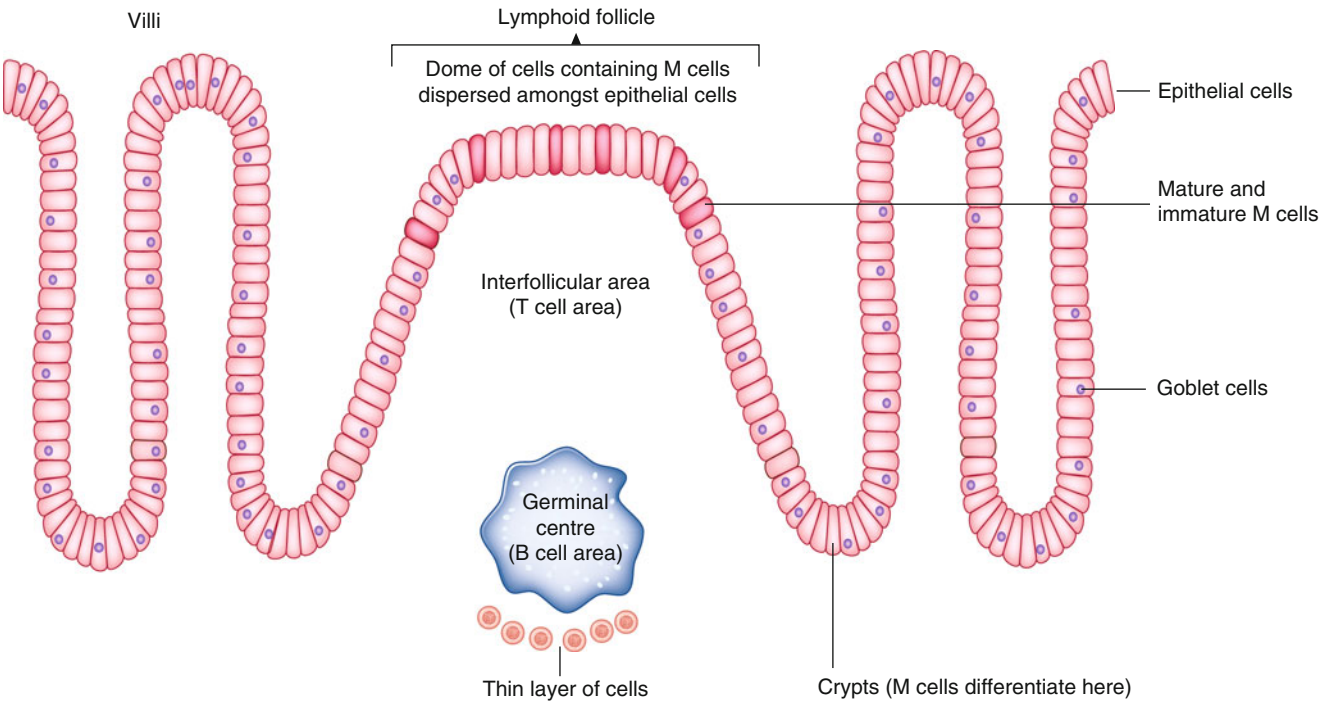


Figure 4.13 ■ Schematic diagram of the structure of intestinal Peyer's patches. M cells within the follicle-associated epithelium are enlarged for emphasis (From O'Hagan (1990)).

peptide bonds distant from the ends of the peptide chain. They preferentially cleave peptide bonds between two hydrophobic amino acids. Other endopeptidases are active in the gastrointestinal tract at neutral pH values, e.g., trypsin, chymotrypsin, and elastase. They have different peptide bond cleavage characteristics that more or less complement each other. Exopeptidases, proteases degrading peptide chains from their ends, are present as well. Examples are carboxypeptidase A and B. In the GI lumen the proteins are cut into fragments that effectively further break down to amino acids, di- and tripeptides by brush border, and cytoplasmic proteases of the enterocytes.

Regarding point 2: Permeability. High molecular weight molecules do not readily penetrate the intact and mature epithelial barrier if diffusion is the sole driving force for mass transfer. Their diffusion coefficient decreases with increasing molecule size. Proteins are no exception to this rule. Active transport of intact therapeutic recombinant proteins over the GI-epithelium has not been described yet.

The above analysis leads to the conclusion that nature, unfortunately, does not allow us to use the oral route of administration for therapeutic proteins if high (or at least constant) bioavailability is required.

However, for the category of oral vaccines, the above-mentioned hurdles of degradation and permeation are not necessarily prohibitive. For oral immunization, only a (small) fraction of the antigen (protein) has to reach its target site to elicit an immune response. The target cells are lymphocytes and antigen-presenting accessory cells located in Peyer's patches (Fig. 4.13). The B-lymphocyte population includes cells that produce secretory IgA antibodies.

These Peyer's patches are macroscopically identifiable follicular structures located in the wall of the gastrointestinal tract. Peyer's patches are overlaid with microfold (M) cells that separate the luminal contents from the lymphocytes. These M cells have little lysosomal degradation capacity and allow for antigen sampling by the underlying lymphocytes. Moreover, mucus-producing goblet cell density is reduced over Peyer's patches. This reduces mucus production and facilitates access to the M cell surface for luminal contents (Delves et al. 2011). Attempts to improve antigen delivery via the Peyer's patches and to enhance the immune response are made by using microspheres, liposomes, or modified live vectors, such as attenuated bacteria and viruses (Kersten and Hirschberg 2004, see also Chap. 22).

■ Alternative Routes of Administration

Parenteral administration has disadvantages (needles, sterility, injection skills) compared to other possible routes. Therefore, systemic delivery of recombinant proteins by alternative routes of administration (apart from the GI tract, discussed above) has been studied extensively. The nose, lungs, rectum, oral cavity, and

Route
+=relative advantage, −=relative disadvantage
Nasal
+ easily accessible, fast uptake, proven track record with a number of "conventional" drugs, probably lower proteolytic activity than in the GI tract, avoidance of first pass effect, spatial containment of absorption enhancers is possible
– reproducibility (in particular under pathological conditions), safety (e.g., ciliary movement), low bioavailability for proteins
Pulmonary
+ relatively easy to access, fast uptake, proven track record with "conventional" drugs, substantial fractions of insulin are absorbed, lower proteolytic activity than in the GI tract, avoidance of hepatic first pass effect, spatial containment of absorption enhancers (?)
– reproducibility (in particular under pathological conditions, smokers/nonsmokers), safety (e.g., immunogenicity), presence of macrophages in the lung with high affinity for particulates
Rectal
+ easily accessible, partial avoidance of hepatic first pass, probably lower proteolytic activity than in the upper parts of the GI tract, spatial containment of absorption enhancers is possible, proven track record with a number of "conventional" drugs
– low bioavailability for proteins
Buccal
+ easily accessible, avoidance of hepatic first pass, probably lower proteolytic activity than in the lower parts of the GI tract, spatial containment of absorption enhancers is possible, option to remove formulation if necessary
– low bioavailability of proteins, no proven track record yet (?)
Transdermal
+ easily accessible, avoidance of hepatic first pass effect, removal of formulation if necessary is possible, spatial containment of absorption enhancers, proven track record with "conventional" drugs, sustained/controlled release possible
– low bioavailability of proteins

Table 4.4 ■ Alternative routes of administration to the oral route for biopharmaceuticals.

skin have been selected as potential sites of application. The potential pros and cons for the different relevant routes are listed in Table 4.4. Moeller and Jorgensen (2009) and Jorgenson and Nielsen (2009) describe "the state of the art" in more detail.

The nasal, buccal, rectal, and transdermal routes all have been shown to be of little clinical relevance if systemic action is required and if simple protein formulations without an absorption-enhancing technology are used. In general, bioavailability is too low and varies too much! The pulmonary route may be the exception to this rule. Table 4.5 (from Patton et al. 1994) presents the bioavailability in rats of intratracheally administered protein solutions with a wide range of molecular weights. Absorption was strongly protein dependent, with no clear relationship with its molecular weight.

In humans the drug should be inhaled instead of intratracheally administered. The first pulmonary insulin formulation was approved by FDA in January 2006 (Exubera®) but taken off the market in 2008 because of poor market penetration. Pulmonary inhalation of insulin is specifically indicated for mealtime glucose control. Uptake of insulin is faster than after a regular SC insulin injection (peak 5–60 min vs. 60–180 min). The reproducibility of the blood glucose response to inhaled insulin was equivalent to SC-injected insulin. Inhalation technology plays a critical role when considering the prospects of the pulmonary route for the systemic delivery of therapeutic proteins. Dry powder inhalers and nebulizers are being tested. The fraction of insulin that is ultimately absorbed depends on (1) the fraction of the inhaled/nebulized dose that is actually leaving the device, (2) the fraction that is actually deposited in the lung, and

Molecule	*M*w kDa	#AA	Absolute Bioavailability (%)
α-Interferon	20	165	>56
PTH-84	9	84	>20
PTH-34	4.2	34	40
Calcitonin (human)	3.4	32	17
Calcitonin (salmon)	3.4	32	17
Glucagons	3.4	29	<1
Somatostatin	3.1	28	<1

Adapted from Patton et al. (1994)

PTH recombinant human parathyroid hormone, *#AA* number of amino acids

Table 4.5 ■ Absolute bioavailability of a number of proteins (intratracheal vs. intravenous) in rats.

Classified according to proposed mechanism of action
Increase the permeability of the absorption barrier:
Addition of fatty acids/phospholipids, bile salts, enamine derivatives of phenylglycine, ester and ether type (non)-ionic detergents, saponins, salicylate derivatives, derivatives of fusidic acid or glycyrrhizinic acid, or methylated β-cyclodextrins
Through iontophoresis
By using liposomes
Decrease peptidase activity at the site of absorption and along the "absorption route": aprotinin, bacitracin, soybean tyrosine inhibitor, boroleucin, borovaline
Enhance resistance against degradation by modification of the molecular structure
Prolongation of exposure time (e.g., bio-adhesion technologies)

Adapted from Zhou and Li Wan Po (1991a)

Table 4.6 ■ Approaches to enhance bioavailability of proteins.

(3) the fraction that is being absorbed, i.e., total relative uptake (TO %) = % uptake from device × % deposited in the lungs × % actually absorbed from the lungs. TO % for insulin is estimated to be about 10 % (Patton et al. 2004). The fraction of insulin that is absorbed from the lung is around 20 %. These figures demonstrate that insulin absorption via the lung may be a promising route, but the fraction absorbed is small and with the Exubera technology, the patient/medical community preferred parenteral administration.

Therefore, different approaches have been evaluated to increase bioavailability of the pulmonary and other non-parenteral routes of administration. The goal is to develop a system that temporarily decreases the absorption barrier resistance with minimum and acceptable safety concerns. The mechanistic background of these approaches is given in Table 4.6. Until now, no products utilizing one of these approaches have successfully passed clinical test programs. Safety concerns are an important hurdle. Questions center on the specificity and reversibility of the protein permeation enhancing effect and the toxicity.

■ Examples of Absorption-Enhancing Effects

The following section deals with absorption enhancement and non-parenteral administration of recombinant proteins. A number of typical examples are provided.

Table 4.7 presents an example of the (apparently complex) relationship between nasal bioavailability of some peptide and protein drugs, their molecular weight, and the presence of the absorption enhancer glycocholate (Zhou and Li Wan Po 1991b).

Molecule	#AA	Bioavailability (%)	
		Without	With glycocholate
Glucagon	29	<1	70–90
Calcitonin	32	<1	15–20
Insulin	51	<1	10–30
Met-hGH[a]	191	<1	7–8

Adapted from Zhou and Li Wan Po (1991b)

[a]See also Chap. 14

Table 4.7 ■ Effect of glycocholate (absorption enhancer) and molecular weight of some proteins and peptides on nasal bioavailability.

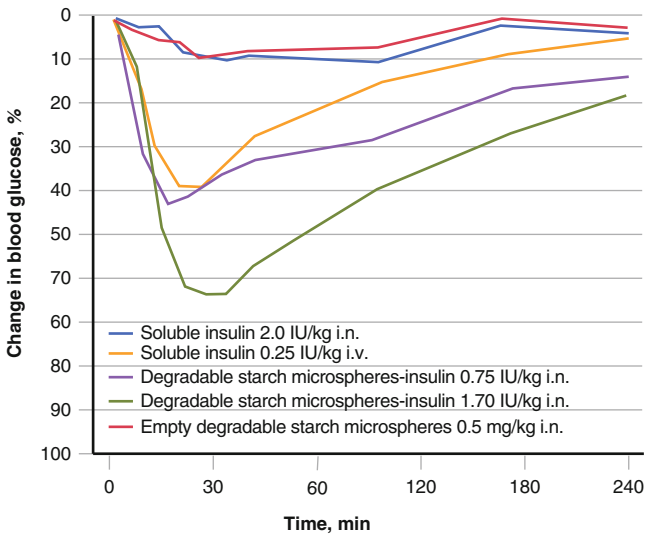


Figure 4.14 ■ Change in blood glucose in rats after intranasal (i.n.) administration of insulin. — Soluble insulin 2.0 IU/kg i.n. — Soluble insulin 0.25 IU/kg IV. — Degradable starch microspheres – insulin 0.75 IU/kg i.n. — Degradable starch microspheres – insulin 1.70 IU/kg i.n. — Empty degradable starch microspheres – insulin 0.5 mg/kg i.n (Discussed by Edman and Björk (1992)).

Figure 4.14 (Björk and Edman 1988) illustrates another case where degradable starch microspheres loaded with insulin were used and where changes in glucose levels were monitored after nasal administration to rats.

In these examples, the effect of the presence of the absorption enhancers is clear. Major issues to be addressed are reproducibility, effect of pathological conditions (e.g., rhinitis) on absorption, and safety aspects of chronic use. Interestingly, absorption-enhancing effects were shown to be species dependent. Pronounced differences in effect were observed between rats, rabbits, and humans.

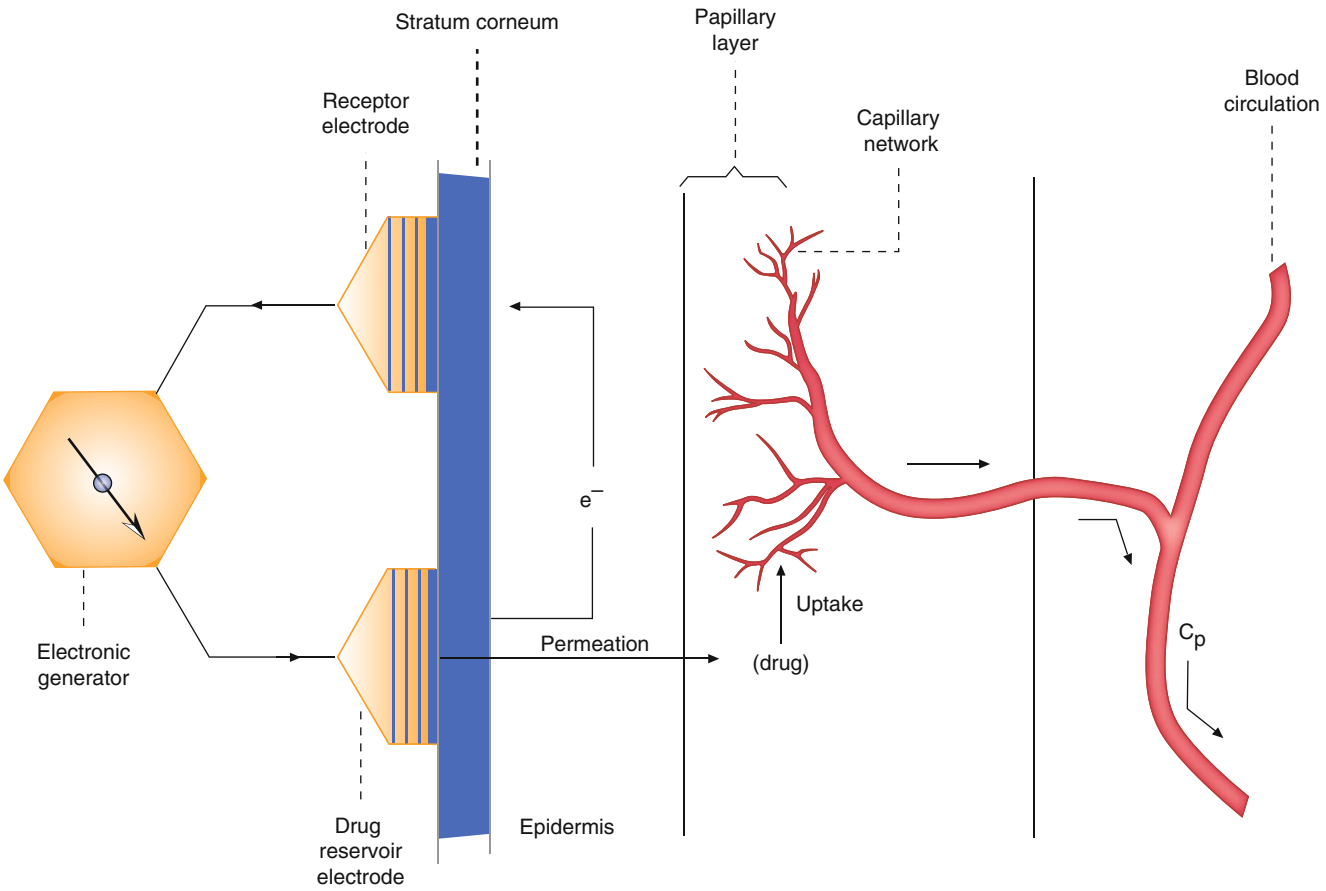


Figure 4.15 ■ Schematic illustration of the transdermal iontophoretic delivery of peptide and protein drugs across the skin (Adapted from Chien (1991)).

With iontophoresis a transdermal electrical current is induced by positioning two electrodes on different places on the skin (Fig. 4.15). This current induces a migration of (ionized) molecules through the skin. Delivery depends on the current (on/off, pulsed/direct, wave shape), pH, ionic strength, molecular weight, charge on the protein, and temperature. The protein should be charged over the full thickness of the skin (pH of hydrated skin depends on the depth and varies between pH 4 (surface) and pH 7.3), which makes proteins with pI values outside this range prime candidates for iontophoretic transport. It is not clear whether there are size restrictions (protein M_W) for iontophoretic transport. However, only potent proteins will be successful candidates. With the present technology the protein flux through the skin is in the 10 μg/cm²/h range (Sage et al. 1995).

Figure 4.16 presents the plasma profile of growth hormone-releasing factor, GRF (44 amino acids, M_W 5 kDa after SC, IV, and iontophoretic transdermal delivery to hairless guinea pigs). A prolonged appearance of GRF in the plasma can be observed. Iontophoretic delivery offers interesting opportunities if pulsed delivery of the protein is required. The device can be worn permanently and

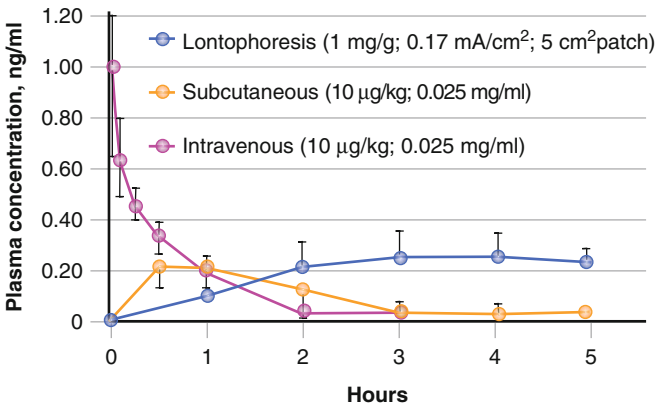


Figure 4.16 ■ Plasma concentration versus time profiles after subcutaneous, intravenous, and iontophoretic transdermal administration of GRF (1–44) to hairless guinea pigs. iontophoresis (1 mg/g; 0.17 mA/cm²; 5 cm² patch). subcutaneous (10 μg/kg; 0.025 mg/ml). intravenous (10 μg/kg; 0.025 mg/ml) (From Kumar et al. (1992)).

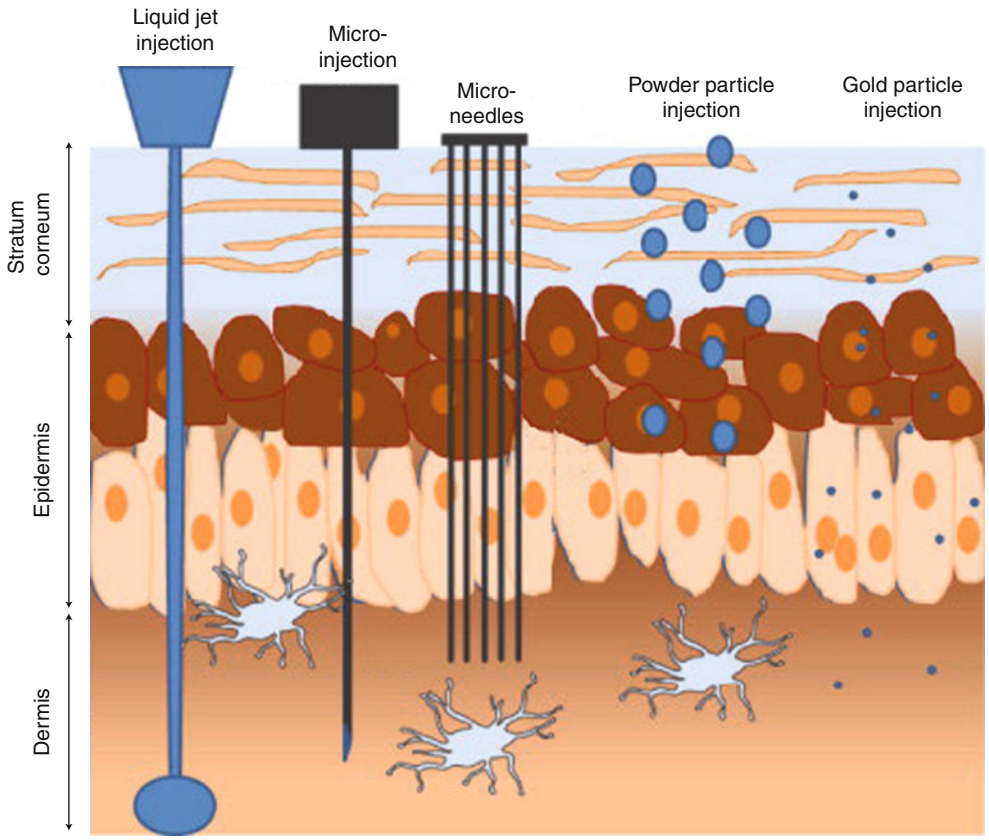


Figure 4.17 ■ Delivery systems enabling the intradermal application of vaccines: liquid jet injection, microinjection, microneedles (*arrays*), powder particle injection, and gold particle injection (Adapted from Kis et al. (2012)).

only switched on for the desired periods of time, simulating pulsatile secretion of endogenous hormones such as growth hormone and insulin. At present, no iontophoretic devices for protein delivery have been approved.

And, last but not least, a number of intracutaneous delivery systems have been developed. Figure 4.17 shows different options. In all approaches shown, the administered volumes were small, microliter level, and basically these approaches are restricted to antigen/adjuvant delivery. The classical liquid jet injectors deliver small amounts of vaccine fluid in the skin with a high velocity. The modern versions use prefilled disposable delivery units for single use to avoid contamination. Ballistic injectors shoot powder particles or gold particles covered with antigen under high pressure into the skin. Finally, microinjectors with microneedles in the (sub) mm range are being used or microneedle arrays with small individual needles in the 100 µm range. The newer versions of these microneedle arrays are self-dissolving. They are made of, e.g., sugar (derivatives) which dissolve rapidly after application (Mitragotri 2005; Kis et al. 2012). Examples are shown in Fig. 4.18a, b.

DELIVERY OF PROTEINS: APPROACHES FOR RATE-CONTROLLED AND TARGET SITE-SPECIFIC DELIVERY BY THE PARENTERAL ROUTE

Presently used therapeutic proteins widely differ in their pharmacokinetic characteristics (see Chap. 5). If they are endogenous agents such as insulin, tissue plasminogen activator, growth hormone, erythropoietin, interleukins, or factor VIII, it is important to realize why, when, and where they are secreted. There are three different ways in which cells can communicate with each other: the endocrine, paracrine, and autocrine pathway (Table 4.8).

The dose–response relationship of these mediators is often not S shaped, but, for instance, bell shaped: At high doses the therapeutic effect disappears (see Chap. 5). Moreover, the presence of these mediators may activate a complex cascade of events that needs to be carefully controlled. Therefore, key issues for their therapeutic success are (1) access to target cells, (2) retention at the target site, and (3) proper timing of delivery.

In particular, for paracrine- and autocrine-acting proteins, site-specific delivery can be highly desirable, because otherwise side effects will occur outside the

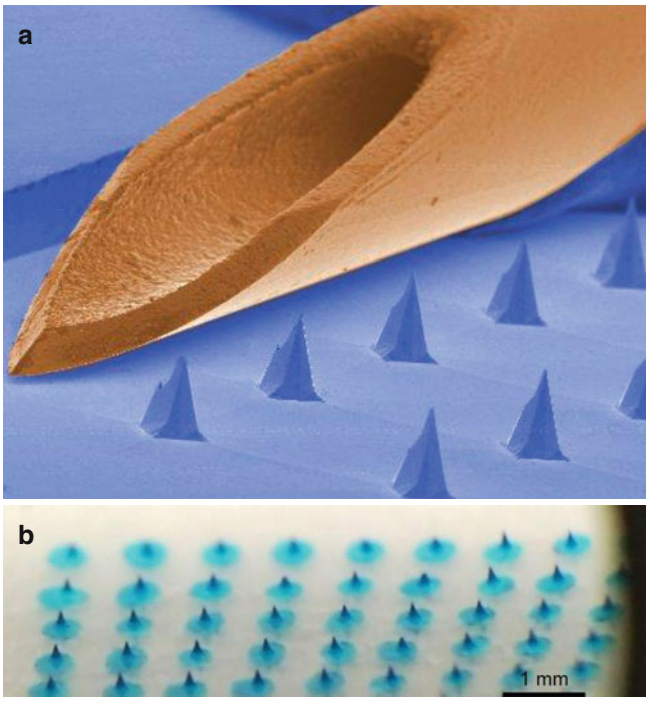


Figure 4.18 ■ (**a**) Hollow 300 μm tall silicon microneedles, (and 26-gauge syringe needle) fabricated using a combination of wet and dry etch micromachining technologies. These microneedles have wide-ranging applications in painless transdermal delivery and physiological sensing (Courtesy: Joe O'Brien & Conor O'Mahony, Tyndall National Institute) (**b**) Example of dissolvable microneedle patches. Dissolvable microneedles, composed of sugars and polymers, were fabricated in PDMS molds of master silicon microneedle arrays. The dimensions of microneedles on the array were 280 μm in height at a density of 144 needles per 1 cm^2. These biodegradable dissolving microneedles were fabricated using a proprietary method (UK Patent Application Number 1107642.9) (Courtesy: Anne Moore, Anto Vrdoljak, School of Pharmacy, University College Cork).

Endocrine hormones:
A hormone secreted by a distant cell to regulate cell functions distributed widely through the body. The bloodstream plays an important role in the transport process
Paracrine-acting mediators:
The mediator is secreted by a cell to influence surrounding cells, short-range influence
Autocrine-acting mediators:
The agent is secreted by a cell and affects the cell by which it is generated, (very) short-range influence

Table 4.8 ■ Communication between cells: chemical messengers.

target area. Severe side effects were reported with cytokines, such as tumor necrosis factor and interleukin-2 upon parenteral (IV or SC) administration (see Chap. 21). The occurrence of these side effects limits the therapeutic potential of these compounds. Therefore, the delivery of these proteins at the proper site, rate, and dose is a crucial part in the process of the design and development of these compounds as pharmaceutical entities. The following sections discuss first concepts developed to control the release kinetics and subsequently concepts for site-directed drug delivery.

APPROACHES FOR RATE-CONTROLLED DELIVERY

Rate control can be achieved by several different technologies similar to those used for "conventional" drugs. Insulin is an excellent example. A spectrum of options is available and accepted. Different types of suspensions and continuous/"smart" infusion systems are marketed (see Chap. 12). Moreover, chemical approaches can be used to change protein characteristics. For example, insulin half-life can be prolonged by using the long circulation time of serum albumin and its high binding affinity for fatty acids such as myristic acid. In insulin detemir (Levemir®) the C-terminal threonine of insulin is replaced by a lysine to which myristic acid is coupled. After subcutaneous injection the myristic acid–insulin combination reaches the blood circulation and binds to albumin. The half-life of insulin is prolonged from less than 10 min to over 5 h. A similar approach is used with glucagon-1-like peptide (GLP-1 (7–37)) for the treatment of diabetes. Attaching myristic acid to GLP-1 (7–37) (liraglutide marketed as Victoza®) increases the plasma half-life from 2 min to over 10 h.

Another approach that has been very successful in prolonging plasma circulation times and dosing intervals is the covalent attachment of polyoxyethylene glycol (PEG) to proteins. Figure 4.19 shows an example of this approach. Commercially highly successful examples that were developed later are pegylated interferon beta formulations (see Chap. 21).

In general, proteins are parenterally administered as an aqueous solution. Only recombinant vaccines and a number of insulin formulations are delivered as (colloidal) dispersions. At present, portable and patient-controlled pump systems are regularly used in practice. As experience with biotech drugs grows, more advanced technologies will definitely be introduced to optimize the therapeutic benefit of the drug. Table 4.9 lists some of the technologically feasible options. They are briefly touched upon below.

■ Open-Loop Systems: Mechanical Pumps

Mechanically driven pumps are common tools to administer drugs intravenously in hospitals (continuous infusion, open-loop type). They are available in different kinds of sizes/prices, portable or not, inside/outside the body, etc. Table 4.10 presents a checklist of issues to be considered when selecting the proper pump. The pump system may fail because of energy

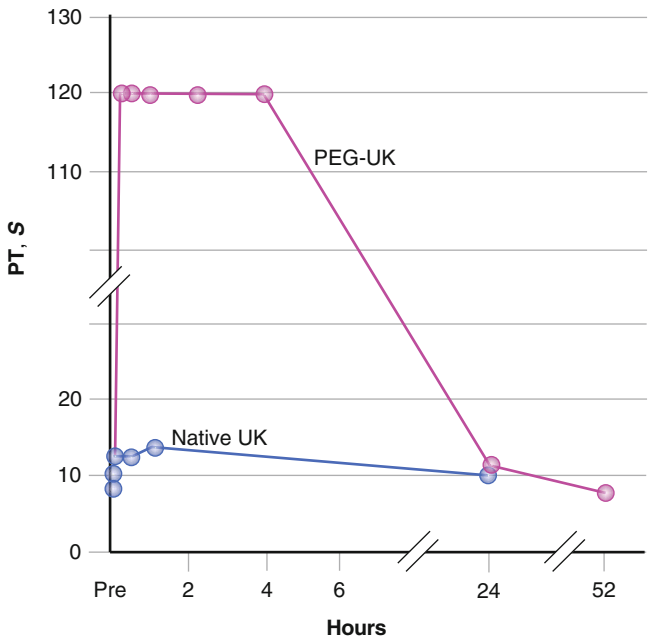


Figure 4.19 ■ Influence of chemical grafting of polyethylene glycol (*PEG*) on the ability of urokinase (*UK*) to affect the prothrombin time (*PT*) in vivo in beagles with time (Through Tomlinson (1987)).

Rate control through open-loop-type approach
Continuous infusion with pumps: mechanically or osmotically driven. Input, constant/pulsatile/wave form
Implants, biodegradable polymers; lipids. Input, limited control
Rate control through closed-loop approach/feedback system
Biosensor–pump combination
Self-regulating system
Encapsulated secretory cells

Table 4.9 ■ Controlled release systems for parenteral delivery.

failure, problems with the syringe, accidental needle withdrawal, leakage of the catheter, and problems at the injection or implantation site (Banerjee et al. 1991). Moreover, long-term protein drug stability may become a problem: The protein should be stable at 37°C or ambient temperature (internal and external device, respectively) between two refills.

Controlled administration of a drug does not necessarily imply a constant input rate. Pulsatile or variable-rate delivery is the desired mode of input for a number of protein drugs, and for these drugs pumps should provide flexible input rate characteristics. Insulin is a prime example of a protein drug, where there is a need to adjust the input rate to the needs of the body. Today by far most experiences with pump systems in an ambulatory setting have been gained with this drug. Even with high-tech pump systems, the

The pump must deliver the drug at the prescribed rate(s) for extended periods of time. It should
Have a wide range of delivery rates
Ensure accurate, precise, and stable delivery
Contain reliable pump and electrical components
Contain drugs compatible with pump internals
Provide simple means to monitor the status and performance of the pump
The pump must be safe. It should
Have a biocompatible exterior if implanted
Have overdose protection
Show no leakage
Have a fail-safe mechanism
Have sterilizable interiors and exteriors (if implantable)
The pump must be convenient. It should
Be reasonably small in size and inconspicuous
Have a long reservoir life
Be easy to program

Table 4.10 ■ Listing the characteristics of the ideal pump (Banerjee et al. 1991).

patient still has to collect data to adjust the pump rate. This implies invasive sampling from body fluids on a regular basis, followed by calculation/setting of the required input rate. The concept of closed-loop systems integrates these three actions and creates a "natural" biofeedback system (see below).

■ Open-Loop Systems: Osmotically Driven Systems

The subcutaneously implantable, osmotic mini-pump developed by ALZA (Alzet mini-pump, Fig. 4.20, Alzet product information 2012) has proven to be useful in animal experiments where continuous, constant infusion is required over prolonged periods of time. The rate-determining process is the influx of water through the rigid, semipermeable external membrane dissolving the salt (osmotic agent), creating a constant osmotic influx over the semipermeable membrane. The incoming water empties the drug-containing reservoir (solution or dispersion) surrounded by a flexible impermeable membrane. The release rate depends on the characteristics of this semipermeable membrane and on osmotic pressure differences over this membrane (osmotic agents/salt inside the pump). Zero-order release kinetics exist as long as the osmotic pressure difference over the semipermeable membrane stays constant.

The protein solution (or dispersion) must be physically and chemically stable at body temperature over the full term of the experiment. Moreover, the protein solution must be compatible with the pump parts to which it is exposed. A limitation of the system is the fixed release rate, which is not always desired (see above). These devices have currently not been used on a regular basis in the clinic.

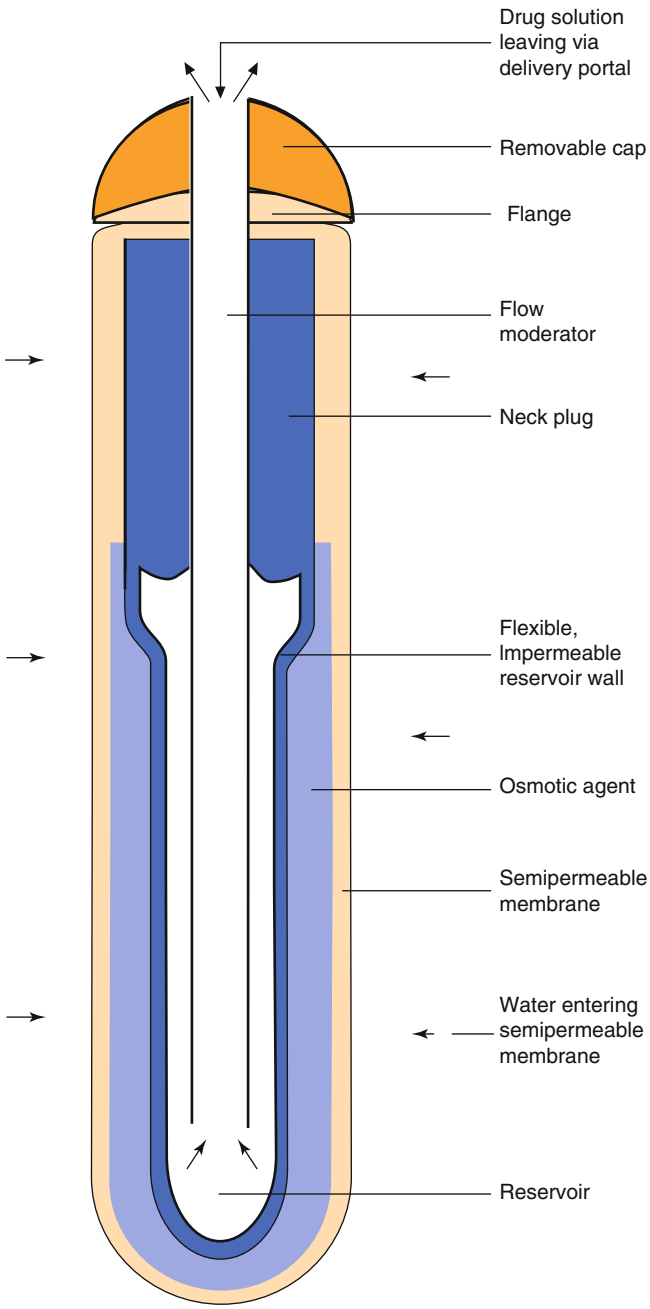


Figure 4.20 ■ Cross section of functioning ALZA Alzet osmotic mini-pump (Through Banerjee et al. (1991)).

■ Open-Loop Systems: Biodegradable Microspheres

Polylactic acid–polyglycolic acid (PLGA)-based delivery systems are being used extensively for the delivery of therapeutic peptides, in particular luteinizing hormone-releasing hormone (LHRH) agonists such as leuprolide in the therapy of prostate cancer. The first LHRH agonist-controlled release formulations were implants containing leuprolide with dose ranges of 1–3 months. Later, microspheres loaded with leuprolide were introduced, and dosing intervals were prolonged to up to 6 months. Critical success factors for the design of these controlled release systems are (1) the drug has to be highly potent (only a small dose is required over the dosing interval), (2) a sustained presence in the body is required, and (3) no adverse reactions at the injection site should occur. A glucagon-like protein-1 (GLP-1, 39 amino acids) slow release formulation (Bydureon™) based on PLGA microspheres for once a week administration to type II diabetics was released in 2012.

New strategies for controlled release of therapeutic proteins are presently under development. For example, Figs. 4.21 and 4.22 describe a dextran-based microsphere technology for SC or IM administration that often has an almost 100 % protein encapsulation efficiency. No organic solvents are being used in the preparation protocol. Thus, a direct interaction of the dissolved protein with an organic phase (as seen in many (e.g., polylactic-co-glycolic acid, PLGA) polymeric microsphere preparation schemes) is avoided. This minimizes denaturation of the protein. Figure 4.22 shows that by selecting the proper cross-linking conditions, one has a degree of control over the protein release kinetics. Release kinetics mainly depend on degradation kinetics of the dextran matrix and size of the protein molecule (Stenekes 2000). Another approach for prolonged and controlled release of therapeutic proteins is to use microspheres based on another biodegradable hydrogel material. PolyActive™ is a block copolymer consisting of polyethylene glycol blocks and polybutylene terephthalate blocks. Results of a dose finding study in humans with PolyActive™ microspheres loaded with interferon-α are shown in Fig. 4.23 (de Leede et al. 2008).

■ Closed-Loop Systems: Biosensor-Pump Combinations

If input rate control is desired to stabilize a certain body function, then this function should be monitored. Via an algorithm and connected pump settings, this data should be converted into a drug-input rate. These systems are called closed-loop systems as compared to the open-loop systems discussed above. If there is a known relationship between plasma level and pharmacological effect, these systems contain (Fig. 4.24):

1. A biosensor, measuring the (plasma) level of the biomarker
2. An algorithm, to calculate the required input rate for the delivery system
3. A pump system, able to administer the drug at the required rate over prolonged periods of time

The concept of a closed-loop delivery of proteins still has to overcome a number of conceptual and practical hurdles. A simple relationship between plasma level and therapeutic effect does not always

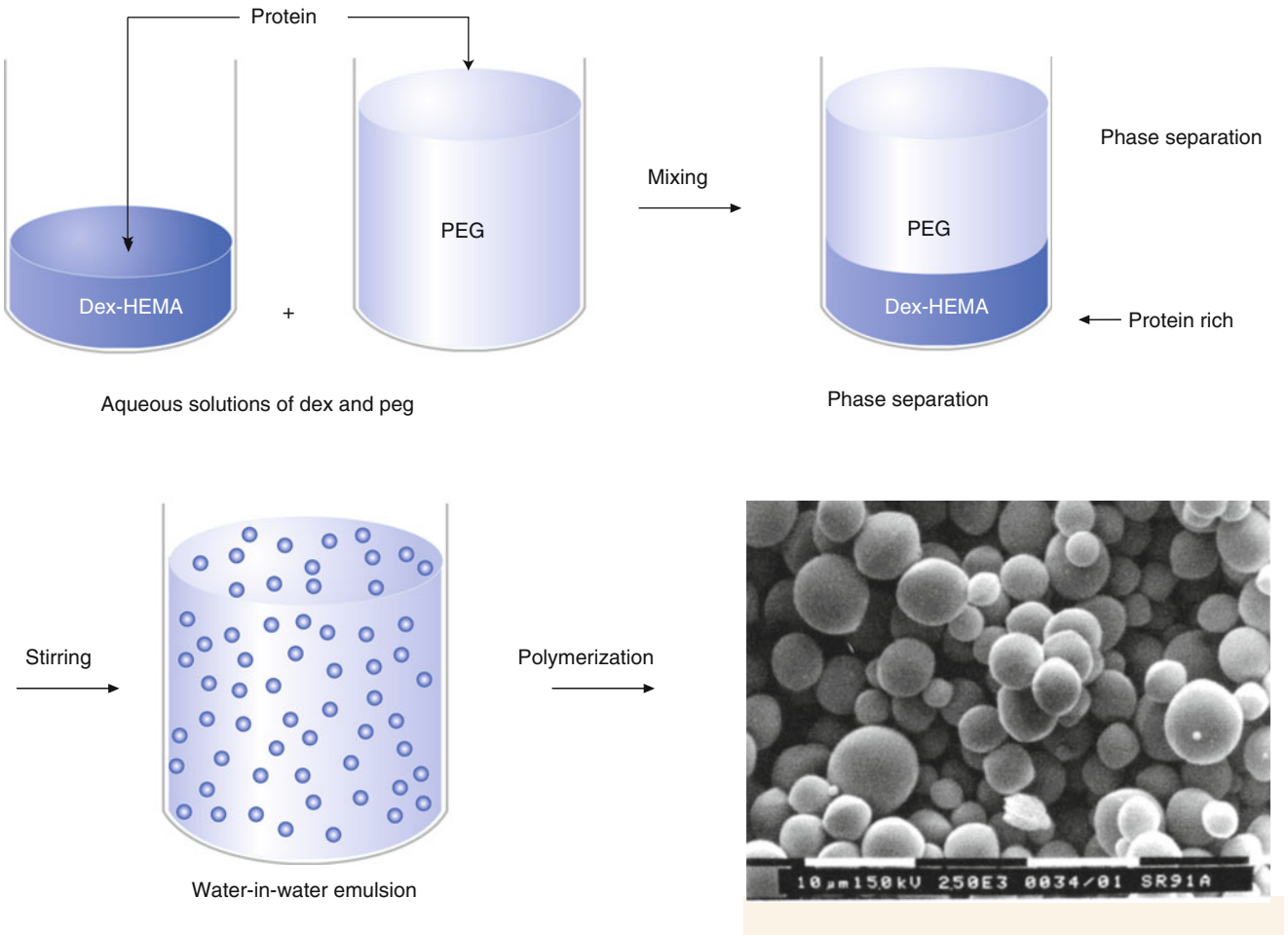


Figure 4.21 ■ Schematic representation of the microsphere preparation process for the controlled release of therapeutic proteins from dextran (DexHEMA = modified dextran = dextran hydroxyethylmethacrylate) microspheres. No organic solvents are involved, and encapsulation efficiency (percentage of therapeutic protein ending up in the microspheres) is routinely > 90 %. Polymerization: cross-linking of dextran chains through the HEMA units (Stenekes 2000).

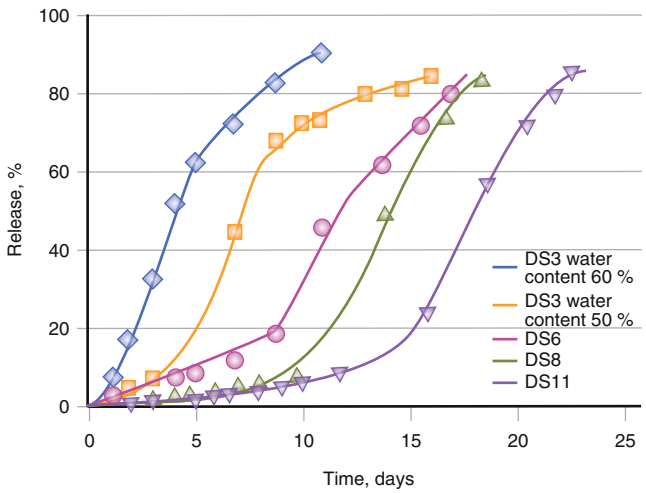


Figure 4.22 ■ Cumulative release of IgG from degrading dex-HEMA microspheres in time in vitro at pH 7, 37 °C. Water content of the dextran microspheres upon swelling: about 60 %, DS 3 (◆), and water content of about 50 %, DS 3 (■), DS 6 (●), DS 8 (▲), and DS 11 (▽). The values are the mean of two independent measurements that deviated typically less than 5 % from each other. *DS* degree of cross-linking (Stenekes 2000).

exist (see Chap. 5). There are many exceptions known to this rule; for instance, "hit and run" drugs can have long-lasting pharmacological effects after only a short exposure time. Also, drug effect–blood level relationships may be time dependent, as in the case of down-regulation of relevant receptors on prolonged stimulation. Finally, if circadian rhythms exist, these will be responsible for variable PK/PD relationships as well.

If PK/PD relationships can be established as with insulin in selected groups of diabetics, then integrated biosensor–pump combinations can be used that almost act as closed-loop biofeedback systems (Schaepelynck et al. 2011; Hovorka 2011). In 2010, FDA approved an integrated diabetes management system (insulin pump, continuous glucose monitoring (CGM), and diabetes therapy management software). The CGM measures interstitial fluid levels every 5 min and sends the outcome (wireless) to a therapy management algorithm. This software program advises the patient to program the insulin pump to deliver an appropriate dose of insulin. In spite of the impressive progress made, it has

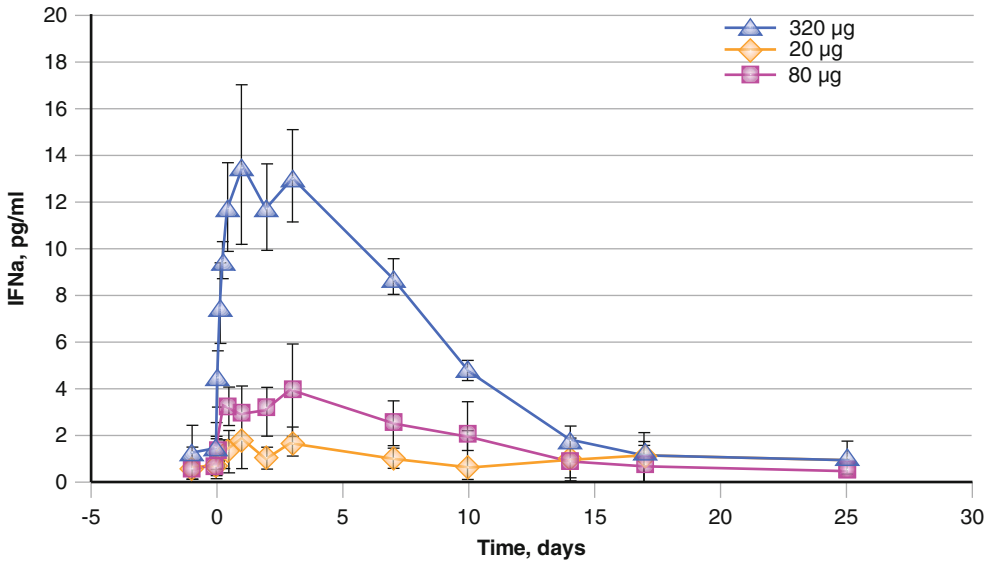


Figure 4.23 ■ Plasma profiles of interferon-α after SC injection of PolyActive™ microspheres loaded with interferon-α in volunteers in a dose finding study. The elimination half-life of "free" interferon-α is about 4–16 h. *Red* alfa interferon 20 µg. *Blue* alfa interferon 80 µg. *Dark blue* (triangles) alfa interferon 320 µg.

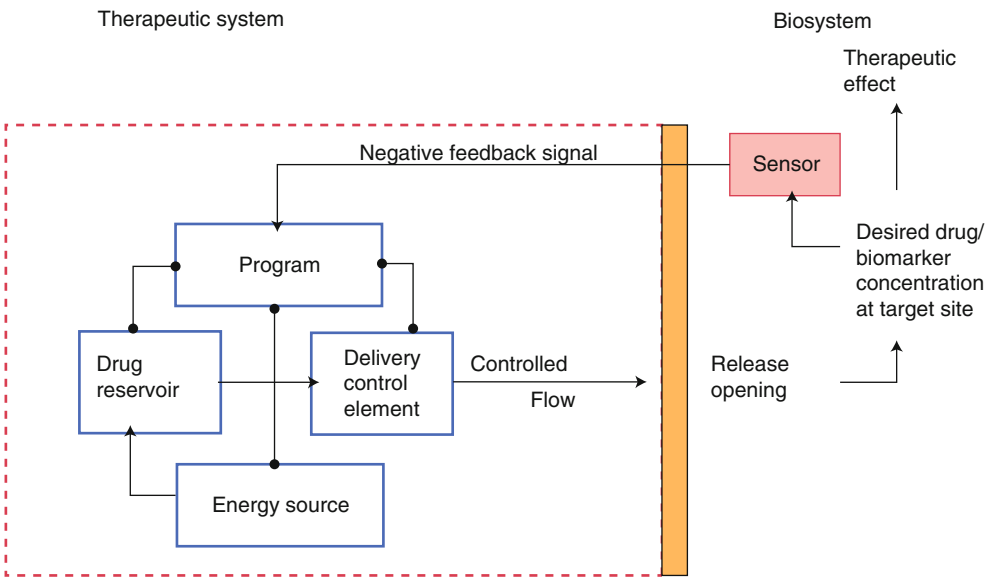


Figure 4.24 ■ Therapeutic system with closed control loop (From Heilman (1984)). (1) A biosensor, measuring the plasma level of the protein. (2) An algorithm, to calculate the required input rate for the delivery system. (3) A pump system, able to administer the drug at the required rate over prolonged periods of time.

not been possible yet to design fully closed-loop biosensors that work reliably in vivo over prolonged periods of time. Biosensor stability, robustness, and absence of histological reactions still pose problems.

■ Protein Delivery by Self-Regulating Systems

Apart from the design of biosensor–pump combinations, two other developments should be mentioned when discussing closed-loop approaches: self-regulating systems and encapsulated secretory cells. Both concepts are still under development (Heller 1993; Traitel et al. 2008).

In self-regulating systems, drug release is controlled by stimuli in the body. By far most of the research is focused on insulin release as a function of local glucose concentrations in order to stabilize blood glucose levels in diabetics. Two approaches for controlled drug release are being followed: (1) competitive desorption and (2) enzyme–substrate reactions. The competitive desorption approach is schematically depicted in Fig. 4.25.

It is based on the competition between glycosylated insulin and glucose for concanavalin (Con A) binding sites. Con A is a plant lectin with a high affinity for certain sugars. Con A attached to sepharose beads and loaded with glycosylated insulin (a bioactive form of insulin) is implanted in a pouch with a semipermeable membrane: permeable for insulin and glucose but impermeable for the sepharose beads carrying the toxic Con A. An example of the performance of a Con A-glycosylated insulin complex in pancreatectomized dogs is given in Fig. 4.26.

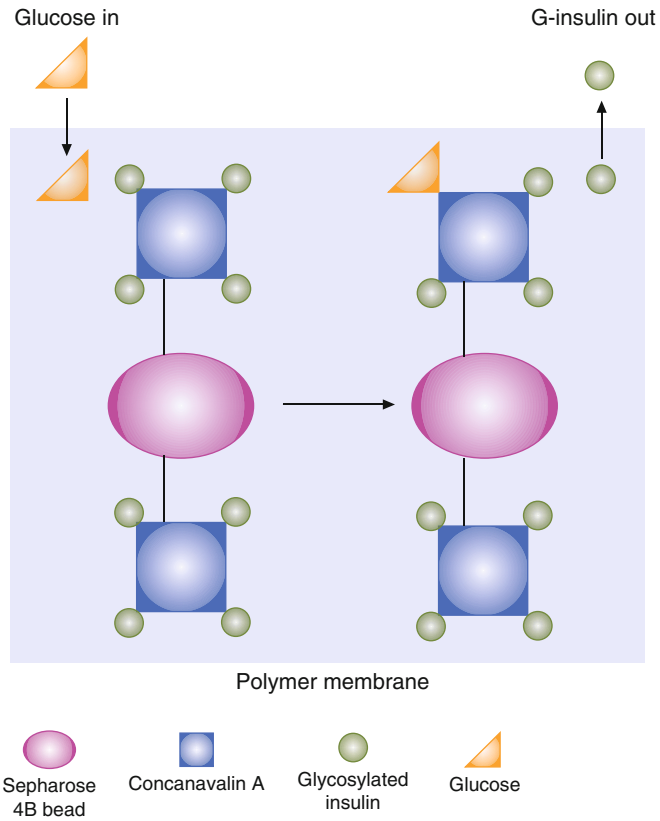


Figure 4.25 ■ Schematic design of the Con A immobilized bead/G(glycosylated)-insulin/membrane self-regulating insulin delivery system (From Kim et al. (1990)).

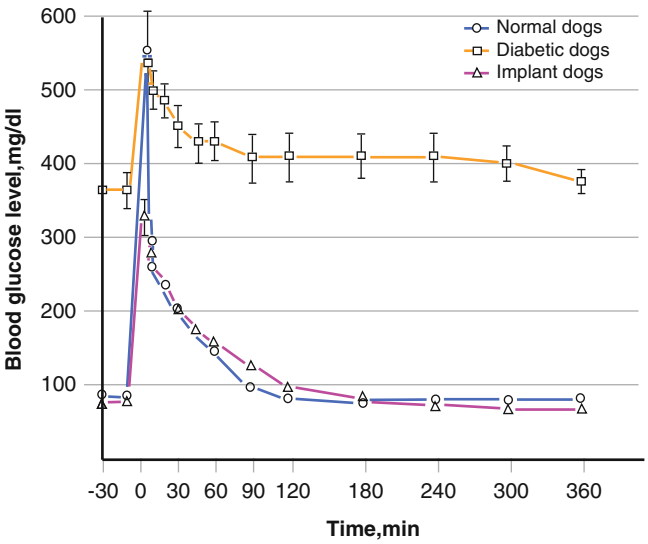


Figure 4.26 ■ Peripheral blood glucose profiles of dogs administered with bolus dextrose (500 mg/kg) during an intravenous glucose tolerance test. Normal dogs (-○-) had an intact pancreas, diabetic dogs (-□-) had undergone total pancreatectomy, and implant dogs (-△-) had been intraperitoneally implanted with a cellulose pouch containing a Con A–G–insulin complex. Blood glucose at $t = -30$ min shows the overnight fasting level 30 min prior to bolus injection of dextrose (Through Heller (1993)).

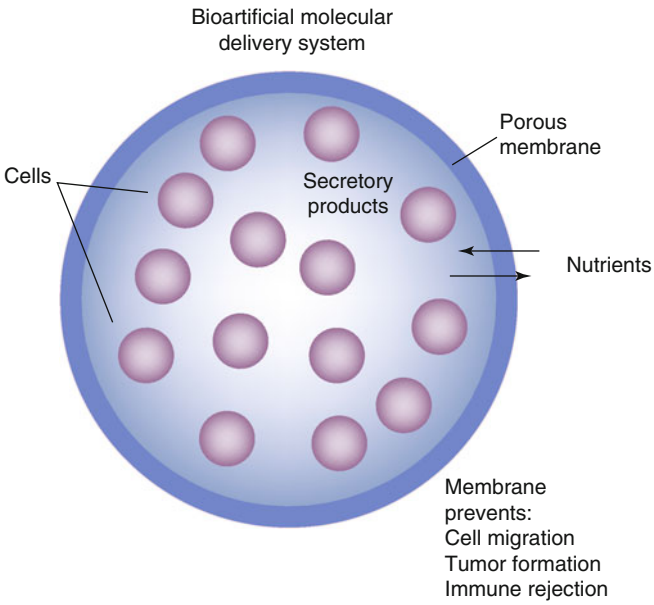


Figure 4.27 ■ Schematic illustration of a "bioartificial molecular delivery system." Secretory cells are surrounded by a semipermeable membrane prior to implantation in host tissue. Nutrients and secretory products passively diffuse through pores in the encapsulating membrane powered by concentration gradients. The use of a membrane that excludes the humoral and the cellular components of the host immune system allows immunologically incompatible cells to survive implantation without the need to administer immunosuppressive agents. Extracellular matrix material may be included depending upon the requirements of the encapsulated cells (From Tresco (1994)).

Enzyme–substrate reactions to regulate insulin release from an implanted reservoir are all based on pH drops occurring when glucose is converted to gluconic acid in the presence of the enzyme glucose oxidase. This pH drop then induces changes in the structure of acid-sensitive delivery devices such as acid-sensitive polymers, which start releasing insulin, lowering the glucose concentration, and consequently increasing the local pH and "closing the reservoir."

■ Protein Delivery by Microencapsulated Secretory Cells

The idea to use implanted, secretory cells to administer therapeutic proteins was launched long ago. A major goal has been the implantation of Langerhans cells in diabetics to restore their insulin production through biofeedback. These implanted secretory cells should be protected from the body environment, since rejection processes would immediately start, if imperfectly matched cell material is used. Besides, it is desirable to keep the cells from migrating in all different directions. When genetically modified cells are used, safety issues would be even stricter. Therefore, (micro) encapsulation of the secretory cells has been proposed (Fig. 4.27).

Thin (wall thickness in micrometer range), robust, biocompatible, and permselective polymeric membranes have been designed for these (micro) capsules (Tresco 1994). The membrane should ensure transport of nutrients (in general low M_W) from the outside medium to the encapsulated cells to keep them in a physiological, "healthy" state and to prohibit induction of undesirable immunological responses (rejection processes). Antibodies ($M_W > 150$ kDa) and cells belonging to the immune system (e.g., lymphocytes) should not be able to reach the encapsulated cells. The polymer membrane should have a cutoff between 50 and 150 kDa, the exact number still being a matter of debate. In the case of insulin, the membrane is permeable for this relatively small-sized hormone (5.4 kDa) and for glucose ("indicator" molecule), which is essential for proper biofeedback processes. Successful studies in diabetic animals were performed, and clinical trials have been initiated (Hernández et al. 2010).

SITE-SPECIFIC DELIVERY (TARGETING) OF PROTEIN DRUGS

Why are we still not able to beat life-threatening diseases such as cancer with our current arsenal of drugs? Causes of failure can be summarized as follows (Crommelin et al. 1992):

1. Only a small fraction of the drug reaches the target site. By far the largest fraction of the drug is distributed over nontarget organs, where it exerts side effects, and is rapidly eliminated intact from the body through the kidneys or through metabolic action (e.g., in the liver). In other words, accumulation of the drug at the target site is rather the exception than the rule.
2. Many drug molecules (in particular high M_W and hydrophilic molecules, i.e., many therapeutic proteins) do not enter cells easily. This poses a problem if intracellular delivery is required for their therapeutic activity.

Attempts are made to increase the therapeutic index of drugs through drug targeting:

1. By specific delivery of the active compound to its site of action
2. To keep it there until it has been inactivated and detoxified

Targeted drug delivery should maximize the therapeutic effect and avoid toxic effects elsewhere. Paul Ehrlich defined the basics of the concept of drug targeting already in the early days of the twentieth century. But only in the last two decades has substantial progress been made to implement this site-specific delivery concept. Recent progress can be ascribed to (1) the rapidly growing number of technological options (e.g., safe carriers and homing devices) for drug delivery; (2) many new insights gained into the pathophysiology of diseases at the cellular and molecular level, including the presence of cell-specific receptors; and, finally, (3) a better understanding of the nature of the anatomical and physiological barriers that hinder easy access to target sites. The site-specific delivery systems presently in different stages of development generally consist of three functionally separate units (Table 4.11).

An active moiety	For: therapeutic effect
A carrier	For: (metabolic) protection, changing the disposition of the drug
A homing device	For: specificity, selection of the assigned target site

Table 4.11 ■ Components for targeted drug delivery (carrier based).

1. Drugs with high total clearance are good candidates for targeted delivery
2. Response sites with a relatively small blood flow require carrier-mediated transport
3. Increases in the rate of elimination of free drug from either central or response compartments tend to increase the need for targeted drug delivery; this also implies a higher input rate of the drug–carrier conjugate to maintain the therapeutic effect
4. For maximizing the targeting effect, the release of drug from the carrier should be restricted to the response compartment

Table 4.12 ■ Pharmacokinetic considerations related to protein targeting.

Nature has provided us with antibodies, which exemplify a class of natural drug-targeting devices. In an antibody molecule one can recognize a homing device part (antigen-binding site) and "active" parts. These active parts in the molecule are responsible for activating the complement cascade or inducing interactions with monocytes when antigen is bound. The rest of the molecule can be considered as carrier.

Most of the drug (protein)-targeting work is performed with delivery systems that are designed for parenteral and, more specifically, intravenous delivery. Only a limited number of papers have dealt with the pharmacokinetics of the drug-targeting process (Hunt et al. 1986). From these kinetic models a number of conclusions could be drawn for situations where targeted delivery is, in principle, advantageous (Table 4.12).

The potential and limitations of carrier-based, targeted drug delivery systems for proteins are briefly discussed in the following sections. The focus is on concepts where monoclonal antibodies are used. They can be used as the antibody itself (also in Chap. 7), in modified form when antibodies are conjugated with an active moiety, or attached to drug-laden colloidal carriers such as liposomes.

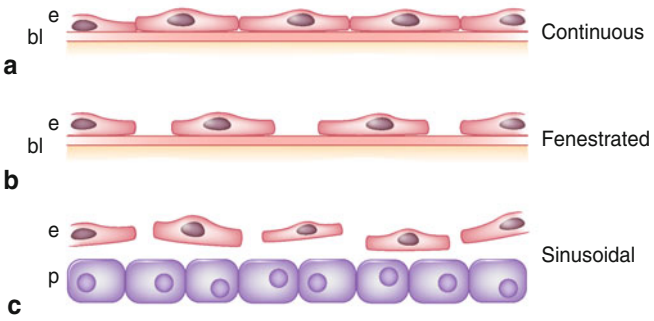


Figure 4.28 ■ Schematic illustration of the structure of different classes of blood capillaries. (**a**) Continuous capillary. The endothelium is continuous with tight junctions between adjacent endothelial cells. The subendothelial basement membrane is also continuous. (**b**) Fenestrated capillary. The endothelium exhibits a series of fenestrae which are sealed by a membranous diaphragm. The subendothelial basement membrane is continuous. (**c**) Discontinuous (sinusoidal) capillary. The overlying endothelium contains numerous gaps of varying size enabling materials in the circulation to gain access to the underlying parenchymal cells. The subendothelial basement is either absent (liver) or present as a fragmented interrupted structure (spleen, bone marrow). The fenestrae in the liver are about 0.1–0.2 μm; the pores in/between the endothelial cells and those in the basement membrane outside liver, spleen, and bone marrow are much smaller (From Poste (1985)).

Two terms are regularly used in the context of targeting: passive and active targeting. With passive targeting the "natural" disposition pattern of the carrier system is utilized for site-specific delivery. For instance, particulate carriers circulating in the blood (see below) are often rapidly taken up by macrophages in contact with the blood circulation and accumulate in the liver (Kupffer cells) and spleen. Active targeting is the concept where attempts are made to change the natural disposition of the carrier by a homing device or homing principle to select one particular tissue or cell type.

■ Anatomical, Physiological, and Pathological Considerations Relevant for Protein Targeting

Carrier-mediated transport in the body depends on the physicochemical properties of the carrier: its charge, molecular weight/size, surface hydrophobicity, and the presence of ligands for interaction with surface receptors (Crommelin and Storm 1990). If a drug enters the circulation and the target site is outside the blood circulation, the drug has to pass through the endothelial barrier. Figure 4.28 gives a schematic picture of the capillary wall structures (under physiological conditions) present at different locations in the body.

Figure 4.28 shows a diagram of intact endothelium under normal conditions. Under pathological conditions, such as those encountered in tumors and inflammation sites, endothelium can differ considerably in appearance, and endothelial permeability may be widely different from that in "healthy" tissue. Particles with sizes up to about 0.1 μm can enter tumor tissue as was demonstrated with long-circulating, nanoparticulate carrier systems (e.g., long-circulating liposomes) (Lammers et al. 2008 and below, Fig. 4.35). On the other hand, necrotic tissue can also hamper access to tumor tissue (Jain 1987). In conclusion, the body is highly compartmentalized. It should not be considered as one big pool without internal barriers for transport.

■ Soluble Carrier Systems for Targeted Delivery of Proteins

Monoclonal Antibodies (MAB) as Targeted Therapeutic Agents: Human and Humanized Antibodies (See Also with More Details: Chap. 7)

Antibodies are "natural targeting devices." Their homing ability is combined with functional activity (Crommelin et al. 1992; Crommelin and Storm 1990). MAB can affect the target cell function upon attachment. Complement can be bound via the Fc receptor and subsequently cause lysis of the target cell. Alternatively, certain Fc receptor-bearing killer cells can induce "antibody-dependent, cell-mediated cytotoxicity" (ADCC), or contact with macrophages can be established. Moreover, metabolic deficiencies can be induced in the target cells through a blockade of certain essential cell surface receptors by MAB. Structural aspects and therapeutic potential of MAB are dealt with in detail in Chap. 7.

A problem that occurs when using murine antibodies for therapy is the production of human anti-mouse antibodies (HAMA) after administration. HAMA induction may prohibit further use of these therapeutic MAB by neutralizing the antigen-binding site; anaphylactic reactions are relatively rare. Concurrent administration of immunosuppressive agents is a strategy to minimize side effects. More in-depth information regarding immunogenicity of therapeutic proteins is provided in Chap. 6.

There are several other ways to cope with this MAB-induced immunogenicity problem. Chapters 1 and 6 discuss immunogenicity issues as well. Here, a brief summary of the options relevant for protein targeting suffices. First of all, the use of $F(ab')_2$ or F(ab') fragments (Fig. 4.29) avoids raising an immune response against the Fc part, but the development of humanized or human MAB minimizes the induction of HAMA even further. For humanization of MAB several options can be considered. One can build chimeric (partly human, partly murine) molecules consisting of a human Fc part and a murine Fab part, with the antigen-binding sites. Or, alternatively, only the six complementarity-determining regions (CDR) of the murine antibody can be grafted in a human

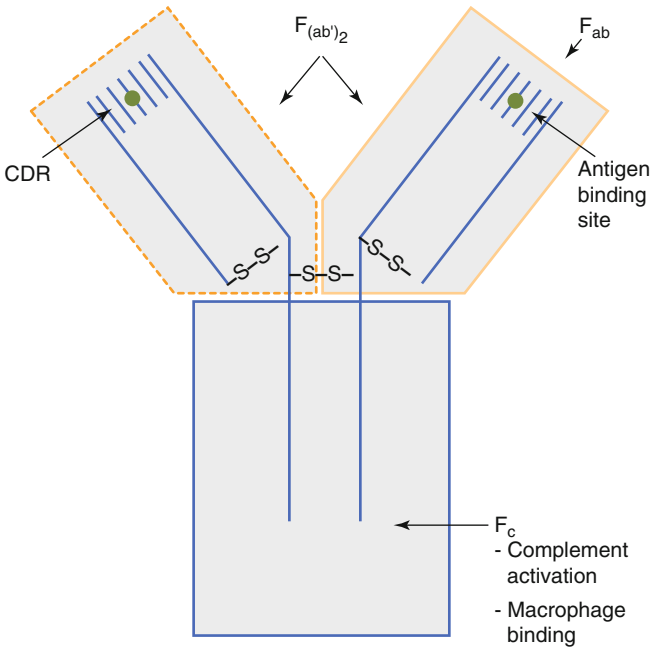


Figure 4.29 ■ Highly simplified IgG1 structure; *CDR* complementarity-determining region (see Figure 7.1 and 7.2).

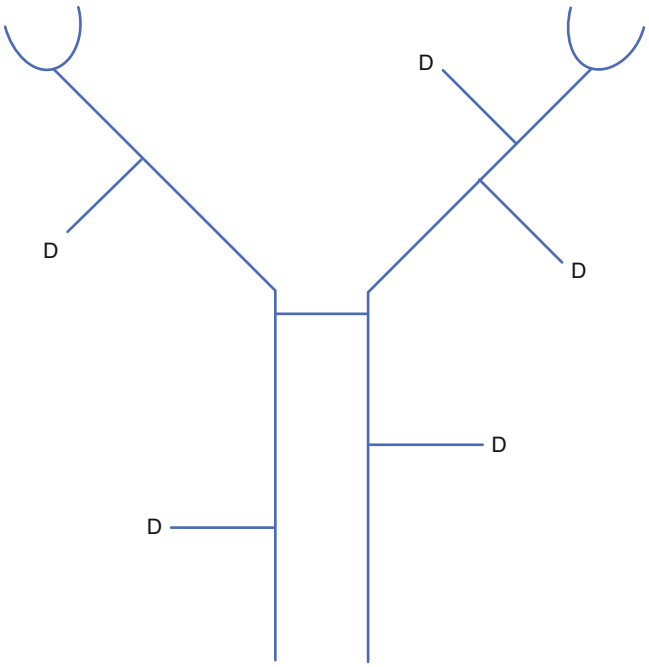


Figure 4.30 ■ A schematic view of an immunoconjugate (*D* drug molecules covalently attached to antibody (fragments)); see Figure 7.3.

antibody structure. CDR grafting minimizes the exposure of the patient to murine-derived material.

Completely human MAB can be produced by transfecting human antibody genes into mouse cells, which subsequently produce the human MAB. Alternatively, transgenic mice can be used (see Chap. 6 and 8). These approaches reduce the immunogenicity compared to the existing generation of murine MAB. But even with all these human or humanized MAB, anti-idiotypic immune responses against the binding site structure of the MAB cannot be excluded.

Bispecific Antibodies (See Also Chap. 7)

To enhance the therapeutic potential of antibodies, bispecific antibodies have been designed. Bispecific antibodies are combinations of two separate antibodies to create a molecule with two different binding sites. Bispecific MABs bring target cells or tissue (one antigen-binding site) in contact with other structures (second antigen-binding site). This second antigen-binding site can bind to effector cells via cytotoxicity-triggering molecules on T cells, NK (natural killer) cells, or macrophages and thus trigger cytotoxicity.

Bispecific antibodies have reached the clinic, e.g., a bispecific antibody that targets CD3 on T cells and CD20 on lymphoma cells (Crommelin and Storm 1990; Holmes 2011). In 2009 catumaxomab (anti-CD3 and antiepithelial cell adhesion molecule) was registered in Europe for the treatment of malignant ascites.

Immunoconjugates: Combinations Between an Antibody and an Active Compound

In many cases antibodies alone or bispecific antibodies showed lack of sufficient therapeutic activity. To enhance their activity, conjugates of MAB and drugs have been designed (Fig. 4.30). These efforts mainly focus on the treatment of cancer (Crommelin and Storm 1990). To test the concept of immunoconjugates, a wide range of drugs has been covalently bound to antibodies and has been evaluated in animal tumor models. As only a limited number of antibody molecules can bind to the target cells and as the payload per MAB molecule is restricted as well, only conjugation of highly potent drugs will lead to sufficient therapeutic activity. So far gemtuzumab ozogamicin (Mylotarg®) was the first immunoconjugate on the market. It is a conjugate of a monoclonal antibody and calicheamicin (see also Chap. 17). The MAB part targets the CD33 surface antigen in CD33-positive acute myeloid leukemia cells (AML). After internalization into the cell, the highly cytotoxic calicheamicin is released. Mylotarg® was launched on the market in 2000. After extensive follow-up evaluations, it was taken off the market in 2010 as serious side effects were reported with no added antitumor benefit compared to standard therapy.

Cytostatics with a high intrinsic cytotoxicity are needed (see above). Because the kinetic behavior of active compounds is strongly affected by the conjugating antibody, not only existing and approved cytostatics

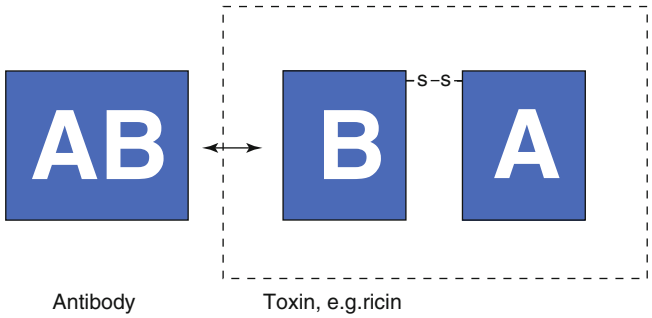


Figure 4.31 ■ Immunotoxins are composed of antibody molecules connected to a toxin, e.g., ricin. Both the integral ricin molecule and the A chain alone have been used. *AB* antibody, *A* and *B* stand for the *A* and *B* chain of the ricin toxin, respectively (not in the list of abbreviations).

1. Covalent binding of the protein/drug to the antibody may change the cytotoxic potential of the drug and decrease the affinity of the MAB for the antigen
2. The stability of the conjugate in vivo may be insufficient; fragmentation will lead to loss of targeting potential
3. The immunogenicity of the MAB and toxicity of the protein/drug involved may change dramatically

Table 4.13 ■ Potential problems encountered with immunoconjugates (Crommelin et al. 1992).

but also active compounds that were never used before as drugs, because of their high toxicity, should now be reconsidered.

Immunoconjugated toxins are now tested as chemotherapeutic agents to treat cancer (immunotoxins). Examples of the toxin family originating from plants are ricin, saporin, gelonin, and abrin. Toxins from bacterial origin are diphtheria toxin and pseudomonas endotoxin. These proteins are extremely toxic; they block enzymatically intracellular protein synthesis at the ribosomal level. For instance, ricin (M_W 66 kDa) consists of an A and a B chain that are linked through a cystine bridge (Fig. 4.31). The A chain is responsible for blocking protein synthesis at the ribosomes. The B chain is important for cellular uptake of the molecule (endocytosis) and intracellular trafficking and is deleted as it is considered redundant.

Table 4.13 lists a number of potential problems encountered with (toxin-based) immunoconjugates (Crommelin et al. 1992). In animal studies with immunoconjugated ricin, only a small fraction of these immunotoxins accumulates in solid tumor tissue (1 %). A major fraction still ends up in the liver, the main target organ for "natural" ricin. Because of the poor tissue penetration, primary targets for clinical use are target cells circulating in blood or endothelial cells. Moreover, in early clinical phase I studies (to assess the safety of the conjugates), the first generation of immunoconjugates turned out to be immunogenic, and these murine MAB have been replaced by human or humanized MAB (see above). Attempts were made to adapt the ricin molecule by genetic engineering so that liver targeting is being minimized. This can be done by blocking (removing or masking) on the ricin molecule ligands for galactose receptors on hepatocytes.

1. Tumor heterogeneity
2. Antigen shedding
3. Antigen modulation

Table 4.14 ■ Factors that interfere with successful targeting of proteins to tumor cells.

Not only MAB or fragments thereof were used as homing device/cell wall translocation moiety. Interestingly, the first and only immunotoxin approved by the FDA (denileukin diftitox) is a fusion protein of IL2 and a truncated diphtheria toxin (DAB389). Its target is the high-affinity IL2 receptor, and it is used in the treatment of cutaneous T-cell lymphoma (Choudhary et al. 2011).

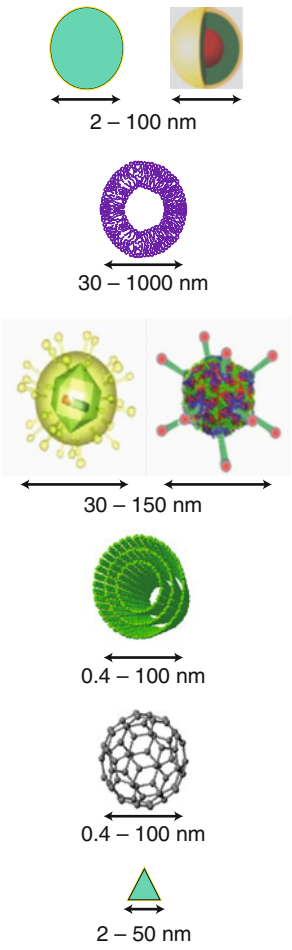
■ Potential Pitfalls in Tumor Targeting

Upon IV injection, only a small fraction of the homing device-carrier-drug complex is sequestered at the target site. Apart from the compartmentalization of the body (see above: anatomical and physiological hurdles) and consequently the carrier-dependent barriers that result, several other factors account for this lack of target site accumulation (Table 4.14).

How successful are MAB in discriminating target cells (tumor cells) from nontarget cells? Do all tumor cells expose the tumor-associated antigen? These questions are still difficult to answer (Hellström et al. 1987). Tumor cell surface-specific molecules used for homing purposes are often differentiation antigens on the tumor cell wall. These structures are not unique since they occur in a lower-density level on non-target cells as well. Therefore, the target site specificity of MAB raised against these structures is more quantitative than qualitative in nature.

Another category of tumor-associated antigens are the clone-specific antigens. They are unique for the clone forming the tumor. However, the practical problem when focusing on clone-specific antibodies for drug targeting is that each patient probably needs a tailor-made MAB.

The surface "makeup" of tumor cells in a tumor or a metastasis is not constant, neither in time nor between cells in the same tumor. There are many subpopulations of tumor cells, and they express different surface molecules. This heterogeneity means that not all cells in the tumor will interact with one, single targeted conjugate.



Nanoparticles Nanoshells	Lipid, polymer, inorganic nanoparticles	• Targeted delivery of therapeutics • *In vivo* navigation devices
Liposomes	Phospholid and polymer-based bilayer vesicles	Targeted delivery of therapeutics
Viral particles	Adenoviruses retroviruses AAV	Gene delivery
Nanotubes	Carbon, peptide, DNA	• Delivery systems • Diagnostics
Fullerenes	Bucky balls	• Delivery systems • Diagnostics
Nanocrystals	Quantum dots	Imaging agents

Figure 4.32 ■ Carrier systems in the nanometer/colloidal size range.

Antigen shedding and antigen modulation are two other ways tumor cells can avoid recognition. Shedding of antigens means that antigens are released from the surface. They can then interact with circulating conjugates outside the target area, form an antigen–antibody complex, and neutralize the homing potential of the conjugates before the target area has been reached. Finally, antigen modulation can occur upon binding of MAB to the cell surface antigen. Modulation is the phenomenon that upon endocytosis of the (originally exposed) surface antigen–immunoconjugate complex, some of these antigens are not exposed anymore on the surface; there is no replenishment of endocytosed surface antigens.

Four strategies can be implemented to solve problems related to tumor cell heterogeneity, shedding, and modulation. (1) Cocktails of different MAB attached to the toxin can be used. (2) Another approach is to give up striving for complete target cell specificity and to induce so-called "bystander" effects. Then, the targeted system is designed in such a way that the active part is released from the conjugate after reaching a target cell but before the antigen–conjugate complex has been taken up (is endocytosed) by the target cell. (3) Not all surface antigens show shedding or modulation. If these phenomena occur, other antigen/MAB combinations should be selected that do not demonstrate these effects. (4) Injection of free MAB prior to injection of the immunoconjugate is a strategy to neutralize "free" circulating antigen. The subsequently injected conjugate should not encounter shedded, free antigen.

In conclusion, targeted (modified) MAB and MAB conjugates are assessed for their value in fighting life-threatening diseases such as cancer. During the last decade, technology has evolved quickly; many different new options became available, and time will tell how successful the concept of immunotoxins and immunoconjugates will be for the patient.

■ Nanotechnology at Work: Nanoparticles for Targeted Delivery of Proteins

A wide range of carrier systems in the colloidal/nm size range (diameters up to a few micrometers) has been proposed for protein targeting. Examples are shown in Fig. 4.32. Upon entering the bloodstream after IV injection, it is difficult for many of these nano-sized

particulate systems to pass through epithelial and endothelial membranes in healthy tissue, as the size cutoff for permeation through these multilayered barriers is around 20 nm (excluding the liver; see above Fig. 4.28). Parameters that control the fate of particulate carriers in vivo are listed in Table 4.15.

As a rule, cells of the mononuclear phagocyte system (MPS), such as macrophages, recognize stable, colloidal particulate systems (< 5 µm) as "foreign body-like structures" and phagocytose them. Thus, the liver and spleen, organs rich in blood circulation-exposed macrophages, take up the majority of these particulates (Crommelin and Storm 1990). Larger (> 5 µm) intravenously injected particles tend to form emboli in lung capillaries on their first encounter with this organ.

Liposomes have gained considerable attention among the colloidal particulate systems proposed for site-specific delivery of (or by) proteins (Gregoriadis 2006). Liposomes are vesicular structures based on (phospho) lipid bilayers surrounding an aqueous core. The main component of the bilayer usually is phosphatidylcholine (Fig. 4.33).

By selecting their bilayer constituents and one of the many preparation procedures described, liposomes can be made varying in size between 30 nm (e.g., by extrusion or ultrasonication) and 10 µm, charge (by incorporation negatively or positively charged lipid molecules), and bilayer rigidity (by selecting special phospholipids or adding lipids such as cholesterol). Liposomes can carry their payload (proteins) either in the lipid core of the bilayer through partitioning, attached to the bilayer, or physically entrapped in the aqueous phase. To make liposomes target site specific, except for passive targeting to liver (Kupffer cells) and spleen macrophages, homing devices are covalently coupled to the outside bilayer leaflet. In Table 4.16 three relative advantages of liposomes over other nanoparticulate systems are given.

After injection, "standard" liposomes stay in the blood circulation only for a short time. They are taken up by macrophages in the liver and spleen, or they degrade by exchange of bilayer constituents with blood constituents. Liposome residence time in the blood circulation

1. Size
2. Charge
3. Surface hydrophilicity
4. Presence of homing devices on their surface
5. Exchange of constitutive parts with blood components

Table 4.15 ■ Parameters controlling the fate of particulate carriers in vivo.

1. Their relatively low toxicity, existing safety record, and experience with marketed, intravenously administered liposome products (e.g., amphotericin B, doxorubicin, daunorubicin) (Storm et al. 1993)
2. The presence of a relatively large aqueous core, which is essential to stabilize the structural features of many proteins
3. The possibility to manipulate release characteristics of liposome-associated proteins and to control disposition in vivo by changing preparation techniques and bilayer constituents (Crommelin and Schreier 1994)

Table 4.16 ■ Liposomes stand out among other particulate carrier systems, because of:

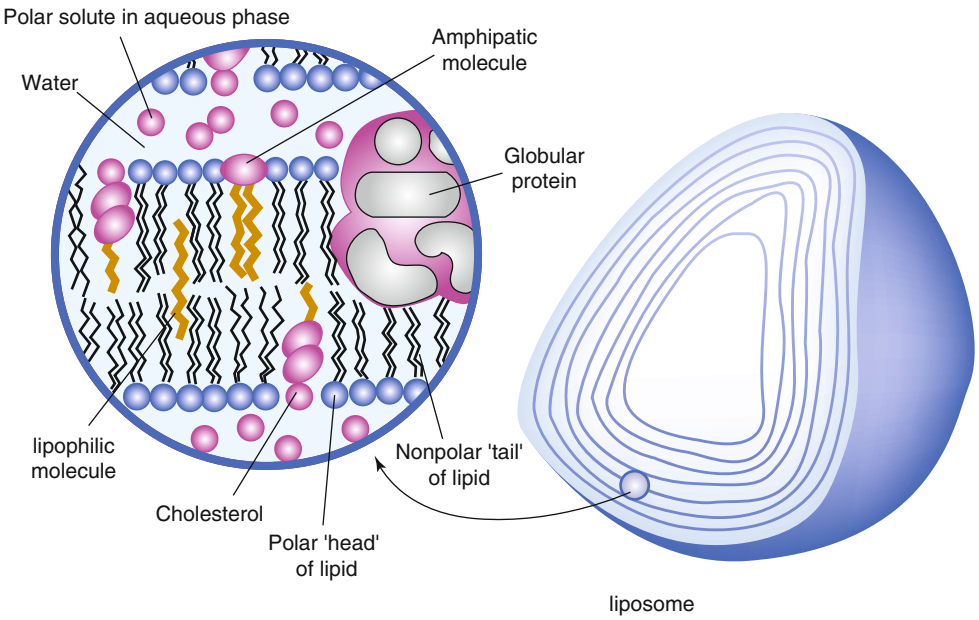


Figure 4.33 ■ An artist's view of what a multilamellar liposome looks like. The lamellae are bilayers of (phospho) lipid molecules with their hydrophobic tails oriented inwards and their polar heads directed to, and in contact with, the aqueous medium. The bilayer may accommodate lipophilic drugs inside. Hydrophilic drugs will be found in the aqueous core and in between the bilayers. Depending on their hydrophilic/hydrophobic balance and tertiary structure, proteins and peptides will be found in the aqueous phase, at the bilayer–water interface, or inside the lipid bilayer (Adapted from Fendler (1980)).

can be extended to many hours and even days upon grafting polyethylene glycol (PEG) chains on the surface and choosing for stable bilayer structures (Fig. 4.34, cf. Fig. 4.19). These long-circulating liposomes escape macrophage uptake for prolonged periods and may then be sequestered in other organs than liver and spleen alone, e.g., tumors and inflamed tissues. The reason for this sequestration is the so-called enhanced permeability and retention (EPR) effect, first described by Maeda et al. (Lammers et al. 2008) (Fig. 4.35). The term refers to the observation that the capillary bed in inflamed or tumor tissue tends to be more permeable than in not-affected, "healthy" beds and that effluent lymphatic transport from the affected beds is hampered. In Fig. 4.36 an example is shown of the use of 99mTc-labelled liposomes in the detection of inflammation sites in a patient. As a caveat, the full therapeutic benefit of the EPR effect is under discussion. For example, tumor heterogeneity with respect to capillary leakage and retarded but still considerable uptake in non-target organs (MPS) question the potential advantage over standard therapies (Bae and Park 2011).

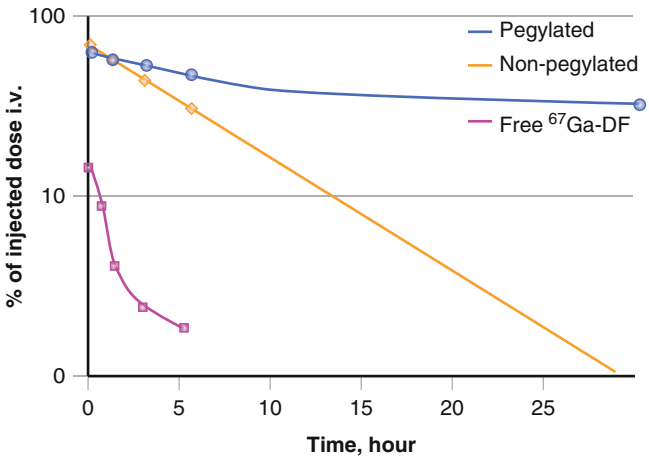


Figure 4.34 ■ Comparison of the blood levels of free label ^{67}Ga-DF, gallium-Desferal with ^{67}Ga-DF-laden pegylated (PEG) and non-pegylated liposomes upon IV administration in rats (From Woodle et al. (1990)).

On the other hand, accumulation of protein-laden liposomes in macrophages (passive targeting) offers interesting therapeutic opportunities. Reaching macrophages may help us to more effectively fight macrophage located in microbial, viral, or bacterial diseases than with our present approaches (Crommelin and Schreier 1994).

Several attempts have been made to sequester immunoliposomes (i.e., antibody (fragment)–liposome combinations) at predetermined sites in the body. Here the aim is active targeting to the desired target site instead of passive targeting to macrophages. The concept is schematically presented in Fig. 4.37.

When designing immunoliposomes, antibodies or antibody-fragments are covalently bound to the surface of liposomes through lipid anchor molecules. Non-pegylated immunoliposomes may have poor access to target sites outside the blood circulation after intravenous injection (see EPR discussion above). Therefore, target sites should be sought in the blood circulation (red blood cells, thrombi, lymphocytes, or

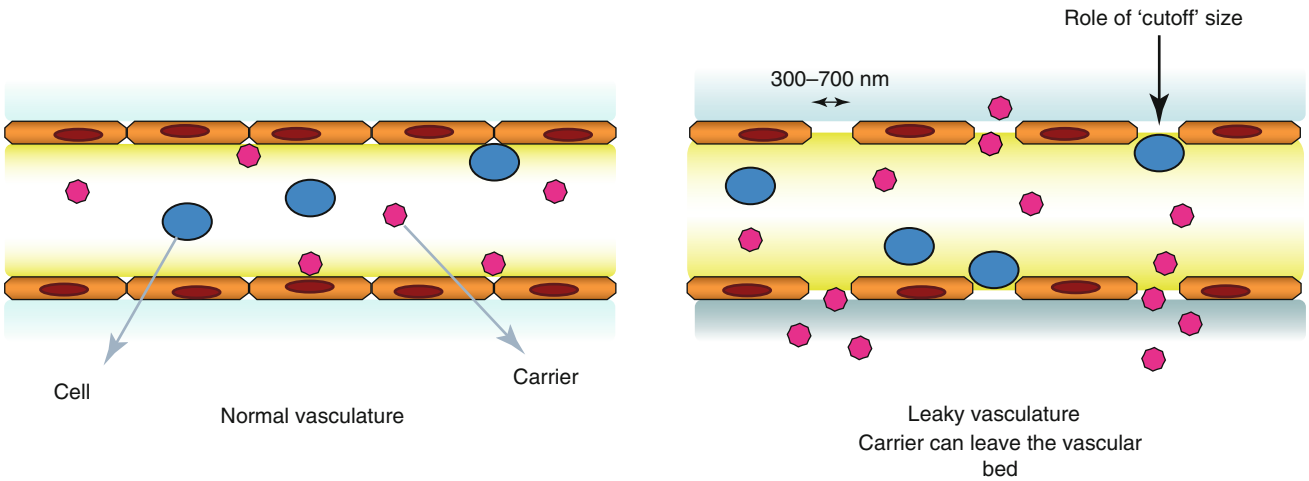


Figure 4.35 ■ The enhanced permeability and retention (EPR) effect refers to the observation that the capillary bed in inflamed or tumor tissue tends to be more permeable than in not-affected, "healthy" beds and that effluent lymphatic transport from the affected beds is hampered.

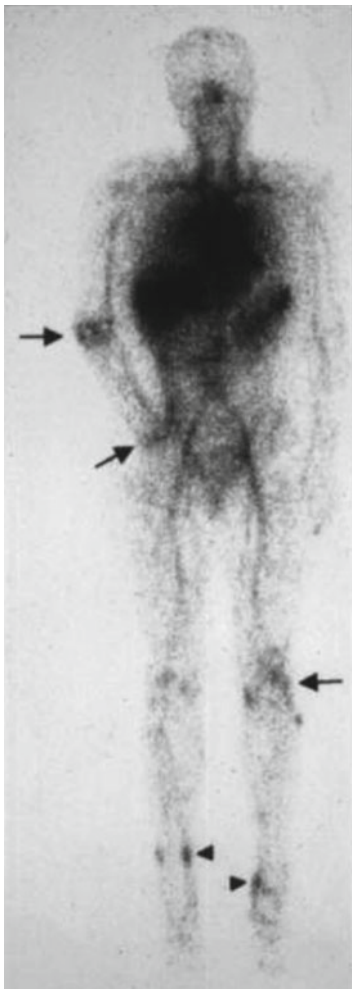


Figure 4.36 ■ ^{99m}Tc-PEG-liposomes scintigraphy of a female patient. Anterior whole body image, 24 h postinjection, shows physiological uptake in the cardiac blood, greater veins, liver, and spleen. Liposome uptake at pathological sites can be noted along synovial lining of the left elbow, left wrist, and right knee (*arrows*) and the medial site of both ankles (*arrow heads*) (Storm and Crommelin 1998).

endothelial cells exposing certain adhesion molecules when under stress, e.g., ICAM-1, intercellular cell adhesion molecule) (Crommelin et al. 1995).

Other interesting target sites are those located in cavities, where one can locally administer the drug–carrier combination. The bladder and the peritoneal cavity are such cavities. These cavities can be the sites where the diseased tissue is concentrated. For instance, with ovarian carcinomas the tumors are confined to the peritoneal cavity for most of their lifetime. After IP injection of immunoliposomes directed against human ovarian carcinomas in athymic, nude mice, a specific interaction between immunoliposomes and the human ovarian carcinoma was observed (Storm et al. 1994) (Fig. 4.38).

Attaching an immunoliposome to target cells usually does not induce a therapeutic effect per se. After establishing an immunoliposome–cell interaction, the protein drug has to exert its action on the cell. To do that, the protein has to be released in its active form. There are several pathways proposed to reach this goal (Fig. 4.39) (Peeters et al. 1987).

When the immunoliposome–cell complex encounters a macrophage, the cells plus adhering liposome are probably phagocytosed and enter the macrophage (option Fig. 4.39a). Subsequently, the liposome-associated protein drug can be released. As this will most likely happen in the "hostile" lysosomal environment, little intact protein will become available. In the situation depicted in Fig. 4.39, option b, the drug is released from the adhering immunoliposomes in the close proximity of the target cell. In principle, release rate control is achieved by selecting the proper liposomal bilayers with delayed or sustained drug release characteristics. A third approach is depicted as Fig. 4.39, option c: drug release is induced from liposomal bilayers by external stimuli (local pH change or temperature change). Finally, one can envision that

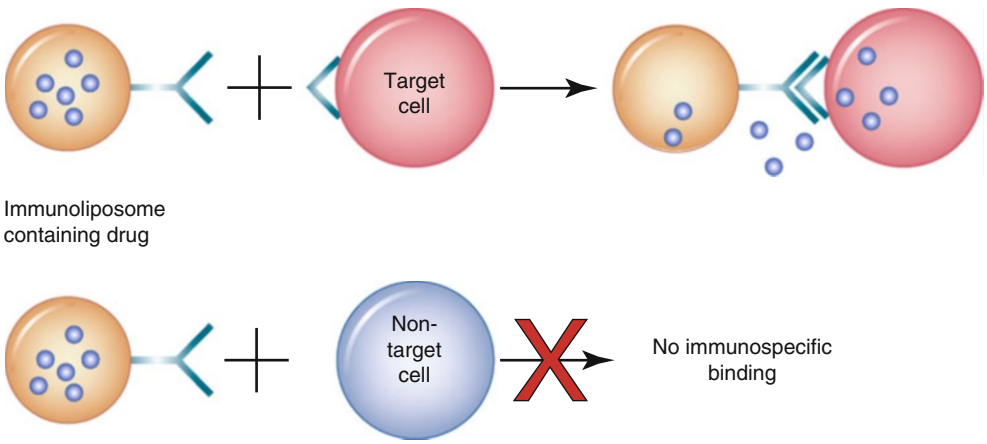


Figure 4.37 ■ Schematic representation of the concept of drug targeting with immunoliposomes (From Nässander et al. (1990)).

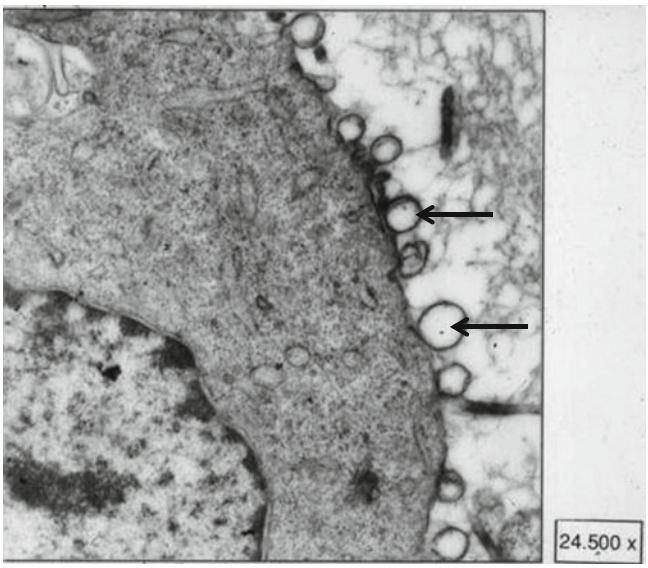


Figure 4.38 ■ Electron micrograph showing immunoliposomes (vesicular structures) attached to human ovarian carcinoma cells (see text).

immunoliposomes are built with intrinsic fusogenic potential, which is only activated upon attachment of the carrier to the target cell. This exciting option, Fig. 4.39d, resembles the behavior of certain viruses. Viruses offer interesting insights in pathways as to how to enter target cells and how to deliver their payload successfully in a target organelle (i.e., for viruses, the nucleus). This virus-mimicking approach led to the design of artificial viruses for targeted delivery of genetic material, but this can be extended to therapeutic proteins (Mastrobattista et al. 2006).

■ Perspectives for Targeted Protein Delivery

Protein-targeting strategies have been developing at a rapid pace. A new generation of homing devices (target cell-specific monoclonal antibodies) and a better insight into the anatomy and physiology of the human body under pathological conditions have been critical factors to achieve this success. A much better picture has emerged, not only about the potentials, but also about the limitations of the different targeting approaches.

Very little attention has been paid to typically pharmaceutical aspects of advanced drug delivery systems such as immunotoxins and immunoliposomes. These systems are now produced on a lab scale, and their therapeutic potential is currently under investigation. If therapeutic benefits have been clearly proven in preclinical and early clinical trials, then scaling up, shelf life, and quality assurance issues (e.g., reproducibility of the manufacturing process, purity of the ingredients) will still require considerable attention.

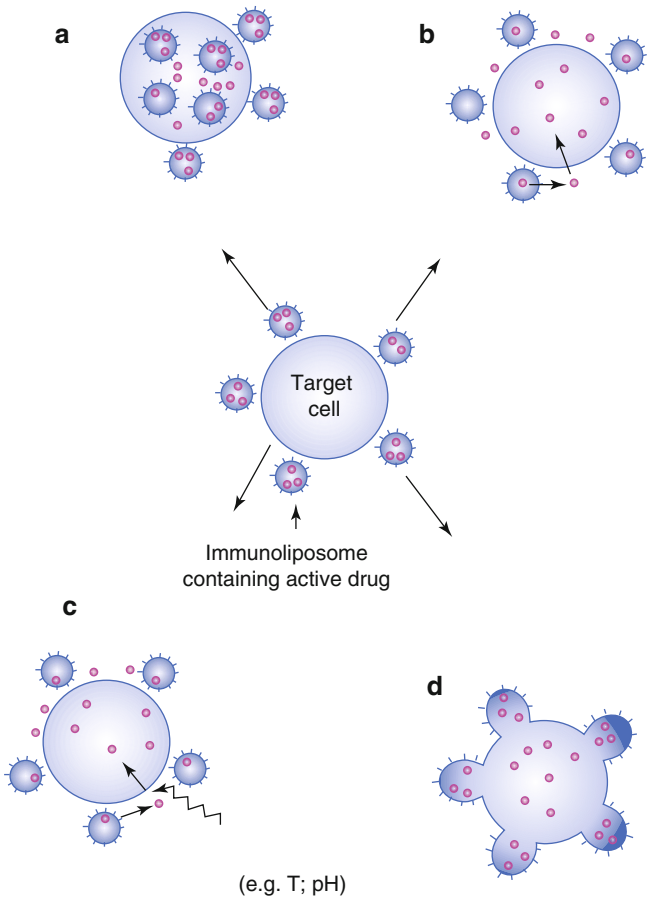


Figure 4.39 ■ Several pathways of drug internalization after immunospecific binding of the immunoliposomes to the appropriate target cell. (**a**) Uptake in liver and spleen macrophages; subsequent drug release.(**b**) Release of drug close to target cell. (**c**) Release of drug close to target cell; external triggering of release. (**d**) Fusion with target cell; subsequent drug release (From Peeters et al. (1987)).

SELF-ASSESSMENT QUESTIONS

■ Questions

1. How does one sterilize biotech products for parenteral administration?
2. A pharmaceutical protein, which is poorly water soluble around its pI, has to be formulated as an injection. What conditions would one select to produce a water-soluble, injectable solution?
3. Why are many biotech proteins to be used in the clinic formulated in freeze-dried form? Why is, as a rule, the presence of lyoprotectants required? Why is it important to know the glass transition temperature or eutectic temperature of the system?
4. Why is it not necessarily wise to work at the lowest possible chamber pressures during freeze-drying?

5. Why are (with the exception of oral vaccines) no oral delivery systems for proteins available?
6. What alternative route of administration to the parenteral route would be the first to look into if a systemic therapeutic effect is pursued and if one does not wish to exploit absorption-enhancing technologies?
7. If one considers using the iontophoretic transport route for protein delivery, what are the variables to be considered?
8. What are the differences between the endocrine, paracrine, and autocrine way of cell communication? Why is information on the way cells communicate important in the drug formulation process?
9. A company decides to explore the possibility to develop a feedback system for a therapeutic protein. What information should be available for estimating the chances for success?
10. Why is the selection of the dimensions of a colloidal particulate carrier system for targeted delivery of a protein of utmost importance?
11. Design a targeted, colloidal carrier system and a protocol for its use to circumvent the three hurdles to achieve successful treatment of solid tumors (mentioned in Table 4.14).
12. What are the options for inducing therapeutic actions upon attachment of immunoliposomes to (tumor) target cells?

■ Answers

1. Through aseptic manufacturing protocols. Final filtration through 0.2 or 0.22 μm pore filters into the vials/syringes further reduces the chances of contamination of the protein solutions.
2. One has to go through the items listed in Table 4.1. As the aqueous solubility is probably pH dependent, information on the preferred pH ranges should be collected. If necessary, solubility enhancers (e.g., lysine, arginine, and/or surfactants) and stabilizers against adsorption/aggregation should be added. "As a last resort," one might consider carriers such as liposomes.
3. Chemical and physical instability of proteins in aqueous media is usually the reason to dry the protein solution.
   Freeze-drying is then the preferred technology, as other drying techniques do not give rapidly reconstitutable dry forms for the formulation and/or because elevated temperatures necessary for drying jeopardize the integrity of the protein.
   The glass transition/eutectic temperature should not be exceeded as otherwise collapse of the cake can be observed. Collapse slows down the drying process rate, and collapsed material does not rapidly dissolve upon adding water for reconstitution.
4. Because gas conduction (one of the three heat transfer routes) depends on pressure and is reduced at low pressure.
5. Because of the hostile environment in the GI tract regarding protein stability and the poor absorption characteristics of proteins (high molecular weight/often hydrophilic).
6. The pulmonary route.
7. Physical characteristics of the protein and medium, such as molecular weight, pI, ionic strength, pH, and, in addition, electrical current options (pulsed, permanent, wave shape) and desired dose level/pattern (pulsed/constant/variable).
8. This information is important because, in particular with paracrine- and autocrine-acting proteins, targeted delivery should be considered to minimize unwanted side effects.
9. Answers:
   - The desired pharmacokinetic profile (e.g., information on the PK/PD relationship/circadian rhythm)
   - Chemical and physical stability of the protein on long-term storage at body/ambient temperature
   - Availability of a biosensor system (stability in vivo, precision/accuracy)
   - Availability of a reliable pump system (see Table 4.10)
10. The body is highly compartmentalized, and access to target sites inside and outside the blood circulation is highly dependent on the size of the carrier system involved (and other factors such as the presence of diseased tissue and surface characteristics such as charge, hydrophobicity/hydrophilicity, ligands).
11. The selection should be based on the induction of bystander effects, "cocktails" of homing devices (e.g., monoclonal antibodies), and selection of non-modulating receptors and non-shedding receptors. Neutralization of free, shed tumor antigens with free, non-conjugated monoclonal antibodies by injection of these free antibodies before the administration of ligand–carrier–drug combinations would be an approach for avoiding neutralization of the carrier–homing device combination by shed antigen.
12. Figure 4.39 gives an overview of these options.

REFERENCES

Alzet product information (2012) http://www.alzet.com/products/ALZET_Pumps/index.html

Arakawa T, Kita Y, Carpenter JF (1991) Protein-solvent interactions in pharmaceutical formulation. Pharm Res 8:285–291

Bae YH, Park K (2011) Targeted drug delivery to tumors: myths, reality and possibility. J Control Release 153:198–205
Banerjee PS, Hosny EA, Robinson JR (1991) Parenteral delivery of peptide and protein drugs. In: Lee VHL (ed) Peptide and protein drug delivery. Marcel Dekker, Inc., New York, pp 487–543
Björk E, Edman P (1988) Characterization of degradable starch microspheres as a nasal delivery system for drugs. Int J Pharm 62:187–192
Brange J, Langkjaer L (1993) Insulin structure and stability. In: Wang YJ, Pearlman R (eds) Stability and characterization of protein and peptide drugs. Case histories. Plenum Press, Inc, New York, pp 315–350
Chien YW (1991) Transdermal route of peptide and protein drug delivery. In: Lee VHL (ed) Peptide and protein drug delivery. Marcel Dekker, Inc., New York, pp 667–689
Choudhari S, Mathew M, Verma RS (2011) Therapeutic potential of anticancer immunotoxins. Drug Discov Today 16:495–503
Constantino HR, Pikal MJ (2004) Lyophilization of biopharmaceuticals. AAPS Press, Arlington
Crommelin DJA, Schreier H (1994) Liposomes. In: Kreuter J (ed) Colloidal drug delivery systems. Marcel Dekker, Inc., New York, pp 73–190
Crommelin DJA, Storm G (1990) Drug targeting. In: Sammes PG, Taylor JD (eds) Comprehensive medicinal chemistry. Pergamon Press, Oxford, pp 661–701
Crommelin DJA, Bergers J, Zuidema J (1992) Antibody-based drug targeting approaches: perspectives and challenges. In: Wermuth CG, Koga N, König H, Metcalf BW (eds) Medicinal chemistry for the 21st century. Blackwell Scientific Publications, Oxford, pp 351–365
Crommelin DJA, Scherphof G, Storm G (1995) Active targeting with particulate carrier systems in the blood compartment. Adv Drug Deliv Rev 17:49–60
De Leede LGJ, Humphries JE, Bechet AC, Van Hoogdalem EJ, Verrijk R, Spencer DJ (2008) Novel controlled-release *Lemna*-derived IFN-α2b (Locteron): pharmacokinetics, pharmacodynamics, and tolerability in a phase I clinical trial. J Interferon Cytokine Res 28:113–122
Delves PJ, Martin MS, Burton DR, Roitt IM (2011) Roitt's essential immunology, 12th edn. Wiley-Blackwell, Oxford
Edman P, Björk E (1992) Nasal delivery of peptide drugs. Adv Drug Deliv Rev 8:165–177
FDA (2010) http://www.fda.gov/BiologicsBloodVaccines/SafetyAvailability/VaccineSafety/UCM096228
Fendler JH (1980) Optimizing drug entrapment in liposomes. Chemical and biophysical considerations. In: Gregoriadis G, Allison AC (eds) Liposomes in biological systems. Wiley, Chichester, p 87
Franks F, Hatley RHM, Mathias SF (1991) Materials science and the production of shelf-stable biologicals. Pharm Technol Int 3:24–34
Gregoriadis G (2006) Liposome technology, 3rd edn. Informa Healthcare, New York
Groves M (1988) Parenteral technology manual. Interpharm Press, Inc., Buffalo Grove
Halls NA (1994) Achieving sterility in medical and pharmaceutical products. Marcel Dekker, Inc., New York
Heilmann K (1984) Therapeutic systems. Rate controlled delivery: concept and development. G. Thieme Verlag, Stuttgart
Heller J (1993) Polymers for controlled parenteral delivery of peptides and proteins. Adv Drug Deliv Rev 10:163–204
Hellström KE, Hellström I, Goodman GE (1987) Antibodies for drug delivery. In: Robinson JR, Lee VHL (eds) Controlled drug delivery. Marcel Dekker, Inc., New York, pp 623–653
Hernández RM, Orive G, Murua A, Pedraz JL (2010) Microcapsules and microcarriers for in situ cell delivery. Adv Drug Deliv Rev 62:711–730
Holmes D (2011) Buy buy bispecific antibodies. Nat Rev Drug Discov 10:798–800
Hovorka R (2011) Closed-loop insulin delivery: from bench to clinical practice. Nat Rev Endocrinol 7:385–395
Hunt CA, MacGregor RD, Siegel RA (1986) Engineering targeted in vivo drug delivery. I. The physiological and physicochemical principles governing opportunities and limitations. Pharm Res 3:333–344
Jain RK (1987) Transport of molecules in the tumor interstitium: a review. Cancer Res 47:3039–3051
Jorgensen J, Nielsen HM (2009) Delivery technologies for biopharmaceuticals: peptides, proteins, nucleic acids and vaccines. Wiley, Chichester
Kersten G, Hirschberg H (2004) Antigen delivery systems. Expert Rev Vaccines 3:89–99
Kim SW, Pai CM, Makino K, Seminoff LA, Holmberg DL, Gleeson JM, Wilson DA, Mack EJ (1990) Self-regulated glycosylated insulin delivery. J Control Release 11:193–201
Kis EE, Winter G, Myschik J (2012) Devices for intradermal vaccination. Vaccine 30:523–538
Klegerman ME, Groves MJ (1992) Pharmaceutical biotechnology: fundamentals and essentials. Interpharm Press, Inc., Buffalo Grove
Kumar S, Char H, Patel S, Piemontese D, Malick AW, Iqbal K, Neugroschel E, Behl CR (1992) In vivo transdermal iontophoretic delivery of growth hormone releasing factor GRF (1–44) in hairless guinea pigs. J Control Release 18:213–220
Lammers T, Hennink WE, Storm G (2008) Tumour-targeted nanomedicines: principles and practice. Br J Cancer 99:392–397
Maberly GF, Wait GA, Kilpatrick JA, Loten EG, Gain KR, Stewart RDH, Eastman CJ (1982) Evidence for insulin degradation by muscle and fat tissue in an insulin resistant diabetic patient. Diabetol 23:333–336
Mastrobattista E, van der Aa MAEM, Hennink WE, Crommelin DJA (2006) Artificial viruses: a nanotechnological approach to gene delivery. Nat Rev Drug Discov 5:115–121
Mitragotri S (2005) Immunization without needles. Nat Rev Immunol 5:905–916
Moeller EH, Jorgensen L (2009) Alternative routes of administration for systemic delivery of protein pharmaceuticals. Drug Discovery Today: Technologies 5:89–94
Nässander UK, Storm G, Peeters PAM, Crommelin DJA (1990) Liposomes. In: Chasin M, Langer R (eds) Biodegradable polymers as drug delivery systems. Marcel Dekker, New York, pp 261–338
Nguyen TH, Ward C (1993) Stability characterization and formulation development of alteplase, a recombinant tissue plasminogen activator. In: Wang YJ, Pearlman R

(eds) Stability and characterization of protein and peptide drugs. Case histories. Plenum Press, New York, pp 91–134

O'Hagan DT (1990) Intestinal translocation of particulates - implications for drug and antigen delivery. Adv Drug Deliv Rev 5:265–285

Patton JS, Trinchero P, Platz RM (1994) Bioavailability of pulmonary delivered peptides and proteins: alpha-interferon, calcitonin and parathyroid hormones. J Control Release 28:79–85

Patton JS, Bukar JG, Eldon MA (2004) Clinical pharmacokinetics and pharmacodynamics of inhaled insulin. Clin Pharmacokinet 43:781–801

Pearlman R, Bewley TA (1993) Stability and characterization of human growth hormone. In: Wang YJ, Pearlman R (eds) Stability and characterization of protein and peptide drugs. Case histories. Plenum Press, New York, pp 1–58

Peeters PAM, Storm G, Crommelin DJA (1987) Immunoliposomes in vivo: state of the art. Adv Drug Deliv Rev 1:249–266

Pikal MJ (1990) Freeze-drying of proteins. Part I: process design. BioPharm 3(8):18–27

Poste G (1985) Drug targeting in cancer therapy. In: Gregoriadis G, Poste G, Senior J, Trouet A (eds) Receptor-mediated targeting of drugs. Plenum Press, New York, pp 427–474

Pristoupil TI (1985) Haemoglobin lyophilized with sucrose: effect of residual moisture on storage. Haematologia 18:45–52

Roy MJ (2011) Biotechnology operations: principles and practices. CRC Press, Boca Raton

Sage BH, Bock CR, Denuzzio JD, Hoke RA (1995) Technological and developmental issues of iontophoretic transport of peptide and protein drugs. In: Lee VHL, Hashida M, Mizushima Y (eds) Trends and future perspectives in peptide and protein drug delivery. Harwood Academic Publishers GmbH, Chur, pp 111–134

Schaepelynck P, Darmon P, Molines L, Jannot-Lamotte MF, Treglia C, Raccah D (2011) Advances in pump technology: insulin patch pumps, combined pumps and glucose sensors, and implanted pumps. Diabetes Metab 37:S85–S93

Stenekes R (2000) Nanoporous dextran microspheres for drug delivery. Thesis, Utrecht University

Storm G, Crommelin DJA (1998) Liposomes: quo vadis? Pharm Sci Tech Today 1:19–31

Storm G, Oussoren C, Peeters PAM, Barenholz YB (1993) Tolerability of liposomes in vivo. In: Gregoriadis G (ed) Liposome technology. CRC Press, Inc, Boca Raton, pp 345–383

Storm G, Nässander U, Vingerhoeds MH, Steerenberg PA, Crommelin DJA (1994) Antibody-targeted liposomes to deliver doxorubicin to ovarian cancer cells. J Liposome Res 4:641–666

Supersaxo A, Hein WR, Steffen H (1990) Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration. Pharm Res 7:167–169

Thurow H, Geisen K (1984) Stabilization of dissolved proteins against denaturation at hydrophobic interfaces. Diabetologia 27:212–218

Tomlinson E (1987) Theory and practice of site-specific drug delivery. Adv Drug Deliv Rev 1:87–198

Traitel T, Goldbart R, Kost J (2008) Smart polymers for responsive drug-delivery systems. J Biomater Sci Polym Ed 19:755–767

Tresco PA (1994) Encapsulated cells for sustained neurotransmitter delivery to the central nervous system. J Control Release 28:253–258

USP 29/NF 24 through second supplement (2006) United States Pharmacopeial Convention, Rockville

Vemuri S, Yu CT, Roosdorp N (1993) Formulation and stability of recombinant alpha1-antitrypsin. In: Wang YJ, Pearlman R (eds) Stability and characterization of protein and peptide drugs. Plenum Press, New York, pp 263–286

Wang YJ, Watson MA (1988) Parenteral formulations of proteins and peptides: stability and stabilizers. J Parenter Sci Technol 42(Suppl 4S):1–26

Woodle M, Newman M, Collins L, Redemann C, Martin F (1990) Improved long-circulating (Stealth®) liposomes using synthetic lipids. Proc Int Symp Contr Rel Bioact Mater 17:77–78

Zhou XH, Li Wan Po A (1991a) Peptide and protein drugs: I. Therapeutic applications, absorption and parenteral administration. Int J Pharm 75:97–115

Zhou XH, Li Wan Po A (1991b) Peptide and protein drugs: II. Non-parenteral routes of delivery. Int J Pharm 75: 117–130