Environmental Chemistry for a Sustainable World

Divya Arora
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Pharmaceuticals from Microbes

The Bioengineering Perspective

Environmental Chemistry for a Sustainable World

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Preface

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them
Sir William Bragg, Nobel Prize in Physics

Microbiology has made a huge contribution to the pharmaceutical industry. Indeed, microbes have been used to produce antibiotics, enzymes, polysaccharides, proteins, vitamins and nucleotides. Further, with the advent of new approaches and modern techniques, pharmaceutical industries are looking for innovative solutions, such as bioengineering, to improve the quality of products and enhance productivity. Bioengineering is a discipline that applies engineering principles of design and analysis to biological systems and biomedical technologies. Bioengineering concepts can be applied to the pharmaceuticals as well as to microbes to generate better quality products. This book presents recent advances in microbial technology with emphasis on drug delivery strategies for healthcare products, vaccine delivery, biotransformation and processing of biopharmaceuticals.

The first chapter by Ageitos and Garcia-Fuentes reviews innovative drug delivery strategies for microbial healthcare products (Fig. 1) with improved pharmacological effects and characteristics, as compared to classical formulations. Kumar then explores live-attenuated bacterial vector applications in the delivery of vaccine antigens to the mucosal immune system, DNA vaccine and immunotherapy and the mechanism of immune responses elicited by live-attenuated bacterial vector-based vaccines in Chap. 2. In Chap. 3, Kumar et al. review the preparation methods of polylactide/polylactide-co-glycolide delivery-based particles, their properties as carriers of bioactive molecules and applications of delivery systems based on polymeric particles against microbes.

Giorgiana discusses drug delivery systems based on pullulan and derivatives and structured function of therapeutic effects of drugs in Chap. 4. Parajuli et al. analyse biotransformation approaches to produce engineered molecules using microbial platforms, with focus on microbial modification of flavonoids, in Chap. 5. Mehta reviews the upstream process and the three chronological steps – initial recovery, purification and polishing – involved in downstream processing of biopharmaceuticals, in Chap. 6. Finally, Shrestha et al. review the fermentation, engineering

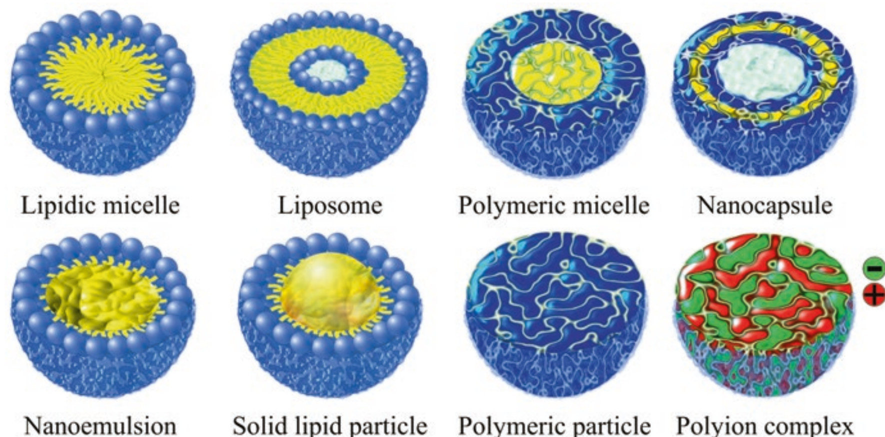

Fig. 1 Nanocarriers for innovative drug delivery. Blue areas are hydrophilic; yellow areas are hydrophobic. In Chap. 1 by Ageitos and Garcia-Fuentes

regulatory genes at the molecular level, with focus on doxorubicin and daunorubicin biosynthesis in *Streptomyces peucetius,* in Chap. 7.

It was our pleasure to interact with all the authors, and we wish to express our gratitude to all the contributors for accepting our invitation. We greatly appreciate their commitment and contribution in shaping the scattered information from diverse fields into their chapters and incorporating the editorial suggestions to produce this venture.

We also extend our thanks to the Springer Nature team for their generous cooperation at every stage of the book production. We hope that the book will serve to update the knowledge and will be helpful to the students, professors, scientists and researchers who have focus on drug discovery. Lastly, we acknowledge God and our family members, who keep motivating and provided all the channels to work in cohesion and coordination right from the conception of the idea to the finalization of the book.

Jammu, India — Divya Arora
Ludhiana, Punjab, India — Chetan Sharma
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Chapter 1
Advances in Drug Delivery Strategies for Microbial Healthcare Products

Jose Manuel Ageitos and Marcos Garcia-Fuentes

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Abstract Biomacromolecules produced by microorganisms have been employed in healthcare ever since ancient times as part of fermented products or natural remedies, but from the discovery of penicillin in 1928 by Alexander Fleming, it is

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impossible to conceive medicine without microbial products. In addition to antibiotics, microorganisms produce secondary metabolites currently employed as anti-inflammatory, immunosuppressant, and antitumoral drugs, among others. As with any other well-established drugs, undesirable side effects may occur with these compounds due to excessive systemic drug concentrations, and their pharmacological activity can be lost by the development of resistance in the target cells. Besides, many microbial drugs have intrinsic physicochemical properties that limit their application in healthcare such as low aqueous solubility, low bioavailability, acute toxicity, and fast systemic and pre-systemic degradation.

Here we review the critical aspects of innovative strategies for microbial products of high interest for academia and healthcare industry. In order to improve some of the current drug limitations, researchers have explored multiple advanced formulation approaches based on disruptive technologies. By means of new biomaterials and nanotechnology, it is possible to maximize the possibilities for functionalization and interfacing with the biological environment, a characteristic that leads to unique properties as drug delivery carriers. These approaches have resulted in improved pharmacological effects and pharmaceutical characteristics as compared to classical formulations, representing the dawn of a new era in microbial healthcare products.

Abbreviations

FDA	Food and Drug Administration
GRAS	generally recognized as safe
MRSA	methicillin-resistant *S. aureus*
PEG	poly(ethylene glycol)
PEGylated	functionalized with PEG
PLGA	poly(lactic-*co*-glycolic acid)
TAT peptide	transactivator of transcription of human immunodeficiency virus (HIV1)
VRE	vancomycin-resistant enterococci
VRSA	vancomycin-resistant *S. aureus*

1.1 Introduction

Microorganisms are a fundamental source of products for human purposes. On the one hand, primary metabolites, such as alcohols, vitamins, amino acids, enzymes, or organic acids, are employed as nutritional supplements and as raw material for industrial biotransformation. On the other hand, secondary metabolites are employed by pharmaceutical industry to produce active pharmaceutical ingredients widely used in healthcare. Only related to healthcare industry, the estimated market of

microbes and microbial products is estimated as $187.8 billion by 2020 (Singh et al. 2017a). As part of these active pharmaceutical ingredients, it can be included anti-inflammatory, antitumoral, and antibiotic drugs, among others.

However, the applicability of these drugs can be hindered by their intrinsic physicochemical properties, such as limited solubility in aqueous environment, reduced intestinal absorption, enzymatic degradation, or interspecific metabolization, and thus can easily conduct to a lack of efficiency or acute toxicity induced by the increase of dose. The conventional approach consists of solubilization by means of surfactants for producing suspensions of these compounds; however, biocompatible surfactants are uneconomic and difficult to synthesize and often unable to eliminate the toxicity or improve the absorption. Another concern for conventional formulations, which makes well-established treatments into obsolete, is the emergence of antibiotic resistance in microorganism (Kalhapure et al. 2015). Antibiotics have been widely employed in medicine since the 1940s; however, due to their prolonged use and abuse, we have conducted the selection of resistant strains of microorganisms (de Miguel et al. 2016; Ageitos et al. 2017). Nowadays, infections with multiresistant microorganisms are becoming the main issue in nosocomial treatments (Inweregbu et al. 2005; WHO 2014). Other than searching for new drugs, researchers are focusing attention in drugs that, while having high antibiotic activity, have high toxicity. Those drugs have not been extensively employed and, therefore, less prone to resistance selection. It is required the reformulation of these drugs to find a therapeutic window where its toxicity is tolerable while maintaining their antibiotic activity. This dichotomy is usually solved with a precise controlled release or using specific carriers to bring the active pharmaceutical ingredients close to where they are needed and, in an ideal case, both solutions (Wong and Choi 2015). The controlled release of drugs was classically conducted by the design of drug delivery systems which allow the sustainable liberation of compounds based on the properties and inner structure of the materials. With the advances in material science, nowadays it is possible to design “smart” drug delivery systems with stimuli-responsive characteristics (Liu et al. 2016).

Nanotechnology is one of the best alternatives for the design of new formulations to improve existing therapies. This emerging area of medicine is based on the use of nanometric carries to significantly reduce the side effects of nonspecific treatments. Drug delivery systems can be classified regarding their properties such as size, composition, structure, and physical properties. There is some controversy regarding the size definition. Nanocarriers are defined materials in nanometric scale (10^{-9} m); however, in biological sciences the concept is dynamic, referring to particles smaller than 500 nm; in the case of microcarriers, the size spans between 0.1 and 100 μm. Regarding composition, the classification is clearer; thereby, metallic devices can be composed by pure metals (usually gold or silver) or metal oxides [iron(II, III)oxide (Fe_3O_4), gadolinium(III) oxide (Gd_2O_3), or titanium dioxide (TiO_2)]. Polymeric devices are generally composed by a polydisperse synthetic polymer, such as poly(lactic-*co*-glycolic acid) (PLGA), poly(lactic acid), poly(ethylene glycol) (PEG), methoxy poly(ethylene glycol), poly(vinyl alcohol), and poly(vinyl acetate), among others. Dendrimers are a special case of polymeric devices where the polymer have a low dispersion, being formed of repetitively branched molecules, such

as poly(propylene imine). Natural polysaccharides, such as chitosan, alginate, hyaluronic acid, starch, or dextran, are employed in formulations, as much as core or covering of polymeric devices. Both metallic and polymeric devices are generally named particles and, depending on size, as nanoparticles or microparticles; while amphipathic polymers produce micelles, lipid-based devices can be composed by a mixture of lipids or oils with emulsifiers in order to produce different structures such as nanoemulsions, lipospheres, solid lipid particles, micelles, liposomes, nanostructured lipid carriers, and cubosomes, among others. The main difference between those devices is the organization of the hydrophobic and hydrophilic regions (Fig. 1.1).

Furthermore, nanocarriers can be functionalized with biomarkers which allow the active delivery of the drugs by targeting ligands specific for a cell surface receptor molecule (Wong and Choi 2015). This mechanism allows a tight adhesion of the nanocarrier to the targeted cell surface, which may lead either to endocytosis or the drug release induced by other factors, such as pH, temperature, redox-responsible, or the presence of enzymes (Liu et al. 2016). The design of drug nanocarriers must be done taking into account some critical parameters, such as size, shape, or surface charge, the biological response they induce, and the employed administration route (Ageitos et al. 2016).

Examples of succeeding drug nanocarriers are the liposomal formulations approved by the US Food and Drug Administration (FDA). Doxil®/Caelyx™, a PEGylated (functionalized with polyethylene glycol) liposomal formulation of doxorubicin developed by Janssen, initially approved by the FDA for Kaposi's sarcoma treatment in 1995, was later approved for ovarian cancer (2005) and multiple myeloma (2008). This formulation improved site-directed delivery to disease and decreased systemic toxicity of free doxorubicin. Also for Kaposi's sarcoma treatment, in 1996, was approved DaunoXome® (Galen), a liposomal formulation of

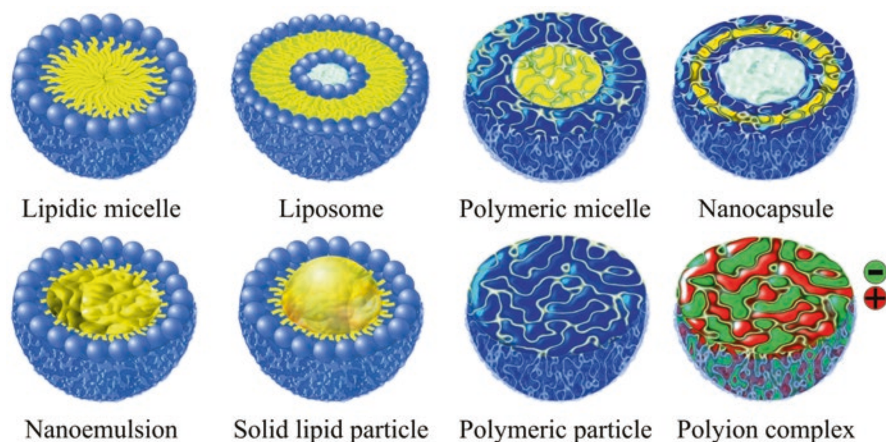

Fig. 1.1 Schematic representation of different nanocarriers described in this chapter. Blue areas represent hydrophilicity and yellow hydrophobicity

daunorubicin, which showed an increased delivery to tumor site and lowered the systemic toxicity arising from side effects of daunorubicin. The antifungal drug amphotericin B was also approved in two liposome formulations, Abelcet® (Sigma-tau) and AmBisome® (Gilead Sciences), both showing a reduction in the toxicity in comparison to free compound. Nowadays there are several microbial drugs based on nanomedicine in clinical trials, and it is expected that those numbers increase soon (Egusquiaguirre et al. 2012; Anselmo and Mitragotri 2016; Bobo et al. 2016; Anselmo et al. 2017).

In the current chapter, we are going to present recent formulations of well-established microbial drugs that can serve as example of the different ongoing approaches, highlighting the critical aspects as compared to classical formulations. It must be considered that the main part of the discussed works are only proofs of concept and will take time to reach the mark, considering that, as explained by Liu and collaborators, "these nanoplatforms are lack of standardized manufacturing method, toxicity assessment experience, and clear relevance between the pre-clinical and clinical studies, resulting in the huge difficulties to obtain regulatory and ethics approval" (Liu et al. 2016). However, authors consider that the works exposed here are an excellent point of reference and a valuable font of knowledge for "advances in drug delivery strategies for microbial healthcare products."

1.2 Anti-inflammatory and Immunosuppressant Drugs

1.2.1 Cyclosporine A

Cyclosporine A (Fig. 1.2) is a non-ribosomally synthesized cyclic peptide isolated from *Tolypocladium inflatum* and is widely used for treating psoriasis and arthritis due its immunosuppressant effect (Thell et al. 2014). Some of the limitations of cyclosporine A are its low bioavailability (Bravo González et al. 2002) derived from poor aqueous solubility and low intestinal permeability (Italia et al. 2007). Cyclosporine A has a narrow therapeutic window where nephrotoxicity, hepatotoxicity, and neurotoxicity have been reported (De Clercq and Holý 2005; Zhang et al. 2013). For these reasons, cyclosporine A is considered a model peptide in nanomedicine, and multiple formulations have been proposed for cyclosporine A delivery through a variety of routes (Wang et al. 2014). Here we will cover the major examples, and we direct the readers seeking further details to a recent review by Guada et al. (Guada et al. 2016b).

PLGA nanoparticles and microspheres have been developed for increasing the stability during the storage of cyclosporine A (Chacón et al. 1999). Orally administered cyclosporine A-loaded poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles showed a cyclosporine A controlled release for 5 days and lower nephrotoxicity than current cyclosporine A formulations (Sandimmune Neoral®) (Italia et al. 2007). Similar results were obtained by Guada and collaborators with cyclosporine A lipid nanoparticles, where these nanoparticles had improved pharmacological response

Fig. 1.2 Chemical structure of cyclosporine A. PubChem CID: 5284373

and less nephrotoxicity than Sandimmune Neoral® (Guada et al. 2016c). Wang et al. have found that lipid-based nanoscale drug delivery systems, such as nanostructured lipid carriers and self-microemulsifying drug delivery systems, were superior to polymeric nanoparticles for enhancing cyclosporine A oral bioavailability (Wang et al. 2014). Interestingly, cyclosporine A lipid-based devices failed to treat inflammatory bowel diseases (Guada et al. 2016a), while cyclosporine A encapsulated in poly(lactic acid) and poly(lactic-*co*-glycolic acid) (PLGA) microspheres showed a reduction in the inflammation of dextran sodium sulfate-induced colitis in animals (Fukata et al. 2011). In addition to the formulations mentioned above for the oral route, cyclosporine A poly-Ɛ-caprolactone nanoparticles showed the ability of penetrating human skin and to reduce inflammation in psoriasis skin models without the appearance of cytotoxicity (Frušić-Zlotkin et al. 2012). Recently, Leung et al. have described cyclosporine A-loaded mannitol porous nanoparticles for pulmonary delivery; those nanoparticles showed good aerosol performance and enhanced dissolution profile compared to spray-dried counterpart (90% in 10 min) (Leung et al. 2017).

Ocular delivery is a specialized type of topical route where other factors such as irritability and mucosal adhesion must be considered. Cationic polymers, such as chitosan, have better adhesion to negatively charged cornea and conjunctiva (Battaglia

et al. 2012), which are critical parameters to ensure an enhanced penetration in the eye of the active pharmaceutical ingredient. Sandri and collaborators investigated cyclosporine A-loaded solid lipid nanoparticles associated with chitosan; these cyclosporine A/solid lipid nanoparticles were biocompatible and enhanced the penetration of cyclosporine A according to in vitro and ex vivo experiments (Sandri et al. 2010). Chitosan nanoparticles have also shown a prolonged cyclosporine A release in vivo, with drug levels being detected both in vitreous and aqueous humor samples (Başaran et al. 2014). Cyclosporine A-poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles have also been studied for ocular delivery. In vitro studies of these nanoparticles confirmed the absence of cytotoxic effects in cell model, while released cyclosporine A retained its anti-inflammatory activity (Hermans et al. 2014). In vivo studies showed that cyclosporine A-loaded PLGA:Eudragit® RL nanoparticles produce a significant increase in cyclosporine A concentrations in rabbit tears in comparison to the commercial formulation (Aksungur et al. 2011). Cyclosporine A has been also assayed in nanomicelle formulations based on polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft copolymer. These micelles showed good biocompatibility and delivered superior levels of cyclosporine A into the cornea in vivo as compared to commercial oil-based cyclosporine A preparations (Guo et al. 2015). Nanomicelles of methoxy poly(ethylene glycol)-hexylsubstituted poly(lactide) copolymers were also instilled ocularly, and authors found higher cyclosporine A cornea levels as compared to the systemic treatment of cyclosporine A (Di Tommaso et al. 2012).

1.2.2 Tacrolimus

Tacrolimus (fujimycin, Fig. 1.3) is an immunosuppressive macrolide lactone, inhibitor of calcineurin-dependent IL2 signaling, isolated from *Streptomyces tsukubaensis*, and is widely employed in clinical practice for prophylaxis of organ rejection (the liver, heart, kidney, pancreas, lung, and bone marrow) in patients

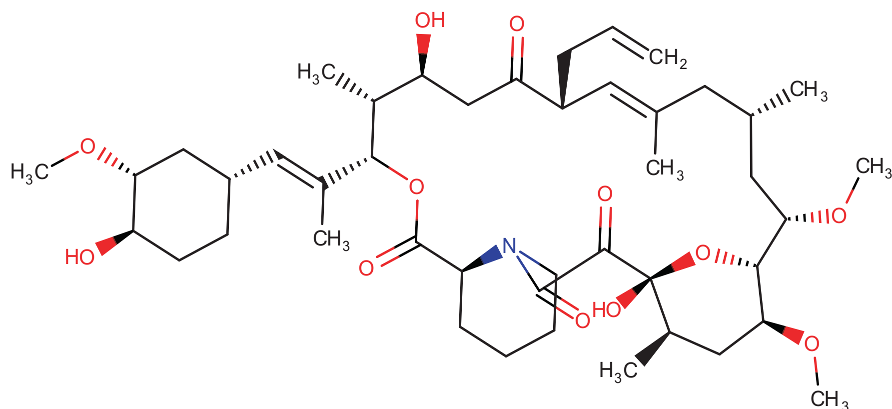

Fig. 1.3 Chemical structure of tacrolimus. PubChem CID: 445643

receiving transplantation (Borhade et al. 2008; Thell et al. 2014). The efficacy of tacrolimus is reduced by its low aqueous solubility and its metabolization in the gastrointestinal tract before absorption. Conventional formulations of tacrolimus have been related to several side effects, including hypertension, nephrotoxicity, and diabetes (Zamorano-Leon et al. 2016).

A few tacrolimus formulations have been tested for improving its bioavailability. For instance, tacrolimus has been formulated in self-microemulsifying drug delivery systems using combinations of various oils, surfactants/cosurfactants, and buffers. Tacrolimus-self-microemulsifying drug delivery systems showed higher bioavailability and immunosuppressive effect than the pure drug and the marketed formulation upon oral administration in an animal model (Borhade et al. 2008). Double-coated tacrolimus-loaded polymethacrylate nanoparticles encapsulated within hydroxypropyl methylcellulose were administered orally to rats and pigs. The researchers detected a 4.9-fold (rats) and a 2.45-fold (pigs) enhancement in relative oral bioavailability as compared to the commercial product (Nassar et al. 2009).

Nanocarriers can also provide targeting for reducing the side effects of tacrolimus. Shin and collaborators studied poly(lactic-*co*-glycolic acid) (PLGA) and PEGylated (functionalized with polyethylene glycol) PLGA nanoparticles loaded with tacrolimus for lymphatic delivery. Tacrolimus nanoparticles and a marketed formulation were intravenously administered to rats; as a result, authors found that concentrations of tacrolimus in mesenteric and axillary lymph nodes were higher for tacrolimus-poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles than for the marketed formulation (Shin et al. 2010). Yoshida et al. proposed another alternative for lymphatic delivery of this active pharmaceutical ingredient using oral oil formulations in rats. Authors found that oil formulations of tacrolimus increased the rate of lymphatic absorption 3- to 15-fold as compared with a solid dispersion formulation while keeping a lower tacrolimus concentration in blood (Yoshida et al. 2016).

Advanced formulations have also been designed to achieve sustained plasmatic levels of drug. For instance, tacrolimus has been loaded in PLGA or poly(lactic acid) microspheres and injected intramuscularly and subcutaneously; after a single injection, it achieves sustained blood levels for 2 weeks, allowing the prolongation of graft survival time in a rat model of heart transplantation (Kojima et al. 2015). Similar results were reported for tacrolimus-loaded in PEG-PLGA nanoparticles administered by gastric perfusion. Tacrolimus/PEG-PLGA nanoparticles produced longer tacrolimus retention time in plasma and increased survival time in a liver transplantation rat model, as compared to tacrolimus capsules (Xu et al. 2014).

Regarding topical applications, tacrolimus has been solubilized in methoxy poly(ethylene glycol)-hexylsubstituted poly(lactide) and loaded into a poly(acrylic acid) gel. The delivery of tacrolimus from this hydrogel formulation was found to be twice that of the commercial formulation in an induced psoriasis model (Gabriel et al. 2016). Another example is a tacrolimus/curcumin-loaded liposphere gel formulation that showed a reduction in lesion markers in an induced psoriasis model as compared to the commercial gel formulation (Jain et al. 2016).

1.3 Cardiovascular Protective Drugs

1.3.1 Lovastatin

Lovastatin (Fig. 1.4) is a highly lipophilic drug isolated from *Aspergillus terreus*, approved as a cholesterol-lowering statin drug by FDA (Chang et al. 2011). Lovastatin is an inhibitor of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase and cholesterol biosynthesis (Jun and Daxin 2016). Lovastatin has been described as an antitumoral drug since it induces cell death in myeloma plasma cells (van de Donk et al. 2002). Due to its lipidic nature, lovastatin has low oral bioavailability (lessthan5%) and short half-life (1–2 h) (Gu et al. 2011); therefore, several formulations have been designed to improve this limitation.

For instance, nanostructured lipid carriers loaded with lovastatin have shown improved pharmacokinetic and pharmacological properties than the free drug (Jun and Daxin 2016). In a similar manner, Gu and collaborators have developed lovastatin-loaded nanostructured lipid carriers functionalized with the major apoprotein of high-density lipoprotein (apoA-I) for targeting foam cells in atherosclerosis. They found that this strategy can deliver lovastatin to foam cells through the very low-density lipoprotein receptor pathway (Gu et al. 2011). Lovastatin has also shown the ability to induce expression of bone morphogenetic protein 2, which can be employed for fracture healing. However, this is only useful upon regional administration, due to oral administration of lovastatin resulted in poor peripheral delivery to the skeleton, since lovastatin was mainly metabolized in the liver. Garret and collaborators developed lovastatin-loaded poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles in the form of nanobeads and observed increased healing in bone fractures after a single injection in the fracture zone as compared to classical oral administration (Garrett et al. 2007).

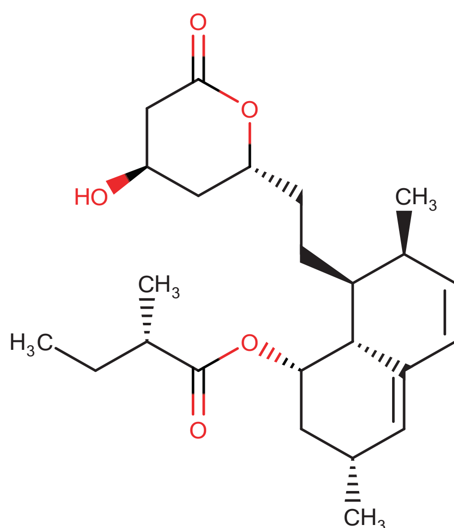

Fig. 1.4 Chemical structure of lovastatin. PubChem CID: 53232

1.3.2 Simvastatin

Simvastatin (Fig. 1.5) is another cholesterol-lowering drug derived synthetically from a fermentation product of *A. terreus*. Simvastatin has been used for the treatment of dyslipidemia, coronary heart disease, and specially hypercholesterolemia. Like lovastatin, simvastatin also shows low oral bioavailability. Simvastatin is a white to off-white, nonhygroscopic, crystalline power that is practically insoluble in water. This profile makes simvastatin a logical candidate for its encapsulation in amphipathic carriers (Wu et al. 2015). Zhang and coworkers have developed simvastatin-loaded solid lipid nanoparticles, which were able to triplicate the oral bioavailability of the unmodified simvastatin in rat models (Zhang et al. 2010). Simvastatin-loaded nanostructured lipid carriers have shown to be more efficient for attenuating the atherogenic risk of erythrocytes in hyperlipidemic rats as compared to a simvastatin suspension. Simvastatin-loaded nanostructured lipid carriers induced enhanced drug absorption and bioavailability, a prolonged half-life of simvastatin (Harisa et al. 2017). Bertha et al. have studied simvastatin-loaded poly(ethylene oxide) electrospinning fibers for the controlled release of this drug. Simvastatin fibers showed the ability of release drug for 12 h following zero-order release rate kinetics, which indicate a release mechanism governed by a non-Fickian diffusion process. Moreover, the release profile could be changed by modifying the drug/polymer ratio (Betha et al. 2015). Simvastatin has also been employed for breast adenocarcinoma, in the form of simvastatin loaded into polymeric nanoparticles, composed by a star-shaped cholic acid core grafted with poly(lactic-*co*-glycolic acid) (PLGA). Simvastatin-loaded cholic acid-PLGA nanoparticles showed higher cytotoxicity than pristine simvastatin or simvastatin loaded in linear PLGA nanoparticles. Simvastatin-loaded cholic acid-PLGA nanoparticles were effective in vitro and in vivo, where they effectively suppressed tumor growth in a BALB/c nude mice xenograft tumor model (Wu et al. 2015).

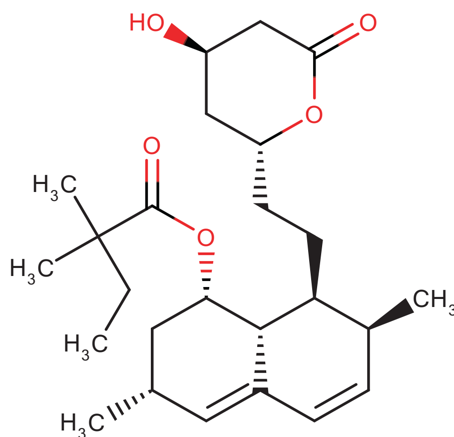

Fig. 1.5 Chemical structure of simvastatin. PubChem CID: 54454

1.4 Antitumoral Drugs

1.4.1 Aclarubicin

Aclarubicin (aclacinomycin A, Fig. 1.6) is an anthracycline anticancer drug isolated from *Streptomyces galilaeus*, employed in China and Japan for cancer treatment. Aclarubicin mainly follows two pathways in its cytotoxic activity; it inhibits DNA topoisomerase activity inhibiting the synthesis of nucleic acids and reduces the oxygen consumption in mitochondria (Iihoshi et al. 2017). Several formulation strategies focused on the active targeting of tumors have been conducted. Aclarubicin has been delivered in cationic albumin-conjugated PEGylated (modified with polyethylene glycol) nanoparticles for glioma chemotherapy in rats (Lu et al. 2007). These in vivo studies showed that aclarubicin concentration in the tumor was 3.3-fold higher than the one reached with a free aclarubicin preparation. This nanomedicine also increased the retention in the glioma, being the concentration of aclarubicin in the tumor 6.6-fold higher than the one reached with free drug 24 h postinjection (Lu et al. 2007). Jia et al. assayed aclarubicin-loaded solid lipid nanoparticles for targeted liver delivery by intravenous administration. This nanomedicine showed a sustained release of aclarubicin, together with high bioavailability; in vivo studies showed that the specific ratio of active pharmaceutical ingredient released in the liver was duplicated as compared to direct aclarubicin injection, while delivery in other organs was reduced significantly. Authors proposed that solid lipid nanoparticles accumulate in the liver by passive mechanisms after intravenous injection; they are absorbed by the reticuloendothelial system, leading to greatest drug accumulation in the liver, due to its size (70 nm) (Jia et al. 2014b).

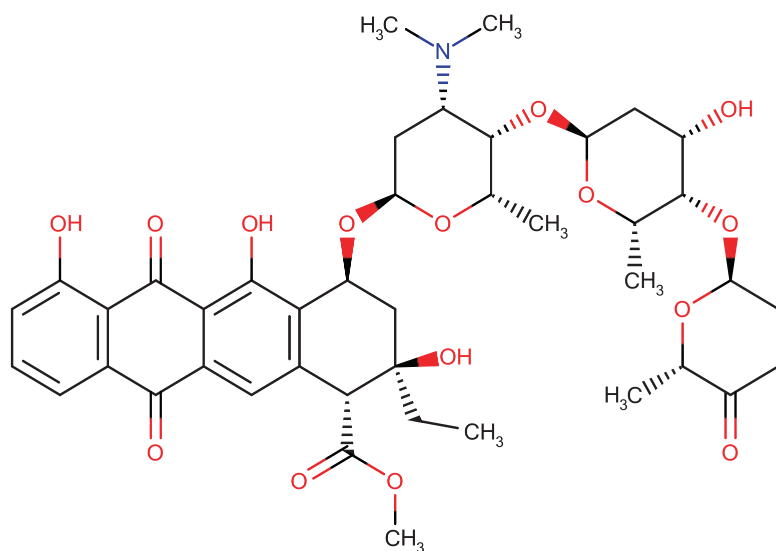

Fig. 1.6 Chemical structure of aclarubicin. PubChem CID: 451415

1.4.2 Bleomycin

Bleomycin (Fig. 1.7) is a mixture of basic glycopeptide antineoplastic antibiotics isolated from *Streptomyces verticillus* (Umezawa et al. 1966) and is commonly used in the treatment of lymphoma, squamous cell carcinomas, germ cell tumors, and malignant pleural effusion (Moeller et al. 2008; Zhang et al. 2011). Bleomycin's major components are bleomycin A_2 (55–70%) and bleomycin B_2 (25–32%) (Zhang et al. 2011; Yu et al. 2015). The cytotoxic activity of bleomycin is based on the free radical degradation of DNA after binding to guanosine-cytosine-rich portions (Dorr 1992). Bleomycin has pulmonary toxicity, and it is employed for the induction of pulmonary fibrosis models in mice (Moeller et al. 2008). Bleomycins are water-soluble molecules, which, combined with their high molecular weight, leads to low cell membrane permeability.

The improvement of bleomycin's action has been extensively studied by different drug delivery strategies (Yu et al. 2015), such as metallic nanoparticles (Shatskaya et al. 2013; Yang et al. 2016), nanoliposomes (Chiani et al. 2017), or microspheres (Nguyen et al. 2011). The objective of those strategies was to improve the cellular uptake, to enhance the lymphatic accumulation (Matsuru et al. 1979), to target cancerous cell receptors through folate receptors (Chiani et al. 2017), or to achieve sustained plasmatic levels and reduced side effects by controlled release formulations (Zhang et al. 2011). For instance, Kullberg and collaborators proposed bleomycin's immunoliposomes conjugated with trastuzumab for recognition of Her-2^+ breast cancer cells. Those immunoliposomes were functionalized with the pore-forming protein listeriolysin O to promote endosomal escape cell internalization. The authors observed that this formulation was able to specifically reduce the viability to Her-2-positive cells with an effective bleomycin concentration that was 57,000-fold lower than the one administered extracellularly (Kullberg et al. 2012).

Fig. 1.7 Chemical structure of bleomycin. PubChem CID:5360373

1.4.3 Doxorubicin

Doxorubicin (Adriamycin, Fig. 1.8a) is a 14-hydroxylated version of daunorubicin (daunomycin, Fig. 1.8b), a chemotherapeutic drug isolated from *Streptomyces peucetius*. Daunorubicin has a broad antitumoral spectrum; however, its application became hindered by its high toxicity and side effects. In order to solve these limitations, novel "smart" stimuli-sensitive drug delivery systems that respond to pH, temperature, and magnetism have been proposed. For instance, Zang and collaborators developed daunorubicin-loaded titanium dioxide (TiO_2) nanoparticles. The fraction of daunorubicin released from daunorubicin-TiO_2nanoparticles increased three- to fourfold in acidic conditions as compared to pH 7.4. As the extracellular pH of tumors is lower than in healthy cell tissues, this allows to trigger daunorubicin release specifically in the tumor. In vitro studies have indicated that daunorubicin-TiO_2 nanoparticles enhance the delivery of daunorubicin to the tumors as compared to conventional administration and that they induce tumor apoptosis in a caspase-dependent manner (Zhang et al. 2012). Daunorubicin has also been investigated for antitumor therapy in combination with nanomedicines integrating also oxaliplatin. The drug combination was integrated in biodegradable amphiphilic polymeric mixed micelles that showed reduced systematic toxicity and greater synergistic effect than the combination of the same free drugs. The mixed micelles demonstrated in vivo lower toxicity and comparable or higher antitumor efficacy compared with the same drugs in small molecule formulation (Xiao et al. 2012).

Doxorubicin has been extensively studied in recent years, for instance, by their encapsulation in doxorubicin-loaded nanoparticles of poly(lactic-*co*-glycolic acid) coated with multilayers of chitosan/alginate. Multilayered nanoparticles showed superior in vivo tumor inhibition rates and decreased toxicity compared to doxorubicin-poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles and doxorubicin in solution (Chai et al. 2017). Malinovskaya et al. have further studied doxorubicin-loaded PLGA nanoparticles in U87 human glioblastoma cells and observed that nanoparticles penetrate cells by means of clathrin-mediated endocytosis and then they accumulate in lysosomes and some ultimately might be released into the

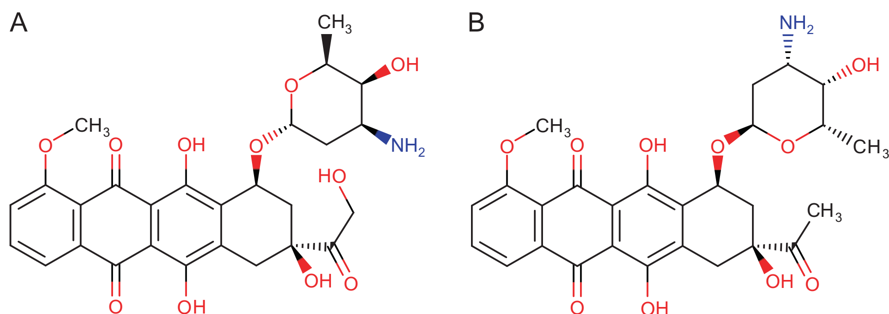

Fig. 1.8 Chemical structure of (**a**) doxorubicin and (**b**) daunorubicin. PubChem CID:31703 and 30323

nucleus (Malinovskaya et al. 2017). Another example is lithocholic acid-polyethylene glycol (PEG)-lactobionic acid nanoparticles loaded with doxorubicin. In vitro, those nanoparticles show high cellular uptake in a human liver cancer cell line triggered by the galactose-asialoglycoprotein receptor interaction; doxorubicin-lithocholic acid-PEG-lactobionic acid nanoparticles were able to suppress in vivo the tumor growth in an orthotopic mouse model of liver cancer (Singh et al. 2017b). Another approach for targeted delivery has been recently reported by Han and collaborators with doxorubicin-loaded angiopep-2 and the transactivator of transcription of the human immunodeficiency virus [TAT peptide, (Ageitos et al. 2016)] peptide dual-modified liposomes. Those complexes showed high binding efficiency to glioma cells, due to specific recognition of angiopep-2 by the low-density lipoprotein receptor-related protein-1 and the cell-penetrating properties of TAT peptide (Han et al. 2017). Pearce and collaborators have employed the prostate-specific membrane antigen receptor, which is overexpressed on many prostate cancers, on lymph nodes, and on bone metastases as therapeutic targets for a doxorubicin-loaded hyperbranched polymer carrier. In addition of their excellent in vitro efficacy, prostate-specific membrane antigen-doxorubicin-polymers did not show adverse toxicity and reduced volume of subcutaneous prostate tumors for in vivo studies (Pearce et al. 2017). An example of the refinement acquired in the design of targeted delivery strategies for doxorubicin is a recently proposed synergistic chemo-photothermal cancer treatment, based on doxorubicin-loaded multi-walled carbon nanotubes coated with poly(N-vinyl pyrrole), functionalized with folic acid and polyethylene glycol. This material allowed doxorubicin release in a pH-dependent manner and allowed the use of combined chemotherapeutical and photothermal treatments in vitro (Wang et al. 2017).

1.4.4 Mithramycin

Mithramycin (plicamycin, Fig. 1.9) is the most representative member of the aureolic acid family of tricyclic polyketides with antitumor activity produced by *Streptomyces argillaceus*, *S. plicatus*, *S. atroolivaceus*, and other *Streptomyces* species (Lombó et al. 2006). It has been described that mithramycin binds preferentially to the minor groove of guanosine-cytosine-rich portions of DNA, inhibiting their transcription (Lee et al. 1990) and inhibiting the binding of transcription factors like Sp1 (Liu et al. 2017). Mithramycin has been employed in the clinical treatment of testicular embryonal carcinoma, glioblastoma (Lombó et al. 2006), or in hypercalcemia in patients with metastatic bone lesions and Paget's disease (Nastruzzi et al. 2012). However, mithramycin has a narrow therapeutic range since it can cause severe hemorrhagic diathesis at doses up to 30 µg/Kg/day (Lee et al. 1990) and produce gastrointestinal, hepatic, kidney, and bone marrow toxicity

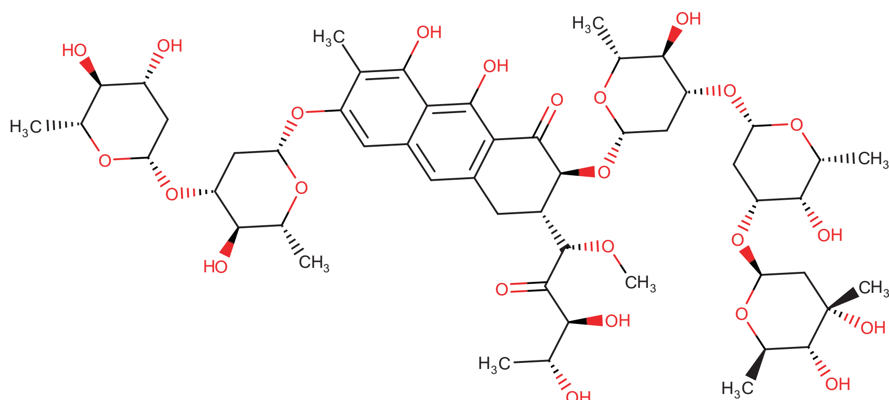

Fig. 1.9 Chemical structure of mithramycin. PubChem CID:163659

(Cohen-Sela et al. 2009). In order to increase absorption and tumor accumulation, Scott et al. designed polyethylene glycol(PEG)-poly(aspartate hydrazide) self-assembled micelles containing mithramycin derivatives that presented increased cytotoxicity to human A549 lung cancer cells. Those micelles had a pH-responsible behavior, inducing the release of mithramycin derivatives at the acidic environment typical of the tumor (Scott et al. 2011). For the treatment of pancreatic carcinoma, mithramycin-loaded methoxy poly(ethylene glycol)-poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles have been recently investigated (Liu et al. 2017). Mithramycin-nanoparticles showed excellent results in vitro and suppressed BxPC-3 tumor growth by 96% in xenograft models.

In addition to the antitumoral activity, new formulations have been assayed with other purposes. Cohen-Sela and coworkers developed a method for the encapsulation of mithramycin in PLGA nanoparticles, with high loading efficiency (80%), and applied this formulation for the treatment of restenosis (Cohen-Sela et al. 2009). Mithramycin-PLGA nanoparticles significantly inhibited RAW264 macrophages and smooth muscle cells and reduced the number of circulating monocytes in rabbits. However, the formulation failed to show a therapeutic effect in a restenosis in rat models. Mithramycin upregulates the expression of human γ-globin genes, which can be associated with a significant improvement in the clinical outcome of the patient with beta-thalassemia. Based on this premise, Nastruzzi and collaborators assayed mithramycin encapsulated in polymeric micellar nanoparticles (Nastruzzi et al. 2012). They found that this advanced formulation was able to reduce the inherent toxicity of the drug and that it induces a more pronounced effect on cell differentiation and γ-globin upregulation when compared to free mithramycin.

1.4.5 Mitomycin C

Mitomycin C (Fig. 1.10) is an antineoplastic antibiotic isolated in Japan in the 1950s from a culture of *Streptomyces caespitosus*. Mitomycin C generates oxygen radicals, alkylates DNA, and produces interstrand DNA cross-links, conducting selective inhibition of DNA synthesis and eliciting genetic recombination, sister chromatid exchange, chromosome breakage, and mutagenesis (Tomasz 1995). Mitomycin C is employed in the treatment of localized bladder cancer and as part of a cocktail chemotherapy of breast, prostate, pancreatic, and non-small cell lung cancers (Bachar et al. 2011). Mitomycin C treatments are associated with number of acute and chronic toxicities, such as severe decrease of blood cells in bone narrow (irreversible myelosuppression), hemolytic uremic syndrome, irritation, or infection (Cheung et al. 2005). Formulation strategies for mitomycin C are focused on improving its bioavailability due to its lipophilic character and on targeted delivery for reducing the side effects of the conventional treatments.

Controlled release formulations of mitomycin C have been designed by encapsulation in poly(lactic acid) nanoparticles upon mitomycin C association with soybean phosphatidylcholine. This association improves the liposolubility of mitomycin C by formation of a mitomycin C-soybean phosphatidylcholine complex (Hou et al. 2009). Besides these strategies aimed at controlling drug levels, other formulations have been developed to control the spatial distribution of the drug. For instance, mitomycin C-loaded hyaluronan-grafted particle clusters were able to produce the specific accumulation of the drug in tumors of epithelial origin (e.g., head and neck cancers) since they express the cell surface glycoprotein receptor for hyaluronan CD44. These hyaluronan clusters showed an increased therapeutic effect on head and neck cancers ex vivo as compared with free mitomycin C, while they did not affect normal cells (Bachar et al. 2011). Another example of targeted delivery is the design of folic acid-tagged (mitomycin C-soybean phosphatidylcholine complex/10-hydroxycamptothecin)-loaded micelles. These micelles have pH-dependent drug release and enhanced cellular uptake mediated by folic acid receptor interaction.

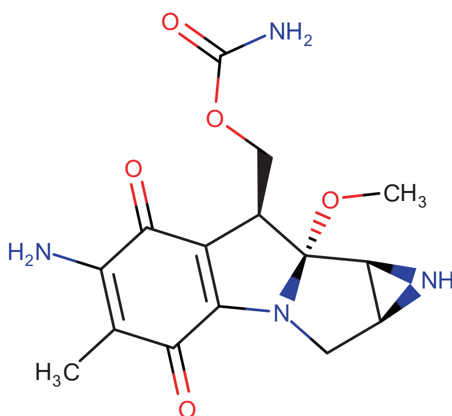

Fig. 1.10 Chemical structure of mitomycin C. PubChem CID:5746

Mitomycin C/10-hydroxycamptothecin-loaded folic acid micelles induced death of tumor cells in vitro and produced the inhibition of growth of tumor tissue in vivo, with low toxicity (Lin et al. 2015). Similar results were obtained with chemical analogs of folic acid such as methotrexate (an inhibitor of dihydrofolate reductase). PEGylated chitosan nanoparticles loaded with mitomycin C/methotrexate were internalized by folic acid receptor-mediated endocytosis and were effective in vivo and in vitro due to the synergic effect of these two drugs (Jia et al. 2014a).

In addition to systemic administration, mitomycin C can be delivered topically using formulations such as the recently reported mitomycin C imprinted poly(2-hydroxyethyl methacrylate-N-methacryloyl-L-glutamic acid) cryogel membranes (Öncel et al. 2017). Cryogel membranes showed low cytotoxicity and released mitomycin C following a non-Fickian diffusion with an initial burst release phase. For intravesical topical treatment focused on bladder tumors, Sun and collaborators proposed mitomycin C loaded onto an in-situ depot of chitosan, β-glycerophosphate, and Fe_3O_4 magnetic nanoparticles (Sun et al. 2016). Fe_3O_4-mitomycin C-chitosan/β-glycerophosphate allowed a sustainable release of mitomycin C in vitro and in vivo, increasing its retention time in the bladder (up to 72 h). Fe_3O_4-mitomycin C-chitosan/β-glycerophosphate increased the survival rate and inhibited the growth of bladder tumors during the in vivo tests, where they observed an improvement in tumor cell apoptosis as compared with conventional administration of mitomycin C (Sun et al. 2016).

1.4.6 Paclitaxel

Paclitaxel (Fig. 1.11) is an anticancer drug isolated in the late 1960s from the western yew, *Taxus brevifolia*; however, the natural abundance of paclitaxel in the bark of yew is only 0.01%–0.05%. Thus, the search for alternative sources of paclitaxel has been a main issue in the past decades. The isolation of several paclitaxel-producing endophytic fungi, such as *Taxomyces andreanae* or *Pestalotiopsis guepinii*, has opened the door to a sustainable paclitaxel source (Zhou et al. 2010). In this way, although the quest is still ongoing (Li et al. 2014; Ismaiel et al. 2017), nowadays paclitaxel can be considered as a drug with microbial origin. Paclitaxel has been approved in many countries for the treatment of ovarian and breast cancers. Its mechanism of action is based on arresting the cell cycle by disrupting the dynamic equilibrium within the microtubule system, inhibiting cell replication. Even though paclitaxel should be functional in most cancer cells, the drug has important solubility problems and side effects and might lack efficacy against some resistant cancers (Steffes et al. 2017).

Traditional formulation of paclitaxel (Taxol®) produces several side effects such as hypersensitivity, nephrotoxicity, neurotoxicity, vasodilatation, labored breathing, lethargy, and hypotension. In order to improve these main issues, several new formulations have been investigated (Nehate et al. 2014). For instance, Danhier and collaborators proposed paclitaxel-loaded polyethylene glycol (PEG)-functionalized

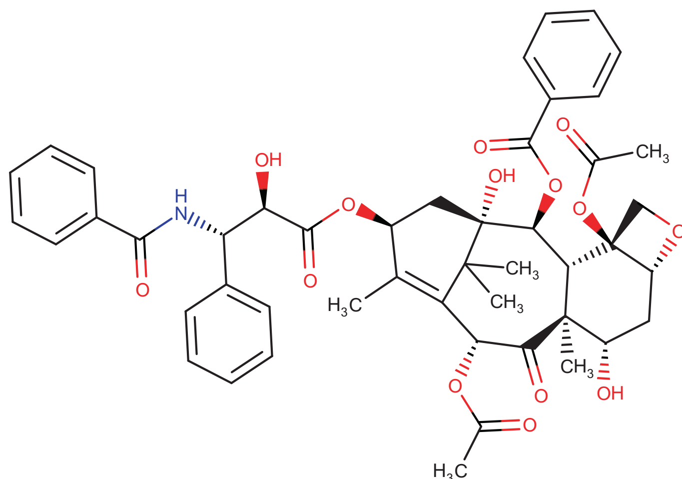

Fig. 1.11 Chemical structure of paclitaxel. PubChem CID:36314

poly(lactic-*co*-glycolic acid) (PLGA)-based nanoparticles. Paclitaxel-loaded nanoparticles showed higher efficacy than commercial paclitaxel formulations in vitro and in vivo, including greater inhibition in the growth of a transplantable lymphoid tumor (Danhier et al. 2009). Paclitaxel-loaded mixed micelles, composed of PEG-*block*-poly(propylene glycol)-*block*-PEG(poloxamer) and poly(ethylene oxide-*co*-propylene oxide) with a diameter of 25 nm, have been studied by Wei and collaborators. Paclitaxel-loaded mixed micelles showed higher toxicity than the commercial paclitaxel formulations in human lung adenocarcinoma cell lines (Wei et al. 2009). Recently, Steffes and collaborators have studied in detail paclitaxel-loaded cationic nanoliposomes with different loading ratio. Nanoliposomes with lower paclitaxel content (1–2 mol%) were more stable and more efficacious than nanoliposomes with higher loading ratio (≥3 mol%), both in release profiles and toxicity against prostate (PC3) and melanoma (M21) human cancer cells lines (Steffes et al. 2017).

In addition to nanoparticle systems, submicron/nanoscale PLGA implants have been assayed for paclitaxel release. Paclitaxel-loaded PLGA nanofiber discs, paclitaxel-loaded PLGA submicron-fiber discs, and paclitaxel-loaded PLGA microspheres entrapped in hydrogel matrices have been intracranially implanted in mice glioblastoma xenograft models. Paclitaxel-loaded nanoscale implants demonstrated optimal drug pharmacokinetics in the brain/tumor and significant tumor inhibition (Ranganath et al. 2010).

1.4.7 Prodigiosin

Prodigiosin (Fig. 1.12) is a natural red pigment produced by several bacterial genera including *Serratia*, *Streptomyces*, *Vibrio*, *Hahella*, *Zooshikella*, and *Pseudoalteromonas* (Dozie-Nwachukwu et al. 2017; Mazzoli et al. 2017), with a wide range of biological activity, including antimicrobial, antimalarial, immunosuppressive, or antitumor properties (Darshan and Manonmani 2015). Prodigiosin can induce apoptosis in cancer cells by several suggested mechanisms of action, such as copper-mediated cleavage of double-stranded DNA, phosphatase inhibition, or disruption of the pH gradient (Rastegari et al. 2017). This versatile pigment has been investigated for different administration routes, for instance, the group of Prof. Soboyejo has studied the controlled release of prodigiosin as breast cancer treatment in a variety of formulations, including thermosensitive poly(N-isopropylacrylamide) hydrogels, implants of poly-di-methyl-siloxane (Danyuo et al. 2014, 2015), biodegradable poly(lactic-*co*-glycolic acid) (PLGA) microparticles (Obayemi et al. 2016), and the free drug (Danyuo et al. 2016). Prodigiosin has also been encapsulated in chitosan microspheres and tested on breast cancer cells with promising results (Dozie-Nwachukwu et al. 2017). Recently, a prodigiosin grafted polysaccharide (β-cyclodextrin and chitosan)-coated magnetic nanoparticles, with lysosome enzymatic-triggered release, have been proposed. These magnetic nanoparticles targeted the GLUT1 receptor, which is overexpressed in cancer cells. Accordingly, prodigiosin-loaded chitosan magnetic nanoparticles showed greater efficacy on cancer cell lines than in noncancerous controls (Rastegari et al. 2017).

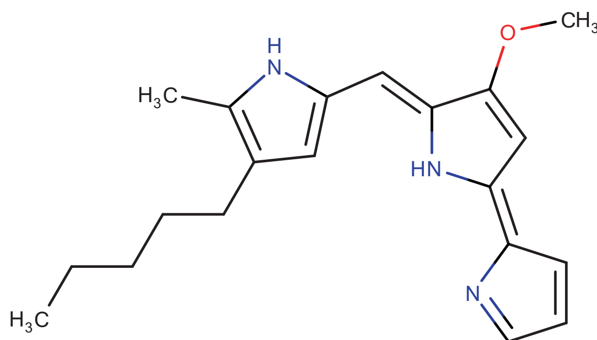

Fig. 1.12 Chemical structure of prodigiosin. PubChem CID:5351169

1.5 Antibiotic Drugs

1.5.1 Amphotericin B

Amphotericin B (Fig. 1.13) is a highly hydrophobic macrolide antifungal antibiotic employed to treat systemic fungal infections and leishmaniasis produced by *Streptomyces nodosus*. This drug has been employed in clinical from more than 60 years as a form of micellar suspension with sodium deoxycholate. However, this formulation produced severe adverse effects such as nephrotoxicity, anemia, and infusion-related side effects. In the 1990s, new formulations based on liposomes were marketed. Liposome-based amphotericin B retained the same activity than micellar amphotericin B while reducing the nephrotoxicity. However, both formulations required an intravenous administration (Serrano and Lalatsa 2017). Current investigations are trying to provide formulations for transmucosal delivery of amphotericin B, and herein we will cover some of the most recent research.

In order to improve oral absorption, amphotericin B-loaded cubosomes have been studied. This formulation increased the bioavailability of amphotericin B by 285% as compared to the commercial micellar suspension, while it did not show nephrotoxicity in animal models (Yang et al. 2012). For bioadhesive mucosal formulations, amphotericin B was encapsulated in core-shell structures formed by monomethoxy polyethylene glycol)-poly(ε-caprolactone) micelles. Amphotericin B/methoxy poly(ethylene glycol)-poly(Ɛ-caprolactone) micelles increased the solubility of amphotericin B yet reduced the overall toxicity, while when loaded in a buccal tablet system, they were able to suppress *Candida albicans* biofilm formation (Zhang et al. 2017a). The same research group also proposed amphotericin B-loaded methoxy poly(ethylene glycol)-poly(Ɛ-caprolactone)-graft-poly(ethylenimine) micelles for local candidiasis treatment. Authors obtained

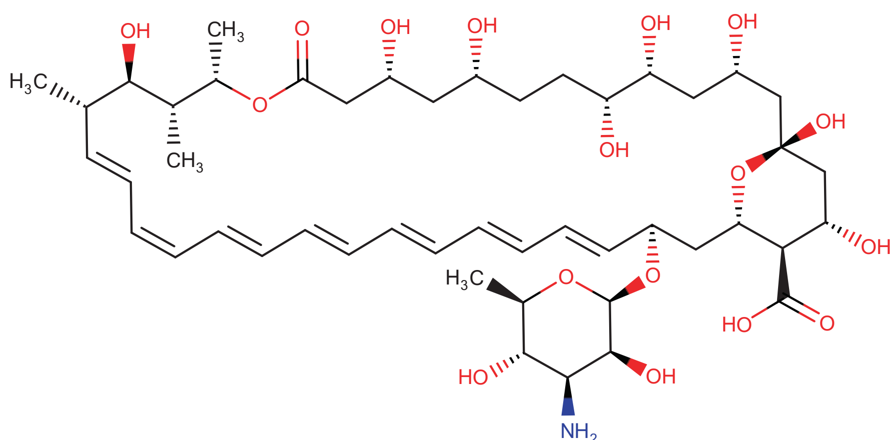

Fig. 1.13 Chemical structure of amphotericin B. PubChem CID:5280965

reduced in vitro cytotoxicity and nonsystemic in vivo toxicity while retaining the same activity against *C. albicans* than Fungizone® (Zhou et al. 2017).

Van De Ven and collaborators developed amphotericin B-loaded poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles and amphotericin B nanosuspensions for intraperitoneal administration, which both systems showed to be more effective in vitro and in vivo against the different *Leishmania* stages and axenic fungi in comparison with the free amphotericin B or marketed formulations (AmBisome® and Fungizone®) (Van De Ven et al. 2012). Jain and collaborators developed amphotericin B-loaded muramyl dipeptide conjugated to multimeric poly(propylene imine) dendrimers for targeting macrophages. These formulations showed a reduction in the hemolytic and cytotoxic effect in erythrocyte and macrophage cultures in comparison to commercial amphotericin B (AmBisome® and Fungizone®). The formulation also showed targeted delivery to macrophages in an in vivo leishmanial model (Jain et al. 2015). Amphotericin B loaded in poly(lactic-*co*-glycolic acid) (PLGA) and dimercaptosuccinic acid nanoparticles showed a preferential tropism for the lungs. This formulation did not produce in vitro hemolysis or in vivo toxicity, while it was able to treat lung fungal infection as commercial amphotericin B, but reduce the number of injections (Souza et al. 2015). Wang and collaborators proposed amphotericin B loaded into polymeric micelles of phenylboronic acid-functionalized polycarbonate/polyethylene glycol and urea-functionalized polycarbonate/PEG diblock copolymers. By means of different blend ratios, authors were able to produce the controlled release of amphotericin B while reducing hemolytic and nephrotoxic effects as compared to commercial amphotericin B colloidal suspension (Fungizone®) (Wang et al. 2016). Recently, it has been reported that amphotericin B-loaded poly(lactic-*co*-glycolic acid) (PLGA)-polyethylene glycol blend nanoparticles had a superior performance to amphotericin B-loaded PLGA nanoparticles, both nanoparticles inhibited in vitro amphotericin B-induced hemolysis and in vivo liver damage (Moraes Moreira Carraro et al. 2017). Another functionalization of PLGA nanoparticles has been performed with amphotericin B-loaded *O*-stearoyl mannose modified PLGA nanoparticles. Mannose-PLGA nanoparticles showed an improvement of intracellular internalization in ex vivo experiments, as compared with pristine amphotericin B-loaded PLGA nanoparticles while effectively treating visceral leishmaniasis in vivo of animal models (Ghosh et al. 2017).

1.5.2 Gentamicin

Gentamicin (Fig. 1.14) is a mixture of three aminoglycoside antibiotics (C1, C1a, and C2), produced by *Micromonospora purpurea*, and is effective against Gram-positive and Gram-negative bacteria; however, like all aminoglycosides, gentamicin is not effective when it is orally provided (Popat et al. 2007). On account of this, gentamicin is often employed in topical applications, especially in bone cements, where it has been employed clinically in various forms for nearly five decades to prevent or treat osteomyelitis (Aviv et al. 2007). This type of infections is difficult

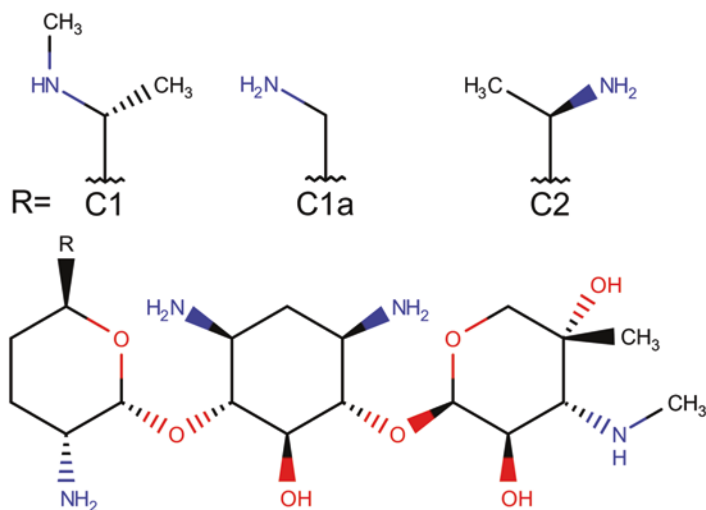

Fig. 1.14 Chemical structure of gentamicin. PubChem CID: 72395, 72396, 72397

to treat because antibiotics cannot readily reach the infection site in bone tissue and because the toxicity and adverse systemic effects of gentamicin, such as ototoxicity and nephrotoxicity, to increment the dosage of drug become infeasible (Swieringa et al. 2008). Besides, most of the classical bone cements were produced with non-biodegradable polymers, which often produce a strong burst release of only hours.

Aviv and collaborators investigated poly(lactic-*co*-glycolic acid) (PLGA) and poly(lactic acid) films for coating of orthopedic implants and observed a sustainable gentamicin release for 12–24 weeks in concentrations, sufficient to inhibit the growth of *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, or *Staphylococcus aureus* (Aviv et al. 2007). Gentamicin-loaded collagen fleeces have been evaluated in acute periprosthetic infections; the release of gentamicin to blood was detected at inhibitory concentrations for *P. aeruginosa*, *S. aureus*, and *Klebsiella* spp., without reaching toxic levels (Swieringa et al. 2008). Gentamicin-PLGA coating on porous magnesium scaffold also showed a controlled release of gentamicin and inhibited adhesion and biofilm formation of *S. epidermidis* and *S. aureus*. Besides this, PLGA-Mg improved the biocompatibility with human bone marrow stromal cells, as compared to porous magnesium scaffold without coating (Li et al. 2015). Pishbin and collaborators have proposed gentamicin-loaded bioactive glass/chitosan composite coatings of metal orthopedic implants. The coating produced an improved cell adhesion and osteogenesis while kept antimicrobial effects against *S. aureus* (Pishbin et al. 2014). Gentamicin has also been encapsulated in *d*-α-tocopheryl polyethylene glycol 1000 succinate micelles and subsequently loaded into a biocomposite material, based on titania nanotubes directly fabricated on titanium surface, coated with chitosan or PLGA. Gentamicin-*d*-α-tocopheryl polyethylene glycol 1000 succinate-chitosan-titania nanotubes showed controlled release of gen-

tamicin (3–4 weeks) and an enhanced osteoblast adhesion and antibacterial properties (Kumeria et al. 2015). Also for titanium coating, gentamicin has been loaded in silk fibroin nanoparticles from *Antheraea mylitta*. Gentamicin-silk fibroin nanoparticles deposited on titanium showed a sustained drug release, enhanced osteoblast adhesion, proliferation, and differentiation in comparison to bare titanium surface (Sharma et al. 2016). A case study of 100 patients with chronic osteomyelitis treated with gentamicin-loaded, calcium sulfate/hydroxyapatite biocomposite has shown that this treatment was able to eradicate the infection in the 96% of the cases with a single procedure (McNally et al. 2016).

Gentamicin-loaded materials have been employed for other purposes, for instance, polyvinyl alcohol/dextran hydrogels have been studied for gentamicin-loaded wound dressing. Gentamicin-loaded gels showed a positive effect of wound healing during in vivo studies and improved the physical properties of pure poly(vinyl alcohol) gels (Hwang et al. 2010). Gentamicin-loaded solid-reversed-micellar-solution-based solid lipid microparticles have been studied for intramuscular injection, being observed diffusion-controlled release profiles in vitro and in vivo rat model (Umeyor et al. 2012). Gentamicin-poly(lactic-*co*-glycolic acid) nanoparticles have shown the controlled release of the drug after intraperitoneal injection and improved the antimicrobial effect of gentamicin toward *P. aeruginosa* infection in a murine model (Abdelghany et al. 2012). In a similar manner, gentamicin-loaded poly(lactic-*co*-glycolic acid) nanoparticles showed a controlled release during 35 days and inhibited the growth of clinical isolates of *S. epidermidis* and *S. aureus* in vitro (Posadowska et al. 2015).

1.5.3 Polymyxin B

Polymyxin B (Fig. 1.15) is a positively charged cyclic antimicrobial peptide produced by *Paenibacillus polymyxa*, with activity against Gram-negative bacteria, especially for multidrug-resistant *P. aeruginosa*. Polymyxin B acts as surfactant, permeabilizing and depolarizing the cell membrane (Ageitos et al. 2017). Even

Fig. 1.15 Chemical structure of polymyxin B. PubChem CID:49800004

though polymyxin B is an effective antibiotic, its clinical use became limited due to its toxicity (mainly nephrotoxicity, ototoxicity, and neuromuscular blockade), protein binding (Brandenburg et al. 2012), and inefficient intestinal absorption (Chifiriuc et al. 2016).

In order to solve the abovementioned drawbacks, polymyxin B has been complexed with liposomes; although in general the bactericidal effect became reduced, it was possible to explore other administration routes, such as the lung. Interestingly, it was not detected in the presence of polymyxin B in the kidney or blood, while bacterial infection was reduced in comparison to conventional polymyxin B (Carmona-Ribeiro and Carrasco 2014; Alipour and Suntres 2014; Martin et al. 2015). Sodium alginate-cross-linked polymyxin B sulfate-loaded solid lipid nanoparticles have been assayed for reducing the toxicity and kept antimicrobial activity, and those solid lipid nanoparticles showed high inhibition capacity against the evaluated strains, with low cytotoxicity (Severino et al. 2015). Recently, it has been reported the vehiculation of polymyxin B in surfactants (poractant alfa); the formulation had a prophylactic in vivo effect on the lung function in neonatal pneumonia of rabbits (Stichtenoth et al. 2017). Sukhishvili and collaborators described an interesting application of polymyxin B/tannic acid films with pH-triggered release induced by bacterial growth. Polymyxin B/tannic acid films inhibited the growth of *S. epidermidis* or *Escherichia coli* while allowing the adhesion and proliferation of murine osteoblast cells (Zhuk et al. 2014). The same group has recently presented polymyxin B-loaded poly(methacrylic acid) hydrogel coatings, which released polymyxin B by the same mechanism (localized pH triggering), and authors proved that these coatings were able to inhibit the growth of *E. coli* even after repeated use or under flowing conditions (Albright et al. 2017). Also for coating applications, polymyxin B has been loaded into 2-hydroxyethyl methacrylate hydrogels on imprinted contact lenses (Malakooti et al. 2015) and as a cationic oral nanoemulsion with dexamethasone acetate for mucosa adhesion (Li et al. 2016). Based on the positive charge of polymyxin B, it is possible to perform polyion complexes based on electrostatic interactions with negatively charged polymers, such as poly(styrene sulfonate). Recently, polymyxin B-polyion complex nanoparticle colloidal suspensions have been reported with antibacterial activity against *P. aeruginosa* similar to free polymyxin B, while polymyxin B-polyion complex nanoparticles showed a gradual release of polymyxin B, which could reduce the toxicity at high doses (Insua et al. 2017a, b).

1.5.4 Nisin

Nisin (Fig. 1.16) is the most prominent member of the lantibiotic family, a group of ribosomally synthetized polycyclic antimicrobial peptides (AMPs) produced by *Lactococcus lactis* subsp. *lactis*, approved as a food preservative by the FDA (Ageitos et al. 2017). Nisins have been formulated in the form of nanoemulsions, nanoliposomes, nanoparticles, and nanofibers or immobilized to produce

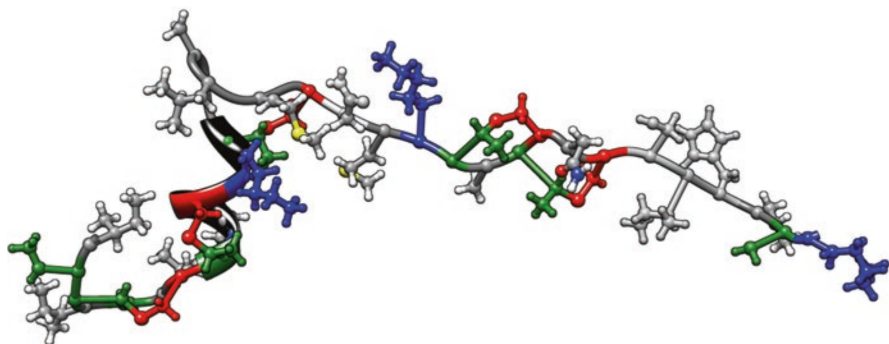

Fig. 1.16 Representation of the structure of nisin (PDB ID: 1WCO) depicting relevant residues. Red, cysteine. Green, uncommon amino acids. Blue, lysine

biodegradable films in order to increase its stability, allow a controlled release, or increase its activity range (Lemes et al. 2016; Khan and Oh 2016). Even if some of the formulation strategies resulted in a partial loss of activity (Carmona-Ribeiro and Carrasco 2014), nisin-loaded solid lipid nanoparticles have shown to inhibit *Listeria monocytogenes* and *Lactobacillus plantarum* grown for up to 20 and 15 days, respectively, compared to 1 and 3 days, respectively, for free nisin (Prombutara et al. 2012). Nisin incorporated with 2,3-dihydroxybenzoic acid in nanofibers of poly($_{D,L}$-lactide) and poly(ethylene oxide) was able to inhibit the biofilm formation by 88% after incubation with a methicillin-resistant *S. aureus* (MRSA) strain (Ahire and Dicks 2014). Based on the synergic effect, nisin-functionalized gold nanoparticles have been studied. The nanoparticles presented low cytotoxicity and lower minimum inhibitory concentration (MIC) (8- to 32-fold) than nisin for clinical isolates of *Enterococcus faecalis* and *S. aureus*, without the appearance of antibiotic resistance (Pradeepa et al. 2017).

1.5.5 Vancomycin

Vancomycin (Fig. 1.17) is a cyclic glycopeptide antibiotic, produced by *Amycolatopsis orientalis*, and is widely used for the treatment of Gram-positive bacterial infection, especially for MRSA. Vancomycin has long been considered as a "drug of last resort"; however, the appearance of vancomycin-resistant *S. aureus* (VRSA) or vancomycin-resistant enterococci (VRE) strains entailed the requirement of higher and more efficient dosage of vancomycin (Singh et al. 2014), which is a major concern, since vancomycin produces nephrotoxicity and hypersensitivity (Honary et al. 2014). Several strategies have been developed for enhancing the efficacy and reducing vancomycin toxicity. Improvement of intestinal viability of vancomycin has been conducted with vancomycin-Eudragit RS100-coated nanoparticles (Loveymi et al. 2012) or vancomycin-poly(lactic-*co*-glycolic acid) (PLGA)

Fig. 1.17 Chemical structure of vancomycin. PubChem CID:14969

nanoparticles (Zakeri-Milani et al. 2013); in both cases, the intestinal absorption was higher than that of vancomycin solutions at the same concentrations.

The group of Concheiro has studied vancomycin loaded in different polymeric films, such as poly(propylene)films with cross-linked poly(acrylic acid) or interpenetrated networks of poly(acrylic acid) and cross-linked poly(acrylic acid) poly(*N*-isopropylacrylamide). All the films showed a pH-dependent vancomycin release and were able to reduce the formation of biofilms by MRSA (Ruiz et al. 2008). Further studies showed that direct grafting of poly(propylene) with gamma radiation produced the smart polymer *net*-poly(propylene)-*g*-poly(acrylic acid)-*inter-net*-poly(*N*-isopropylacrylamide), with temperature- and pH-responsible swelling; vancomycin-loaded-*net*-poly(propylene)-*g*-poly(acrylic acid)-*inter*-*net*-poly(*N*-isopropylacrylamide) films released vancomycin at adequate levels for killing bacteria attempting to adhere the surface of the film; this strategy seems suitable for functionalizing the surface of medical devices based on poly(propylene) (Muñoz-Muñoz et al. 2009). The group of Hu et al. has studied derivatized chitosan, vancomycin-loaded N-trimethyl chitosan nanoparticles (Xu et al. 2015), and vancomycin/N-trimethyl chitosan nanoparticles associated with composite beads of poly(trimethylene carbonate) (Zhang et al. 2017b) for the controlled release of van-

comycin. Both vancomycin/N-trimethyl chitosan nanoparticles and vancomycin/N-trimethyl chitosan nanoparticle-poly(trimethylene carbonate) had excellent antibacterial activity, while the combination of vancomycin/N-trimethyl chitosan nanoparticle-poly(trimethylene carbonate) was able to promote bone repair. Hachicha et al. studied vancomycin-loaded poly(lactic-*co*-glycolic acid) (PLGA) microparticles for continuous release in intraocular route (Hachicha et al. 2006). Vancomycin-loaded folic acid-tagged chitosan nanoparticles were able to reduce the minimum inhibitory concentration and minimum bactericidal concentration for VRSA strains. Authors concluded that folic acid tag was required for nanoparticle action (Chakraborty et al. 2010). Gu and collaborators described (vancomycin)-capped gold nanoparticles with an improved activity against VRE strains and Gram-negative bacteria (Gu et al. 2003). Comparable results were obtained by Mohammed Fayaz and collaborators with vancomycin-bound gold nanoparticles, which were able to reduce four times for *E. coli* and six times for VRSA the minimum inhibitory concentration as compared with free vancomycin (Mohammed Fayaz et al. 2011). Argenziano and collaborators have proposed an interesting approach for vancomycin delivery. Authors developed vancomycin-loaded nanobubbles, a core-shell nanostructure filled with a gas (perfluoropentane), with shell of dextran sulfate, where vancomycin was loaded. Vancomycin-nanobubbles allowed a controlled release of the active pharmaceutical ingredient and were generally more effective against MRSA than free vancomycin while reducing in vitro cytotoxicity on human keratinocytes (Argenziano et al. 2017).

1.6 Probiotic Microorganisms

Even though this chapter is focused on microbial compounds, it must also be taken into account that microorganism themselves can exert an important role in healthcare, as it is the case of probiotics. The Food and Agriculture Organization defines probiotic microorganisms as “live microorganisms, which when consumed in adequate amounts, confer a health effect on the host” (Morelli and Capurso 2012). Probiotics must survive to gastrointestinal tract in order to exert beneficial health effects (Calo-Mata et al. 2016); however, they lose viability due to the extreme conditions they are subjected (gastric acids, bile salts, proteases) (Arslan-Tontul and Erbas 2017). In view of the above, several formulations for the protection of probiotics have been developed. One example of coating technology is Phloral™, a combination of an anionic polymer based on starch, which cannot be digested by mammal amylases, but does bacterial ones, and Eudragit® S, a pH-activated polymer (Ambrogi et al. 2008; Dodoo et al. 2017). Dodoo and collaborators observed that lyophilized *Lactobacillus acidophilus* LA-5 has poor tolerance to simulated gastric fluid, while after encapsulation with Phloral®, viabilities of 90% were observed after the same treatment (Dodoo et al. 2017). Microencapsulation can produce single- or double-layered structures using techniques such as spray drying or

spray chilling. Arslan-Tontul and Erbas showed that the structure of the microcapsules will confer different survival rates to heat and gastric conditions of several probiotic microorganisms *Saccharomyces boulardii*, *L. acidophilus*, and *Bifidobacterium bifidum* (Arslan-Tontul and Erbas 2017). In general terms, single-layered microcapsules produced by spray drying (gum arabic and β-cyclodextrin) showed improved survivability, for instance, encapsulated microorganism survived after incubation at 80 °C, while non-encapsulated ones only bore at 50 °C. In order to increase the viability of probiotics during storage and gastrointestinal environment, pea protein-alginate microcapsules have been recently reported (Varankovich et al. 2017). *Lactobacillus rhamnosus* R0011 and *L. helveticus* R0052 were encapsulated in pea protein-alginate microcapsules with or without a chitosan coating, being observed that both formulations were able to protect the viability of probiotic microorganisms after incubation in simulated gastrointestinal environment. Moreover, chitosan-coated pea protein-alginate microcapsules were able to keep the viability of bacteria up to 9 weeks at room temperature (Varankovich et al. 2017). Besides the intrinsic prophylactic effect of probiotics, these microorganisms have been assayed for mitigating toxic side effects of chemotherapeutic agents; thus, Sharma et al. prepared microparticles containing 5-fluorouracil (pyrimidine analog that is an antineoplastic antimetabolite) and selected probiotic strain with high free radical scavenging activity. In vivo studies showed that microparticles with *L. rhamnosus* protected the colonic epithelium from the cytotoxic effect of 5-fluorouracil; these results open the door to a new solution for the problems associated with traditional chemotherapy (Sharma et al. 2017).

1.7 Conclusion

In the current chapter, we have summarized several novel strategies for the formulation of clinically relevant products with a microbiological origin, paying special attention to nanocarriers for advanced drug delivery. Through review of ongoing approaches, we have explored current academic and industrial interests toward the design of safer and better formulations. Besides some new emerging compounds, the current catalog of microbial drugs can alleviate most of the diseases for which they are described, but sometimes, at an excessive cost. Therefore, significant efforts have been paid for the development of new delivery systems, being among the most promising, the ones based on nanotechnology. These systems aim to improve some of the current drug limitations. Nanodevices maximize the surface by unit of mass, thus maximizing the possibilities for functionalization and interfacing with the biological environment, a characteristic that leads to unique properties as drug delivery carriers. In this way, new strategies attempt to enhance the bioavailability, minimize toxic effects, control the release of drugs, or broaden the range of treatable diseases with conventional drugs, among others. However, it is important to notice that nanomedicine is not a panacea since the properties of the materials drastically change at nanometric scale. On the one hand, new undesirable effects may emerge, even from

GRAS (generally recognized as safe) bulk materials at nanoscale, such as undesired accumulation in organs or even inside cells. On the other hand, the same formulation is not always universally applicable for different drugs; this means that every single compound requires a tremendous research effort in order to arise clinical market. It should be pointed out that there is a clear mismatch between the vast quantity of literature regarding this topic and the limited number of treatments approved so far. Notwithstanding, there is little doubt that after clinical approval, nanocarriers are safer and more efficacious than their traditional counterparts. Microbial drugs are a paradigm of variety and effectiveness, and if considered their synergic effect with a disruptive technology such as nanomedicine, each time more refined and accurate, we can boldly speculate that we are at the dawn of a new era that will utterly flourish in the next decades.

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The chemical structures were obtained from the PubChem Database (https://pubchem.ncbi.nlm.nih.gov/) and represented using the MarvinSketch software (ChemAxon Ltd, Budapest, Hungary).

Protein molecular models were obtained from the Protein Data Bank (PDB; http://www.rcsb.org/pdb; 1WCO). Molecular graphics and analyses were performed with the UCSF Chimera package (Pettersen et al. 2004). Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

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Chapter 2
Live-Attenuated Bacterial Vectors for Delivery of Mucosal Vaccines, DNA Vaccines, and Cancer Immunotherapy

Sudeep Kumar

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Abstract Vaccines save millions of lives each year from various life-threatening infectious diseases, and there are more than 20 vaccines currently licensed for human use worldwide. Moreover, in recent decades immunotherapy has become the mainstream therapy, which highlights the tremendous potential of immune response mediators, including vaccines for prevention and treatment of various forms of cancer. However, despite the tremendous advances in microbiology and immunology, there are several vaccine preventable diseases which still lack effective vaccines. Classically, weakened forms (attenuated) of pathogenic microbes were used as vaccines. Although the attenuated microbes induce effective immune response, a significant risk of reversion to pathogenic forms remains. While in the twenty-first

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century, with the advent of genetic engineering, microbes can be tailored with desired properties.

In this review, I have focused on the use of genetically modified bacteria for the delivery of vaccine antigens. More specifically, the live-attenuated bacteria, derived from pathogenic bacteria, possess many features that make them highly suitable vectors for the delivery of vaccine antigens. Bacteria can theoretically express any heterologous gene or can deliver mammalian expression vectors harboring vaccine antigens (DNA vaccines). These properties of live-attenuated microbes are being harnessed to make vaccines against several infectious and noninfectious diseases. In this regard, I have described the desired features of live-attenuated bacterial vectors and the mechanisms of immune responses manifested by live-attenuated bacterial vectors. Interestingly anaerobic bacteria are naturally attracted to tumors, which make them suitable vehicles to deliver tumor-associated antigens thus I have discussed important studies investigating the role of bacterial vectors in immunotherapy. Finally, I have provided important discussion on novel approaches for improvement and tailoring of live-attenuated bacterial vectors for the generation of desired immune responses.

2.1 Introduction

Vaccines provide protection against numerous life-threatening infectious diseases, by activating the adaptive immunity against specific pathogen-derived antigens. Since the introduction of active immunization, several vaccines have been licensed for human use. These include some subunit vaccines, which are preferred for their superior safety profile. However, their success is limited by their poor immunogenicity, as multiple booster immunizations and adjuvants are required to achieve an adequate level of protective immunity. Moreover, a subunit vaccine is only applicable for pathogens where a well-defined protective antigen has been discovered. Subunit vaccines are also limited in their ability to induce cell-mediated immunity. In contrast, the live-attenuated/live-inactivated vaccines exhibit superior immunogenicity and induce humoral as well as cell-mediated immunity. Although attenuated viruses and bacteria are both utilized as vaccine vectors, this review will focus only on attenuated bacterial vaccine vectors. Bacteria harbor natural adjuvants in the form of pathogen-associated molecular patterns (PAMPs) (Fig. 2.1). PAMPs, which are recognized by components of the innate immune system including Toll-like receptors (TLRs), facilitate the release of pro-inflammatory mediators and recruitment of antigen-presenting cells (Fig. 2.2). Furthermore, even after attenuation, a limited degree of proliferation and dissemination capacity is retained in the attenuated pathogens. Overall, these factors contribute to the superior immunogenicity of live-attenuated bacteria, which consequently elicit a robust and durable immunity against the cognate antigens.

With the advent of molecular biology and genetics, it is feasible to effectively excise or insert desired genes into bacteria. Bacterial vectors can be engineered to

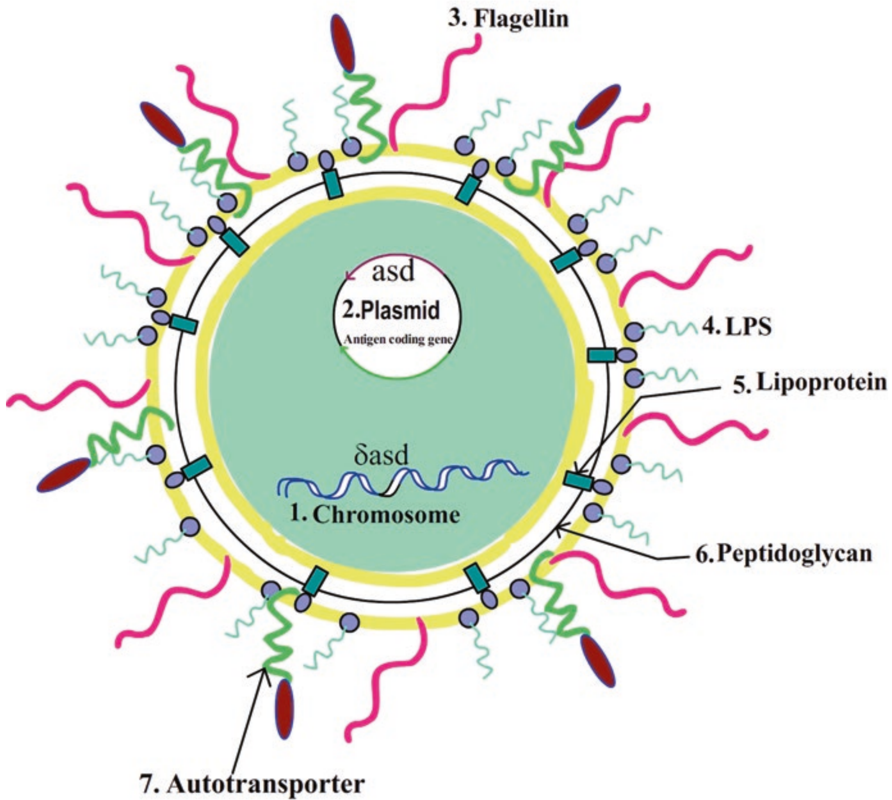

Fig. 2.1 Essential components of live-attenuated bacterial vectors: To construct live-attenuated bacterial vectors, the pathogenicity of bacteria is attenuated by creating mutations in various virulence genes (*1*). The *asd* mutation in the chromosome (*1*) is complemented with a functional copy of *asd* gene, inserted into the plasmid (*2*); this feature ensures antibiotic-free maintenance of plasmids. The plasmid (*2*) also carries genes encoding antigenic proteins. Various pathogen-associated molecular patterns including flagellin (*3*), lipopolysaccharide (*4*), lipoprotein (*5*), and peptidoglycan (*6*) facilitate the interaction with and signal the activation of antigen-presenting cells, while the additional appendages like autotransporters (*7*) facilitate surface display of antigens

express and deliver heterologous proteins, such as antigens or therapeutic proteins, in mammalian hosts. Moreover, by genetic manipulation, bacterial vectors can be engineered with properties including reduced virulence, high immunogenicity, properties which are desirable in a vaccine vector. Thus far, a variety of live-attenuated bacterial vectors including *Mycobacterium bovis* strain *Bacillus* Calmette-Guérin (BCG), *Salmonella* spp., *Listeria monocytogenes* (*Lm*), *Vibrio cholerae*, *Escherichia coli*, and *Shigella* spp. have been utilized for the delivery of heterologous proteins into mammalian hosts as vaccine antigens or therapeutic proteins. Such bacteria are called, live-attenuated bacterial vectors (LABVs).

Vaccines elicit distinct immune responses depending on the route of immunization. Mucosal immunization induces strong systemic as well as mucosal immune

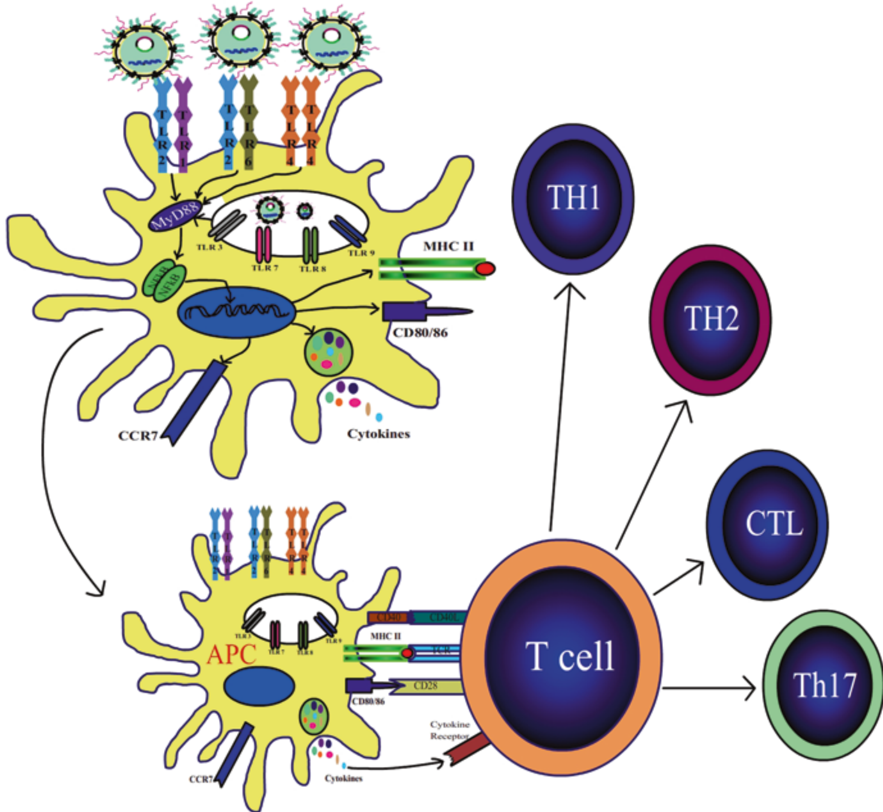

Fig. 2.2 Activation of antigen-presenting cells by live-attenuated bacterial vectors leads to adaptive immune response: Various pathogen-associated molecular patterns present in the live-attenuated bacterial vectors interact with Toll-like receptors expressed on the surface or in endosomal membranes. The signaling initiated by this interaction leads to the activation of antigen-presenting cells. Activated antigen-presenting cells express costimulatory molecules CD80, CD86, and CD40 as well as enhance expression of MHC-II. Costimulatory molecules are required to deliver the essential second signal for T-cell activation, while the first signal is received via TCR-MHC-peptide interaction. Importantly, CCR7 expressed by activated APCs help migration to draining lymph node. Moreover, the type of cytokines directs the fate of T-cell polarization to Th1, Th2, or TH17. Cytosolic delivery of antigens gives rise to CTL response

response, whereas parenteral immunization induces potent systemic but a poor mucosal immune response. Since birth, mucosal surfaces of the human body are constantly challenged with agents of the external environment that are either completely harmless (food ingredients and nonpathogenic microbes) or pathogenic (pathogenic microbes). Thus, in order to restrict pathogenic insults at mucosal surfaces, mucosa-associated lymphoid tissues (MALT) are organized. In fact, MALT constitutes the largest immune system of the human body. The oral route is the most favored route for mucosal immunization over other mucosal routes including nasal,

vaginal, and rectal. Upon oral administration, antigens travel through the gastrointestinal tract and reach the mucosal inductive sites called Peyer's patches. Peyer's patches are lined with specialized epithelial cells called M cells which serve as a point of entry into the lamina propria. In the lamina propria, dendritic cells take up the antigens and migrate to the draining lymph nodes where they present the antigens to T cells. A specialized feature of dendritic cells from Peyer's patches and mesenteric lymph nodes is that they induce gut-homing receptors α4/β7 and CCR9 on T and B cells. This feature is not found in the dendritic cells from cervical lymph nodes and spleen. Thereby, T and B cells primed at the mucosal sites are destined to migrate to mucosal tissues (Pasetti et al. 2011) (Fig. 2.3). Live-attenuated microbes exhibit superior ability to deliver vaccine antigens to the mucosal immune system, as many of them are derived from natural mucosal pathogens, including *Salmonella* spp., *Lm*, *E. coli*, *V. cholerae*, and *Shigella* spp.

This review explores the current knowledge about the LABV application in the delivery of vaccine antigens (to the mucosal immune system), DNA vaccine, and immunotherapy. Mechanism of immune responses elicited by LABV-based vaccines, the recent advances, and future perspectives have been discussed.

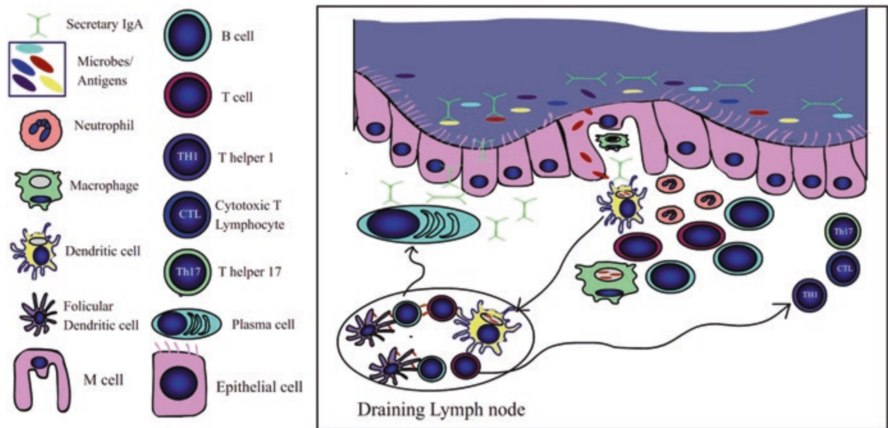

Fig. 2.3 Mucosal immune response elicited by live-attenuated bacterial vectors: Mucosal inductive sites including gut-associated lymphoid tissues and nasal-associated lymphoid tissues facilitate sampling of antigens through M cells. M cells allow the passage of bacteria through the mucosal epithelium, where they are taken up by antigen-presenting cells including DCs and macrophages. APCs undergo activation upon interaction with live-attenuated bacterial vectors, and the associated antigens are presented to T cells in the lymphoid follicles or the draining lymph nodes. Activated T cells help B cells differentiate into IgA-producing plasma cells. The secreted IgA provide effective protection against mucosal pathogens. The T and B cells primed at mucosal sites migrate back to mucosal sites where they perform their effector functions

2.2 Desired Features of Live-Attenuated Bacterial Vectors

Nonpathogenic commensals including the lactic acid bacteria and *Bacillus subtilis* as well as the attenuated versions of the pathogenic bacteria including *Salmonella* spp., *E. coli, Shigella* spp., *Lm*, and *V. cholerae* have been utilized as LABVs. While the commensal microbes are generally regarded as safe or food grade, the virulence of the pathogenic microbes needs to be significantly attenuated before they can be considered safe to deliver vaccine antigens into humans (Fig. 2.1).

Generally, plasmids are employed for the expression of heterologous antigens in LABVs. Plasmids can be easily manipulated in *E. coli* (a universal tool for genetic engineering) and subsequently introduced into the desired bacterial strains. Shuttle vectors (plasmids) carry genetic elements for replication in *E. coli* and promoter elements for gene expression in other bacterial or mammalian cells. Thus, mammalian expression vectors can be first manipulated and propagated in *E. coli* and then delivered via LABVs to mammalian cells, where the desired antigens are expressed (Fig. 2.1).

2.2.1 Attenuation

In early days, attenuation of the pathogenic microbes was achieved by in vitro cultivation for several generations, followed by the evaluation of virulence in successive generations. The classic example is BCG, where Albert Calmette and Camille Guerin, by culturing a virulent strain of *M. bovis* for more than 230 serial passages in vitro (between 1908 and 1921), generated the attenuated strain BCG. BCG is still the only vaccine available for prevention against tuberculosis. Subsequent genetic analysis revealed that BCG lack multiple virulence factors associated with *M. bovis* (Zheng et al. 2015). Similarly, the search for vaccines against typhoid fever led to the generation of attenuated live vaccine strains of *Salmonella*. *Salmonella enterica* serotype Typhi (ST) strain Ty21a (ST-Ty21a) was generated by chemical mutagenesis of wild-type ST strain Ty2. The ST-Ty21a is considerably attenuated which is now licensed for humans use as an oral vaccine (Wang et al. 2001). However, the strain exhibits low immunogenicity, as 3–4 doses are required to achieve adequate levels of protection. With the advances in microbial genetics and genetic engineering techniques, it has now become routine to identify and inactivate virulence genes. Interestingly, various auxotrophic mutant *Salmonella* strains, which lack the ability to synthesize aromatic amino acids, were found to be avirulent (Hoiseth and Stocker 1981). It is known that in *Salmonella*, the gene products of *aroA*, *aroC*, and *aroD* are required for the biosynthesis of aromatic amino acids, as well as several essential vitamins. Hoiseth and Stocker note that these factors are not found in mammalian hosts in sufficient amount; thus *Salmonella* aroA mutants cannot proliferate in mammalian hosts (Hoiseth and Stocker 1981). Harnessing this knowledge, various *Salmonella enterica* serovar Typhi (*ST*) or Typhimurium (*STm*) have been created,

with mutations in *aroA* (Dalla Pozza et al. 1998; Roberts et al. 2000; Arnold et al. 2004), *aroC* (Khan et al. 2003; Capozzo et al. 2004), *aroD* (Capozzo et al. 2004; Sevil Domènech et al. 2008), or *aroAD* (Strugnell et al. 1992; Roberts et al. 2000). Notably, the ST strain CVD908 which carries *aroC* and *aroD* mutations exhibits residual virulence in humans (Wang et al. 2001), while other investigators have also targeted genes in nucleotide biosynthesis pathways for creating attenuated *Salmonella*. Wang et al. engineered a *Salmonella* strain with a mutation in *gua*BA operon, which interferes in the guanine nucleotide biosynthesis. The resultant strain, called CVD 915, exhibits safety profile comparable to that of the typhoid vaccine strain ST-Ty21a. Importantly, ST-Ty21a, CVD-908-*htrA* (harboring mutations at *aroC*, *aroD*, and *htrA*), and CVD 915 all exhibit a high level of immunogenicity (Wang et al. 2001). Not surprisingly, *ST* strains with *gua*BA mutations have been widely utilized as LABV (Pasetti et al. 1999, 2000; Wang et al. 2001). Another approach of attenuation of *Salmonella* is to introduce mutations in *cya* (adenylate cyclase) and *crp* (cyclic AMP) receptor genes. These proteins are transcriptional regulators of many important genes. Although cAMP is found in mammalian cells, their concentrations in gastrointestinal tissues are below the requirement of *Salmonella*. Thus *cya* and *crp* mutants show reduced virulence (Tacket et al. 1992; Chen and Schifferli 2003; Wyszyńska et al. 2004; Ferreira Oliveira et al. 2012). Mutations in the two-component regulatory system PhoP/PhoQ, which controls more than 40 virulence genes involved in resistance to antimicrobial peptides, nutrient scavenging, and lipid A modifications, significantly decrease *Salmonella* virulence (Raupach and Kaufmann 2001). *Salmonella* strains with PhoP/PhoQ mutations have been used in a number of studies as LABV (Angelakopoulos and Hohmann 2000; Kotton et al. 2006; Galen et al. 2009; Wang et al. 2013). *RpoS* is an alternate sigma factor that regulates resistance under stress induced during gastrointestinal infection such as pH, nutrient starvation, change in osmolarity, and temperature. ST-Ty21a contains multiple mutations including *rpoS* (Wang et al. 2001). *SsaV* is a component of *Salmonella* type III secretion system, which is required for secretion of SPI-2 genes (essential for growth in macrophages). The ST strain ZH9 which contains *aroC* and *ssaV* mutations is highly attenuated and immunogenic in humans (Hindle et al. 2002). Tacket et al. generated mutations in *htrA* gene, which encodes a heat shock protein. The resultant strain was avirulent because of reduced ability to survive and replicate in host tissues (Tacket et al. 2000). *HtrA* mutant *Salmonella* strains have been used in multiple studies as LABVs (Galen et al. 1999; Roberts et al. 2000; Pasetti et al. 2002; Capozzo et al. 2004; Fraillery et al. 2007).

Similarly, for attenuation of *Lm*, multiple virulence factors have been targeted. *ActA* which encodes for a surface protein required for actin polymerization in host cells and helps in intracellular migration has been a prominent target for *Lm* attenuation. Together with the mutation in internalin B (*inlB*), the *actA* mutation renders *Lm* unable to infect hepatocytes; thus these strains are highly attenuated (Brockstedt et al. 2004). Phospholipase-C B (PlcB) is required for efficient escape from phagosomal vacuoles. *PlcB* mutants are thus attenuated due to defect in escape from secondary vacuoles (Peters et al. 2003; Starks et al. 2004; Stevens et al. 2004; Johnson et al. 2011; Jia et al. 2012; Liang et al. 2014). Cell wall biosynthesis genes

specifically *dal* (alanine racemase) and *dat* (D-amino acid aminotransferase) have been mutated in several attenuated *Lm* vaccine vectors (Friedman et al. 2000; Verch et al. 2004; Jiang et al. 2007; Im et al. 2013). The double mutant of *Lm* requires D-alanine for cell wall biosynthesis and is highly attenuated. Recently McLaughlin et al. demonstrated that deletion of *Lm* fur-regulated virulence factor A (*frvA*) results in attenuation in murine models of infection, due to the inability of iron homeostasis (McLaughlin et al. 2013).

Attenuated strains of *Shigella* are also being used as LABV. Noriega et al. (Noriega et al. 1996) generated *gua*BA mutant of *Shigella* CVD1204, which is highly attenuated in animals and is widely used as LABV. Other approaches of mutations include SC602, with deletions on *ics*A (mediate intra- and intercellular spread) and *iucA* (aerobactin); this strain is highly attenuated and immunogenic (Ranallo et al. 2005).

2.2.2 Plasmid Maintenance

Introduction of heterologous genes into bacterial vectors is facilitated by plasmids. Plasmids are extrachromosomal circular DNA, which are introduced into bacteria by a process called transformation. Generally, bacteria maintain the plasmids utilizing antibiotic resistance mechanism. During in vitro growth, antibiotic selection pressure ensures stable plasmid maintenance; however, in the in vivo conditions, the lack of antibiotic selection pressure plasmid-less bacteria outgrows plasmid-bearing bacteria. Moreover, the use of antibiotic markers are also discouraged, due to the risk of horizontal gene transfer to other microbes with pathogenic potential (Lin et al. 2015; Mignon et al. 2015). Novel antibiotic-free approaches of plasmid maintenance have been devised to mitigate these concerns. One such approach, known as the balanced lethal system, utilizes mutation in an essential gene in the bacterial chromosome, while the plasmid carries the functional copy of the same gene, thereby ensuring its maintenance by the bacteria (Fig. 2.1). Galen et al. generated a balanced lethal system for *STm* based on mutation in *asd* gene. *Asd* encodes aspartate semialdehyde dehydrogenase, an enzyme required in the biosynthesis pathway of DAP (diaminopimelic acid), which is an essential component of bacterial cell wall. DAP is needed for growth and maintenance of *asd* mutants. A copy of *asd* gene is inserted into the plasmid; thus *asd*-deficient bacteria are forced to maintain the plasmid in order to survive in DAP-deprived conditions, such as in the mammalian tissues. The resultant *Salmonella typhimurium* (*STm*)-based balanced lethal system exhibits high degree of plasmid stability. This system also exhibits stable expression of the associated heterologous genes (Galán et al. 1990). Balanced lethal system has been most widely used in various LABVs including *ST* (Tacket et al. 1997), *STm* (Kang et al. 2002), and *S. flexneri* (Zheng et al. 2005). Similarly, thymidine auxotrophy has also been utilized in *ST* (Bumann et al. 2010), *STm* (Mignon et al. 2015), and lactic acid bacteria (Bermúdez-Humarán et al. 2011) for balanced lethal system approach of plasmid maintenance. Glutamine auxotroph *V. cholerae* complemented with *glnA* gene is another example of the balanced lethal system utilized for antibiotic-free plasmid maintenance (Ryan et al. 2000).

2.3 Immune Mechanisms of Vaccines Delivered by Live-Attenuated Bacterial Vectors

Various mucosal pathogens and nonpathogenic food grade microbes have been extensively utilized for LABV development. *Salmonella* infect via orogastric route and enter the intestinal lamina propria by transcytosis via M cells, which are present in the mucosal inductive sites (Peyer's patches). In the lamina propria, *Salmonella* is taken up by various phagocytes including neutrophils, macrophages, and dendritic cells. The infected phagocytes then carry *Salmonella* to various organs including the liver and spleen via blood or to the mesenteric lymph nodes via lymph. The virulence factors, clustered in *Salmonella* pathogenicity islands (SPI-1 and SPI-2), facilitate invasion, survival, and proliferation in the intracellular spaces of macrophages (Pham and McSorley 2015). *Salmonella* possess a variety of pathogen-associated molecular patterns (PAMPs) including lipoprotein, lipopolysaccharide (LPS), flagellin (FliC), and CpG. These PAMPs are recognized by host pattern recognition receptors (PRRs) including TLR 2(1/6) (lipoproteins), TLR4 (LPS), TLR 5 (FliC), or TLR9 (CpG). Activation of these PRRs leads to the expression and secretion of cytokines such as TNFα, IL1β, IL6, IL8, IL12, IL-18, and IL-23 (Broz et al. 2012). These pro-inflammatory factors recruit neutrophils, macrophages, and dendritic cells. On the other hand, upon interaction with *Salmonella* LPS and flagellin, dendritic cells increase the expression of CCR7, CD80, CD86, and CD40. These mature dendritic cells with enhanced capability to process and present antigens can migrate to T-cell areas and initiate adaptive immune responses to cognate antigens. Studies suggest that *Salmonella* induces humoral as well as $CD4^+$-, $CD8^+$-, and Th17-dependent cell-mediated immune responses (Pham and McSorley 2015). Heterologous antigens carried by *Salmonella* elicit serum IgG (Frey et al. 2013), mucosal IgA (Allen et al. 2000; Ferreira Oliveira et al. 2012; Pei et al. 2015; Lalsiamthara and Lee 2017), $CD4^+$ (Ramirez et al. 2009; Ashraf et al. 2011), and $CD8^+$ T cells (Luria-Perez et al. 2007; Sevil Domènech et al. 2008; Berchtold et al. 2009). Due to the versatile immune response elicited by *Salmonella*, *ST*- and *STm*-based LABVs have been utilized to develop vaccines against numerous viral, bacterial (extracellular and intracellular), and parasitic pathogens. Importantly, it is well known that neutralizing antibodies and CTL responses confer adequate protection against viral pathogens; thus *ST*- and *STm*-based LABVs are capable of eliciting adequate antiviral immune responses. Antigens from viral pathogens including H1N1, H5N1, HIV, and SARS virus, when delivered by *ST* or *STm*, elicit antigen-specific antibody response (Karpenko et al. 2004; Luo et al. 2007; Pei et al. 2015; Hajam and Lee 2017). On the other hand, CTL response is generated against dengue virus (NS3) and HIV antigens (Karpenko et al. 2004; Luria-Perez et al. 2007). LABVs based on *ST* and *STm* elicit serum IgG, mucosal IgA, $CD4^+$ T-cell, and $CD8^+$ T-cell responses against a variety of bacterial pathogens including *B. anthracis* (Galen et al. 2010), *B. pertussis* (Dalla Pozza et al. 1998), *E. coli* (Ferreira Oliveira et al. 2012), *Helicobacter pylori* (Angelakopoulos and Hohmann 2000), *L. monocytogenes* (Igwe et al. 2002), *Pseudomonas aeruginosa* (Bumann et al. 2010), *Streptococcus pneumoniae* (Shi et al. 2010), and *Yersinia pestis* (Branger et al. 2010). While antigen-specific Th1

responses are generated against some parasitic pathogens including *Leishmania mexicana* (González et al. 1998), *Schistosoma japonicum* (Chen et al. 2011), and *Taenia solium* (Ding et al. 2013), mucosal IgG and IgA are generated against *Giardia lamblia* (Abdul-Wahid and Faubert 2007) and *Cryptosporidium parvum* (Benitez et al. 2009) by *ST*- and *STm*-based LABVs carrying related antigens.

Besides *Salmonella*, *Listeria* is the most extensively studied bacteria as LABV. Similar to *Salmonella*, *Listeria* infection begins with orogastric infection. *Lm* moves across gastrointestinal epithelial barrier by first attaching to and invading epithelial cells. Adhesion and internalization require *Lm* protein Ami and internalin A (inlA), respectively. Once in the lamina propria *Lm* rapidly spreads systemically. *Lm* primarily targets liver with the help of fibronectin binding protein (FbpA). FbpA recognizes fibronectin on the surface of hepatocytes, and at this point another molecule, called internalin (inlB), facilitates *Lm* internalization. On the other hand, phagocytes specifically macrophages and monocytes recognize lipoteichoic acid via scavenger receptors. After the phagocytosis *Lm* escapes phagocytic vesicles by synergistic activities of listeriolysin O (LLO) and two phospholipase C (PlcA and PlcB). Another virulence factor is ActA, which has actin polymerization activity and helps *Lm* migrate from cell to cell (Liang et al. 2014). *Lm* expresses various TLR agonists including peptidoglycan, flagellin, and bacterial DNA, which induces pro-inflammatory cytokines including TNFα, IFNγ, IL1β, and IL12. *Lm*-induced cell death results in secretion of IL6, which helps in recruitment of neutrophils. IL12 helps in induction of IFNγ by NK cells and $CD8^+$ T cells. *Lm* can also induce type-I IFNs (IFNαand IFNβ), which is desirable as antiviral immunity. Evidences suggest that *Lm* induces both $CD4^+$ and $CD8^+$ T-cell-mediated immune response (Zenewicz and Shen 2007; Liang et al. 2014). *Lm*-carrying viral antigens such as HIV/SIV-gag (Frankel et al. 1995; Friedman et al. 2000; Im et al. 2013), HPV-E7 (Jia et al. 2012), and LCMV-NP118-126 (Tvinnereim et al. 2002) induces CTL immune response. *Lm*-based LABV also induces neutralizing antibody against HIV-gp160 (Lakhashe et al. 2011). However, there are limited reports on *Lm* as LABV against bacterial and parasitic pathogens. In one study, *Lm*-carrying *Coxiella burnetii* antigen T4SS (epitopes) induced $CD8^+$ T-cell immune response (Xiong et al. 2017). In another study *Lm*-carrying *Francisella tularensis* antigen IglC induced IFNγ producing $CD4^+$ and $CD8^+$ T-cell-mediated immune response.

BCG, an attenuated *M. bovis*, has also exhibited potential as LABV. Following immunization, BCG interacts with phagocytes such as macrophage, dendritic cells, and neutrophils. Various PRRs of macrophages involved in interaction with BCG include CR3, TLR2 (1/6) and TLR-4. However, dendritic cells utilize a different set of phagocytic receptors including CR3, CR4, DC-SIGN (CD209), and DEC 205. Infected dendritic cells upregulate expression of MHC-II and costimulatory markers CD80, CD86, CD40, and CD54 which are involved in activation of adaptive immune response (Moliva et al. 2017). BCG is known to induce humoral as well as T-cell-mediated immune response (Abomoelak et al. 1999). The T-cell responses induced by BCG include polyfunctional $CD4^+$ T cells that secret TNF, IL-2, and IFNγ (Moliva et al. 2017). BCG expressing IL12 and two *M. tuberculosis* (*Mtb*) antigens (secreting antigen Ag85B and culture filtrate antigen CFP10) induce antigen-specific Th1-type immune response including IFNγ-producing cells and

IgG2a (Chen et al. 2017). Antigen-specific humoral immune response is induced by BCG expressing a hepatitis-B surface antigen (Rezende et al. 2005). BCG-induced CD8$^+$ T cells also secrete IFNγ (Moliva et al. 2017). In a mouse model, BCG carrying *Mtb* antigen (Ag85B) exhibit antigen-specific Th17 immune response (Hatano et al. 2016). Pertussis toxin subunit S1 expressed by BCG induces IFNγ producing CD4$^+$ T cells which completely protects against lethal *Bordetella pertussis* challenge (Nascimento et al. 2008). Furthermore, in a mouse model of an intracellular pathogen *Lm,* BCG expressing *Lm* antigen p60 induced CD4$^+$ and CD8$^+$ T-cell-dependent protection (Grode et al. 2002). Studies have demonstrated that BCG induces long-lived mycobacteria-specific memory B cells. Moreover, following BCG immunization, hosts secrete robust *Mtb*-specific serum antibodies including IgG isotypes IgG1, IgG2, and IgG3. However, it is not known if specific mucosal IgA is induced by BCG (Moliva et al. 2017).

S. flexneri infection occurs through orogastric route. Once in the colon, *S. flexneri* crosses epithelial layer through highly endocytic M cells. *S. flexneri* then adhere to and infect colonic epithelium through the basolateral surface. Colonic epithelial cells engulf *S. flexneri* by macropinocytosis, and through the activity of IpaB and IpaC, they are released from macropinocytic vacuoles to the cytosol (Mellouk and Enninga 2016). *S. flexneri* invasion causes activation of innate immune system and release of a variety of cytokines including IL-1, TNF-α, IL6, TGF-β, and IL-8 (Fernandez and Sansonetti 2003; Jennison and Verma 2004). Though *Shigella* has the capacity of cell-to-cell translocation, its infection is limited to lamina propria of the intestine, and it doesn't migrate to other organs (Maurelli and Sansonetti 1988). In the lamina propria *S. flexneri* is phagocytosed by macrophages and dendritic cells. Infected macrophages undergo apoptosis, which leads to the release of pro-inflammatory cytokine IL1, IL18, and IFNγ (Fernandez and Sansonetti 2003). *S flexneri* induces both systemic and mucosal antibody response including IgM, IgG, and secretory IgA (Jennison and Verma 2004). *S. flexneri 2a* with *gua*BA mutation (CVD 1204) has limited invasiveness, and proliferative capacity. Attenuated *S. flexneri* expressing ETEC antigens CFA-I, LTB, CS2, CS3, and CS4 induce antigen-specific serum IgG and mucosal IgA (Koprowski et al. 2000; Barry et al. 2003; Strain et al. 2003; Ranallo et al. 2005; Zheng et al. 2005).

Food grade bacteria including *B. subtilis* and *Lactobacillus lactis* are considered important candidates for LABV, due to their superior safety profile. Upon oral administration, *B. subtilis* spores can safely transit through the stomach, germinate, and proliferate in the upper intestine and finally undergo re-sporulation in the colon (Cutting et al. 2009). Nevertheless, the mechanism of immune response in response to *B. subtilis* delivered antigens is not fully understood. Antigens delivered by *B. subtilis* have been shown to induce humoral as well as Th1-mediated immune response (Cutting et al. 2009). *B. subtilis* has been used as LABV for various bacterial and parasitic pathogens including pathogenic *E. coli*, *H. pylori*, *Mtb*, *Clonorchis sinensis*, and *S. japonicum. B. subtilis* induces systemic IgG (Amuguni and Tzipori 2012; Zhou et al. 2015), mucosal IgA (Amuguni and Tzipori 2012; Zhou et al. 2015), and Th1/Th17 (Sibley et al. 2014; Stasilojc et al. 2015) immune response against cognate antigens. The lactic acid bacteria are among the microbes, which occur physiologically in animal digestive tracts and like other natural microflora

through their metabolites and interaction with macrophages can stimulate cytokine production. Peptidoglycan of the lactic acid bacteria induces secretion of IL1, IL6, and TNF, by monocytes (Bermúdez-Humarán et al. 2011; Szatraj et al. 2017). Unlike attenuated strains of otherwise pathogenic microbes used as LABV, *B. subtilis* and *L. lactis* do not invade through the gut mucosa and serve mainly as protein (antigen) factories, which supply vaccine antigens to gut-associated lymphoid tissue (GALT). Protective antigens of pathogenic viruses H1N1 (HA) and H5N1 (HA) expressed by *L. lactis* induce mucosal antibodies. *L. lactis* expressing bacterial antigens *Campylobacter jejuni* (cjAD) (Kobierecka et al. 2016), *Clostridium difficile* (TcdA) (Yang et al. 2013), *Clostridium perfringens* (epsilon toxoid) (Alimolaei et al. 2016), *H. pylori* (omp22, HpaA, cag12, and UreaseB) (Kim et al. 2006; Gu et al. 2009; Zhang et al. 2016b), and *V. cholerae* (WZM) (Zamri et al. 2012) also induce mucosal antibodies.

2.4 Delivery of DNA Vaccines by Live-Attenuated Bacterial Vectors

In preclinical models, DNA vaccines have proven to confer protective immunity against a variety of infectious agents including HIV, herpes simplex virus (HSV), *Plasmodium* spp., and *Mtb* (Schoen et al. 2004). An attractive feature of DNA vaccine is that it can induce humoral as well as cell-mediated immune response. While antibodies alone can protect against many pathogens and toxins, cell-mediated immunity is required for protection against intracellular pathogens and cancer. The DNA vaccines in the form of eukaryotic expression plasmids are delivered either by intramuscular injection of naked DNA, intradermal bombardment using DNA coated on gold particles with help of a gene gun, or electroporation following needle injection. However, most of these methods induce only moderate levels of protection in animal models and fail to show efficacy in clinical trials (Schoen et al. 2004). In recent years many bacterial vectors have been utilized to deliver plasmids into the host cells (Schoen et al. 2004). As many attenuated strains are being developed for delivery of vaccine antigens, similar strains can also be utilized to deliver plasmids as DNA vaccines. Attenuated strains of gut pathogens including *ST*, *STm*, or *L. monocytogenes* are of particular importance, as they colonize and infect mucosal epithelial cells.

As discussed above, *Listeria* infection begins at gastrointestinal tract, and after invasion through intestinal mucosa, *Listeria* migrate through blood vessels and lymph to other organs. *Listeria* can infect a wide array of cell types including intestinal epithelial cells, hepatocytes, dendritic cells, and macrophages. *Listeria* escape phagocytic vesicles and multiply in cytosol where they release the plasmids. Listeriolysin O helps *Listeria* lyse and escapes the phagosomal vacuoles (Liang et al. 2014). Miki et al. engineered a self-destructing *Lm*-based vaccine delivering a eukaryotic expression plasmid encoding *Mtb* antigens Ag85a/Ag85b and MPB/MPT51. The vaccine induced protective immune response against *Mtb* in a mouse model (Miki et al. 2004).

Salmonella also infects via gastrointestinal tract, and after crossing epithelial barrier through M cells, *Salmonella* is taken up by macrophages (Pham and McSorley 2015). *Salmonella* has the capability of surviving and replicating in phagocytic vacuoles (Pham and McSorley 2015). However, through unknown mechanisms, they can release plasmid DNA into the cytosol (Schoen et al. 2004). *Salmonella* strains expressing listeriolysin O have been shown to escape the phagosome vesicles to the cytosol, thus making gene transfer by *Salmonella* more efficient (Schoen et al. 2004). HIV-1 T-cell epitopes in the form of eukaryotic expression plasmid delivered by attenuated *STm* induced CTL as well as antibody immune response (Karpenko et al. 2004). Another study targeting an *S. pneumoniae* protective antigen PsaA and PspA delivered by *STm* induced mucosal IgA against both antigens. Thus immunized mice were protected against nasopharyngeal colonization by *S. pneumoniae* (Zhang et al. 2011). Pathogenic parasites *Trichinella spiralis* and *Trypanosoma cruzi* have also been targeted for *STm*-mediated DNA vaccination. Yang et al. constructed a DNA vaccine against *T. spiralis* using antigen Ts87 and *STm* as the delivery vehicle. Mice immunized orally with this vaccine induced antigen-specific mucosal IgA which correlated with protection against *T. spiralis* larval challenge. *Salmonella*-delivered *T. spiralis* DNA vaccine induced a Th1-/Th2-type immunity and IL5, IL6, and IL10 cytokines (Yang et al. 2010). In another study, Matos et al. using *STm* delivered *T. cruzi* antigens (Tc-52) into mice via the oral route. Immunized mice elicited specific antibodies with higher IgG2a/IgG1 ratio, suggesting a Th1 bias. The vaccinated group also induced strong cell-mediated immunity and mucosal IgA (Matos et al. 2014).

Most bacteria used as DNA delivery vehicles were designed to disintegrate after infecting host cells. If the bacterial DNA vaccine vectors are destroyed in the phagolysosomes, before reaching the cell cytoplasm, it will lead to inefficient delivery of the plasmid. To circumvent this problem various approaches have been devised. One such approach takes advantage of phage lysin to disintegrate ΔaroA-*Lm* after reaching host cell cytosol. The inclusion of phage lysin significantly improved bactofection (bacteria-mediated delivery of plasmid DNA into mammalian cells) efficiency in phagocytic as well as non-phagocytic cells (Pilgrim et al. 2003). Recently, Kong et al. developed a universal DNA vaccine delivery platform, which includes several modalities for enhanced delivery and immune response to cognate antigens. The attenuated *STm* includes the capability to escape the phagosomal compartment to the cytosol of the host cells, before phagolysosomal degradation (Kong et al. 2012). SifA proteins direct *Salmonella*-induced filament formation when *Salmonella* is contained in the endosomal vacuoles, and the deletion of *sif*A gene results in the release of *Salmonella* into the cytosol. Hence, mutation of *sif*A gene in *Salmonella* plasmid carriers allowed successful transfer of plasmid DNA into the cytosol of the host cells (Kong et al. 2012). Kong et al. also incorporated elements that guide the plasmid into the nucleus. Transcription factors such as NF-κB and AP2 bind to plasmids carrying NF-κB and AP2 binding sequences and transport them to the nucleus where the desired antigens are transcribed (Kong et al. 2012). *Salmonella* induces apoptosis/pyroptosis in infected cells that diminishes the overall transfection efficiency. Deletion of *tlpA* and *sseL* genes significantly reduces apoptosis in

host cells (Kong et al. 2012). Moreover, *Salmonella* degradation is delayed due to the regulated expression of the *Salmonella* lysis program. This allows a limited number of replication and invasiveness, thereby ensuring optimal delivery of plasmids. An influenza antigen (HA) delivered by this platform induced enhanced HA-specific IgG, which correlated with protection against influenza virus challenge (Kong et al. 2012).

2.5 Immunotherapy Against Cancer Using Live-Attenuated Bacterial Vectors

A nineteenth-century physician, William B. Coley, for the first time observed regression of malignant tumor in one of his patients after a bacterial infection. Coley went on to develop the first bacterial therapy against cancer using killed gram-positive bacteria streptococci and a gram-negative bacteria *Serratia marcescens*. This mixture called "Coley's toxins" when injected into patients suffering from various forms of cancer resulted in partial to complete regression. In cases of soft tissue sarcoma, long-term disease-free survival was achieved in approximately 50% of the patients. Nevertheless, despite the remarkable success of "Coley's toxins," with the advent of chemotherapy and radiotherapy, this line of investigation was prematurely abandoned (Bickels et al. 2002). However, in recent years this approach is regaining attention. In fact, BCG is currently being used as immunotherapy for bladder cancer and exhibits superiority over epirubicin and IFNα2b, mitomycin, and epirubicin alone (Fuge et al. 2015). Since the first report of BCG's use in cancer treatment in 1936, preclinical and clinical investigations of BCG have also been reported for other forms of cancer. Mice preimmunized with BCG exhibited slower tumor growth compared to control (Zheng et al. 2015). Morton et al. reported complete regression of tumor lesions in melanoma patients, upon intralesional injection of BCG in 684 out of 754 lesions. Similarly, survival benefits against cancer were also reported in other clinical trials. See Zheng et al. for a detailed review on application of BCG in cancer therapy (Zheng et al. 2015).

Bacteria, specifically anaerobes, exhibit natural tropism toward solid tumors. This phenomenon, although poorly understood, is theorized that certain characteristics of tumor microenvironment facilitate this phenomenon. The deeper pockets of tumors, which are devoid of new blood vessels, are poorly oxygenated and show limited accessibility to chemotherapeutic drugs (Lee 2012; Lin et al. 2015). Forbes et al. demonstrated that *STm* accumulate at a rate of 2000-fold more compared to other organs including the liver, spleen, lung, heart, and skin (Forbes et al. 2003). Using an in vitro model, Kasinkas and Forbes demonstrated that *STm* exhibits chemotaxis. Depending on the availability of specific receptors (tsr, tar, and trg), *STm* were differentially attracted to corresponding chemoattractants expressed in the tumor microenvironment (serine, aspartate, and ribose/glucose), while the wild-type strains accumulate around necrotic zones inside tumors (Kasinskas and Forbes 2007). Moreover, various immunosuppressive mechanisms manifested by the tumor microenvironment also support the proliferation of microbes (Lin et al. 2015).

Distinct tumor-homing property of microbes, including *Lm* and *Salmonella*, has been harnessed to deliver various tumor therapeutic modalities, including therapeutic vaccine antigens, DNA vaccines, and anticancer drugs. Various tumor-associated antigens (TAAs) have been targeted for therapeutic vaccines using LABV as delivery vehicles. PSA (prostate-specific antigen) is secreted by prostate epithelial cells and is overexpressed in malignant prostate cells. Attenuated *Lm* expressing PSA (*Lm*-LLO-PSA) antigen was tested as therapeutic vaccine in mouse tumor models expressing human PSA. Immunization with *Lm*-LLO-PSA completely regressed tumors in five out of eight mice and induced PSA-specific cellular immune response. Immunization of *Lm*-LLO-PSA significantly increased infiltration of PSA-specific $CD8^+$ T cells in tumors and decrease in CD4/CD25/$FoxP3^+$ T_{reg} cells (Wallecha et al. 2009). HER2/neu is overexpressed in about 25–30% of breast cancers and is a potential target for immunotherapy. Shahabi et al. engineered an *Lm*-based vaccine incorporating HER2/neu as antigen (ADXS31-1642). ADXS31-164 elicited HER2-specific $CD8^+$ T cells. The vaccine caused a significant delay in the formation of mammary tumors, and 50% of mice were tumor-free till 45 weeks of the experiment, whereas all sham-treated mice developed tumors and succumbed to the disease. This vaccine also resulted in significant increase in tumor-infiltrating $CD8^+$ T cells and a decrease in the intratumoral $FoxP3^+T_{reg}$cells (Shahabi et al. 2011). *P. aeruginosa* can also deliver heterologous antigens using its type III secretion system. In an experimental model of B-cell melanoma expressing ovalbumin (OVA), Chauchet et al. demonstrated antitumor efficacy of *P. aeruginosa*-based vaccine expressing OVA. *P. aeruginosa* induced a long-lasting and polyfunctional $CD8^+$ T-cell immune response against the cognate antigen, wherein antigen-specific $CD8^+$ T cells expressed IFNγ, TNFα, and IL2 simultaneously. These $CD8^+$ T cells also showed enhanced tumor infiltration property and a greater ratio between effector versus regulatory T cells (Chauchet et al. 2016). Recently Mei et al. utilized a composite approach of DNA vaccine and bacterial surface expression to achieve $CD8^+$ and $CD4^+$ T-cell-mediated immunity targeted to a tumor-associated antigen. The *Salmonella*-based vaccine included AIDA-I autotransporter-Melan A (a murine melanoma antigen) fusion protein and a DNA vaccine element encoding two murine melanoma epitopes (Mei et al. 2017).

2.6 Novel Technologies for Tailored and Enhanced Immune Response

2.6.1 Control of Gene Expression: Use of Plasmid Copy Number and In Vivo Promoters

High levels of antigen synthesis by multicopy plasmids exert metabolic burden to LABV, which results in hyperattenuation, low colonization, loss of viability, and most importantly poor immunogenicity. Various strategies have been adapted to circumvent this problem including the use of low-copy plasmid, use of in vivo

inducible promoters (IVIP), and use of arabinose-inducible promoters (Loessner et al. 2007). Among the first promoters introduced in LABV is $P_{nir}B$, which is activated under anaerobic conditions. $P_{pag}C$ and $P_{ssa}G$ are macrophage-inducible promoters from *Salmonella*. Dunstan directly compared the immunogenicity of antigens upon expression of antigens regulated by $P_{nir}B$ and $P_{pag}C$ and found significantly higher antibody response with $P_{pag}C$ compared to $P_{nir}B$ (Dunstan et al. 1999). Arnold et al. achieved differential antigen expression in vivo using in vivo inducible promoters $P_{pag}C$, comprising variable ribosomal binding site (RBS). By this approach, strains with a high level of expression of heterologous protein exhibited low level of colonization, while a moderate amount of expression resulted in a significantly improved infection rate in mesenteric lymph nodes. A very low level of in vivo inducible antigen expression resulted in unhampered infectivity compared to the parent strain. Immunogenicity was dependent on the rate of infection, as well as the level of antigen expression. Notably, the best immune response was achieved with moderate level of antigen expression and infectivity, while high antigen-expressing strain resulted in little to no immune response. On the other hand, a moderate level of immune response was generated with high infectivity and low antigen expression (Arnold et al. 2004). Wang et al. developed a regulated delayed antigen synthesis system, consisting of *LacI* repressor to repress transcription from P_{trc} during in vitro cultivation. The arabinose-regulated promoter P_{BAD} drives LacI expression in vitro in medium supplemented with arabinose. Upon immunization and lack of external arabinose supplementation, P_{trc} is derepressed, leading to the synthesis of antigens. The regulated delayed antigen synthesis system induced equivalent levels of antibody and protection to that of $P_{pag}C$-controlled antigen synthesis and better than that of $P_{ssa}G$-controlled antigen synthesis (Wang et al. 2011).

2.6.2 Acid Resistance

Upon oral immunization, LABV must withstand acidic environment of the stomach for successful colonization. Enteric pathogens including *E. coli*, *L. monocytogenes*, *Shigella* spp., and *L. lactis* can tolerate extreme acidic pH (below pH 2.5) because they possess the most potent acid resistance (AR) system known as GDAR (glutamate-dependent acid resistance) pathway. Attenuated strains of *ST* and *STm* have limited acid tolerance and exhibit moderate immunogenicity (Dharmasena et al. 2016a). By engineering AR components from *Shigella* spp., Dharmasena et al. significantly (10^5-fold) enhanced acid tolerance of attenuated *ST*-Ty21a (Dharmasena et al. 2016a).

2.6.3 Detoxification of Lipopolysaccharide

LPS plays important role in survival and infectivity of bacteria. However, it is also involved in toxicity to the host. Various attempts at the use of LPS O- antigen mutants of *STm* resulted in poor attachment and intestinal invasion and survival

following oral immunization. By regulated expression of LPS O- antigen components such that they are expressed in vitro and at the time of immunization, but soon after colonization their synthesis is stopped, it is expected to achieve maximal infectivity and minimal toxicity (Wang et al. 2013). Kong et al. engineered a *Salmonella* strain where LPS O- antigen synthesis genes *rfc* and *rfaH* are kept under the control of the promoter *araC*-P_{BAD}, which is tightly regulated by arabinose. This strain is highly attenuated nevertheless exhibits superior immunogenicity (Kong et al. 2009, 2010). Another approach of detoxification of *Salmonella* LPS included removal of 1-phosphate group from lipid A of LPS. Kong et al. introduced an inner membrane phosphatase LpxE from *F. tularensis*, which can selectively remove the 1-phosphate group from *Salmonella* lipid A. The resultant LPS had reduced toxicity while preserved adjuvant activity(Kong et al. 2011). See Wang et al. for a detailed review on LPS modifications in *Salmonella*-based LABV (Wang et al. 2013).

2.6.4 Optimization of Virulence: Control of Safety and Immunogenicity

Many methods employed for attenuation, although make the LABV strains less pathogenic and safe to administer at high doses, it often renders them poorly immunogenic due to their inability to circumvent physicochemical defense of the host. Moreover, inability of penetration through mucosal barrier also makes them poorly immunogenic. To circumvent this problem, Curtiss et al. generated a regulated delayed attenuation system (RDAS), which retains full virulence till the passage through gastrointestinal tract and infection of epithelial cells. In the modified RDAS strains, *Salmonella* virulence genes *fur*, PhoP/Q, *rpoS*, and *crp* are expressed under the control of *araC*-P_{BAD} promoter. Arabinose concentration in human tissues is very less. Thus, in vitro these strains express all the virulence genes in medium supplemented with arabinose, whereas in vivo under the arabinose deprivation, many virulence genes are suppressed, resulting in attenuation of *Salmonella*. This approach results in high immunogenicity combined with tolerance at high doses (Curtiss 3rd et al. 2009).

2.6.5 Cytosolic Delivery of Antigen

In order to evoke $CD8^+$ T-cell (CTL) response, antigens need to be delivered into the cytoplasm of host cell. Various approaches are in use to accomplish the cytosolic delivery of antigens including the use of a type III secretion system that can directly deliver vaccine antigens into the host cell cytoplasm and use of a-hemolysin (HlyA) secretion system of *E. coli* which is fully active in *Salmonella* (Gentschev et al. 1996). On the other hand, escape from endocytic vacuoles is also a feasible approach. Unlike *Lm*, *ST* and *STm* do not reach cytoplasm of infected cells and elicit $CD4^+$ T-cell response more effectively compared to $CD8^+$ T-cell response to cognate

antigens. Chen et al. used secretion signal of a type III secretion system *Salmonella* outer protein E (SopE) and HlyA (secretion signal) to deliver *S. japonicum* antigen Sj23-LHD-GST. The *Salmonella* vaccine constructs carrying Sj23 LHD-GST fused to HlyA (secretion signal) or SopE effectively expressed and delivered antigens into cytoplasm of murine macrophages in vitro. This vaccine construct induced Sj23-LHD-GST-specific Th1 type response and protected against *S. japonicum* infection (Chen et al. 2011). Gentschev et al. reported that two Listerial antigens delivered by *STm* using HlyA (secretion signal) generated protection against *Listeria* infection (Gentschev et al. 1996). Simultaneous delivery of two Listerial antigens (LLO and p60) by *STm* using *Yersinia* outer protein E (YopE) as a carrier molecule for *Salmonella* type III secretion system developed LLO- and p60-specific T cells and protection against murine listeriosis (Igwe et al. 2002). SopE-mediated delivery of Listerial antigen p60 generated $CD8^+$ T-cell-mediated protection against *Listeria* infection (Berchtold et al. 2009).

2.7 Conclusion

What makes bacteria an excellent vaccine delivery vehicle is their natural ability to induce potent and long-lasting immune response. LABVs possess the capacity to induce humoral as well as cell-mediated immune response. While the humoral immune response includes serum IgG and mucosal IgG and IgA, the cell-mediated immunity is characterized by Th1-, Th2-, and Th17-type CD4+ T cells and CD8+ CTLs. IgA and IL17 have been specifically implicated in mucosal protection against various mucosal pathogens. The cell-mediated immunity is required for intracellular pathogens. It should be noted that subunit vaccines have a poor capacity to evoke mucosal as well as cell-mediated immunity. LABVs have also shown the capacity to overcome immunosuppressive nature of various forms of tumors. These characteristic of LABVs, together with their tumor-tropic capacity, makes them a highly suitable vector for cancer immunotherapeutic vaccines. In the past two decades, tremendous progress has been made regarding LABV-mediated delivery of vaccine antigens for prevention of a variety of viral, bacterial, and parasitic diseases. Recent advances have further improved the safety and immunogenicity profile of several LABV platforms. The new-generation LABVs can withstand harsh physicochemical conditions of gastrointestinal tract, exhibit regulated attenuation, regulated antigen expression, and targeted antigen delivery. LABVs have exhibited effectiveness in various preclinical and preliminary clinical trials (Table 2.1). However, a limited number of clinical trials have been conducted to date using LABVs, due to potential safety concerns. Further optimization would result in a versatile, safe, and highly immunogenic vaccine delivery platforms.

Table 2.1 Examples of vaccines delivered by live-attenuated bacterial vectors

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
Viral pathogens					
BCG		Hepatitis B	Surface antigens	Antibodies	Rezende et al. (2005)
BCG		HIV	SIV-Gag and CD8+ T-cell epitopes	CD 8+ T cell	Venkataswamy et al. (2014) and Mahant et al. (2017)
BCG		HIV and SIV	gp120, Gag	T cells	Hart et al. (2015)
L. lactis		HPV-16-E7	LL-E7	Th1 immune response	Almeida et al. (2016)
L. lactis		H1N1	HA	IgA, Antibodies	Joan et al. (2016)
L. lactis		H5N1	HA	IgA	Bobek et al. (2010)
Lm	Δ*actA*/ Δ*plc*Band Δ*actA*/ Δ*inl*B	H1N1	NP	Th1	Johnson et al. (2011)
Lm	Δ*dal*, Δ*dat*	HIV	Gag, gp160	CD8+, CTL, nAb	Frankel et al. (1995), Friedman et al. (2000), Rayevskaya and Frankel (2001), Rayevskaya et al. (2002), Jiang et al. (2007) and Lakhashe et al. (2011)
Lm	Δ*act*A, Δ*plc*B	HPV17	E7	CTL	Jia et al. (2012)
Lm	Δ*act*A	LCMV	NP118-126	CD8+ T cells	Tvinnereim et al. (2002)
Lm	Δ*dal*, Δ*dat*	SIV	Gag	CD8+, Cellular immune response	Sciaranghella et al. (2011) and Im et al. (2013)
STm	Δ*aro*A	Dengue virus	NS3-MisL	CTL	Luria-Perez et al. (2007)
STm	Δ*cpx*R, Δ*lon*, Δ*asd* and Δ*wba*P	H1N1	HA and M2e	IgG1 and IgG2a and Th1 cell response	Hajam and Lee (2017)
STm	Δ*aro*A	H5N1	HA, NA, NP	IgG and mucosal IgA and gamma-producing T cells	Ashraf et al. (2011) and Pei et al. (2015)

(continued)

Table 2.1 (continued)

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
STm	Δ*aro*C	HIV-1	10- E8, Gag	Antibody, CTL	Karpenko et al. (2004), Chin'ombe and Ruhanya (2013), Li et al. (2016)
STm	Δ*aro*A	Measles virus	B-cell and T-cell epitopes	IgG	Spreng et al. (2000)
ST	Δ*pil*S	SARS	Nucleocapsid protein	IgG2a and IgA	Luo et al. (2007)
STm	Δ*aro*A	TGEV	N gene, C and A epitopes	IgG	Chen and Schifferli (2003, 2007) and Zhang et al. (2016a)
Bacterial pathogens					
BCG		*B. pertussis*	Pertussis toxin Subunit S1	Th1	Nascimento et al. (2008, 2009)
BCG		*Lm*	p60 Ag	CD4 and CD8 T cell	Grode et al. (2002)
BCG		*Mtb*	Ag 85B	IL 17A T cells	Hatano et al. (2016)
BCG		*B. pertussis*, tetanus, *Mtb*	Pertussis-tetanus toxin fusion	Humoral and cellular	Abomoelak et al. (1999)
BCG		*S. pneumoniae*	PspA	IL-17A and IFNg	Goulart et al. (2017)
B. subtilis		ETEC	CfaB	Sera and mucosal Ab	Amuguni and Tzipori (2012)
B. subtilis		*H. pylori*	Urease B	IgG, IgA, Th1/Th17	Stasilojc et al. (2015) and Zhou et al. (2015)
B. subtilis		*Mtb*	MPT64	Th1	Sibley et al. (2014)
B. subtilis		Tetanus	TT C fragment	IgG, IgA	Amuguni et al. (2011)
E. coli	Δ*intimin*, Δ*stx*1 and Δ*stx*2	ETEC	CFA-I, LThK63	IgG and IgA	Byrd and Boedeker (2013)
E. coli	Δ*ler*	EHEC	Stx1B	Antibody	Zhu et al. (2006)
L. casei		*C. perfringens*	Epsilon	IgG, IgA	Alimolaei et al. (2016)
L. lactis		*C. jejuni*	cjAD	Antibody	Kobierecka et al. (2016)
L. lactis		*C. difficile*	TETC-TcdA	Antibodies	Yang et al. (2013)
L. lactis		*H. pylori*	Omp22 or HpaA, cag12, urease B	Antibody	Gu et al. (2009), Kim et al. (2009), Li et al. (2014), and Zhang et al. (2016b)

(continued)

Table 2.1 (continued)

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
L. lactis		*L. monocytogenes*	Listeriolysin O	CD8+ T cells	Bahey-El-Din et al. (2008)
L. lactis		*S. pyogenes*	M protein (CRR)	Mucosal IgA	Mannam et al. (2004) and Mannam et al. (2004)
L. lactis		*V. cholera*	Wzm	IgG and IgA	Zamri et al. (2012)
Lm	Δ*act*A, Δ*inl*B	*C. burnetii*	T4SS (Epitopes)	CD8+ T cells	Xiong et al. (2017)
ST	Δ*aro*C, Δ*aro*D, and Δ*htr*A	*B. anthracis*	PA83, PAd4	IgG, Ifng	Galen et al. (2004, 2010)
STm	Δ*aro*A	*B. pertussis*	Pertussis toxins S1, S2, S3, S4, and S5	IgG	Dalla Pozza et al. (1998)
STm	Δ*lon*, Δ*cpx*R	*B. abortus*	BCSP31, Omp3b, and SOD	IgG and sIgA	Kim et al. (2016) and Lalsiamthara and Lee (2017)
STm	Δ*aro*C, Δ*aro*D and Δ*htr*A	EHEC O157:H7	Intimin, CFA-I, CS3, STx2	Mucosal IgG and IgA	Girón et al. (1995), Rojas et al. (2010) and Ferreira Oliveira et al. (2012)
STm	ΔPhoP/Q	*H. pylori*	Urease B, hpaA, adhesin AB, babA2/ureI, CagA, and VacA	Antibodies	Angelakopoulos and Hohmann (2000), Bai et al. (2004), Xu et al. (2005) and Liu et al. (2011)
STm	Δ*aro*A/ Δ*spt*P	*L. monocytogenes*	Listeriolysin and p60	CD8+ T cell, IFNg	Igwe et al. (2002), Sevil Domènech et al. (2008) and Berchtold et al. (2009)
STm	ΔaroA	*Mtb*	ESAT6-Ag85B	T cell, IFNg	Wang et al. (2009)
STm	Δ*aro*C, Δ*aro*D, and Δ*htr*A	*P. aeruginosa*	OprF/OprI, LPS	Antibodies and TH1, mucosal IgG, and IgA	Arnold et al. (2004), Digiandomenico et al. (2004) and Bumann et al. (2010)
STm	Δ*cya* and Δ*crp*	*P. gingivalis*	HagB	IgG and IgA and mucosal IgA	Isoda et al. (2007) and Pathangey et al. (2009)
STm	Δ*gal*E	*S. dysenteriae*	LPS	Serum antibody	Dharmasena et al. (2016b)
STm	Δ*gal*E	*S. sonnei*	LPS	Serum antibody	Dharmasena et al. (2013)

(continued)

Table 2.1 (continued)

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
STm	Δ*crp* and Δ*asd*A	*S. pneumoniae*	PspA, PspC	Th1/Th2, IgG, and IgA	Kang et al. (2002), Xin et al. (2009), Shi et al. (2010), Wang et al. (2010, 2011), Kong et al. (2011) and Frey et al. (2013)
ST	Δ*aro*C, Δ*aro*D, and Δ*htr*A	Tetanus	TetC	IgG, IgA	Dunstan et al. (1999), Allen et al. (2000), Orr et al. (2001) and Capozzo et al. (2004)
STm	Δ*aro*A	*Y. enterocolitica*	HSP-60	T cell, IFNg	Kramer et al. (2003)
STm	ΔPhoP/Q	*Y. pestis*	F1, V, YadC, YadBC, PsaA, LcrV, Psn, and HmuR	IgG, CD4, and CD8+ T cells	Ramirez et al. (2009), Branger et al. (2010), Torres-Escobar et al. (2010), Sizemore et al. (2012), Sun et al. (2014) and Galen et al. (2015)
S. flexneri	Δ*gua*BA	ETEC	CFA-I and LTB, CS2, CS3, CS4, and CFA/A	Serum IgG and mucosal IgA	Koprowski et al. (2000), Barry et al. (2003), Ranallo et al. (2005) and Zheng et al. (2005)
V. cholerae	Δ*ctx*	*C. difficile*	TcdA	IgG	Ryan et al. (1997)
V. cholerae	Δ*att*RS1	*E. coli*	LT	Serum IgG and mucosal IgA	Ryan et al. (1999)
V. cholerae	ΔCTA	*H. pylori*	HpaA	IgG	Tobias et al. (2017)
V. cholerae	Naturally attenuated	Tetanus toxin, *B. pertussis*	TetC, BP-TCF	IgG	Chen et al. (1998)
Parasitic pathogens					
B. subtilis		*C. sinensis*	Enolase	Mucosal IgG and IgA	Yu et al. (2015)
B. subtilis		*S. japonicum*	GST protein	Mucosal IgG and IgA	Li et al. (2009)
E. coli	Δ*ler*	Malaria	NANP	Antibody	Zhu et al. (2006)
Lm	Δ*act*A	*L. major*	LJM11, p36, and LACK	Th1	Soussi et al. (2002), Saklani-Jusforgues et al. (2003) and Abi Abdallah et al. (2014)

(continued)

Table 2.1 (continued)

Vaccine vector	Attenuation	Target pathogen	Target antigen	Immune response	References
STm	Δ*pmi*, Δ*fur* and Δ*crp*	*G. lamblia*	α1-Giardin, CWP2	Th1/Th2, mucosal IgG, and IgA	Abdul-Wahid and Faubert (2007) and Jenikova et al. (2011)
STm	Δ*aro*C, Δ*aro*D, and Δ*htr*A	*C. parvum*	Cp23, CP15, and Cp40	Antibody	Benitez et al. (2009) and Roche et al. (2013)
STm	Δ*aro*A	*E. histolytica*	Gal/Gal-NAC lectin	Antibody	Mann et al. (1997)
ST	Δ*aro*C and Δ*aro*D	*L. mexicana*	gp63	Th1 type immunity	González et al. (1998)
STm	Δ*purl* and Δ*msb*B	*S. japonicum*	Sj23LHD-GST	Th1 type immunity	Chen et al. (2011)
STm	Δ*crp* and Δ*cya*	*T. solium*	TSOL18	CD4 and CD8 T cells	Ding et al. (2013)

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Chapter 3
Poly-lactide/Poly-lactide-co-glycolide-Based Delivery System for Bioactive Compounds Against Microbes

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Abstract Infectious diseases caused by pathogenic microorganisms are one of the leading causes of mortality worldwide. For many of these diseases, prophylactic and therapeutic treatments are available in the form of vaccines and drugs. Novel discoveries in the pathophysiology and immunology of these diseases have led to the identification of contributing factors to the progress of these diseases. Our immune system puts forth a strong defense against these infections, but the microbes develop strategies to evade the immune system and survive inside the host. There is an ongoing hunt to look for potent therapeutic agents against these harmful bugs. These therapeutic drugs also need to be delivered effectively for long-lasting protection. This has led to the enhanced emphasis on the type of a suitable delivery system that can carry these agents inside the human body in its bioactive form. Hence, to achieve newer ways to deal with infection, we need better delivery systems as powerful tools for infection control and treatment.

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Biodegradable and biocompatible polymeric particles such as poly(lactic acid) and poly(lactic-co-glycolic acid) have emerged as one of the efficient delivery systems for many life-saving drugs. These polymers offer several advantages such as targeted delivery, sustained release, and maintenance of bioactivity; it also leads to dose sparing by reducing the exposure of bioactive molecules in the circulation. The polymeric particles are being extensively studied in several applications as delivery systems due to their ability to exhibit a broad range of desirable properties. The present review focuses on the polymer-based particulate delivery system as a plausible solution to circumvent the shortcomings of conventional therapeutic and prophylactic systems, and it discusses some of the methods for their preparation as well as mechanisms of action against infection. Here we also review the cellular interaction of nanoparticles because this interaction influences the effectiveness of the particles. The present review aims at different preparation methods of poly-lactide/poly-lactide-co-glycolide-based particles, their properties as carriers of bioactive molecules, and applications of polymeric particle-based bioactive delivery systems against microbes with an emphasis on recent findings. This review sheds light on the latest applications of particle-based delivery systems attempting to provide an updated study about the field.

3.1 Introduction

Poly(lactic acid) and poly(lactic-co-glycolic acid) are Food and Drug Administration-approved biodegradable and biocompatible polymers used for various applications in human healthcare (Peres et al. 2017). The development of poly-lactide/poly-lactide-co-glycolide formulations as potential antigen/drug carriers emerges from the fact that these polymeric particles can be tuned in multiple ways to deliver the cargo selectively to specific sites and at controlled rates (Fig. 3.1). The mechanical properties of these polymers can be modified to develop efficient delivery system for different biomedical applications (Anderson and Shive 2012; Danhier et al. 2012), and enormous amounts of literature support that the poly-lactide/poly-lactide-co-glycolide polymers have extensive applications for entrapping bioactive molecules such as antigens, drugs, antibiotics, etc. (Mitragotri et al. 2014). Polymers of different molecular weights can be used to fabricate nano- or microparticles, and these particles show good stability upon storage and when administered in vivo. Since the release profiles of particles can be easily modulated, long-circulating polymeric particles are synthesized which are capable of releasing the drug at the site of action and possess desirable release kinetics. Particle properties can also be tailored accordingly by controlling the degradation rates or size, porosity, etc. (James et al. 2016).

Particle properties play a very important role in governing their interaction with the cells. Bulk and surface properties of these particles can also be changed to achieve desirable characteristics. Bulk properties are primarily changed by using

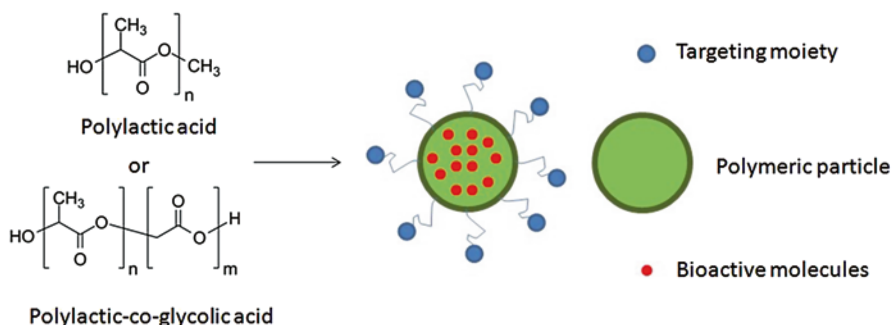

Fig. 3.1 Chemical structure of polylactic acid and polylactic-co-glycolic acid along with an indication of polymeric particle formulation to deliver a different kind of bioactive molecules. The chemical structure of these particles allows the flexibility of tailoring the functional groups on the surface, which subsequently brings about changes in the particle property. This can facilitate modulation of the particle properties for specific end application of these particles

poly-lactide/poly-lactide-co-glycolide with other polymers. Surface properties can be modulated by changing the surface coating of polymer particles. This approach can be used to attain targeted delivery of these particles at specific sites. Entrapment efficiency, drug/antigen load, and release profiles of these particles can be easily modified as per the requirements. Modifications such as pegylation of these polymeric particles can add to the stability of the entrapped bioactive molecule. Properties such as size and shape of particles can be altered to attain optimum pharmacokinetic and biodegradable profiles. The large surface to volume ratio of these particles provides more reaction sites to interact with the surrounding environment (Coelho et al. 2010). Owing to their safety profiles, biocompatibility, biodegradability, and the ease with which the particle properties can be changed for a particular application, these polymers are gaining attention for a plethora of applications (Murariu and Dubois 2016).

The huge burden of infectious agents on human population and the high demand of therapeutic molecules against these diseases need an effective solution. Biocompatible polymeric particle entrapping bioactive molecules such as proteins, peptides, and antibiotics are capable of releasing these molecules over a desired period of time, thereby decreasing the frequency of their administration (Pagels and Prud'homme 2015). Protection of bioactive molecules from degradation and enhancing the bioavailability of these molecules for a long time are additional advantages for considering it as a delivery system. Having these properties, poly-lactide/poly-lactide-co-glycolide particles also provide a great platform to be explored as adjuvants for development of new vaccines that can enhance the immune response of specific antigens against various diseases without compromising their safety (Gutjahr et al. 2016). This review reports different techniques to make polymer-based particle formulation, the cellular interaction of these formulations, and recent advancement in their application for delivering bioactive molecules against microbes.

3.2 Preparation of Poly-lactide/Poly-lactide-co-glycolide Particles

Different methods are employed to synthesize nanoparticles and microparticles from polymers such as poly(lactic acid) and poly(lactic-co-glycolic acid). Double emulsion solvent evaporation is a commonly used method to encapsulate bioactive molecules into particles. The aqueous phase containing the water-soluble compound is added to the polymer-containing organic phase to create primary w/o emulsion. This emulsion is then added to the aqueous phase containing stabilizers to create a w/o/w double emulsion. Solvent evaporation then yields particles. Antibiotics like ciprofloxacin and other novel antimicrobial peptides have been entrapped into poly-lactide-co-glycolide nanoparticles using this method to check biofilm development (Cruz et al. 2017; Thomas et al. 2016). It was reported that entrapping the bioactive molecules into polymeric particles allowed local and sustained concentrations of these molecules, thus enhancing their activity at the site. Other bioactive molecules such as lysozyme have been successfully entrapped into poly-lactide-co-glycolide nanoparticles using this method (Dinarvand et al. 2011). Double emulsion solvent evaporation method allows tailoring the particle properties in endless ways, exploiting the different variables influencing the process.

Nanoprecipitation (also known as solvent displacement method) is a simple one-step process for the preparation of poly-lactide-based nanoparticles and requires two miscible solvents. An important prerequisite is that both the polymer and the bioactive molecule must be soluble in one (same) solvent but should not be soluble in the second one. When the polymer-drug containing solvent diffuses into the second solvent which is present in an excess amount, sudden desolvation of the polymer allows entrapment of bioactive molecule inside the polymeric particles. If the process parameters are kept constant, consistent batches of particles can be produced using nanoprecipitation (Sahin et al. 2017). Using this method, anticancer drugs have been successfully entrapped using nanoprecipitation with high drug load (El-Hammadi et al. 2017; Pandey et al. 2016). Tamoxifen was entrapped into poly-lactide-co-glycolide nanoparticles using nanoprecipitation, and an entrapment efficiency as high as 71% was observed.

Other methods like spray drying, microfluidic technique, and template−/mold-based method are now being used for more uniform particles and large-scale production. Spray drying is a single-step process of transforming a liquid feed into a dry form by passing the liquid through a medium (inert gas such as nitrogen) (Wan and Yang 2016). It is a useful, scalable particle formulation method, where an organic phase containing drug dissolved or dispersed in a polymer is sprayed as ultrafine droplets in dry air flow. Microspheres entrapping antimicrobial drugs such as cefquinome sulfate can be synthesized using spray drying and has been shown to be targeted to the lungs where they showed a sustained release of the drug in vivo (Qu et al. 2017). The drug against tuberculosis such as rifapentine has been successfully entrapped into poly-lactide-co-glycolide nanoparticles and used for inhaled therapy (Parumasivam et al. 2016). Microfluidics-based devices are used to produce

uniform particles with precisely controlled release profiles. Polymers are dissolved in organic solvents and single/double emulsions are made in microfluidic devices, by droplet solidification through solvent evaporation (Ekanem et al. 2017b). Microfluidic devices can entrap multiple components in a one-step emulsification process and can also generate core-shell structures. Bipolymer microparticles have also been synthesized using microfluidic emulsification and solvent evaporation using poly(lactic acid) and polycaprolactone (Ekanem et al. 2017a). Surface properties of poly-lactide-co-glycolide microparticles can be changed in a desired way using the flow-focusing microfluidic method. These surface properties have been shown to influence the entrapment efficiency and release kinetics from these particles (Hussain et al. 2017). Microparticles using poly(lactic-co-glycolic acid) and amphiphilic poly(styrene-alt-maleic anhydride) were made by the capillary-focused microfluidic technique that entrapped anticancer drug doxorubicin. The particles were found to be pH sensitive releasing the drug in a sustained manner. Using template-based methods, gels can be made that can change their sol-gel phase upon receiving their specific cue such as temperature. The warm gel solution is put in the hard cast. Once the mold solidifies, it is extracted from the mold and the solvent is evaporated (Basu et al. 2016; M Saffer et al. 2011). The advantages associated are reproducibility in the batches, monodispersed particle formation with high drug load. Another variant of the template-based method is molecular imprinting which imparts a "memory" to the polymer. This is an emerging technology and has to be exploited to yield maximal use of the approach (Gagliardi et al. 2017).

Several parameters can influence these processes of particle making such as the amount of compound to be entrapped, initial polymer concentration, type of solvent, surfactant concentration, the ratio of aqueous phase to organic phase, stirring rate, and other variables. The parameters that affect the particle formation also include the injection rate of the organic phase into the anti-solvent, the compound/polymer ratio, solvent evaporation rate, and the solubility of the compound. The choices of a combination of the drug/polymer and solvent/non-solvent system are yet other important parameters.

3.3 Different Facets of an Interaction of the Polymeric Particles with the Cells

The cells can internalize polymeric particles of subcellular size range and the interaction between particles and cells are influenced by particle properties. The cells can take up these particles by endocytosis, which is a broad term that encompasses several energy-dependent mechanisms such as phagocytosis, pinocytosis, macropinocytosis, and receptor-mediated endocytosis (Zhao et al. 2011) (Fig. 3.2). Exocytosis extrudes a major proportion of the internalized particles out. A very small fraction of the remaining internalized fraction escapes the endosomal compartment and provides the therapeutic benefits owing to particle retention in the

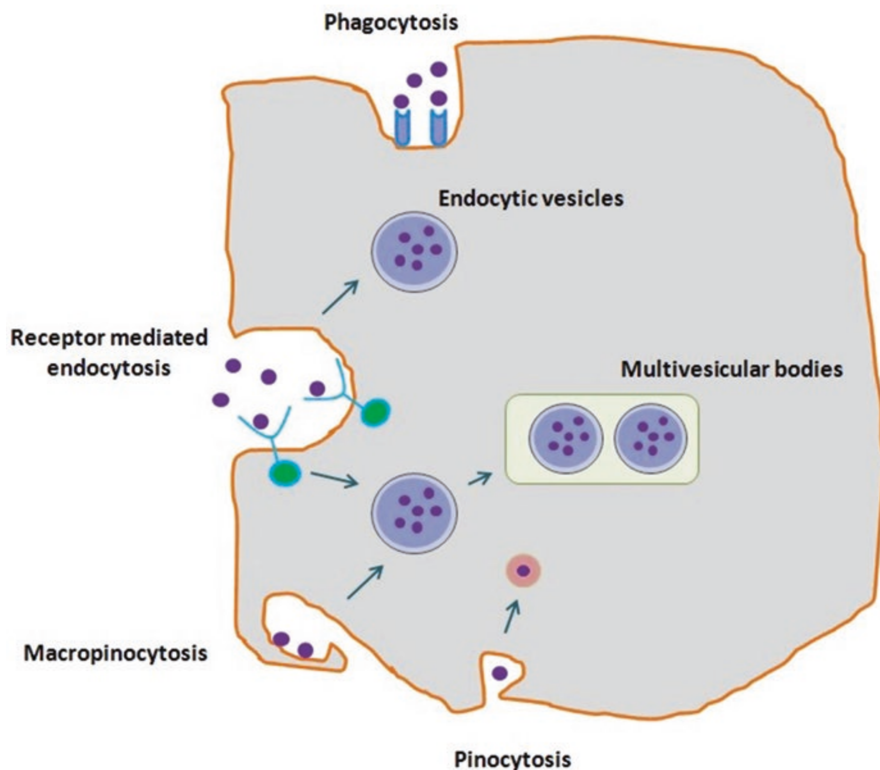

Fig. 3.2 Different mode of cellular uptake of polymeric nano- and microparticles. The cells, in multiple ways, can take up polymeric nanoparticles such as phagocytosis, pinocytosis, micopinocytosis, and receptor-mediated endocytosis. Particle properties play a major role in governing the mode of uptake of these particles into the cells. Physical properties such as size and shape and chemical modifications on the surface affecting the surface charge or hydrophobicity of these particles have a huge impact in mediating the interaction of these particles with the cells

cytosol (Panyam and Labhasetwar 2003; Panyam et al. 2002). Hence, for the maximal efficacy of a drug delivery particulate system, the mode of uptake of these particles becomes an important concern (Hillaireau and Couvreur 2009).

To enhance the internalization of poly-lactide/poly-lactide-co-glycolide nanoparticles inside the cells, different factors are taken into account such as size, shape, charge, and ligands (Saraiva et al. 2016) (Fig. 3.3). Particle properties such as size and shape influence their cellular uptake (Roointan et al. 2018; Zhao et al. 2011). The particle size of around 500 nm is found to be optimal for efficient phagocytosis of these particles (Hillaireau and Couvreur 2009). It is also reported that charged particles either positive or negative have better internalization into the cells than the neutrally charged polymeric particles (Fröhlich 2012; Hillaireau and Couvreur 2009). It is also observed that the mode of entry of poly-lactide particles inside the cells may vary depending on their surface being cationic or anionic (Fröhlich 2012).

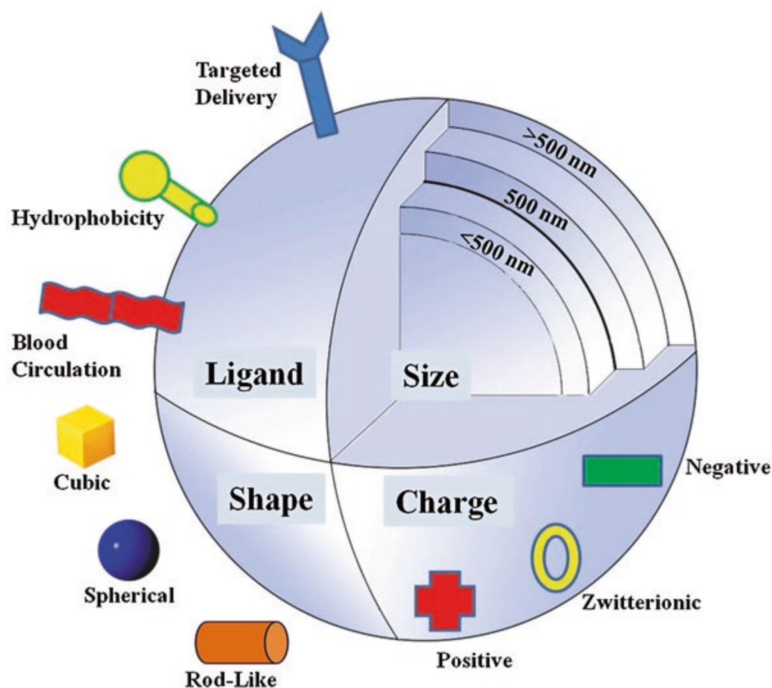

Fig. 3.3 Factors influencing the polymeric particle-mediated bioactive delivery: Different-sized particle can be formulated for efficient delivery of bioactive molecules. Shape (spherical, cubic, and rodlike) and charge (negative, zwitterionic, and positive) can be varied to enhance entrapment and cellular interaction; poly(lactic-*co*-glycolic acid) and poly(lactic acid) particles are negatively charged. Surface functionalization of a particle is another important parameter that can influence the bioactive delivery; ligands such as poly(ethylene glycol) improve particle presence in circulation, amphiphilic peptide increases hydrophobicity, and receptors/antibodies help in targeted delivery. (Adapted from Saraiva et al. 2016)

Poly-lactide-co-glycolide nanoparticles also find their use in treating inner ear drug delivery. Modulating the sizes of these particles and their surface modification using polyethylene glycol/chitosan have shown to enhance drug delivery to the inner ear (Cai et al. 2017). Drugs against methicillin-resistant *Staphylococcus aureus* infections such as vancomycin generally possess low bioavailability. To enhance the availability, the drug is entrapped into pegylated poly-lactide-co-glycolide particles, which have better results when compared to the soluble drug only (Pei et al. 2017).

Surface modification of the particles has also a great impact in affecting biodistribution, cellular uptake, and endosomal escape of these particles (Vasir and Labhasetwar 2007). Surface functionalization of particles can be used to target dual cellular populations (Roointan et al. 2018). The surface charge of the pegylated poly-lactide nanoparticles is seen to also affect the oral bioavailability of particles after their administration (Du et al. 2018). Pegylation of the nanoparticles is a commonly used approach to confer hydrophobicity on the particles and hence enhance

the interaction with the cells (Aldrian et al. 2017; Suk et al. 2016). Surface decoration of the ligands to specific markers or receptors on the surface of the cells can improve the particle uptake by the cells. One of the approaches to enhance the internalization involves the surface modification of these particles by cell-penetrating peptides which are hydrophobic and cationic (Steinbach et al. 2016). Peptides being smaller in size, having lower immunogenicity, and with ease of synthesis serve as good targeting molecules for improving the bioavailability of poorly adsorbed drugs (Gourdon et al. 2017). Sun et al. 2017 reported designing of abalone peptide functionalized poly-lactide nanoparticles entrapping doxorubicin and showed that both tumor cells and vascular endothelial cells took up these particles more efficiently. This may be a very promising approach for the development of anticancer drug delivery systems. Koutsiouki et al. 2017 reported the use of cysteine-modified TAT peptide (HCys-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-NH2) when conjugated on the surface of poly(lactide)-poly(ethylene glycol) nanocapsules as anticancer drug delivery system for paclitaxel. This surface modification led to an increased cellular uptake and subsequent better anticancer response by these particles into the cells. Polymeric nanoparticles are being currently exploited as regimen against neurodegenerative diseases like Alzheimer's. Bhatt et al. 2017 encapsulated enzyme nattokinase into poly-lactide-co-glycolide nanoparticles and conjugated these particles with Tet-1 peptide of 12 amino acids (HLNILSTLWKYR) to increase its affinity toward the neurons. Huang et al. 2017 showed that poly-lactide-co-glycolide particles, when conjugated to a brain-targeting peptide such as a cyclic CRTIGPSVC peptide (CRT), penetrated blood-brain barrier easily in Alzheimer's disease mice models.

Stabilizers used during the particle synthesis may be used to target ligand coupling on the particle surface, which ultimately influences the internalization of these particles into the cells. Raudszus et al. 2018 reported the use of a stabilizer vinyl sulfone-modified poly(vinyl alcohol) derivative to prepare poly-lactide nanoparticles which showed different uptake behavior in cerebral endothelial cells owing to different functional groups present on the surface of these particles. Thus specific stabilizers may be used for specific targeting applications.

In case of many diseases, direct antigen delivery is not practically useful because of the limitations such as low immunogenicity, low stability, as well as the lesser half-life of these antigens in vivo. Polymeric particles offer solutions to these limitations and are emerging as promising delivery systems in immunotherapy (Tran et al. 2018). Using polymeric particles not only allow slow and sustained release of antigens after their administration but also allow targeted delivery of these particles, incorporation of multiple components in a single assembly, enhancing uptake, as well as modulating the immune response (Silva et al. 2016). It is observed that antigen entrapped into poly-lactide-co-glycolide nanoparticles, when immunized into the animals, could elicit the higher antigen-specific immune response than the particles that had antigens adsorbed upon the particles (Liu et al. 2016b). Surface charge is an important parameter that governs the interaction of these particles with the cells. Poly-lactide-co-glycolide nanoparticles that contained antigen and were coated with polyethyleneimine were readily taken up by the antigen-presenting

cells primarily through phagocytosis and macropinocytosis. These particles could facilitate cross-presentation of the antigen through endosomal escape. This approach could be adopted for designing immunotherapeutic vaccines (Song et al. 2016). Similarly, another study reported that hyaluronic acid-modified cationic lipid dioleoyltrimethylammoniumpropane poly-lactide-co-glycolide nanoparticles were able to process exogenous antigens via both major histocompatibility complex (MHC-I and MHC-II) antigen presentation pathways (Liu et al. 2016a). Poly-lactide-co-glycolide nanoparticles can encapsulate multiple leishmanial antigenic peptides that can undergo cross-presentation by MHC-I molecules via endosomal escape (Athanasiou et al. 2017). Several strategies are developed against the pathogens which invade through the mucosal lining of the host requiring surface modification such as the coating of poly-lactide-co-glycolide particles with appropriate agents for targeted delivery to M-cells (Allahyari and Mohit 2016). Meng et al. (2018) developed Huperzine A-entrapped poly-lactide-co-glycolide nanoparticles and coated it with lactoferrin (Lf)-conjugated N-trimethylated chitosan to make it a targeted nasal drug delivery system against Alzheimer's disease. The modified drug delivery carrier showed lower toxicity and higher uptake behavior when compared to unmodified particles.

Poly-lactide-co-glycolide nanoparticles are also being used to deliver antifungal compounds against mycosis. Amphotericin B that is commonly used to treat paracoccidioidomycosis has serious side effects. Entrapment of single-chain variable fragments into nanoparticles was shown to confer protection against experimental paracoccidioidomycosis (Jannuzzi et al. 2017). Opportunistic pathogens such as *Pseudomonas aeruginosa* are responsible for commonly occurring secondary infections by forming biofilms. Poly-lactide-co-glycolide-entrapped amikacin have shown effective clearance of the biofilms (Sabaeifard et al. 2017). Pegylated and mannosylated poly-lactide/poly-lactide-co-glycolide nanoparticles were synthesized using nanoprecipitation, entrapping an anti-infective surfactant protein, SP-A. The study reported a potential drug delivery system to enhance the availability of the drug in the lungs (Ruge et al. 2016). Poly-lactide-co-glycolide particles that could withstand acid hydrolysis were synthesized encapsulating CCF, which was a construct comprising a dual-antigen epitope and dual-adjuvant vaccine, to confer protection against *Helicobacter pylori* infections (Tan et al. 2017). Using polymeric particles offer the ease of tailoring the particle properties to meet specific end-use application.

3.4 Poly-lactide/Poly-lactide-co-glycolide Particulate Delivery System of Bioactive Therapeutics

Among the polymeric delivery system, biodegradable and biocompatible poly-lactide/poly-lactide-co-glycolide-based particulate system has been extensively investigated for therapeutic bioactive molecules. Biodegradable polymeric particulate system can be used as a delivery system and has an ability to entrap and present

different bioactive molecules of interest on their surface. However polymeric particles have many challenges to overcome such as low entrapment efficiency, low loading capacity, and product sterility for animal use. The recent development of a novel or improved method for bioactive molecule-loaded polymeric particle formulation will lead to the successful application of polymeric particle-based delivery system. The size of polymeric particles can modulate the immune response against antigen delivered through particles (Oyewumi et al. 2010; Shah et al. 2014). Depending on required immune response or effective drug treatment against the specific infectious agent, different-sized particles can be formulated to deliver therapeutic molecules (Fig. 3.3). Several new poly-lactide/poly-lactide-co-glycolide-based delivery systems have now been developed (Fig. 3.4) and showed great promise for various kinds of bioactive molecules alone and in combination to overcome the global problem of infectious diseases (Table 3.1).

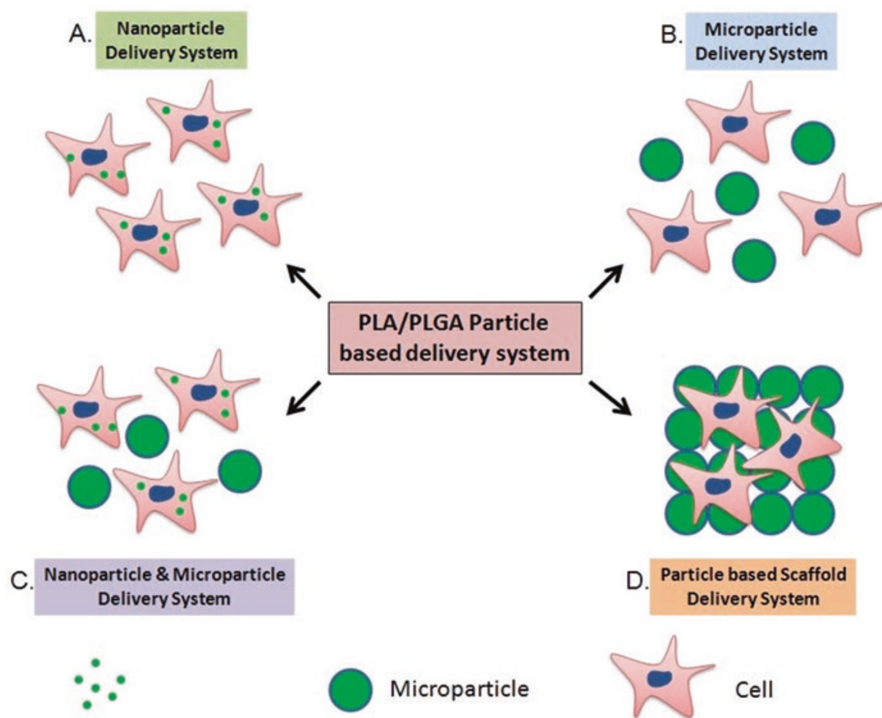

Fig. 3.4 Schematic illustration of various approaches for poly-lactide/poly-lactide-co-glycolide particle-based delivery system: (**a**) nanoparticle-based delivery system; (**b**) microparticle-based delivery system; (**c**) nanoparticle and microparticle-based delivery system; and (**d**) particle-based scaffold delivery system. Nanoparticle and microparticle-based methods are used for years but have seen recent advancements in their applications owing to our greater understanding of these methods. Also, newer methods have come up that take care of the limitations associated with the older methods, allowing a larger range of use of these particles. PLA/PLGA, poly-lactide/poly-lactide-co-glycolide

Table 3.1 Poly-lactide/poly-lactide-co-glycolide-based delivery system against various infectious agents

Bioactive delivery system	Key components	Bioactive molecules	Related microorganism/ disease	References
Nanoparticle	PLA and PLGA	GIBIM-P5S9K peptide	*Pseudomonas aeruginosa*, *Escherichia coli O157:H7*, and methicillin-resistant *Staphylococcus aureus*	Cruz et al. (2017)
	PLGA	Ciprofloxacin	*Pseudomonas aeruginosa*	Türeli et al. (2017)
	PLGA	Vancomycin	Methicillin-resistant *Staphylococcus aureus*	Pei et al. (2017)
	PEG-PLGA			
	Eudragit E100			
	ZWC			
	HP55/PLGA	CCF	*Helicobacter pylori*	Tan et al. (2017)
	PLGA/PEI	H1N1 DNA vaccine	H1N1 virus	Seok et al. (2017)
	PLGA-PLL/γPGA	Ebola DNA vaccine	Ebola virus	Yang et al. (2017)
	PLGA	CNA19	*Staphylococcus aureus*	Genta et al. (2016)
	PLGA	HA, NP, PA, and M2e-PP peptide	H1N1 virus	Hiremath et al. (2016)
	PLGA	KAg	Swine influenza virus	Dhakal et al. (2017)
	PLGA	LCP-1	Group A streptococcus	Marasini et al. (2016)
	PLGA	rROP18	*Toxoplasma gondii*	Nabi et al. (2017)
	PLGA	Dengue virus E-protein	Dengue virus	Metz et al. (2016)
	PLGA	TLR7 agonist and DTaP vaccine	Diphtheria, tetanus, and pertussis	Bruno et al. (2016)
	PLGA	sLiAg, MPLA, TNFα mimicking peptide	Leishmaniasis	Margaroni et al. (2016)
	PLGA	CPA160–189, MPLA	*Leishmania infantum*	Agallou et al. (2017)
	PLGA	Envelope gp140, Gag p55 protein, and TLR4 and TLR7/8 agonists	Simian immunodeficiency virus	Kasturi et al. (2017)
	PLA	FNIII9/10 protein and p24	Human immunodeficiency virus	Dalzon et al. (2016)

(continued)

Table 3.1 (continued)

Bioactive delivery system	Key components	Bioactive molecules	Related microorganism/ disease	References
Microparticle	PLGA	Outer membrane proteins (porins)	*Salmonella Typhi*	Carreño et al. (2016)
	PLGA	HCV-E2	Hepatitis C virus	Roopngam et al. (2016)
	PLGA	rSAG1 and rGRA2	*Toxoplasma gondii*	Allahyari et al. (2016)
	PLGA	rCDPK6 and rROP18	*Toxoplasma gondii*	Zhang et al. (2016)
	PLGA	Inactivated polio vaccine	Polio virus	Tzeng et al. (2016)
	PLGA	rOMVs	Influenza A virus	Watkins et al. (2017)
	PLGA	NH36 and 3M-052	*Leishmania donovani*	Wang et al. (2017)
Nanoparticle/ microparticle	PLGA	Pertussis toxoid	Pertussis	Li et al. (2016)
Scaffold	PLA and PLGA	Gentamicin, neomycin	*Staphylococcus aureus*	Admane et al. (2017)

Table abbreviations: PLA, poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); PEG, polyethylene glycol; ZWC, zwitterionic chitosan; HP55, hydroxypropyl methyl cellulose phthalate; CCF, a construct comprising a dual-antigen epitope and dual-adjuvant vaccine; PEI, polyethylenimine; H1N1, an influenza A virus; DNA, deoxyribonucleic acid; PLL/γPGA, poly-l-lysine/poly-γ-glutamic acid; CNA19, a purified recombinant collagen-binding bacterial adhesion fragment; HA, NP, PA, and M2e-PP peptide, conserved peptides of influenza A virus; KAg, inactivated swine influenza virus H1N1 antigens; LCP-1, lipid core peptide; rROP18, recombinant rhoptry protein 18; DTaP, diphtheria-tetanus-pertussis; sLiAg, soluble leishmania antigens; TNFα, tumor necrosis factor alpha; CPA160–189, cysteine protease A; MPLA, monophosphoryl lipid A; FNIII9/10, human fibronectin recombinant proteins; p24, an HIV antigen; HCV-E2, E2 envelope glycoprotein of hepatitis C virus; rSAG1 and rGRA2, recombinant surface antigen 1 and recombinant dense granular protein; rCDPK6, recombinant calcium-dependent protein kinase 6; rOMVs, recombinant outer membrane vesicles; NH36, *Leishmania donovani* nucleoside hydrolase antigen; 3M-052, a toll-like receptor 7/8 agonist

3.4.1 Nanoparticle-Based Delivery System

In recent times, biocompatible polymeric nanocarriers have been widely explored as a delivery system for bioactive molecules such as an antigen, nucleic acid, and antibiotics. These nanocarriers help them to sustain for longer duration in the host system and avoid getting degraded rapidly. Considering this, poly-lactide and poly-lactide-co-glycolide nanoparticles entrapping GIBIM-P5S9K peptide were formulated using double emulsion method (Cruz et al. 2017). GIBIM-P5S9K peptide, a new antimicrobial molecule, inhibited the growth of *Pseudomonas aeruginosa*, *Escherichia coli O157:H7*, and methicillin-resistant *Staphylococcus aureus* at very low peptide concentration when it was delivered using nanoparticle. It enhanced the

antimicrobial activity by 20-fold compared to the soluble peptide. In vivo study also suggested that these particles are hemocompatible. In another study, Türeli et al. (2017) showed that cystic fibrosis lung infected with *Pseudomonas aeruginosa* can also be treated using antibiotic-loaded poly-lactide-co-glycolide nanoparticles. Ciprofloxacin-loaded nanoparticles were able to penetrate the barrier of biofilm/ mucus and showed enhanced antimicrobial activity compared to ciprofloxacin complex. These nanocarriers were nontoxic to human epithelial cell lines at a minimum inhibitory concentration of antibiotic required against bacteria. It could be a new strategy to treat *P. aeruginosa* infections in cystic fibrosis lung.

For making an effective delivery vehicle, Pei et al. (2017) formulated a nanoparticle delivery system using a blend of polymers with distinct features for vancomycin against methicillin-resistant *Staphylococcus aureus* infection. Vancomycin is ineffective against this intracellular pathogen due to poor cellular uptake. Considering this limitation, four polymers were used for nanoformulation: poly-lactide-co-glycolide as a main delivery system, polyethylene glycol-poly-lactide-co-glycolide conjugate for maintaining vancomycin polarity, Eudragit E100 for enhancing entrapment, and a chitosan derivative for pH-sensitive drug release. Result showed that this nanoparticle was effective against intracellular pathogens than free vancomycin and could be more promising for treatment. Tan et al. (2017) have shown the role of hypromellose phthalate HP55/poly-lactide-co-glycolide nanoparticle as an oral delivery system for *H. pylori* recombinant antigen CCF, a combination of a multi-epitope vaccine CTB-UE and a chimeric flagellum that protects antigen from the gastrointestinal environment. Also immunized mice with these nanoparticles induced urease-specific antibody and T cell-mediated response. Moreover immunized mice were protected after *H. pylori* challenge. These results indicated that use of these nanoparticles as antigen delivery system for gastrointestinal infection could be a promising strategy. In the recent study, Seok et al. (2017) designed an influenza A (H1N1) vaccine delivery system using polyplexes of poly-lactide-co-glycolide/polyethyleneimine nanoparticles coated on microneedle. This system was optimized for high transfection efficiency in mammalian cells. Intradermally immunized mice induced enhanced humoral immune response than an intramuscular delivery of polyplex containing H1N1 deoxyribonucleic acid using microneedles. However, immunogenicity against H1N1 protein was still weak; therefore such systems need to be improved for a desired immune response against influenza virus. A deoxyribonucleic acid vaccine coated on the surface of polylactic-co-glycolic acid-poly-L-lysine/poly-γ-glutamic acid nanoparticles was formulated for Ebola vaccination using microneedle patch (Yang et al. 2017). Nanoparticles were administered in mice through intramuscular or with microneedle patch to the skin. Although antibody titer value was not significantly different for both routes, neutralizing activity of antibody against Ebola virus was higher after microneedle immunization. That concluded the use of polylactic-co-glycolic acid-poly-L-lysine/poly-γ-glutamic acid nanoparticles may be needed for development of a robust and immunogenic microneedle patch for Ebola vaccine.

Food and Drug Administration-approved biodegradable and biocompatible poly-lactide and poly-lactide-co-glycolide polymers have gained huge attention for

vaccine development due to its ability to modulate immune response specific to the antigen. Genta et al. (2016) have shown the adjuvant property of poly-lactide-co-glycolide-based nanoparticle for a purified collagen-binding fragment (CNA19 peptide) from *Staphylococcus aureus*. In vivo study in mice showed that polymeric nanoparticle has more potential as an adjuvant with respect to alum for subcutaneous route of CNA19 immunization. Furthermore study proved that compared to intranasal route, subcutaneous immunization will be the preferential route for developing CNA19-loaded nanoparticle vaccine. In another study, Hiremath et al. (2016) have developed poly-lactide-co-glycolide nanoparticle delivery system for influenza virus-conserved peptides. Influenza virus-conserved peptides are potentially able to elicit the cross-protective immune response but are poorly immunogenic. Pigs immunized with nanoparticle-entrapped peptides induced T cell-specific response, not enhanced antibody response. Challenge with virulent influenza virus showed no fever and flu symptoms in nanoparticle-loaded peptides-immunized pig. In summary, polymeric nanocarrier could be effective to develop a vaccine with high T immune response. In a similar way, Dhakal et al. (2017) formulated a poly-lactide-co-glycolide nanoparticle-entrapped inactivated swine influenza virus H1N1 antigens formulation against influenza flu. Pigs immunized with antigen-loaded nanoparticle induced strong antigen-specific T cell response and had no flu symptoms after challenge with heterologous swine influenza virus. In summary, inactivated influenza virus-loaded nanoparticle can be a solution for making influenza virus vaccine. Recently, Marasini et al. (2016) also designed a poly-lactide-co-glycolide nanoparticle-loaded lipopeptide vaccine to prevent group A streptococcus infections. Study showed that lipopeptide-entrapped nanoparticle improved uptake of antigen in antigen-presenting cells and enhanced immunoglobulin (Ig) level; mucosal IgA, and systemic IgG response against lipopeptide. That helps in bacterial growth inhibition and clearance.

Nabi et al. (2017) have shown a noninvasive approach for developing poly-lactide-co-glycolide-based vaccine against *Toxoplasma gondii*. The nanocarriers were formulated to encapsulate recombinant rhoptry protein (rROP18) and administered in mice through intranasal immunization. Immunized mice elicited enhanced IgG2a and IgA response compared to control. However immunological study still needs to be explored to translate these findings into a potential vaccine. New method like particle replication in non-wetting template (PRINT) technology was also explored to make effective subunit vaccine against infectious agents. Development of dengue vaccine is tricky because the vaccine should protect against all four infectious dengue virus serotypes. Several vaccine formulations including live attenuated tetravalent virus vaccine showed only partial efficacy. Considering this limitation, Metz et al. (2016) used the PRINT technology to form dengue virus E-protein-adsorbed nanoparticles. Immunization study showed that nanovaccine induced higher IgG that was more effective compared to soluble antigen protein. These results indicate that poly-lactide-co-glycolide nanoparticles using PRINT technology seem a promising platform for subunit vaccine delivery.

Further enhancement in its adjuvant property needs incorporation of immune potentiator molecules like toll-like receptor (TLR) agonists. More advanced

polymeric delivery systems have been developed to incorporate both antigen and immune potentiator. This system can deliver both antigen and immunepotentiator together at the particular site. Bruno et al. (2016) have combined the adjuvant role of poly-lactide-co-glycolide nanoparticle with a synthetic immune potentiator molecule that targets TLR7. Double emulsion method was used to formulate nanoparticle-entrapped TLR7 agonist and then diphtheria-tetanus-pertussis (DTaP) vaccine was adsorbed onto the nanoparticles. To see the adjuvanticity of this nanoformulation, mice model was used and evaluated for the improvement in the efficacy of DTaP vaccine. Co-delivery of TLR7 agonist through nanoparticle and antigens resulted in high IgG and IgG2a antibody titers compared to non-entrapped form. That concluded that poly-lactide-co-glycolide can be used along with other immunomodulators to get the desired immune response against antigens. In another study, soluble leishmania antigens and monophosphoryl lipid A adjuvant-entrapped poly-lactide-co-glycolide nanoparticles were formulated with tumor necrosis factor alpha mimicking peptide on their surface (Margaroni et al. 2016). Efficient uptake of nanoparticles by dendritic cells helped in inducing its maturation and differentiation. That resulted in high level of the co-stimulatory molecules and stimulated the production of interleukins: IL-12 and IL-10 cytokines. Nanoparticles-primed dendritic cells promoted T cell activation and differentiation which was characterized by T cell-specific transcriptional factors and cytokine expression. These results indicated that this new nanoformulation could be considered as a potential vaccine candidate against leishmaniasis. Similarly, a peptide-based poly-lactide-co-glycolide nanovaccine was also reported to enhance the immunogenicity against leishmaniasis (Agallou et al. 2017). Cysteine protease A (CPA160–189), a synthetic peptide containing overlapping epitopes of a highly immunogenic leishmania protein, was entrapped along with monophosphoryl lipid A adjuvant in poly-lactide-co-glycolide nanoparticles. In vivo study in the visceral leishmaniasis-susceptible BALB/c mice model showed a significant reduction in parasite burden compared to control group, suggesting that well-designed peptide-loaded nanocarriers could be a promising vaccine candidate against leishmaniasis. Recently, Kasturi et al. (2017) investigated the adjuvant role of poly-lactide-co-glycolide nanoparticles containing TLR4 and TLR7/8 agonists for envelope gp140 and Gag p55 protein against simian immunodeficiency virus (SIV): SIVmac239. This formulation was immunized with virus-like particles containing gp140 and Gag p55 in rhesus macaques. Nanoparticles-adjuvanted formulation induced robust immune response and showed high protection against intravaginal challenges with heterologous strain of SIV in animals. Such formulation can also be designed for other viral infection.

Targeted delivery of antigens is one of the major concerns to make an effective vaccine. In order to develop an efficient vaccine delivery system for an HIV antigen (p24), Dalzon et al. (2016) developed a human fibronectin protein (FNIII9/10)-coated poly-lactide nanoparticle system to target an integrin α5β1-positive cells. Human fibronectin FNIII9/10 proteins have an integrin α5β1-binding site, the RGDS sequence (Arg-Gly-Asp-Ser) that enhanced cellular uptake of nanoparticles by α5β1-positive cells. Subcutaneously immunized mice with nanoparticles co-

coated with p24 and FNIII9/10 proteins showed no significant improvement in humoral response compared to nanoparticles coated with p24. However, the presence of FNIII9/10 proteins helped in increasing avidity index of p24 antibody.

3.4.2 Microparticle Delivery System

Poly-lactide/poly-lactide-co-glycolide microparticle can be very useful for entrapping high concentration of bioactive molecule. Moreover, it can be used for the long and sustained delivery of therapeutic molecules in blood circulation. These microparticles provide thermostability and maintain the nature of loaded bioactive molecules such as antigenicity of a vaccine and bioactivity of antibiotic. Currently, there are two vaccines available for *Salmonella Typhi* infection, but both are thermolabile. Carreño et al. (2016) formulated the *S. Typhi* outer membrane protein (porin)-entrapped poly-lactide-co-glycolide microparticle for making a thermostable oral vaccine. An orally administered vaccine was protected from an acidic environment and showed strong B cell immune response in mesenteric lymph nodes and Peyer's patches. In general, microparticle encapsulation improved the efficacy of *S. Typhi* oral vaccine.

Microparticles not only protect antigen degradation in circulation but also help in the modulation of an immune response against antigens. Recently, Roopngam et al. (2016) have reported E2 envelope glycoprotein of hepatitis C virus type (HCV-E2)-loaded poly-lactide-co-glycolide microparticle as a potential vaccine against hepatitis C virus. This virus causes hepatitis and liver carcinoma and there is a need to develop a vaccine. HCV-E2 helps the virus to bind with host cells, and antibody against it is very effective in neutralizing the virus. Results suggested that vaccinated animals had the high number of CD8+ T cells and interferon gamma (IFNγ)-secreting immune cells. Moreover, the antigen-specific antibody titer was also high for HCV-E2 microparticles. Thus antigen delivery using microparticle can be a good strategy for a vaccine development. Poly-lactide-co-glycolide microparticle was also used to design protein-based subunit vaccine against *T. gondii* using a recombinant surface antigen 1 (rSAG1) and recombinant dense granular protein (rGRA2) (Allahyari et al. 2016). rSAG1- and rGRA2-adsorbed microparticles were used to enhance the immune response in BALB/c mice. All immunized mice were able to induce strong humoral and cellular response compared to control. rSAG1 and rGRA2 microparticles-vaccinated mice were also able to survive for longer times and partially protected against acute toxoplasmosis compared to a single antigen. Similarly, Zhang et al. (2016) developed the recombinant calcium-dependent protein kinase 6 (rCDPK6)- and rhoptry protein 18 (rROP18)-entrapped poly-lactide-co-glycolide microparticle for generating long-lasting immune response against *T. gondii*. Mice immunized with this formulation showed T helper cell type 1(Th1) response with enhanced IFNγ and IL-2 cytokines and specific antibody response. Also vaccinated mice conferred high protective immunity after *T. gondii* challenge compared to control. These studies hold the great promise of

polymeric microparticle as a delivery system in the development of a vaccine against *T. gondii*.

Microparticle delivery system has also been investigated for a single-shot vaccine development. In order to resolve the requirement of multiple doses of inactivated polio vaccine (IPV) to obtain full protection, Tzeng et al. (2016) developed IPV-entrapped poly-lactide-co-glycolide microparticle. Rat immunized with IPV-entrapped microparticle showed robust and long-lasting antibody response compared to multidose soluble IPV. This technique can be used to eradicate infectious diseases such as polio using single-dose vaccine. For influenza A virus, Watkins et al. (2017) described a single-dose poly-lactide-co-glycolide microparticle entrapping recombinant outer membrane vesicles (rOMVs) which has the heterospecies tandem sequence of the M2 protein. Immunized BALB/c mice elicited high IgG titers and produced the high level of IFNγ for rOMV microparticle. After challenge with lethal dose of influenza virus, vaccinated mice were also protected against influenza. These data support the role of poly-lactide-co-glycolide microparticle for making a single-shot vaccine against influenza.

The role of additional adjuvant has also been evaluated along with polymeric particle to improve the efficacy of potential vaccines. In a recent study, Wang et al. (2017) formulated poly-lactide-co-glycolide microparticle co-entrapping a 36-kDa nucleoside hydrolase recombinant protein antigen (NH36) from *Leishmania donovani* along with the TLR7/8 agonist 3M-052 to generate Th1 immune response against leishmaniasis. NH36 and 3M-052 loaded particles elicited enhanced Th1 response associated with IgG2a and IgG2b antibodies and have more IFNγ-producing splenocytes compared to other groups in BALB/c mice. These results indicate that polymeric particles along with 3M-052 could be used as an adjuvant for leishmaniasis vaccines.

3.4.3 Nanoparticle/Microparticle Delivery System

Both nanoparticle- and microparticle-based vaccine delivery systems have been demonstrated for different immune modulation against antigen (Gregory et al. 2013; Johansen et al. 2000; Peyre et al. 2004). Nanoparticles are reported to promote cellular immune response, whereas microparticle enhanced humoral response (Chong et al. 2005; Gutierro et al. 2002). Recently, Li et al. (2016) investigated poly-lactide-co-glycolide-based nanoparticle/microparticle as a vaccine delivery system for pertussis antigen. In vitro study showed higher uptake of antigen in macrophage cells for polymeric nano−/microparticles and immunized mice significantly enhanced INF-γ and IL-17 cytokine level in splenocytes after priming with heat-killed *Bordetella pertussis*, indicating a Th1/Th17 response. Also, vaccinated mice were protected against subsequent pertussis infection. This finding suggests that poly-lactide-co-glycolide nanoparticle/microparticle may serve as an alternative to acellular pertussis vaccine for balanced Th1/Th2 immune response.

3.4.4 Biodegradable Polymeric Scaffold

Biodegradable and biocompatible polymers have now been explored for making particle-based fabrication of bioactive molecule-loaded self-assembled scaffold. It offers many advantages such as safety, desirable loading, and sustained release of loaded molecules in comparison to other implanted delivery systems. This polymeric scaffold-based system is now serving as a platform for many applications such as localized delivery of antibiotics, wound healing, and tissue engineering. Admane et al. (2017) have described a new method to design gentamicin and neomycin loaded scaffold using poly-lactide particles. Firstly antibiotics were loaded in poly-lactide during microparticle formulation, and then these particles were fused using methanol to form scaffold at room temperature. The bioactivity of antibiotic from scaffold was tested and confirmed against *Staphylococcus aureus*. The scaffold was nontoxic to mammalian cells and supported the attachment and proliferation of cells. Subcutaneous implantation of scaffold in mice demonstrated the cellular infiltration and vascularization in scaffolds. Thus, this study provides an easy method to design a poly-lactide-based scaffold for various applications.

3.5 Conclusion

Biopolymers are making their progress in a wide range of biomedical applications. It is expected that the suitability of these polymers are going to rocket up owing to endless ways of tailoring the particle properties to suit the desired application. Newer methods are coming up to design these poly-lactide/poly-lactide-co-glycolide nano- and microparticles for specific end-use properties. Blends are made using these polymers in combination with other polymers to overcome the limitations such as low glass transition temperature or fast degradation rate. Other natural and synthetic fibers are also used with these polymers. Thus these polymers are seen as promising materials to suit a diverse range of possibilities in biomedical applications. Poly-lactide and poly-lactide-co-glycolide polymer slowly degrade and form lactic acid and glycolic acid as degradation products which are components of various metabolic pathways in animal model (Brady et al. 1973). It makes these polymers more suitable for various biomedical applications including drug, antigen, and antibiotic delivery system. Requirement of safe and reproducible vehicle, targeted delivery system, personalized medicine, and delivery of specific bioactive molecule have led to the exploitation of biodegradable and biocompatible poly-lactide and poly-lactide-co-glycolide polymers.

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Chapter 4
Drug Delivery Systems Based on Pullulan Polysaccharides and Their Derivatives

Anca Giorgiana Grigoras

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Abstract The remarkable physical and chemical properties of pullulan, especially the biodegradability, biocompatibility, and nontoxicity, have been exploited in the past few decades and adapted in order to design more efficient drug delivery systems. This polysaccharide itself and its derivatives, which possess more reactive functional groups generated by functionalization of pullulan, were able to form conjugates or complexes with a variety of drugs, especially with hydrophobic drugs. By modulating the hydrophilic-hydrophobic balance in the support macromolecule structure and favoring various types of physical interactions between drug and carrier, the researchers attempted to optimize the charging and subsequent transport of drugs to target cells such as liver cell receptors or cancer cells.

In this chapter, beside the pullulan-based systems with antibacterial, antifungal, antitumor, antioxidant, anti-inflammatory, immunomodulatory, antilipidemic, or antiglycemic properties, other pharmaceutical formulations potentially useful to

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treat heart or bone diseases were reviewed. All studies highlighted the versatility of pullulan derivatives to form micelles, films, hydrogels, microparticles, and nanoparticles. Also, the results from in vivo and in vitro tests of cytotoxicity and the profiles of drug release from these carriers were encouraging such that the usage of pullulan polysaccharides for the future medical applications remains an open field.

4.1 Introduction

Resulted as an amorphous slime matter from the aerobically fermentation broth of *Aureobasidium pullulans* polymorphic fungus, pullulan is a neutral linear homopolysaccharide consisting almost of regularly repeating α-(1 → 4)–maltotriosyl units (3-D-glucopyranosyl) joined through α-(1 → 6) linkages (Singh et al. 2008; Cheng et al. 2011). The molecular weight of this exopolysaccharide varies from 4.5×10^4 to 6×10^5 Da, function of the yeast cultivation parameters. Due to the chain flexibility and hydroxyl functional groups of pullulan, susceptible for chemical modifications, a series of derivatives could be synthesized: cholesterol-bearing pullulan, pullulan acetate, carboxymethyl pullulan, pullulan succinylate, and pullulan amine (Singh et al. 2015). In this way, some of chemical and physical properties of pullulan like nonreducing agent, water solubility, formation of oxygen-impermeable films, hygroscopicity, biodegradability, and viscosity were improved due to the increased solubility in organic solvents or enrichment with new reactive functional groups.

Since its introduction in Japanese food industry as food additive, pullulan and its derivatives recorded numerous applications in food, pharmaceutical, and medical industries. The conceiving of drug delivery systems based on natural polymers has registered increasingly in pharmaceutical industry. Polysaccharides, in particular pullulan, were chosen as part of drug delivery systems because most of them are moldable, spinnable, edible, biodegradable, nontoxic, and biocompatible.

The present work brings updated data regarding drug delivery systems based on pullulan and its derivatives, structured function of therapeutic effects of drugs, and complete information from other reviews (Singh et al. 2008, 2015, 2017; Cheng et al. 2011; Prajapati et al. 2013).

4.2 Pullulan-Based Systems with Antibacterial or Antifungal Activity

The interaction between the pathogens from surrounding world such as bacteria, viruses, fungi, or algae and different substances depends on factors like surface chemistry of cell, surface charge presence, temperature, pH of medium, age of culture, and concentration of substances, such that there is a distinction between antibacterial, antiviral, antifungal, or antialgae substances and bactericidal, virucidal, fungicidal, or algaecidal agents. Regarding the heterogeneous bacterial cell outer layer,

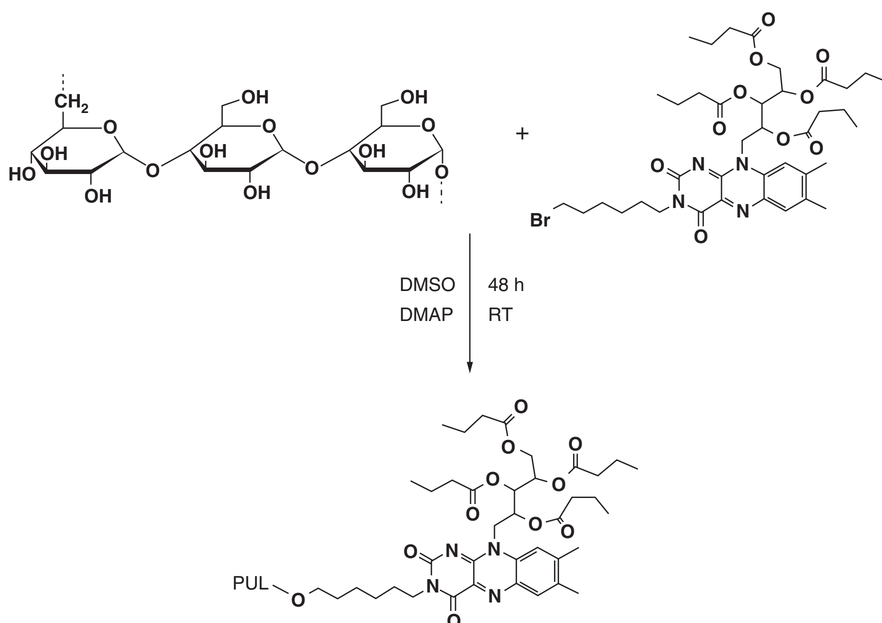
complex biological molecules like peptidoglycan, lipopolysaccharides, phospholipids, and lipoteichoic and teichoic acids possess carboxyl, phosphoryl, or amine functional groups placed in several discrete binding sites that ensure the interactions with antibacterials via dipole interactions, covalent or ionic bonding, steric interactions, and hydrophobic associations. Excepting the presence of different types of proteins in the bacterial cell wall, the morphological differences between Gram-positive and Gram-negative cells are significant: (a) *murein* layer from periplasmic space between outer and inner membranes of Gram-negative bacteria is thin unlike the murein layer in the Gram-positive cell wall which is thick, cross-linked, reinforced with teichoic and lipoteichoic acids, and exposed to the external part of cell wall; (b) predominant phospholipids in bacterial membrane consist from *cardiolipin* in the case of Gram-positive bacteria or *phosphatidylethanolamine* and *phosphatidylglycerol* in the case of Gram-negative cells; and (c) outer membrane is covered with a layer of *lipopolysaccharides* only in the case of Gram-negative microorganisms (Grigoras et al. 2016).

Based on studies in a number of fungi, the cell wall has been shown to be primarily composed of chitin, glucans, mannans, and glycoproteins. It was demonstrated that the chitin, glucans, and glycoproteins are covalently cross-linked together (Bowman and Free 2006).

Ciprofloxacin, an antibiotic for localized treatments applicable to a wide range of human bacterial infections, is prescribed in its hydrophilic or hydrophobic form to treat keratitis caused by Gram-positive and Gram-negative bacteria. It seems that the antibacterial activity of ciprofloxacin hydrochloride is lower compared with that of native drug. On the other hand, the amphiphilic polymers like poly(DL-lactide-*co*-glycolide)-*graft* pullulan (Jeong et al. 2006) or *N*-octyl-*O*-glycol chitosan (Huo et al. 2010) improved water solubility of hydrophobic drugs. In order to prepare therapeutic contact lens from poly(hydroxyethylmethacrylate) pHEMA, using poly(ethylene glycol) dimethacrylate PEGDM as UV-cross-linker, Garhwal et al. (2012) encapsulated the hydrophobic free base form of ciprofloxacin in core-shell nanospheres based on pullulan-poly(caprolactone) (PULL-PCL) block copolymer. Ciprofloxacin-containing nanospheres were uniform in size with diameters of 142 nm. The antimicrobial activity of hydrogel lenses incorporating ciprofloxacin-containing nanospheres was tested concluding that the proliferation of cultures inoculated with 10^7 or 10^8 bacteria/mL of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively, was effectively inhibited by less than 2 μg/mL of nanosphere-ciprofloxacin conjugate solution.

Di Meo et al. (2015) designed a new nanohydrogel platform in order to test the loading and release of ***Levofloxacin*** with a role of model hydrophilic molecule. The amphiphilic hydrogel was prepared based on the activation of neutral pullulan chains with 4-(dimethylamino)pyridine (DMAP), followed by the reaction with bromohexyl derivative of riboflavin tetrabutyrate in the presence of dimethyl sulfoxide (DMSO) (Scheme 4.1).

It was observed that, by esterification reaction involving pullulan-DMAP system, only 20% of the used Br-tetrabutyrilriboflavin reacted and 1 pullulan-repeating unit out of 20 was derivatized. Then, using a combined procedure for nanohydrogel



Scheme 4.1 Synthesis of nanohydrogel platform for levofloxacin based on pullulan and riboflavin derivate, in the presence of 4-(dimethylamino)pyridine (DMAP) and dimethyl sulfoxide (DMSO). (Reprinted from Di Meo et al. 2015 with permission of Elsevier)

formation, namely, autoclaving process for spontaneously self-assembling of the polymer chains, followed by bath sonication, polymeric suspensions with the size of 210 nm, polydispersity index of 0.2, and zeta potential in water of −18 mV were obtained. The cytocompatibility of pullulan-riboflavin derivative, tested on mammal fibroblasts ATCC BalbC 3T3, revealed that the nanohydrogels in the form of undiluted or diluted suspensions were completely safe for cells.

To treat the vulvovaginal candidiasis, a novel hydrogel containing ***Clotrimazole***-loaded nanocapsules of Eudragit® RS100 was developed by association with pullulan, a mucoadhesive polymer with high molecular weight, and an anionic emulsifier derivative of poly(acrylic acid) named Pemulen® TR1. Different semisolid formulations were tested, and one of them showed in vitro slow release of drug-loaded nanocapsules from the hydrogel formulations (20.14 μg/cm^2 in 8 h). In addition, the ex vivo cow vaginal mucosa permeation/penetration study showed that hydrogels containing nanoencapsulated drug had a low penetration of only 14.00 μg/cm^2, suggesting that the nanoencapsulation helped to retain clotrimazole on the mucosa surface and minimized the drug systemic absorption (de Lima et al. 2017).

A pullulan nanobased nail formulation for onychomycosis treatment was developed by mixing pullulan solution with suspension of ***Tioconazole***-loaded lipid nanocapsules coated with a cationic chitosan. The nanoencapsulated drug was released from a lipid core composed from medium-chain triglycerides in a controlled and sustained manner in the nail plate, while the bioadhesive pullulan promoted

a good hydration of the nail and increased the drug permeability. The new pullulan nanobased nail formulation was less irritant than other commercial formulations used for the treatment of onychomycosis because nanoencapsulated systems permitted the incorporation of a smaller quantity of drug but with comparable therapeutic effects (Flores et al. 2017).

Another antibiotic used over 20 years to treat onychomycosis is ***Terbinafine***. To overcome the side effects induced by topical administration, this drug was included in liposome-loaded pullulan films. The optimum film formulation for antifungal activity was chosen on the basis of drug release profile (~72%); also, the accumulated drug in the nail plates recorded a maximum of 31% (Tuncay Tanriverdi et al. 2016).

To dissolve and enhance the bioavailability of poorly water-soluble drug ***Griseofulvin***, an oral drug used to treat fungal infections, Krull et al. (2016) prepared pullulan-based strip films via casting-drying of the wet-milled drug suspensions. For this purpose, the following substances were implied: pullulan as steric stabilizer in suspension, xanthan gum as thickening agent, glycerin as plasticizer, and sodium dodecyl sulfate as surfactant. A film-forming pullulan-glycerin-xanthan gum solution represented the dispersant medium for griseofulvin. The drug suspension prepared via mixing was subsequently milled. The milling time was varied to obtain different drug particle sizes. The authors observed that the film properties can be modulated by controlling the thickness, xanthan gum, and drug loadings. In addition, sodium dodecyl sulfate content of films (~0.2%) was considered minimum toxic for formulations, and most of films exhibited fast or immediate release of drug (higher than 80% for films dissolved in 30 min).

4.3 Pullulan-Based Systems with Antitumor Properties

According to the Ringsdorf model, a polymeric drug delivery system consists of a definite number of *drug* molecules, bonded to a *macromolecule* or polymeric backbone through a *spacer*. The spacer molecule incorporates a predetermined breaking point which ensures the drug release after cellular uptake of the conjugate. The system can also contain *targeting moieties*, e.g., antibody fragments, tumor-specific antibody, or saccharides (Kratz et al. 2002). Pullulan-based delivery systems for anticancer drugs like doxorubicin, paclitaxel, camptothecin, methotrexate, alendronate, cisplatin, and combretastatin A4 include pH-sensitive pullulan nanoparticles, bioconjugates, and self-assembling hydrophobized pullulan. Usually, the design of bioconjugates supposed using of an acid-sensitive hydrazone bond, stable at physiological pH but hydrolysable under acidic conditions, in order to conjugate the drug to the pullulan backbone (Scomparin et al. 2015). Also, much effort has been invested to find the best ligand for selective targeting of drug delivery systems to a tumor tissue.

Doxorubicin (DOX), the most popular anthracycline produced and extracted from the *Streptomyces peucetius* bacteria, represents a chemotherapy medication approved for various medical uses that include Kaposi's sarcoma, lymphoma, acute

lymphocytic leukemia, breast cancer, bladder cancer, and hepatocellular carcinoma. The effectiveness of this drug could be improved when it is prepared in nanoparticulated or liposomal forms and it is used together with other chemotherapy agents, respectively.

A chemotherapeutic drug is expected to have reduced side effects, to have enhanced therapeutic efficacy, and to be specifically transported to the tumor region. In order to treat the hepatocellular carcinoma, Li et al. (2015a) prepared pullulan-doxorubicin nanoparticles with a diameter of 50–170 nm by conjugating the drug onto polysaccharide molecule via pH-responsive hydrazone bond and using spacers with different alkane chain lengths. It was observed that, in aqueous solution, the conjugates were self-assembled so those spontaneously resulted in core-shell structures with doxorubicin as core and pullulan as shell. In acidic medium (pH = 5), these doxorubicin-pullulan nanoparticles released the drug in 2 h, but in neutral conditions, they have been kept relatively stable. In vitro cell coculture experiments revealed that doxorubicin was specifically internalized by hepatic carcinoma cells through receptor-mediated endocytosis via asialoglycoprotein receptor (ASGPR) from the surface of these cells and then rapidly diffused into the nuclei of cells.

When the chemoresistance restricts the curative effect of a drug, it is necessary to use *combination chemotherapy*. In this regard, new approaches supposed the adding to doxorubicin in pharmaceutical formulation of an inhibitor drug like pyrrolidine dithiocarbamate (PDTC) for nuclear factor kappa B (Li et al. 2015b) or sorafenib for tyrosine protein kinases (Sui et al. 2017). In this way, pullulan-adipodihydrazide-doxorubicin conjugate became a carrier to co-load pyrrolidine dithiocarbamate or co-load sorafenib such that the synergistic combinatorial delivery systems could be used against hepatocellular and murine breast carcinoma, respectively. Pullulan-doxorubicin-pyrrolidine dithiocarbamate nanoparticles, synthesized according to the route presented in Fig. 4.1, had fairly stable sizes during 4 h of drug releasing study in pH 7.4 (Fig. 4.2). Adipohydrazine was employed instead of hydrazine hydrate because it has higher loading capacity and better biocompatibility. Within 2 h in pH 5.0 buffer, the particle size increased from 160 to 240 nm since the detachment of some hydrophobic doxorubicin decreased the hydrophobic interaction in the nanoparticle core. After another 30 min, nanoparticles collapsed due to complete fractions of hydrazone bonds.

Asialoglycoprotein receptor, a hepatic lectin, internalizes sugars like galactose or lactose and glycoproteins with terminal galactose or *N*-acetylgalactosamine by endocytosis, and it is overexpressed on the surface of hepatocytes in the case of patients with hepatocarcinoma, being thus a target for chemotherapy. In the case of PES-Gantrez AN 119-doxorubicin-pullulan nanoparticle design, pullulan plays the role of hydrophilic ligand between doxorubicin and asialoglycoprotein receptor, the biodegradable poly(ethylene sebacate) (PES) ensures the hydrolytic stability of formulations, and anhydride side chain of Gantrez, as synthetic alternating copolymer of methyl vinyl ether and maleic anhydride, hydrolyzes to produce free diacid solutions which are directly useable in oral care products (Guhagarkar et al. 2010). There are also improved formulas based on ligand combination of pullulan with arabinogalactan which promotes doxorubicin anchoring by adsorption

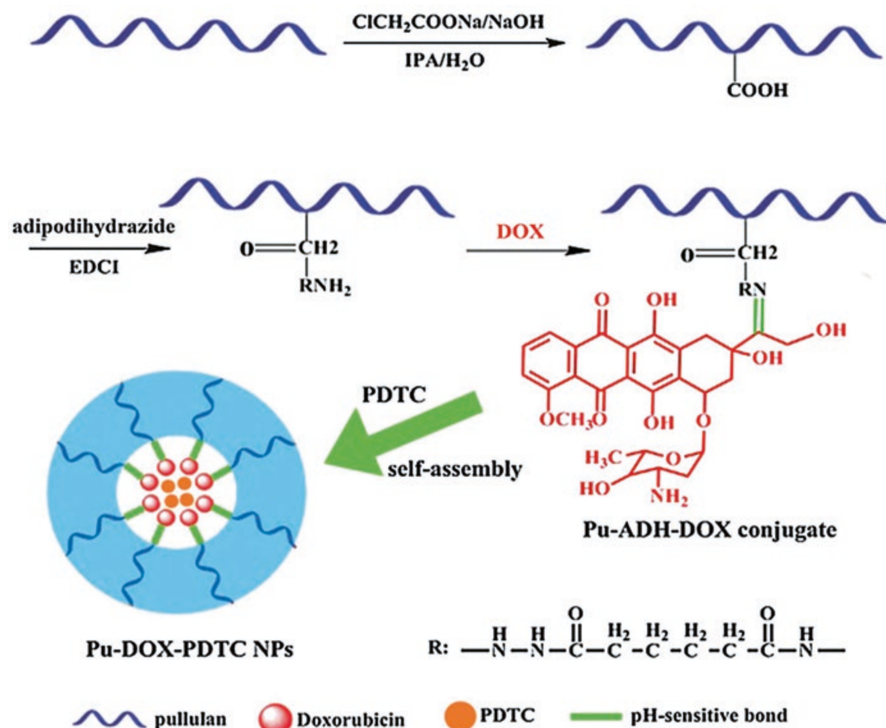


Fig. 4.1 Schematic illustration of the synthesis route for pullulan-doxorubicin-pyrrolidine dithiocarbamate nanoparticles (Pu-DOX-PDTC NPs) based on functionalization of pullulan and subsequently self-assembly of pullulan-adipodihydrazide-doxorubicin (Pu-ADH-DOX) conjugate. (Reprinted from Li et al. 2015b with permission of Royal Society of Chemistry)

(Pranatharthiharan et al. 2017). In this case, nanoparticles prepared by modified nanoprecipitation recorded an average size of 220 nm with polydispersity index lower than 0.62 and about 20% doxorubicin loading. Although higher concentration of doxorubicin was recorded in the kidneys, the entrapment of drug in poly(ethylene sebacate)-Gantrez-doxorubicin-pullulan nanoparticles reduced the cardiotoxicity compared to doxorubicin solution.

The bioconjugates represent a current approach to design anticancer drug delivery. Balasso et al. (2017) performed in vitro cell uptake investigations in order to test a novel bioconjugate for hepatocellular carcinoma, obtained by conjugation of PreS1, a peptide of hepatitis B surface antigen, and rhodamine as label. Using poly(ethylene glycol) (PEG) spacers, the conjugation of PresS1 and rhodamine to the aldehyde groups present along the oxidized pullulan backbone was realized through reductive animation. Afterward, a hydrazone pH-sensitive bond was exploited to conjugate doxorubicin to the polymer backbone. It was found that PreS1-pullulan-doxorubicin

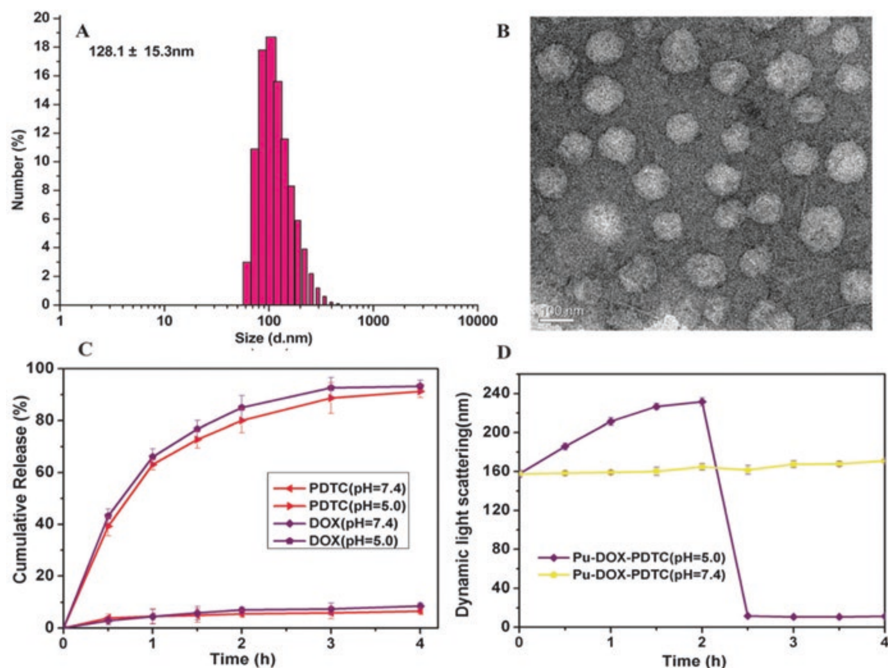
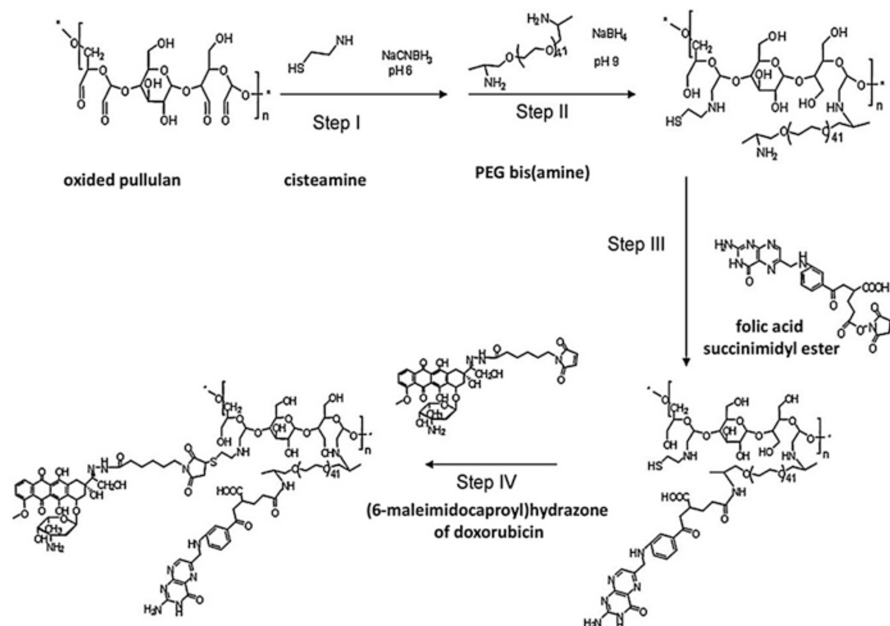


Fig. 4.2 Characterization of pullulan-doxorubicin-pyrrolidine dithiocarbamate (PULL-DOX-PDTC) nanoparticles; (**a**) representative dynamic light scattering (DLS) and (**b**) transmission electron microscopy (TEM) images of PULL-DOX-PDTC nanoparticles (DOX/PDTC: 21.26%/4.63%); (**c**) in vitro drug release profiles and (**d**) size change of PULL-DOX-PDTC nanoparticles (DOX/PDTC, 21.26%/16.26%) at different pH values (n = 3). (Reprinted with permission of Royal Society of Chemistry, Li et al. 2015b)

conjugate showed a twofold increase of anticancer activity with respect to the control pullulan-doxorubicin toward HepG2/SERPINB3 cells.

Folic acid (FA), a small non-immunogenic molecule, represented an attractive targeting agent to a large number of cancer cell types that overexpress the folate receptor (FR). By introducing folate functions in the supramolecular structure of anticancer bioconjugate, the internalization of the carriers was promoted, the therapeutic activity of drug carriers increased, and cardiotoxicity was reduced.

Scomparin et al. (2011) synthesized some antitumor bioconjugates: a folic acid-free derivative tagged as (NH_2-PEG)-PULL-(Cyst-DOX) containing 6.3% (w/w) doxorubicin, while folic acid-doxorubicin-coupled derivative labeled (FA-PEG)-PULL-(Cyst-DOX) was contained 6% (w/w) doxorubicin and 4.3% (w/w) folic acid. First, oxidized pullulan was functionalized with cysteamine (step I) and poly(ethylene glycol) bis(amine) PEG(NH_2)$_2$ (step II) and then conjugated with folic acid (step III) and doxorubicin (step IV) (Scheme 4.2). In continuation to this, Scomparin et al. (2015) compared the performance of supramolecular doxorubicin carriers depending on the nature of the nanocarriers: bioconjugates like (NH_2-PEG)-PULL-(Cyst-DOX) and (FA-PEG)-PULL-(Cyst-DOX), liposomes like PLD



Scheme 4.2 Synthesis of poly(ethylene glycol) bis(amine)-pullulan-cysteamine (NH_2-PEG)-PULL-(Cyst) and folic acid-poly(ethylene glycol)-pullulan-cysteamine-doxorubicin (FA-PEG)-PULL-(Cyst-DOX) bioconjugates. (Reprinted with permission of Elsevier, Scomparin et al. 2011)

named Doxil® and containing doxorubicin-loaded PEGylated liposomes, and Doxil®-folic acid conjugates. They concluded that folate receptor-targeted version of each nanocarrier specifically interacted in vitro with tumor cells via the folate ligand and reduced cardiotoxicity of drug delivery system.

Other pullulan derivatives were decorated with folic acid to develop doxorubicin carriers, too. Thus, maleilated pullulan (MP) was chosen as a polysaccharide support because it contained vinyl carboxylic acid groups susceptible for doxorubicin conjugation via primary amine bonds and pendant hydroxyl groups able to conjugate with folic acid (Zhang et al. 2011). The cellular uptake of folic acid-maleilated pullulan-doxorubicin conjugate was presented in Fig. 4.3. Also, in vitro cytotoxicity of bioconjugate was tested on an ovarian cancer cell line A2780. The resulted folic acid-maleilated pullulan-doxorubicin conjugate enhanced the therapeutic potential and reduced the systemic side effects of the drug.

The spherical self-assembled nanoparticles based on folate-decorated maleilated pullulan and having the average diameter of ~150 nm represented a co-delivery system for doxorubicin and pyrrolidine dithiocarbamate, successfully used in combination with chemotherapy due to an increased amount of doxorubicin transported within cells (Li et al. 2013).

Because pullulan itself, a linear, hydrophilic polymer, cannot load drugs, several attempts have been made for the introduction of the hydrophobic segments like poly(lactide) (PLLA) or poly(DL-lactide-*co*-glycolide) (PLGA) into its backbone,

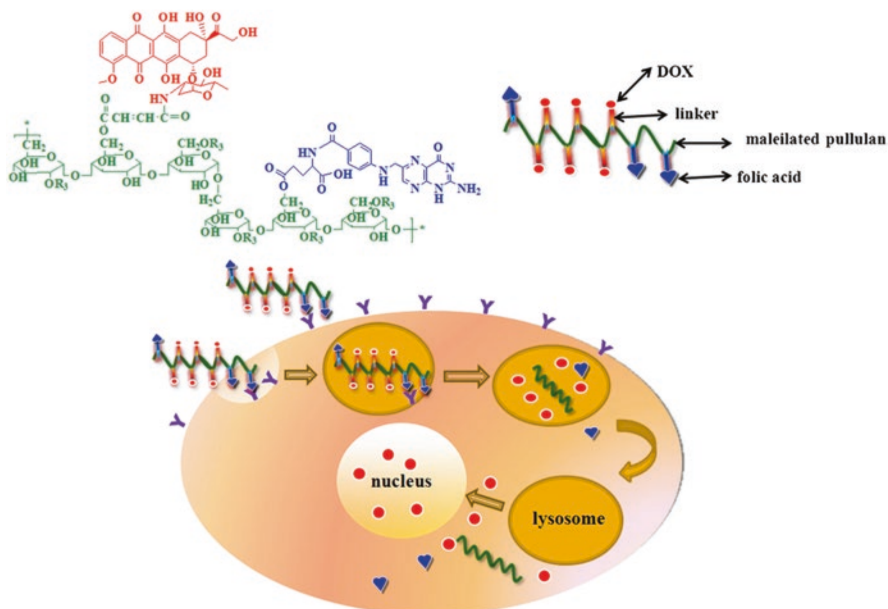


Fig. 4.3 Receptor-mediated endocytosis of folic acid-maleilated pullulan-doxorubicin (FA-MP-DOX) conjugate. (Reprinted with permission of Royal Society of Chemistry, Zhang et al. 2011)

resulting in amphiphilic polymers able to self-assemble in aqueous solution into micelle-type structures with poly(lactide) hydrophobic inner core and pullulan hydrophilic outer shell. Thermoresponsive nanogels from poly(L-lactide)-*g*-pullulan copolymers with different lactide contents can be used as a long-term doxorubicin delivery system in cancer treatments (Seo et al. 2012). As stimuli-responsive biomaterials, these grafted copolymers have the ability to change its molecular structure in accordance with the environmental conditions. Triggered by higher temperatures (e.g., 43 °C), these nanogels with hydrodynamic diameter between 121 and 163 nm at 25 °C and drug content of about 4% w/w may have increased rates of doxorubicin release, being thus a more efficient and effective system for killing cancer cells. In this way, higher temperatures facilitated the internalization of doxorubicin-loaded copolymer nanogels in the cells (Fig. 4.4).

Since the folate receptor is universally overexpressed on the tumor cell membrane, it represents a target for folic acid conjugated on different drug delivery systems. Lee et al. (2015) fabricated nanoparticles based on folic acid-conjugated pullulan and poly(DL-lactide-*co*-glycolide) graft copolymer for folate receptor-mediated drug delivery. Thus, model drug doxorubicin was incorporated in these nanoparticles, which self-assembled in water and have a diameter lower than 200 nm and drug content of about 7% w/w, in order to treat the folate receptor-overexpressing KB human carcinoma cells.

Beside folic acid, there are other hydrophobic molecules like biotin, retinoic acid, lipoic acid, stearic acid, and cholesterol which were introduced into the com-

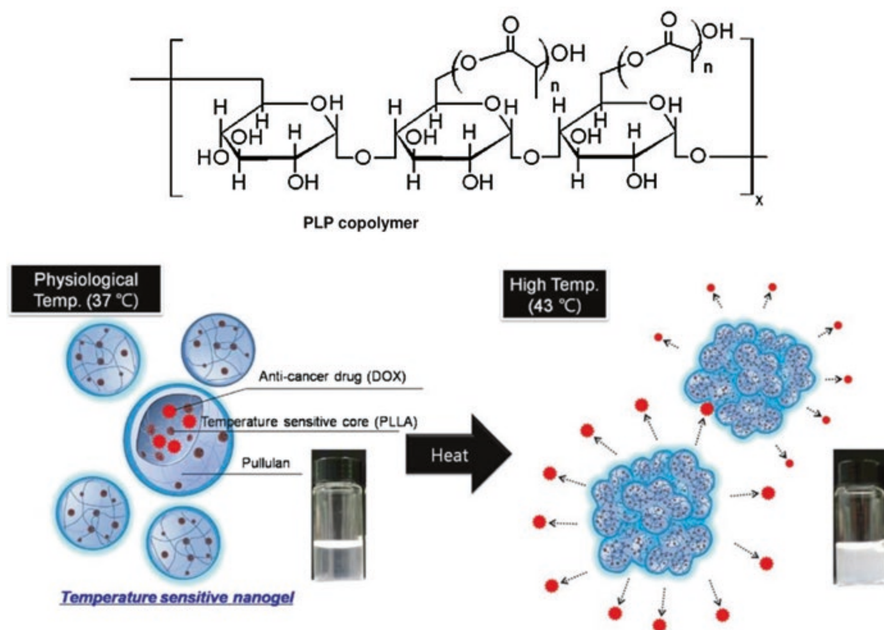


Fig. 4.4 Molecular structure of poly(L-lactide)-*g*-pullulan (PLP) copolymer and schematic diagram for doxorubicin (DOX) release from thermosensitive PLP nanogels by triggering temperature. (Reprinted with permission of Elsevier, Seo et al. 2012)

position of pullulan-based antitumor drug delivery systems in order to induce them an amphiphilic character. Biotin, the water-soluble vitamin B_7, a necessary element for cell growth and fatty acid production, involved in metabolism of the amino acids and lipids, represents a cofactor responsible for CO_2 transfer in some carboxylases. By a facile one-pot synthesis, Wang et al. (2016) prepared folate-biotin-pullulan (FBP) nanoparticles for encapsulation of doxorubicin. By conjugation with pullulan, the water solubility of biotin dramatically decreases because of the loss of hydrophilic carboxyl group and acts as a hydrophobic moiety, being responsible for the self-assembling of nanoparticles. Then, the conjugated folate was introduced into pharmaceutical formula, by conjugation of folate with hydroxyl groups of pullulan, as a targeting ligand for specific interaction with folate receptors which are overexpressed in various tumors of the breast, ovary, endometrium, kidney, lung, head, neck, brain, and bone marrow. Doxorubicin was encapsulated in self-assembled nanoparticles by membrane dialysis method and thus recorded a diameter of about 170 nm and zeta potential of –4.9 mV. The release behavior of drug, studied by dialysis against phosphate-buffered saline (PBS) solution, revealed a drug-loading content of 1.72% and 69% loading efficiency.

To avoid the general toxicity of anticancer drugs appeared by the distribution of these drugs in non-cancerous organs and cells, Hassanzadeh et al. (2016) adopted a better approach, with fewer side effects, namely, polymeric micelle design. For

biotin-targeted delivery of doxorubicin in breast cancer chemotherapy, they synthesized pullulan-retinoic acid-biotin conjugate using carbodiimide activation ester bond formation strategy. These amphiphilic structures possessing a hydrophilic and a hydrophobic moiety represented by pullulan and retinoic acid, respectively, had become suitable candidates to develop doxorubicin delivery systems due to their high colloidal stability in water, biodegradability, small particle size, and high loading capacity; zeta potential of −9.45 mV, particle diameter of about 192 nm, and entrapment efficiency of 92% were recorded. The negative zeta potential was related with the presence of numerous unreacted hydroxyl groups on the final conjugates. The micellar size originated from the hydrophobic forces which formed the hydrophobic chain into the core and the volume repulsion between the chains. It seems that the hydrophobic core of micelles acted as a vehicle for hydrophobic doxorubicin, but it had a limited entrapping capacity because the week noncovalent bonds formed into core were broken and the drug was fast released.

Retinoic acid, an active metabolite of retinol in the biological metabolic pathway, is used in chemotherapy because it controls the proliferation, differentiation, and apoptosis of the cells. On the other hand, the anticancer activity of retinoic acid is controlled by retinoic acid receptor on the nuclear membrane. The antineoplastic or cytotoxic effect of all-trans-retinoic acid (ATRA), also named Tretinoin, was exploited by Lee et al. (2013). They designed and synthesized biodegradable nanogels PURA based on all-trans-retinoic acid-pullulan conjugate for doxorubicin delivery. The long carbon chain and the carboxylate group of retinoic acid make it compatible for conjugation with pullulan. For different degrees of substitution, e.g., 1.59, 2.78, or 4.00, different doxorubicin-loaded PURA nanogels were resulted with 230–260 nm hydrodynamic diameters, 38–47% drug-loading efficiency, and 1.1–1.4% drug contents. Although the biopolymer has low drug-loading capacity, high cellular uptake efficiency was shown. When the nanogels were internalized to cancer cells, pullulan was enzymatically degraded such that retinoic acid bounded to nuclear membrane receptor and doxorubicin was released in cytosol.

Amphiphilic self-assembled polymeric micelles represent suitable nanocarriers for anticancer drugs because they enhance their solubility in water, prolong the blood circulation in time, and favor the permeation and retention in tumor cells. Also, it is desirable to improve the therapeutic efficacy by a rapid drug release after micelle endocytosis in tumor cell. In this regard, the researchers studied smart stimuli-responsive systems able to trigger the drug release only at targeting site (Fig. 4.5). Usually, these sensitive micelles contain characteristic disulfide S–S bonds, which are stable in the mildly oxidizing extracellular medium but susceptible to be prone to rapid cleavage through thiol-disulfide exchange reactions with intracellular reducing molecules, especially with glutathione (GSH). The significant difference in glutathione level between extracellular and intracellular environments is the premise of design redox-sensitive micelles (Li et al. 2012). Thus, Wang X. and co-workers developed a novel intracellular reduction-sensitive delivery system of doxorubicin, based on pullulan-stearic acid conjugates with reduction-sensitive disulfide bonds P-ss-SA (Wang X. et al. 2014b). Pullulan succinate was linked to stearic acid by cystamine in the case of pullulan-disulfide-stearic acid (P-ss-SA)

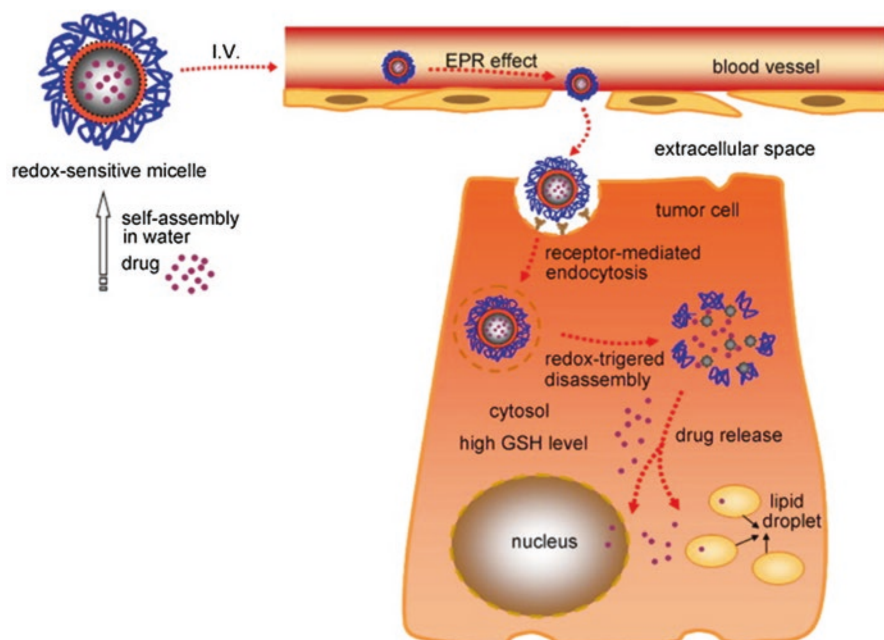
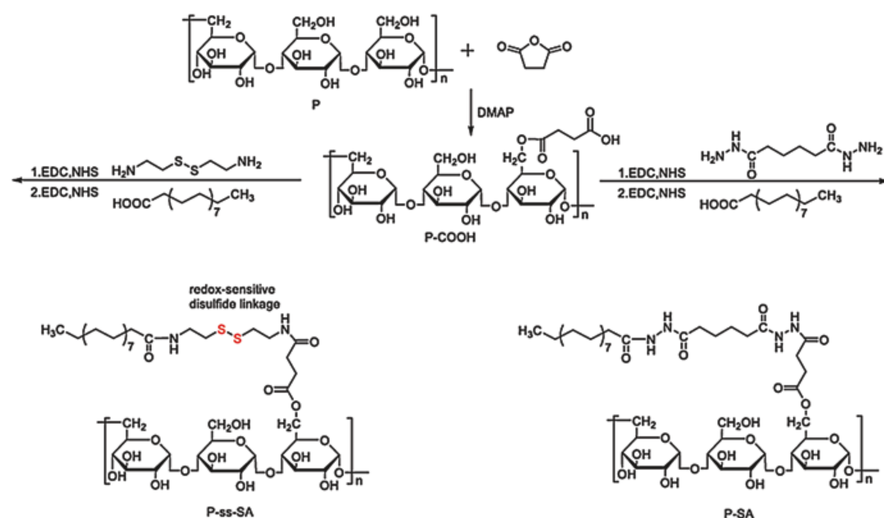


Fig. 4.5 Self-assembly, accumulation at tumor tissue, and intracellular trafficking pathway of redox-sensitive micelles; receptor-meditated cellular internalization, endo- or lysosomal escape, reduction-triggered micelle disassembly, and drug release (I.V. (intravenous) administration of drug; EPR (enhanced permeability and retention) effect; GSH (glutathione) level). (Reprinted with permission of Elsevier, Li et al. 2012)

conjugate or by adipic dihydrazide in the case of its structural analog P-SA synthesized as control (Scheme 4.3). Pullulan-disulfide-stearic acid conjugates could self-assemble into micelles in aqueous media and encapsulate doxorubicin, but as a response to dithiothreitol, a similar medium with the reduction condition in human body, the disulfide bonds broke the micelles and triggered the fast release of doxorubicin.

The blank and doxorubicin-loaded micelles recorded dimensions around 190 nm, while the drug-loading content and encapsulation efficiency of pullulan-disulfide-stearic acid micelles were around 6.19% and 65.53%, respectively. In vitro tests revealed negligible cytotoxicity of blank micelles against HepG2 and MCF-7 cells, but an excellent hemocompatibility. Both types of doxorubicin-loaded micelles could be effectively internalized in MCF-7 cells after 4 h. These results recommended the biocompatible reduction-sensitive pullulan-disulfide-stearic acid micelles as potential carrier systems for the intracellular delivery of doxorubicin.

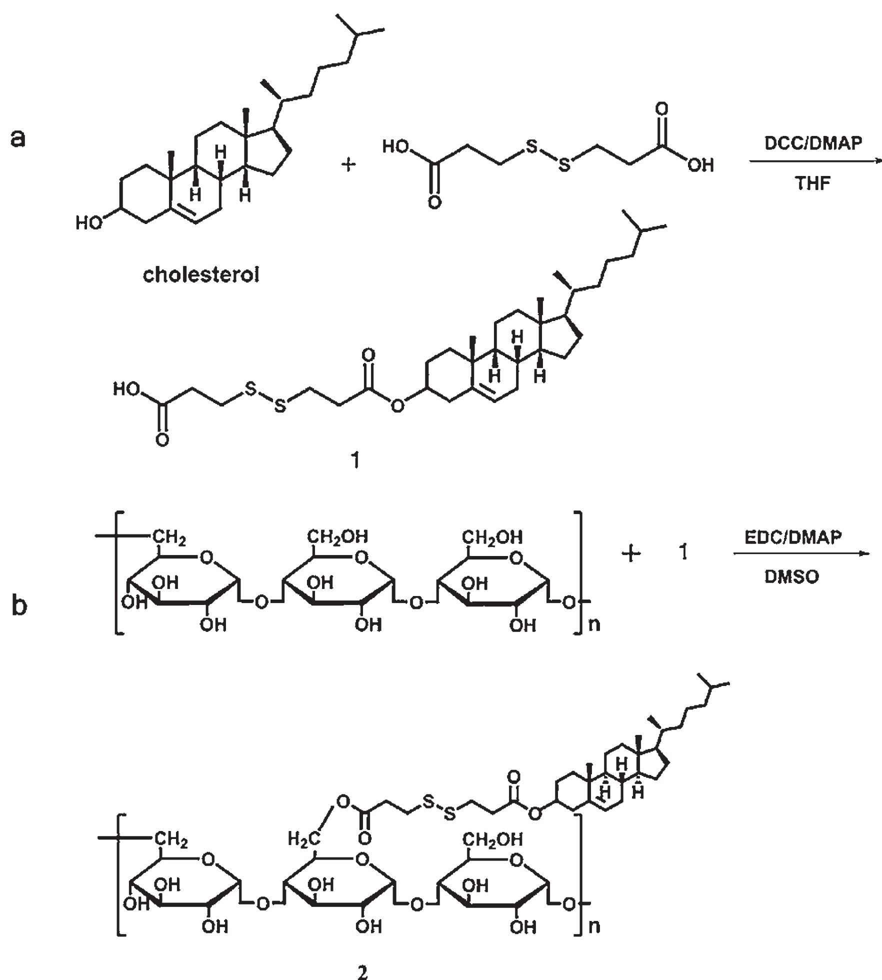
In order to combine the chemotherapy with the gene therapy, researchers have to conceive biocompatible carriers capable for simultaneous loading of therapeutic drug and gene. Chen et al. (2015) prepared amphiphilic bifunctional pullulan derivative which contained stearic acid and low molecular weight branched



Scheme 4.3 Synthesis of pullulan-disulfide-stearic acid (P-ss-SA) conjugate and its structural analog pullulan-stearic acid (P-SA) in the presence of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (EDC), 4-dimethylaminopyridine (DMAP), and *N*-hydroxysuccinimide (NHS). (Reprinted with permission of Royal Society of Chemistry, Wang et al. 2014b)

poly(ethylenimine) for the co-delivery of doxorubicin and p53 protein. This new system was able to self-assemble in water into core-shell-type micelles having an average size of 189 nm, a positive zeta potential of 18 mV, doxorubicin-loading content of about 5.10%, and encapsulation efficiency for doxorubicin of 56.07%, respectively. The co-delivery of doxorubicin and therapy gene p53 using these amphiphilic micelles, successfully internalized by MCF-7 cells, displayed higher cytotoxicity and induced a higher apoptosis rate of tumor cells in vitro compared with single doxorubicin or p53 delivery. Later, a similar carrier based on amphiphilic bifunctional pullulan derivative was design to use in cancer co-delivery of doxorubicin and p53 gene (Chen et al. 2017). In this case, stearic acid was replaced with lipophilic desoxycholic acid and grafted on pullulan backbone, along with the same low molecular weight branched poly(ethylenimine) of 1 kDa. It has been found that the resulted nanomicelles have a great potential in delivering hydrophobic anticancer drugs and therapeutic genes simultaneously for improved cancer therapy, too.

By coupling cholesterol to pullulan via disulfide bond, Li et al. (2014) prepared a series of doxorubicin-encapsulated multifunctional nanoparticles, specifically targeted to hepatic carcinoma cells and able of triggered drug release. Cholesterol, as small endogenous molecule of the body's metabolic process, was chosen to hydrophobize and stabilize the self-assembled nanoparticles. The cholesterol-modified pullulan (CHP) conjugates were developed following a two-step synthesis: (a) synthesis of cholesterol-dithiodipropionic acid monoester (CDE) by reaction of cholesterol with 3,3′-dithiodipropionic acid (DTDPA) and (b) conjugation of cholesterol-dithiodipropionic acid monoester with pullulan (Scheme 4.4).



Scheme 4.4 Synthesis route of reducible cholesterol-modified pullulan (2); (**a**) synthesis of cholesterol-dithiodipropionic acid monoester (CDE) (1) by reaction of cholesterol with 3,3′-dithiodipropionic acid (DTDPA) in the presence of *N*,*N*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), and tetrahydrofuran (THF); (**b**) conjugation of CDE with pullulan in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), 4-dimethylaminopyridine (DMAP), and dimethyl sulfoxide (DMSO). (Reprinted with permission of Royal Society of Chemistry, Li et al. 2014)

The new method of synthesis has proposed to replace the insensitive exogenous 1, 6-hexyl diisocyanate with 3,3′-dithiodipropionic acid. The reducible cholesterol-modified pullulan (rCHP) self-assembled in water to form stable nanoparticles with diameter ranging from 57 to 89 nm and narrow size distributions with polydispersity index less than 0.2 depending on the substitution degree (3.6–8.3). Doxorubicin-loaded rCHP nanoparticles resulted by dialyzing the dimethyl sulfoxide solution of

rCHP and doxorubicin against water were spherical in shape, mono-distributed in size, and homogeneous. The authors suggested that the hydrophobic interactions and π–π stacking interactions between doxorubicin molecules were responsible for drug-loaded nanoparticle formation. The small dimensions of drug-loaded nanoparticles having diameter ranging between 80 and 160 nm contributed to extravasation into hepatic tumors through enhanced permeability and retention effect but also to avoid its capture by macrophages. In vitro and in vivo investigations of antitumor effects confirmed that doxorubicin was not only concentrated in tumor but also internalized by hepatoma carcinoma cell by receptor-mediated endocytosis and fast released in HepG2 cells.

A novel series of amphiphilic and pH-sensitive nanoparticulated carriers based on pullulan labeled as UCPA were prepared by conjugation of cholesterol succinate, a hydrophobically modified moiety, and urocanic acid, a pH-sensitive grafted moiety, to the polysaccharide backbone (Wang Y. et al. 2014c). UCPA nanoparticles prepared by the precipitation method were roughly spherical with sizes in a range of 150–300 nm and represented a good candidate for physically loading of doxorubicin. It was observed that these nanoparticles with a degree of substitution of urocanyl and cholesterol moieties of 6.8% and 3.5%, respectively, recorded relatively high doxorubicin-loading capability strong in vitro pH-induced drug release; their responding pH value was around 6.5. Also, they realized the intracellular delivery of doxorubicin after internalization and enhanced cytotoxicity of doxorubicin against MCF-7 cells.

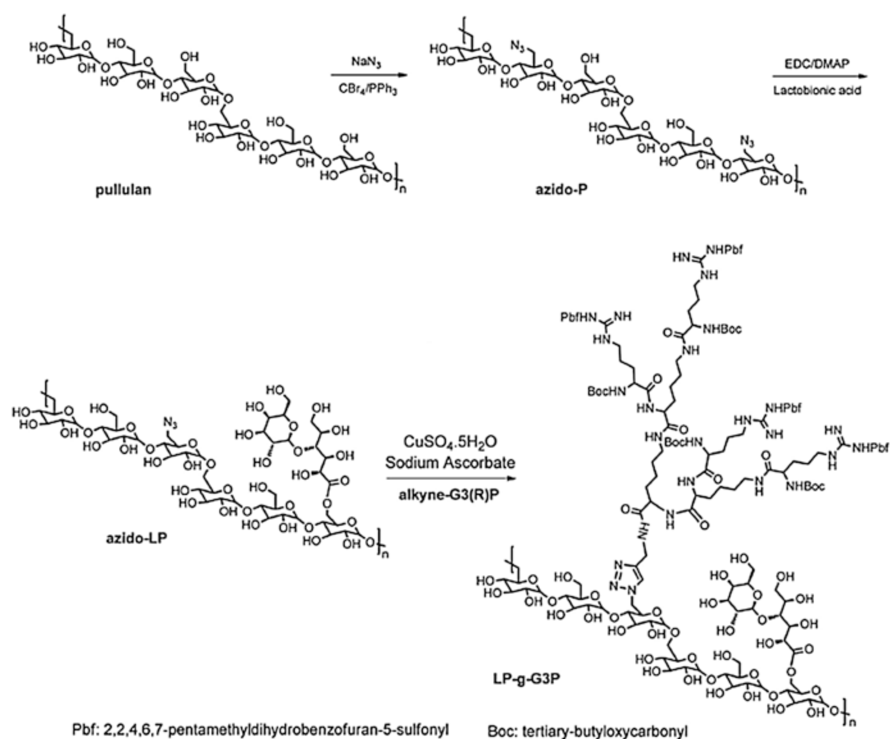
In the last decade, the self-assembly capacity of another biocompatible pullulan derivatives, like carboxymethylated, thiolated, or lactosylated pullulan, was exploited to design multifunctional compounds able to load or co-load different anticancer molecules, doxorubicin or doxorubicin, together with gene. For some of them, the complex supramolecular structure was tailored to increase the drug-loading capacity of such systems.

In order to prevent undesired interaction between cationic carboxymethylated pullulan (CMP) with serum proteins and blood components, the electrostatic interaction with anionic poly(allylamine) (PAA) was permitted, resulting in self-assembled nanocomplexes (Vora et al. 2014). Initially, the pH-sensitive doxorubicin-carboxymethylated pullulan conjugate was synthesized using the hydrazone bonds, and then complexation with polyanion was performed in aqueous solution. Resulted nanocomplexes self-assembled into spherical shape with dimensions lower than 250 nm. These nanocomplexes were the subject of a cancer combination therapy which in addition involved the conjugation of a nucleotide like plasmid DNA to the drug delivery system. Thus, it was contemplated a single nanodelivery system and the synergistic effects of different therapeutic agents that make it up. The cytotoxic effects of doxorubicin-carboxymethylated pullulan-poly(allylamine) nanocomplexes have been monitored on a human embryonic kidney cell line HEK293 using MTT cell viability assay. Also, it was pointed out the beneficial effect of combination therapy by reducing the doxorubicin-induced off-target side effects.

Because the polysaccharides bear a large number of reactive groups like –OH, –COOH, or $-NH_2$, they are susceptible for chemical modification in order to obtain

various amphiphilic derivatives involving hydrophobic moieties. Mohamed Wali et al. (2017) developed a targeted drug delivery systems based on arginine third-generation dendrons grafted on lactosylated pullulan labeled as LP-*g*-G3P according to Scheme 4.5. Supramolecular structure of LP-*g*-G3P spontaneously self-assembled into core-shell nanoarchitectures in the aqueous media, resulting in medium dimensions of 54 nm and polydispersity index of 0.13, and was tested as a carrier for doxorubicin delivery. Doxorubicin was selectively and stably encapsulated in the hydrophobic core of dendrons through multiple hydrophobic, hydrogen bonding and π–π interactions. From applicative point of view, it was observed the internalization of doxorubicin-loaded LP-*g*-G3P nanoparticles into the hepatoma carcinoma cells and inhibition of HepG2 and NIH3T3 cell proliferation.

Different thiolated conjugates of pullulan with poly(ethyleneimine) (PEI) were synthesized by conjugation with varying amounts of mercaptosuccinic acid (MSA). The formed conjugate was oxidized to obtain reversible disulfide linked cationic pullulan labeled as PPMSS. Nanoplexes between PPMSS and calf thymus DNA, labeled as ctDNA, were prepared in different weight ratios. PPMSS condensed with DNA and formed nanostructured entities with dimensions lower than 150 nm and



Scheme 4.5 Reaction of synthesis for amphiphilic lactosylated pullulan-*graft*-propargylamine third-generation dendrons LP-*g*-G3P. (Reprinted with permission of Elsevier, Mohamed Wali et al. 2017)

zeta potential between +10 and +19 mV. The viability of C6 rat glioma cells pretreated with PPMSS-ctDNA nanoplexes and later treated with doxorubicin has been monitored. Mercaptosuccinic acid acted as a ligand to target cancer cells effectively via EPR effect. Also, the synergetic effect of p53 plasmid in augmenting doxorubicin sensitivity in C6 glioma cells treated with PPMSS-p53-doxorubicin dual delivery system was analyzed. All observations have been summed up to the conclusion that the polymer PPMSS represented a promising vector for gene and drug delivery applications (Priya et al. 2017).

Another anthracycline drug able to inhibit DNA and RNA synthesis is ***Epirubicin***, a molecule primarily used against gastric cancer, breast and ovarian cancer, lung cancer, and lymphomas, with a reduced toxicity and a faster elimination compared with its isomer doxorubicin. This chemotherapeutic drug is favored over doxorubicin because it appears to cause fewer side effects like vomiting, rush, mouth inflammation, hair loss, bone marrow suppression, anaphylaxis, and heart damage.

Pullulan acetate (PA) nanoparticles with nearly spherical shape, dimensions of 200–450 nm, and low zeta potentials both in distilled water and in 10% FBS were prepared by hydrophobization of pullulan in order to obtain self-assembled carriers able to encapsulate the hydrophobic epirubicin in their core (Tang et al. 2010). The safety tests of pullulan acetate nanoparticles realized on mice showed that these model animals tolerated a dose of 200 mg/kg. In addition, the imperceptible pathological modifications and nonexistent inflammatory reactions in heart, liver, spleen, lung, and kidney sections stained with hematoxylin and eosin suggested no apparent toxicity of pullulan acetate nanoparticles. Epirubicin-loaded pullulan acetate nanoparticles recorded a much longer circulation time and a half-time of about 17 h, 2.12 times that of free epirubicin.

In order to improve the cancer-targeting activity of epirubicin, Zhang et al. (2010) additionally conjugated folic acid molecules on pullulan acetate chains. Consequently, the epirubicin-loaded and folate-modified pullulan acetate nanoparticles exhibited a faster drug release than epirubicin-pullulan acetate nanoparticles in vitro. The total amount of drug released at 37 °C (PBS, pH 7.4) from epirubicin-pullulan acetate nanoparticles and epirubicin-folic acid-pullulan acetate nanoparticles over 72 h was 52% and 92%, respectively. Folate moieties enhanced hydrophilicity of epirubicin-loaded nanoparticles in such a manner phosphate-buffered saline medium easily accessed the core of nanoparticles and increased the drug release rate. Also, folate moieties increased the intracellular uptake of nanoparticles as a consequence of specific binding of folic acid-epirubicin-pullulan acetate nanoparticles with folate receptors on KB cells.

To deliver epirubicin to Hela and MCF-7 cell lines, other researchers tried to optimize some micellar structures based on pullulan, tocopherol succinate, and folic acid. Because these micelles showed the particle size of 149.5 nm, zeta potential of −6.49 mV, a polydispersity index of 0.26, loading efficiency of 88%, and release efficiency of 63%, they could be recommended as a promising candidate for epirubicin treatment of cancers (Hassanzadeh et al. 2018).

The amphiphilic pullulan derivatives represent a class of substances intensively used in pharmaceuticals. Thus, cholesterol-modified pullulan (CHSP) was synthesized

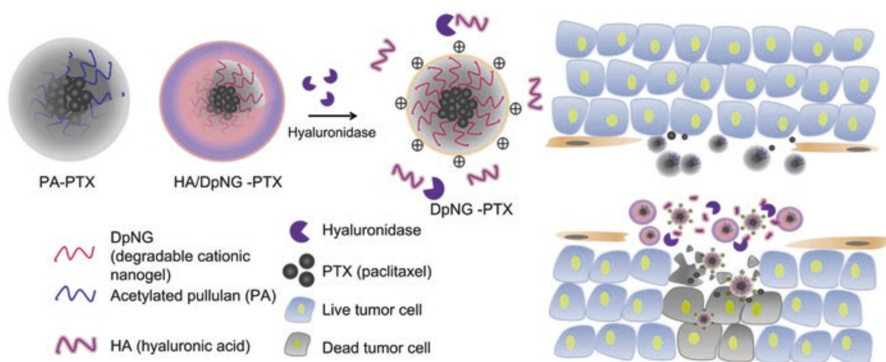
using succinic acid as a linker, a safely compound involved in the body's metabolic process (Shen et al. 2014). Cholesterol-modified pullulan served as carrier for epirubicin. The size of cholesterol-modified pullulan nanoparticles with a mean diameter of 52 nm increased to about 160 nm when they were loaded with epirubicin. Often the pharmacokinetic profiles of the parent drug are different compared with those of the drug encapsulated in nanoparticles. Also, the physicochemical properties of nanoparticles influence their behavior in vivo. In the case of the abovementioned study, the more than twice higher half-time of epirubicin-cholesterol-modified pullulan nanoparticles in the blood plasma, namely, about 19 h compared with parent drug, suggested that the bioavailability of epirubicin was improved when it was loaded into cholesterol-modified pullulan nanoparticles.

Paclitaxel (PTX), also known as Taxol, a lipophilic substance extracted for the first time from *Taxus brevifolia* in 1971 and approved for medical use in 1993, is a chemotherapy molecule intensively used by specialists to treat cancers. Few years later it was discovered that paclitaxel was actually synthesized by endophytic fungi in the shrub bark.

Lee et al. (2012) succeeded to encapsulate paclitaxel in a pullulan derivative by the nanoprecipitation method. Previously, they hydrophobically modified the polysaccharide using acetic anhydride and resulting pullulan acetate. The viability of RAW264.7 macrophage cell line (more than 93%) in the presence of pullulan acetate nanoparticles having dimensions lower than 100 nm, tested in different concentrations, indicated the safety of particle usage in human subjects, too. The sustained paclitaxel release profile of nanoparticles tested in vitro against HCT116 human colon carcinoma cells, together with reduction in tumor growth observed in vivo experiments on the same type of carcinoma cells, indicated paclitaxel-incorporated pullulan acetate nanoparticles as a hopeful candidate for antitumor drug delivery.

Keeping in mind that the tumors secrete hyaluronidase, some researchers designed, in the first step, biodegradable nanoparticles consisted of acetylated pullulan (Yim et al. 2013). Then, the surface of particles was coated with hyaluronic acid (HA) and was loaded with paclitaxel in such a manner that nanoparticles recorded size of 200–250 nm and surface charge of 0 mV, respectively. After enzymatic degradation of hyaluronic acid, resulted cationic nanoparticles easily penetrated into tumor tissues by electrostatic interactions, and paclitaxel-loaded pullulan acetate nanoparticles were located only at the tumor site by the enhanced permeability and retention (EPR) effect (Scheme 4.6). It was found that, after intravenous injections with control and test samples to laboratory mice bearing heterogeneous cancer cells, the antitumor efficiency of drug-loading hyaluronic acid-treated nanoparticles was increased compared with the paclitaxel-pullulan acetate nanoparticles. This was due to the fact that degradable cationic nanogel has exerted a synergistic action on paclitaxel.

To replace sorafenib, an antiangiogenic molecule with unsatisfactory results for patients with hepatocellular carcinoma, the researchers have proposed an alternative treatment based on a combination of paclitaxel and combretastatin A4 labeled as CA4, two microtubule-associated inhibitors which displayed synergistic effects on tumor cells and tumor vasculature (Zhang et al. 2016). In this regard, they explored

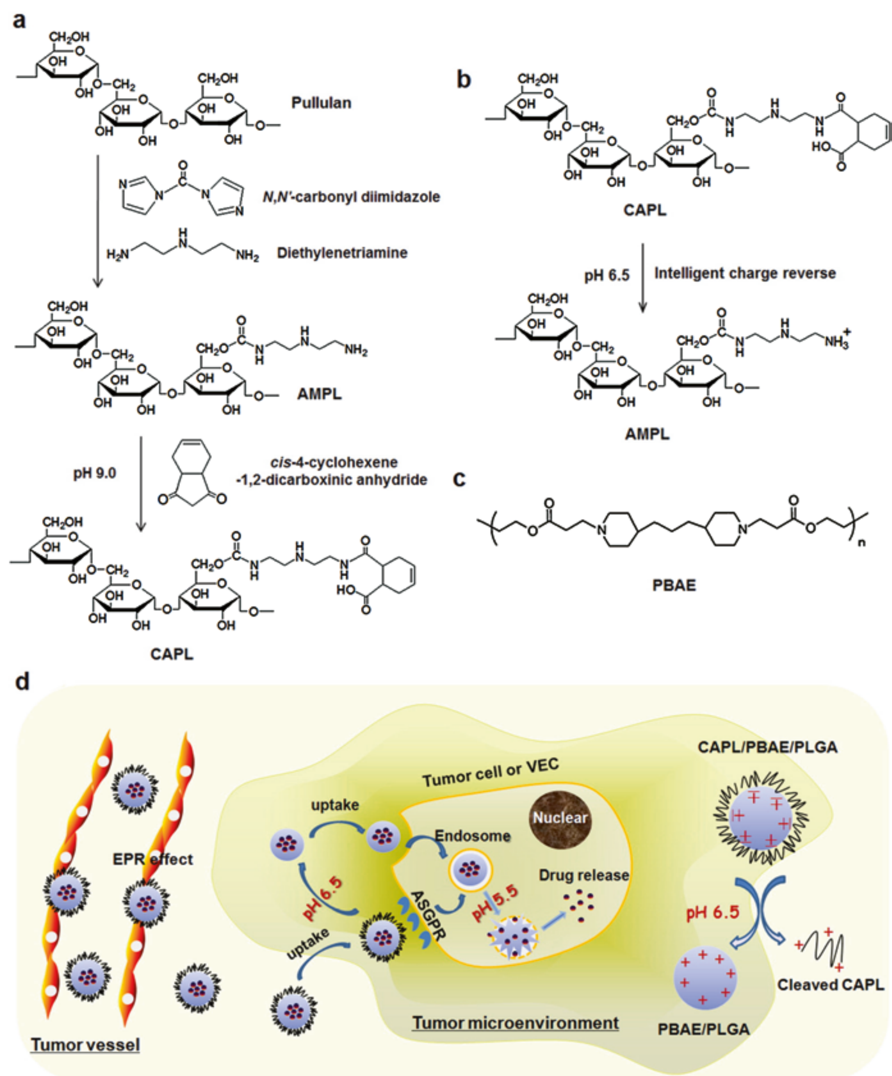


Scheme 4.6 A schematic illustration showing the composition of the degradable deep penetrating cationic nanoparticle (DpNG) and how it can be used to penetrate into tissue. DpNG was able to penetrate deep into the tissue via paracellular transport. Acetylated pullulan-paclitaxel nanoparticles (PA-PTX NPs) were located only at the tumor site by the enhanced permeability and retention (EPR) effect. (With permission of Elsevier, Yim et al. 2013)

in vivo and in vitro potentials of some complex nanoparticles with a role of carriers for each one therapeutic molecule, which could improve the drug solubility and delivery to the target tumors. These nanoparticulated systems contained charge-reversible pullulan CAPL-based shells and poly(β-amino ester)-poly(lactic-*co*-glycolic acid) PBAE-PLGA cores. The synthesis and the action mechanism of them in tumor cells were presented in Scheme 4.7. β-Carboxylic amide groups from the structure of charge-reversible pullulan were able to spontaneously break in acidic extracellular tumor microenvironment and subsequently detached from the nanoparticle surfaces due to the electrical repulsion between charge-reversible pullulan and positively charged poly(β-amino ester)-poly(lactic-*co*-glycolic acid) nanocores. Beside the cleavage of β-carboxylic amide bond in charge-reversible pullulan, the "proton sponge" effect of poly(β-amino ester) realized the efficient and orderly releases of combretastatin A4 and paclitaxel, too.

Hong et al. (2011) aimed to evaluate in vitro the antitumor effect of combinatorial targeted therapy against CT 26 colon carcinoma cell lines. They used paclitaxel-incorporated pullulan acetate nanoparticles, prepared by the nanoprecipitation-solvent evaporation method and having a medium size of 160 nm, and all-trans-retinoic acids-incorporated nanoparticles, with a diameter of 60 nm resulted from dialysis, using a methoxy poly(ethylene glycol)-grafted chitosan (ChitoPEG) copolymer. By incorporation in nanoparticles, both anticancer agents, possessing hydrophobic character in their original non-encapsulated state, have exercised their synergistic anticancer effects, being an inhibitor of tumor cell invasion mediated by all-trans-retinoic acids and an antiproliferative agent represented by paclitaxel, respectively.

Huang et al. (2017) developed novel reversibly stabilized core-cross-linked pullulan nanoparticles as a "natural" and intelligent platform for active asialoglycoprotein receptor-targeted delivery of paclitaxel, a highly hydrophobic diterpenoid.



Scheme 4.7 Composition and structure of charge-reversible pullulan-poly(*β*-amino ester)-poly(lactic-*co*-glycolic acid) (CAPL-PBAE-PLGA) nanoparticles and their mechanisms for in vivo delivery of antitumor drugs; (**a**) synthesis route of amine pullulan (AMPL) and CAPL; (**b**) pH-responsive charge-reversal mechanism of *β*-carboxyl amide bond in CAPL; (**c**) chemical structure of PBAE; (**d**) in vivo hepatoma-targeting and stepwise pH-responsive mechanisms of CAPL/PBAE/PLGA nanoparticles (enhanced permeability and retention (EPR) effect; vascular endothelial cell (VEC)). (Reprinted with permission of Elsevier, Zhang et al. 2016)

These core-cross-linked nanoparticles were prepared from pullulan-lipoic acid derivatives and then cross-linked in the presence of a catalytic amount of 1,4-dithio-DL-threitol. Lipoic acid, a natural product of human metabolic pathway, was chosen to be a part of pharmaceutical formulation because it only contains carboxyl chains, so the formation of by-products during esterification is negligible. But in alkaline environment with 1,4-dithio-DL-threitol, the disulfide-containing lipoic ring is prone to ring-opening polymerization, thereby forming inter-disulfide-bond linkage. The conjugation with lipoic acid transformed the hydrophilic pullulan into hydrophobic polysaccharide, facilitating nanoparticle formation and core-cross-linking. The resulted disulfide-cross-linked nanoparticles were reversibly stable under the extracellular environment but also can rapidly de-cross-link in intracellular reductive matrix and release the drug cargo. Thus, 1,4-dithio-DL-threitol was the trigger for paclitaxel release from cross-linked nanoparticles. The authors concluded that, in general, the combination of self-targeting and reversible cross-linking can serve as a promising strategy for polymer-based delivery of targeted hydrophobic or lipophilic drugs.

Mitoxantrone, also known as mitozantrone, is an anthracenedione antineoplastic agent able to block the topoisomerase 2 such that the cancer cell's DNA gets tangled up, finally stopping them to grow and reproduce. It was used in monotherapy to treat certain types of cancer, such as acute myeloid or lymphoblastic leukemia, non-Hodgkin's lymphoma, and metastatic breast cancer, or in combination with prednisone for metastatic hormone-refractory prostate cancer.

The entrapment or entanglement in drug mono- or co-delivery systems based on pullulan represents a viable approach even for mitoxantrone. To overcome the multidrug resistance of mitoxantrone, Mitha and Rekha (2014) conceived a nanoplatform labeled as PPEICD by coupling β-cyclodextrin and cationic poly(ethyleneimine) to hydrophilic pullulan for co-delivery of mitoxantrone and gene encoding tumor-suppressor protein p53. In this complex system, β-cyclodextrin was the nanocontainer for the drug, while the cationic moiety had the role to condense pDNA. The tests revealed that the conjugate was nontoxic and hemocompatible. The authors observed that combined drug and gene-loaded nanoplexes recorded a more apoptotic effect than either the drug or gene individually. Cell viability studies on HepG2 and C6 cell lines demonstrated that the PPEICD nanoplatform can efficiently and selectively deliver both p53 and mitoxantrone to cancer cells inducing high cell death.

Tao et al. (2012) were concerned about how biological systems respond to administration of drug-loaded nanoparticles. They suggested to take into consideration the effect of human serum albumin (HSA) binding on drug release. For this purpose, they prepared a pullulan-based nanocarrier for mitoxantrone and then studied the complexation with human serum albumin. Firstly, carboxyethyl pullulan (CEP) was synthesized with additive reaction using acrylic acid and pullulan. Then, cholesterol was introduced into carboxyethyl pullulan by esterification reaction, resulting in cholesterol-modified carboxyethyl pullulan (CHCP) conjugates. For comparison, cholesterol-modified pullulan conjugate was also synthesized. In this way, carboxyl groups conjugated to amphiphilic polysaccharide which generated a negative surface charge to cholesterol-modified pullulan nanoparticles, cholesterol-modified pullulan

and cholesterol-modified carboxyethyl pullulan, consisted of a hydrophobic core able to encapsulate hydrophobic drugs and hydrophilic shell responsible for solubility and stability of nanoparticles in aqueous solutions. Nanoparticles were loaded with mitoxantrone, recording thus an encapsulation efficiency of 58% and 50%, dimensions of 168 and 192 nm, and loading capacity of 7.12% and 6.14% in the case of cholesterol-modified pullulan and cholesterol-modified carboxyethyl pullulan, respectively. Also, cholesterol-modified carboxyethyl pullulan formed self-aggregated nanoparticles in aqueous solution with a spherical structure and zeta potential of −20 mV, in contrast to −1.2 mV of cholesterol-modified pullulan nanoparticles. The complexation of human serum albumin with mitoxantrone-loaded nanoparticles has been highlighted by structural changes in protein, meaning reduction of α-helical content after human serum albumin addition to the release media.

Yang et al. (2010) designed novel cholesterol-modified pullulan (CHSP) conjugates with succinyl linkages with different degrees of substitution of cholesterol, from 3.87 to 5.70 cholesterol groups per hundred glucose units. These almost spherical in shape nanoparticles with sizes in a range of 51–73 nm, depending on the degree of substitution, represented the carriers for the model anticancer drug mitoxantrone. Mitoxantrone-loaded cholesterol-modified pullulan self-aggregated nanoparticles recorded increased dimensions, from 153 to 174 nm, and mitoxantrone-loading capacity from 4.35% to 14.29%. Generally, it was observed a sustained release of mitoxantrone from cholesterol-modified pullulan nanoparticles and a decreased mitoxantrone release rate with increasing the pH value of media. Starting from cholesterol-modified pullulan, Yang et al. (2014) synthesized a series of biotin-conjugated compounds and explored their self-aggregation behavior in aqueous media and drug-loading capacity. The biotin-modified cholesteryl pullulan (Bio-CHSP) nanoparticles had the ability to load mitoxantrone with of an efficiency of 80%, 85%, or 53% and recorded dimensions of 205 nm, 170 nm, or 146 nm, in accordance with the degrees of substitution of biotin moiety, 20, 29, or 39. In vivo toxicity tests, realized on mice, revealed no representative histopathological changes in selected tissues derived from slaughtered animals and previously treated with biotin-modified cholesteryl pullulan nanoparticles.

Recent studies presented less explored pullulan-based systems involved in targeted administration of other antitumor drugs like 5-fluorouracil, gemcitabine, methotrexate, cassiarin A, or hydroxycamptothecin.

Beside plasmid DNA-expressing green fluorescent protein (pEGFP), a model gene, ***Methotrexate*** (MTX), as a model chemotherapy agent, was part of a complex nanoparticulated system with core-shell structure based on pullulan and poly(*β*-amino) ester (PBAE) proposed for hepatocarcinoma treatment (Liu et al. 2014). The gene was fully condensed with cationic poly(*β*-amino) ester polymer and formed the inner core of poly(*β*-amino) ester-pEGFP polycomplex. The prodrug methotrexate-pullulan, synthesized by conjugation of a drug to pullulan by ester bond, was then adsorbed on the surface of poly(*β*-amino) ester-pEGFP polycomplex and formed methotrexate-pullulan-poly(*β*-amino) ester-pEGFP nanoparticles. These nanoparticles realized the efficient transfection of pEGFP in HepG2 cells and exhibited significant inhibitory effect on the cell proliferation.

To maximize the therapeutic efficiency of ***10-Hydroxycamptothecin***, a poor water-soluble derivative of camptothecin, with low bioavailability and nonselective cytotoxicity to healthy cells or tissues, that inhibits DNA topoisomerase by enacting strand breaks in chromosomal DNA, Wang J. et al. (2014a) developed a convenient and safe delivery system based on self-assembled core-shell nanomicelles. These amphiphilic nanomicelles consisting of α-tocopheryl succinate-modified pullulan were used for the encapsulation of the hydrophobic anticancer drug 10-hydroxycamptothecin. The size of micelles varied from about 160 nm in empty or blank state to 170–250 nm in the case of nanomicelles loaded with 10-hydroxycamptothecin, but polydispersity index was maintained relatively constant (about 0.2). All drug-loaded polymeric micelles showed a sustained and pH-sensitive drug release pattern; a low pH accelerated the drug release rate. Depending on the molar ratio tocopheryl/pullulan and the weight ratio between a drug and copolymer, the loading capacity and entrapment efficiency of nanomicelles varied between 1.65–16.42% and 56–95%, respectively. MTT assay indicated that the blank polymeric nanomicelles were nontoxic to normal HEK293 and T lymphocyte cells. In vitro antitumor activity studies using MCF-7 cells showed that 10-hydroxycamptothecin-loaded nanomicelles having molar ratio tocopheryl/pullulan of 0.3 and weight ratio between drug and copolymer of 0.1 were more cytotoxic than free drug due to the higher uptake of drug-loaded nanomicelles compared with free 10-hydroxycamptothecin. The cellular uptake of micelles was an energy-dependent and actin polymerization-associated endocytic process which favored the rapid transport of drug from micelles into the cell nuclei.

Gemcitabine, known as 2′,2′-difluorodeoxycytidine, is a nucleoside analog used as chemotherapy which inhibits thymidylate synthetase, leading to inhibition of DNA synthesis and cell death. More exactly, as with fluorouracil and other analogs of pyrimidines, gemcitabine replaces one of the building blocks of nucleic acids, in this case cytidine, during DNA replication. The process arrests tumor growth, as new nucleosides cannot be attached to the "faulty" nucleoside, resulting in apoptosis or cellular "suicide" (https://www.drugbank.ca/drugs). This chemotherapy medication is effective in the treatment of solid tumors in non-small cell lung, pancreatic, bladder, ovarian, breast, or esophageal cancer and lymphomas.

Preparation of pharmaceutical formulations in the case of this drug with excellent antitumor proprieties is a challenge taking into account that the achievement of both drug solubility and stability in a solution state is required. Even that liposomal or particulated systems were tested in the past for delivery of gemcitabine, very few proved be effective for local or systemic controlled delivery of gemcitabine. A non-invasive alternative to surgery for the treatment of benign and malignant esophageal, gastrointestinal, and bile duct strictures or unresectable malignant obstructions supposes using nonvascular drug-eluting stents. Starting from these considerations, Moon et al. (2011) designed self-expandable metallic stents covered by a double-layered polymeric membrane composed from poly(tetrafluoroethylene) (PTFE) as a primary membrane and pullulan acetate. Polysaccharidic compound represented the drug-loading controlled-release matrix. While the thickness of the pullulan acetate-

poly(tetrafluoroethylene) membrane increased from 37.7 to 52.3 mm with the degree of acetylation ranging from 1.18 to 2.10, the gemcitabine loading in the membrane was also increased from 186 to 216 mg/cm^2. Pullulan acetate with a higher degree of acetylation had greater drug-loading capacity with more extended release of gemcitabine over 30 days. Pullulan acetate increased the hydrophilicity and smoothness of the membrane and possibly increased the tissue compatibility. In vivo tests revealed that subcutaneous CT-26 colon tumors totally regressed following the treatment with gemcitabine-pullulan acetate-poly(tetrafluoroethylene) film without systemic exposure of gemcitabine or its related toxicities (Fig. 4.6).

It seems that all experimental results recommended this type of stent for the treatment of malignant gastrointestinal cancer as well as cancer-related stenosis.

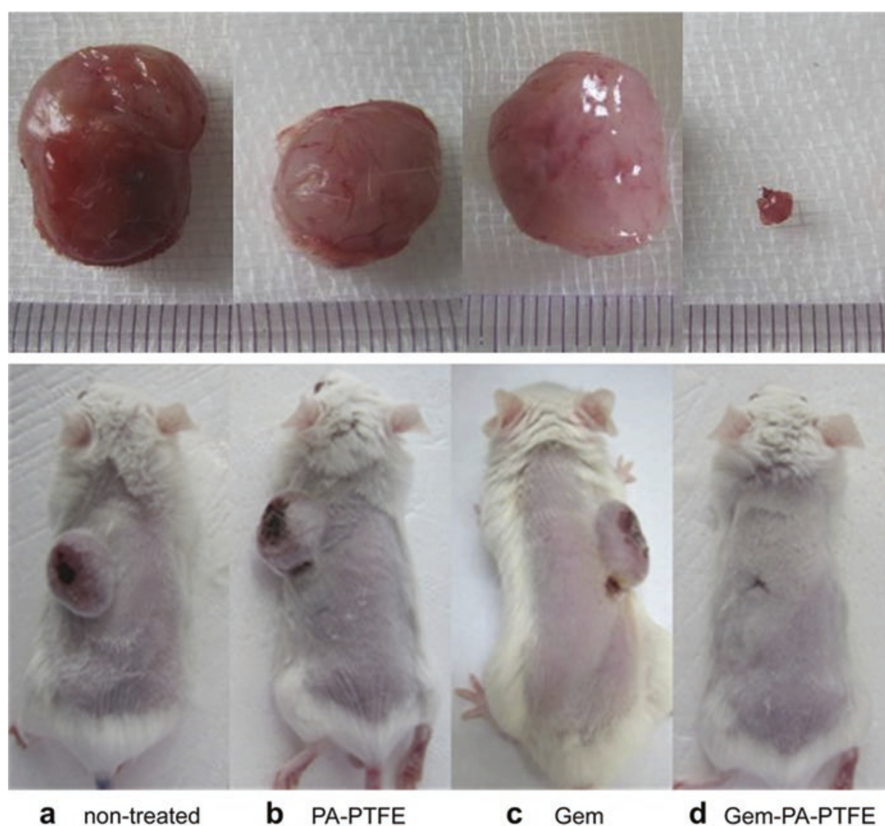


Fig. 4.6 In vivo inhibition of tumor growth. Stent membranes based on gemcitabine-pullulan acetate-poly(tetrafluoroethylene) (Gem-PA-PTFE) (**d**) or PA-PTFE (**b**) and Gem solution (**c**) were inserted or injected at subtumoral sites and were compared with non-treated specimens (**a**) Gem-eluting stent membranes (Gem-PA-PTFE) have exerted total regression of subcutaneous tumors induced by a murine colorectal carcinoma cell line CT-26 (**d**) (Reprinted with permission of Elsevier, Moon et al. 2011)

4.4 Pullulan-Based Systems with Antioxidant Activity and Free Radical Scavenging Properties

Under oxidative stress conditions, the excessive levels of reactive oxygen species (ROS), constantly generated and eliminated in the biological system under normal physiological conditions by regulatory pathways, can damage the cellular proteins, lipids, and DNA, leading to fatal lesions in cell that contribute to carcinogenesis (Dickinson and Chang 2011).

To overcome the aging and carcinogenesis of cells, it is important to maintain a balanced diet with compounds having antioxidant activity and free radical scavenging properties like rutin or curcumin. These types of substances need to be protected by pH variations until they get into blood circulation. Curcumin and rutin are polyphenolic compounds known to have antioxidant and anti-inflammatory activities. A combination therapy that included the two substances was tested to improve inflammatory bowel diseases.

Rutin, also called rutoside, quercetin-3-O-rutinoside, or sophorin, is a glycoside of flavonoid quercetin, endowed with hydroxyl functional groups; founded in a wide variety of plants, e.g., capers, citrus fruits, and apple; and used as an antioxidant, anticoagulant, or anti-inflammatory agent. This bioflavonoid was the subject of the fabrication of some electrospun antioxidant pullulan nanofibers containing 4% w/v Pluronic F127 solid dispersions and rutin (Lee et al. 2017). Compared with raw rutin, it seems that the version of the drug loaded on smooth and porous nanofibers presented an enhanced solubility and UV stability but at the same time retained its antioxidant capacity, recording a fast drug release profile.

Curcumin, the active ingredient of turmeric, is a substance that has been attributed in recent years to anti-inflammatory, antioxidant, antimicrobial, anticancer, and cholesterol-lowering properties, which could be efficiently adsorb in organism in the presence of Bioperine, the active ingredient extracted from the *Piper nigrum* plant. To improve its therapeutic index, the researchers developed new pullulan-based delivery systems such as micelles, nanogels, or nanoparticles in order to prolong the protection of curcumin during circulation or to improve its toxicity toward cancer cells.

Pullulan acetate nanoparticles loaded with curcumin had been proposed to treat liver diseases (Ganeshkumar et al. 2016). These curcumin-loaded pullulan acetate nanoparticles improved the encapsulation efficiency, stability, and therapeutic efficiency of curcumin with sustained release, under physiological conditions, and thus countered its poor bioavailability rendered by its physiochemical properties like low solubility and stability in aqueous medium. These new nanoparticles have been proposed as an effective hepatoprotective agent against diethyl nitrosamine, a compound founded in tobacco smoke which induced liver damage.

Sarika et al. (2015) developed pullulan-curcumin conjugates with and without targeting ligand in order to analyze and compare their cytotoxicity difference in HepG2 cells. The entire study was realized in few steps: (1) oxidation of pullulan-to-pullulan aldehyde (Pu Ald), (2) modification of lactobionic acid (LbA) with ethylene-

diamine to introduce amino groups required for Schiff's base reaction with pullulan aldehyde resulting in $LbANH_2$, (3) preparation of $LbANH_2$-pullulan aldehyde conjugate, (4) modification of curcumin by the addition of succinic anhydride SA, and (5) preparation of $LbANH_2$-pullulan aldehyde-curcumin succinic anhydride and pullulan-curcumin succinic anhydride conjugates. In this work, galactose-containing lactobionic acid is selected as the targeting ligand. Galactose-terminal molecules or conjugates are selectively recognized by asialoglycoprotein receptor present on the sinusoidal surface of the hepatocytes and transport them to lysosomes inside the liver cells.

The self-assembled amphiphilic micelles of $LbANH_2$-pullulan aldehyde-curcumin succinic anhydride conjugate and pullulan-curcumin succinic anhydride conjugate exhibited unimodal distribution with hydrodynamic diameter in the range of 355 nm and 363 nm, respectively. These values were recorded by dynamic light scattering (DLS) measurements and were slightly different from these from scanning electron microscopy (SEM): 290 nm and 320 nm, respectively. The negative zeta potential of pullulan-curcumin succinic anhydride of −15 mV and −10 mV in $LbANH_2$-pullulan aldehyde-curcumin succinic anhydride conjugate was derived from the presence of unreacted succinic acid groups on the conjugated curcumin molecules and indicated the absence of cross-linking reaction between pullulan and curcumin succinic anhydride.

In vitro drug release studies performed for 48 h at two different pH revealed a fast release of curcumin at acidic pH and relatively slower at pH 7.4. $LbANH_2$-pullulan aldehyde-curcumin succinic anhydride conjugate showed better and selective toxicity toward HepG2 cells compared to pullulan-curcumin succinic anhydride conjugate and manifested the targeting efficacy of galactose moiety. The cellular uptake studies of $LbANH_2$-pullulan aldehyde-curcumin succinic anhydride and pullulan-curcumin succinic anhydride conjugates demonstrated ameliorated accumulation of the galactosylated conjugate in HepG2 via asialoglycoprotein receptor-mediated pathway. All these results showed that both conjugate micelles are suitable candidates for delivery of curcumin toward hepatocarcinoma cells.

The polymeric micelles, liposomes, polymeric nanoparticles, lipid-based nanoparticles, and hydrogels represented solutions for loading of low molecular weight and hydrophobic polyphenols like curcumin. This chemopreventive and therapeutic agent is widely used in liver pathologies due to its potent antioxidant and anti-inflammatory properties. To prevent its extremely low aqueous solubility, rapid systemic elimination, inadequate tissue absorption, and degradation at alkaline pH, Yuan et al. (2014) synthesized a novel carrier for curcumin based on glycyrrhetinic acid-pullulan (GAP) with different degrees of substitutions (6.2, 4.5, 1.2) of glycyrrhetinic acid, an important bioactive compound in traditional Chinese medicine liquorice with anti-inflammatory, antiviral, antimicrobial, antioxidative, and anticancer activities and immunomodulatory, hepatoprotective, and cardioprotective properties. The self-assembled spherical nanoparticles were prepared by the dialysis method and were tested for their capacity to load curcumin in a simulated physiological environment; thus, liquid chromatography recorded values of 10%, 7%, and 4% for the same w/w ratio of 10 for drug/carrier, depending on the

degree of substitution of each nanoparticle. The curcumin release was sustained and pH-dependent. A MTT study showed curcumin-glycyrrhetinic acid-pullulan nanoparticles with higher cytotoxicity in HepG2 cells than free curcumin, while glycyrrhetinic acid-pullulan nanoparticles had no significant cytotoxicity. Furthermore, the curcumin-GAP nanoparticles could significantly improve the water solubility, stability, and cytotoxicity of curcumin in HepG2 cells in vitro, which may due to the liver target of glycyrrhetinic acid and the inherent affinity of pullulan biomolecules for the liver cells.

D'Souza et al. (2013) have proposed to compare *in silico* to in vivo the asialoglycoprotein receptor ligand binding for hepatic targeting of curcumin-loaded Gantrez (GZ) nanoparticles. For this purpose, carbohydrate-based hepatocyte asialoglycoprotein receptor ligands were selected: kappa carrageenan KC, arabinogalactan AG, and pullulan P. Arabinogalactan and kappa carrageenan are galactose based, pullulan is a glucose-based polymer, and Gantrez is the free acid form of the copolymer of methyl vinyl ether and maleic anhydride (PVM/MA) with excellent film-forming capacity and mucosal bioadhesivity. Curcumin-Gantrez nanoparticles with dimensions between 530 and 550 nm and polydispersity index of 0.31–0.38 were prepared by nanoprecipitation and anchored with the ligands by nonspecific adsorption onto preformed nanoparticles; the change in zeta potential values from −12 to 30 mV confirmed adsorption of the ligands. After 1 h from intravenous administration of nanoparticles to rats, the in vivo hepatic accumulation lowered in the order: curcumin-Gantrez-arabinogalactan, curcumin-Gantrez-kappa carrageenan, and curcumin-Gantrez-pullulan. At the end of 6 h, pullulan exhibited maximum hepatic accumulation and arabinogalactan minimum accumulation. In turn, *in silico* study revealed maximum hepatic accumulation for arabinogalactan and pullulan. The authors concluded that arabinogalactan ligand could enable to a rapid and high uptake of curcumin nanoparticles, while pullulan ligand facilitated prolonged hepatic retention of nanoparticles.

4.5 Pullulan-Based Systems with Anti-inflammatory and Immunomodulatory Properties

Anti-inflammatory drugs like indomethacin, diclofenac, naproxen, piroxicam, and etanercept and immunomodulatory drugs such as silymarin and cucurbitacin B were the subject of entrapment or encapsulation in pullulan-based systems.

Beside analgesic and antipyretic effects, ***Indometacin*** is a nonsteroidal anti-inflammatory agent with anti-inflammatory activity that inhibited the activity of cyclooxygenase and consequently the synthesis of prostaglandins involved in pain, fever, and inflammation. Indomethacin was used as hydrophobic model drug to be incorporated by different methods like nanoprecipitation and dialysis at room temperature, using N,N-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO) as organic solvents, into thermoresponsive pullulan-grafted-poly(*N*-isopropylacrylamide) (P-*g*-pNIPAM) copolymers. The higher loading and entrap-

ment efficiency were obtained by dropping in water of a *N*,*N*-dimethylformamide solution containing both the copolymer and the drug. For 1/1 weight ratio of indomethacin/polymer and 10 g/L polymer concentration, the drug entrapment efficiency was up to 80%. The formation of indomethacin-loaded pullulan-*g*-poly(*N*-isopropylacrylamide) nanoparticles was attributed to hydrogen bondings between indomethacin-poly(*N*-isopropylacrylamide) and pullulan. In vitro release of indomethacin from the indomethacin-loaded pullulan-*g*-poly(*N*-isopropylacrylamide) nanoparticles in phosphate-buffered saline solution at pH = 7.4 and acetate buffer at pH = 5 depended on the molecular weight of poly(*N*-isopropylacrylamide) units in the copolymer, indomethacin content, and temperature (Constantin et al. 2017).

Diclofenac or [2-(2,6-dichloroanilino)phenyl]acetic acid), an acetic acid nonsteroidal anti-inflammatory drug primarily available as the sodium salt, has a similar mechanism of action and effects as indomethacin. It is usually used to treat various symptoms of arthritis and spondylitis.

When they wanted to delay the delivery of diclofenac up to 30 min in rat organism, some researchers (Cristescu et al. 2012) coated the drug pellets with triacetate-pullulan thin films by matrix-assisted pulsed laser evaporation method. In this experiment, the radiation generated by a pulsed excimer KrF*laser source (λ = 248 nm, τ = 20 ns) operating at 2 Hz repetition rate was used for ice targets evaporation.

Other authors (El-Malah and Nazzal 2013) prepared some films from a tertiary mixture of pullulan, poly(vinylpyrrolidone), and hypromellose as a matrix for sodium diclofenac loading. They were interested to apply the real-time spectroscopy in conjunction with statistical design for the optimization and development of nonconventional intraoral delivery system such as fast-dissolving films. In this regard, they compared the mechanical properties of blank film having 2–49 MPa for tensile strength and 1–21 MPa% for Young's modulus, over time interval of 21–105 s, with those of diclofenac-loaded films which recorded tensile strength and Young's modulus of the film of 11.21 MPa and 6–78 MPa%, respectively. In addition, the dissolution of the optimized film was found to commence almost immediately with 50% of the drug released within 1 min.

Mocanu et al. (2014) succeeded to prepare anionic pullulan nanoparticles for diclofenac delivery. The thermoassociative nanoparticles were obtained through the cross-linking reaction of periodate oxidized carboxymethyl pullulan with two difunctional Jeffamines: ED-600 and ED-2003. Nanoparticles had spherical shape, amphiphilic character, and dimensions of about 200 nm, suitable properties for diclofenac entrapment. Diclofenac release occurred gradually; the less cross-linked nanoparticles released the drug faster than more cross-linked ones. In the first 30 minutes, the release was fast of about 18–32% diclofenac due to the "burst effect," and then a slower sustained and controlled release occurred throughout the incubation period, the release amount being of 72–99.5%. At 40 °C, diclofenac was released much faster, with about 70% being released in 1 h.

Naproxen or (2S)-2-(6-methoxynaphthalen-2-yl) propanoic acid, an anti-inflammatory agent with analgesic and antipyretic properties, is a drug used in both

its acid and sodium salt forms to treat pain and inflammation in primary dysmenorrhea, rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, tendinitis, bursitis, and acute gout. Bishwambhar and Vuppu (2012) synthesized pH-sensitive pullulan acetate microparticles as biocompatible support for naproxen. In vitro drug release study showed that naproxen was released from naproxen-loaded microspheres with a drug content of 50 (wt %) much faster at pH 7.4 (PBS) than at pH 1.2 (HCI in PBS). If at acidic pH, the protonated carboxylic groups of pullulan acetate not permitted the swelling of pullulan acetate microspheres; at neutral pH the microsphere swelling effectively enhanced due to the ionization of –COOH groups. In this way, even the drug release was influenced by pH changes. In the first 4 h, at initial pH 1.2, no naproxen release was observed. When the pH of the aqueous environment was changed to 7.4, the drug was released from microspheres quickly, because at pH 1.2, there is not any disintegration of microsphere, while at pH 7.4, the disintegration of the microspheres facilitated the rapid sustainable release of naproxen.

To suppress the disadvantages derived from the poor aqueous solubility and the gastrointestinal side effects associated with oral naproxen administration, Choi et al. (2017) used the host-guest chemistry to tailor hydroxypropyl cyclosophoraose-pullulan (HPCys-pull) microspheres as a novel hybrid system for controlled naproxen delivery. Cyclosophoraoses are macrocyclic carbohydrate host molecules isolated from *Rhizobium* strains of soil bacteria. In this study, hydroxypropyl cyclosophoraose-conjugated pullulan microspheres were prepared using the emulsion cross-linking method. Due to pendant hydroxypropyl cyclosophoraose moieties on the matrix of pullulan, the naproxen complexation ability of microspheres increased 4.2-fold time compared with pullulan microspheres. In addition, in vivo naproxen release from microsphere was observed in Sprague-Dawley rat; the naproxen levels in the plasma after oral administration of naproxen-loaded microsphere were maintained for 72 h.

Piroxicam or 4-hydroxy-2-methyl-1,1-dioxo-*N*-(pyridin-2-yl)-2H-1λ^6,2-benzothiazine-3-carboxamide, a nonsteroidal anti-inflammatory drug that blocks the enzymatic activity of cyclooxygenase-1 and suspends the production of prostaglandins and inhibits the migration of leukocytes into sites of inflammation, has a long half-life which enables it to be administered once daily in the case of postoperative pain, rheumatoid arthritis, and osteoarthritis. The anti-inflammatory piroxicam was loaded by the solvent casting technique followed by autoclave treatment in nanohydrogel platform composed from pullulan modified with the hydrophobic and fluorescent molecule riboflavin tetrabutyrate (Di Meo et al. 2015). The nanohydrogels were shown to be able to load hydrophobic piroxicam, acting as solubility enhancers for a drug. The purification of the system from unloaded drug was obtained by mild centrifugation, and the free piroxicam was spectrophotometrically quantified, resulting in thus an entrapping efficiency of 11% w/w.

Etanercept, a drug commercialized as Enbrel and used as an emerging therapeutic protein for rheumatoid arthritis and plaque psoriasis, binds specifically to tumor necrosis factor (TNF), a natural cytokine involved in normal inflammatory and immune responses, and thereby modulates biological processes which are induced

or regulated by tumor necrosis factor. The clinical applications of etanercept are limited by its instability and low bioavailability. To stabilize etanercept and prolong its therapeutic effect against rheumatoid arthritis, Jung et al. (2013) applied a sophisticated approach by using temperature-induced noncovalent interaction controllable complex without covalent cross-linking. They developed complexes based on succinylated pullulan-*g*-oligo(L-lactide) labeled as SPL, a temperature-sensitive amphiphilic polyelectrolyte, and the positively charged etanercept. The complexation was realized via electrostatic interactions at 4 °C below the clouding temperature of polymer such that the resulting complex significantly improved salt and serum stability of drug with increased hydrophobic interactions at temperatures (physiological condition, 37.5 °C) above the clouding temperature. Because of the exposure of the functional active site and the molecular chaperone-like effect of the hydrophobic copolymer, the long-term stability of etanercept in an aqueous environment was improved by temperature-induced noncovalent interaction controllable complex.

Cucurbitacin B, a triterpenoid constituent of Cucurbitaceae plant species, has a broad range of biological activity and effects: anti-inflammatory, antitumor, hepatoprotective, and hepatocurative. Because the phospholipid-bile salt-mixed micelles (PL-BS-MMs) are potent carriers for oral adsorption of poorly soluble in water drugs, Lv et al. (2015) prepared fast-dissolving oral films (FDOFs) containing phospholipid-bile salt-mixed micelles (Fig. 4.7). First, the phospholipid-sodium deoxycholate (PL-SDC)-mixed micelles incorporated cucurbitacin B and then were mixed with a film former (50 mg/mL pullulan PI-20 grade, M_w = 2,000,000), a plasticizer (40 mg/mL poly(ethylene glycol) PEG 400), and a sweetener (aspartame). By solvent casting method, FDOF containing nanoparticles resulted. In these structures, cucurbitacin B was embedded into the hydrophobic core of nanomicelles, making it released as a nanoparticle. The concentration of cucurbitacin B was determined to be 5 mg/mL. Results showed that narrow size distributed nanomicelles with a mean particle size of 86 nm and zeta potential of −31 mV w obtained in the optimized cucurbitacin B-phospholipid-sodium deoxycholate-mixed micelle formulation. The FDOFs containing phospholipid-sodium deoxycholate-mixed micelles not only kept the absorption properties as same as phospholipid-sodium deoxycholate-mixed micelles but also significantly increased the oral bioavailability of cucurbitacin B compared to the cucurbitacin B suspension. An uncertainty about how much of the cucurbitacin B is absorbed buccally and how much from the intestine after unintentional swallowing of cucurbitacin B containing saliva remained in work.

Silymarin, a hepatoprotective drug extracted from *Silybum marianum* fruits, has antioxidant and membrane-stabilizing properties and acts as a defensive material in different toxic forms of liver diseases like liver cirrhosis and alcoholic liver disease. It is broadly used as a food additive and is considered to be safe for human use. This hydrophobic drug was encapsulated in pullulan acetate with an efficiency of about 90% resulting in silymarin-loaded pullulan acetate nanoparticles with a medium size of 720 nm. The in vitro release of silymarin was carried out in phosphate-buffered saline solution with pH 7.4: in the first hour these released only 19.05% of

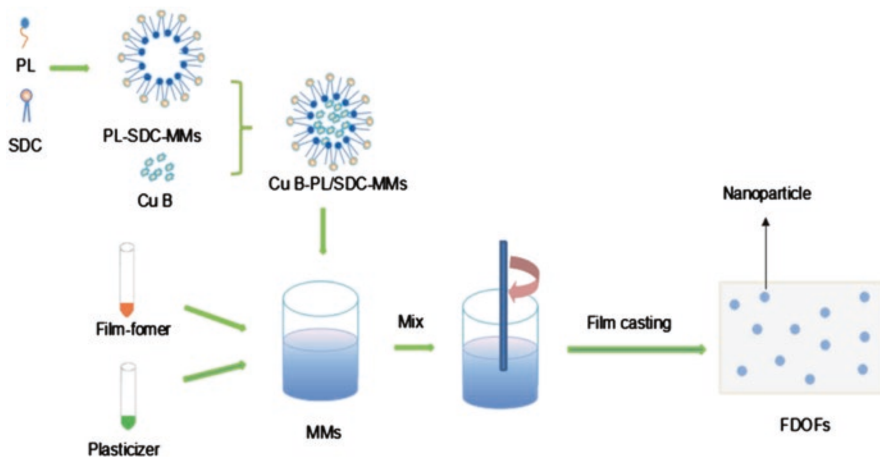


Fig. 4.7 Process schematic for preparation of fast-dissolving oral films (FDOFs) containing drug nanoparticles. CuB = Cucurbitacin B; PL-BS-MMs = phospholipid-bile salt-mixed micelles; SDC = sodium deoxycholate (detergent); PL = pullulan PI-20 grade (M_w 2,000,000) film former; poly(ethylene glycol) 400 (PEG 400) = plasticizer; aspartame = sweetener. (Reprinted with permission of Springer Nature, Lv et al. 2015)

a drug; after the seventh hour, the drug release was 61.14%. The release of a drug at the 24th hour was in therapeutic level to inhibit hepatic cancer cells (Santhosh Kumar et al. 2012).

Guhagarkar et al. (2015) evaluated the role of pullulan as hepatic targeting agent. In this regard, they designed poly(ethylene sebacate)-silymarin (PES-SIL) nanoparticles and then modified their surface with pullulan by nanoprecipitation. Resulted poly(ethylene sebacate)-silymarin-pullulan nanoparticles were evaluated for hepatoprotective activity in a model of carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats. In the case of rats pretreated with poly(ethylene sebacate)-silymarin-pullulan nanoparticles, the levels of serum transaminases and alkaline phosphatase were reduced compared with the group treated with CCl_4 confirming in this way the role of pullulan as hepatic targeting agent.

4.6 Pullulan-Based Systems with Antilipidemic and Antiglycemic Properties

The anomalies of lipid and carbohydrate metabolisms influence the normal functioning of some organs and systems. The correction of them with the least adverse effects and as targeted as possible was the premise of designing revolutionary drug delivery systems.

Apolipoproteins B, namely, Apo B-48 produced by the small intestine and Apo B-100 secreted by the liver, are amphipathic glycoproteins implied in the metabolism

of plasma lipoproteins, mainly very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL). Elevated levels of Apo B and LDL increase the risk of atherosclerotic diseases such as coronary artery disease and heart disease. The inhibition of Apo B synthesis by short-interfering RNA targeting of the Apo B gene labeled as Apo B siRNA efficiently has reduced the serum LDL. Kang et al. (2012) combined the chemically modified Apo B siRNA, possessing increased enzymatic stability, with a liver-targeted gene delivery system to reduce both serum LDL and Apo B mRNA in the liver. More precisely, they developed a poly(etyleneimine)-pullulan (PEI-PUL) conjugate for efficient delivery of chemically modified Apo B siRNA into the liver. The sizes of poly(etyleneimine)-pullulan-Apo B-siBNA complexes were lower than 260 nm, and their zeta potentials were almost neutral. The poly(etyleneimine)-pullulan carrier prepared with high molecular weight pullulan (M_w = 107,000) was more stable in the bloodstream and more efficiently reduced the serum LDL and Apo BmRNA than the carrier prepared with low molecular weight pullulan (M_w = 5900).

Fenofibrate or propan-2-yl 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoate is an antilipidemic agent which reduces elevated LDL cholesterol, total cholesterol, triglycerides, and Apo B and increases HDL cholesterol in adult patients with primary hypercholesterolemia or mixed dyslipidemia, both being risk factors of cardiovascular disease. In the treatment of hypercholesterolemia and hypertriglyceridemia, this fibrate drug is also indicated to diet as adjunctive mono- or co-therapy, alone or along with statins.

The pharmaceutical industry tries to produce micro- and nanocrystals of drugs in order to increase their solubility and in vivo bioavailability. In this regard, the addition of additives which greatly improve the stability of a drug suspension system is just an empiric or heuristic step with unpredictable results. The combination of a biocompatible polymers and surfactants has been widely used in controlling the crystal growth of drugs, but understanding of the cooperative behavior between them remains unexplained most of the time. Zhu et al. (2011) used atomistic molecular dynamic simulations to investigate and predict the additive interactions and, thus, to evaluate the stabilization potential of individual and multiple surface-active additives on the surface of the model drug fenofibrate. It has poor intrinsic water solubility (lower than 0.29 mg/L at 37 °C), so the additions of nonionic surfactant Tween 80, anionic surfactant sodium dodecyl sulfate, neutral pullulan, and thermogelling hydroxypropyl methylcellulose were examined. The examination of three distinct starting configurations of the dual-additive systems like side-by-side, surfactant on polymer, and polymer on surfactant revealed that a synergism between additives which interacted with the crystal surface, in some cases, is significantly stronger than individual additives alone. The predictions of mixed additives indicated a decrease of effectiveness as follows: hydroxypropyl methylcellulose-sodium dodecylsulfate, hydroxypropyl methylcellulose-Tween 80, pullulan-Tween 80, and pullulan-sodium dodecylsulfate. The predicted computationally results were validated using a series of anti-solvent crystallization measurements. For example, the snapshots of the simulations, shown in Fig. 4.8, visually exhibit the stronger binding of hydroxypropyl methylcellulose on the surface of fenofibrate when compared to pullulan.

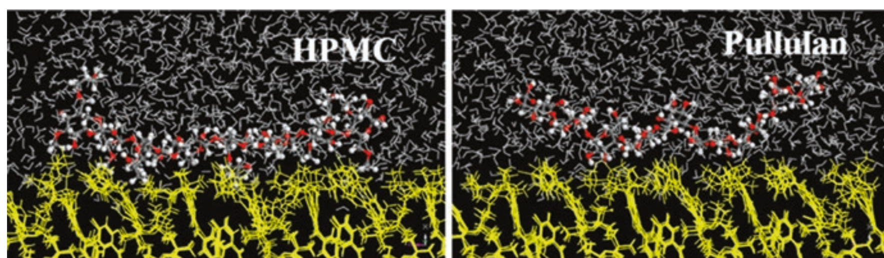


Fig. 4.8 Snapshots of binding configurations of hydroxypropyl methylcellulose (HPMC) and pullulan on the fenofibrate crystal surface. (Reprinted with permission of Elsevier, Zhu et al. 2011)

Pramlintide, traded as *Symlin*, is a synthetic analog of amylin, a hormone that is released into the bloodstream, in a similar pattern as insulin, after a meal. Chemical structure of pramlintide differs from human amylin by the replacement of alanine, serine, and serine at positions 25, 28, and 29, respectively, with proline. Used as a relatively new adjunct treatment for diabetes type 1 and type 2, pramlintide acts to improve the glycemic control through modulation of the rate of gastric emptying, through prevention of postprandial rise in glucagon levels, and by increasing sensations of satiety, thereby reducing caloric intake and potentiating weight loss (https://www.drugbank.ca/drugs). Patel and Patel (2012) investigated the feasibility of prolonged delivery of this synthetic peptide from biodegradable polymer microsphere depot formulation. They prepared pramlintide microspheres by water-in-oil-in-water double emulsion method. The encapsulation with biodegradable polymers has been considered as an alternative to injectable multi-dose liquid formulation of drug. The aqueous phase consisted from pramlintide in water was added to oil phase composed from pullulan acetate in dichloromethane and sonicated. The obtained emulsion was emulsified in continuous phase of poly(vinyl alcohol) (PVA) in water and after removing of dichloromethane and hardening the polymer; the resultant microspheres with a size of 70–98 μm and entrapment efficiency of ~80% were collected by centrifugation, washed three times with water for injection, and freeze-dried. It seems that, during the drying process, the pramlintide molecules migrated from the inner phase to the surface of the microspheres. In addition, with the degradation of pullulan acetate, the microsphere morphology changed from an original smooth surface to a porous surface after 7 days, and, finally, microsphere disruption was observed after 14 days. Thus in vitro drug release profile of pramlintide-loaded microspheres exhibited a low "burst effect" and a slow drug release rate for over 14 days, too.

4.7 Pullulan-Based Systems for Heart Diseases

The heart and blood circulation systems are the sources of nutrients and oxygen to all cells in the body. When the lipid and carbohydrate metabolisms or hormones work badly, even the heart and blood vessels are affected. Thus, the most visible and

encountered effect is high blood pressure. Over time, the researchers have developed various molecules for the treatment of hypertension.

Atenolol or 1-p-carbamoylmethylphenoxy-3-isopropylamino-2-propanol is a cardioselective *β*-adrenergic blocker with properties and potency similar to propranolol, but without a negative inotropic effect, used in the management of hypertension and edema. It can also help to prevent heart attack or heart damage after a heart attack. Like metoprolol, atenolol competes with sympathomimetic neurotransmitters such as catecholamines for binding at *β1*-adrenergic receptors in the heart and vascular smooth muscle, inhibiting sympathetic stimulation. This results in a reduction in resting heart rate, cardiac output, systolic and diastolic blood pressure, and reflex orthostatic hypotension. Higher doses of atenolol also competitively block *β2*-adrenergic responses in the bronchial and vascular smooth muscles (https://www.drugbank.ca/drugs). Sushmitha et al. (2014) developed by solvent casting method mucoadhesive fast melt-away wafers based on hydroxypropyl methylcellulose and pullulan as oral drug delivery system for atenolol. The formulation with hydroxypropyl methylcellulose E6 400 mg has shown better in vitro dissolution profile compared with other formulations. The in vitro drug release study showed a drug release of 98.83% in 150 seconds from the wafer.

Nebivolol or 1,1′-[bis(6-fluoro-3,4-dihydro-2H-1-benzopyran-2-yl)]-2,2′-iminodiethanol, a new third-generation *β1*-adrenergic blocker, mainly used in the treatment of hypertension, induces endothelium-dependent arterial relaxation in a dose-dependent manner, by stimulation of the release of endothelial nitric oxide. Consequently, nitric oxide acts to relax the vascular smooth muscle cells and inhibits the platelet aggregation and adhesion (https://www.drugbank.ca/drugs). Using the solvent casting technique and an optimized formulation, Parejiya et al. (2013) designed a quick-dissolving film of nebivolol hydrochloride composed from hydroxypropyl methylcellulose, pullulan, and poly(vinylpyrrolidone), alone or in combination, glycerol as plasticizer, aspartame as sweetener, and *passion fruit* flavor. The high procent of drug release from the film in simulated saliva and simulated gastric fluid indicated that the simplex lattice design of optimized film can be successfully used in drug delivery systems for drugs with high first-pass metabolism.

Metoprolol or 1-[4-(2-methoxyethyl)phenoxy]-3-[(propan-2-yl)amino]propan-2-ol is a cardioselective *β1*-adrenergic blocking agent used for the management of acute myocardial infarction, heart failure, angina pectoris, and mild to moderate hypertension. It may also be used for supraventricular tachyarrhythmias and prophylaxis for migraine headaches. Metoprolol is structurally similar to bisoprolol, acebutolol, and atenolol in that it has two large *β1*-selective substituents in the *para* position of the benzene ring. Metoprolol possesses a single chiral center and is administered as a racemic mixture. At low doses, metoprolol selectively blocks cardiac *β1*-adrenergic receptors with little activity against *β2*-adrenergic receptors of the lungs and vascular smooth muscle. Receptor selectivity decreases with higher doses. Unlike propranolol, metoprolol does not exhibit membrane-stabilizing or intrinsic sympathomimetic activity. The membrane-stabilizing effects are only observed at doses much higher than those needed for *β*-adrenergic blocking activity (https://www.drugbank.ca/drugs). Metoprolol succinate was loaded in different mixed polymeric films like pullulan-poly(acrylamide) (Vishwanath et al. 2012a) or

chitosan-pullulan (Vishwanath et al. 2012b). In the case of both types of films, the fast drug release kinetic was fitted to the Higuchi and Korsmeyer-Peppas model indicating a Fickian-controlled diffusion mechanism.

Captopril or (2S)-1-[(2S)-2-methyl-3-sulfanylpropanoyl]pyrrolidine-2-carboxylic acid is a competitive inhibitor of angiotensin-converting enzyme responsible for the conversion of angiotensin I to angiotensin II, used for the treatment of hypertension and some types of congestive heart failure and nephropathy (https://www.drugbank.ca/drugs). Considering the short half-life of this drug, the researchers explored an alternative to prepare gastroretentive matrix tablets of captopril. They prepared floating matrix tablets by direct compression technique using different polymers and natural gums: karaya gum, gellan gum, pullulan gum, and hydroxypropyl methylcellulose. The drug release tests showed that the formulations containing pullulan gum and gum karaya in a ratio of 3:1 as polymer matrix exhibited the better release of drug (Reddy et al. 2011).

4.8 Pullulan-Based Systems for Bone Diseases

Pamidronate, named pamidronic acid or (3-amino-1-hydroxy-1-phosphonopropyl) phosphonic acid, belongs to the class of drugs called bisphosphonates which are adsorbed in bone to hydroxyapatite or calcium phosphate crystals and may directly block dissolution of this mineral component of bone. In vitro studies also suggest that the inhibition of osteoclast activity contributes to inhibition of bone resorption. In postmenopausal women, it reduces the elevated rate of bone turnover, leading to, on average, a net gain in bone mass. Because pamidronate reduces the breakdown of bones, it is used in the treatment of Paget's disease of bone, to prevent osteoporosis, to reduce high levels of calcium in the blood associated with malignancy, and to reduce the breakdown of bone due to metastases of breast cancer or multiple myeloma (https://www.drugbank.ca/drugs).

The bone, cartilage, skin, and nerve tissues could be regenerated and repaired using cells and biomaterials for the drug delivery system of growth factors and cell scaffolds. The healing and repairing of bones are generally monitorized by computer tomography (CT), X-ray, and magnetic resonance imaging (MRI). A new trend tries to visualize the bone tissue regeneration by bone-specific multimodal imaging. Considering the high affinity of pamidronate for the hydroxyapatite of bone tissue, Liu et al. (2012) prepared pamidronate-pullulan conjugates containing two imaging probes: Cy5.5 monofunctional *N*-hydroxysuccinimidyl ester Cy5.5/DTPA as fluorescent moiety and gadopentetate dimeglumine (Gd^{3+}-DTPA) as MRI agent (Fig. 4.9). The solutions of resulted conjugates, namely, pamidronate-pullulan-F/M labeled as Gd^{3+}-chelated pamidronate-pullulan-Cy5.5/DTPA, were intravenously injected at different time intervals for 4 weeks into the mouse model of ectopic bone formation, prepared by subcutaneous implant with a gelatin hydrogel incorporating bone morphogenetic protein BMP-2. As expected, the histology examination confirmed the pamidronate functioned well to enhance the

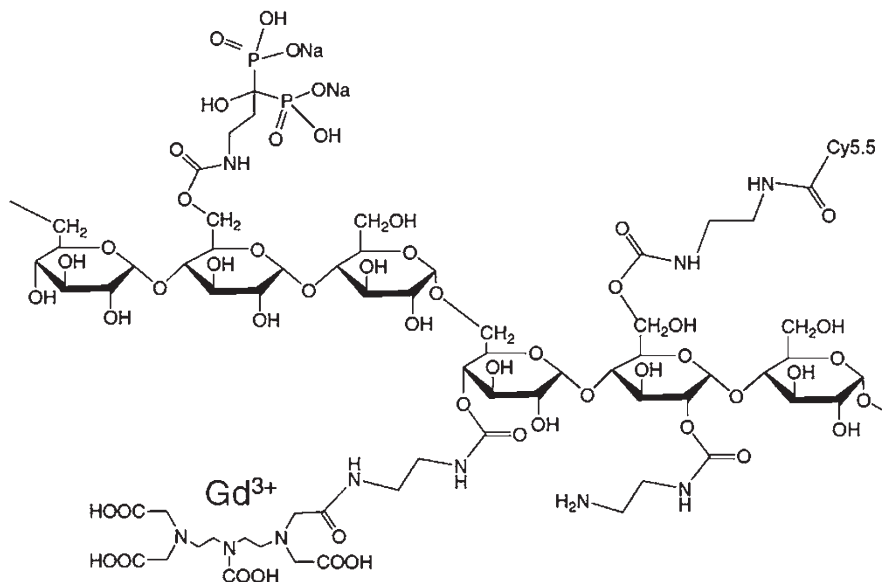


Fig. 4.9 Chemical structure of pamidronate-pullulan conjugate PA-pullulan-F/M bearing Cy5.5 monofunctional *N*-hydroxysuccinimidyl ester (Cy5.5/DTPA) as fluorescent (F) moiety and Gd^{3+}-DTPA (gadopentetate dimeglumine) as magnetic resonance imaging (MRI) agent. (Reprinted with permission of Elsevier, Liu et al. 2012)

hydroxyapatite affinity of pullulan and the in vivo accumulation at the site of bone tissue newly formed. Thus, this drug delivery system proved to be noninvasive multimodal imaging system for monitorization of bone regeneration, too.

Risedronate or [1-hydroxy-1-phosphono-2-(pyridin-3-yl)ethyl]phosphonic acid, traded as Actonel, is a pyridinyl bisphosphonate which inhibits osteoclast-mediated bone resorption and modulates bone metabolism, and it is indicated for the treatment and prevention of osteoporosis in postmenopausal women and treatment of Paget's disease of bone (https://www.drugbank.ca/drugs).

Usually, the patients treated with oral tablets of risedronate should swallow the drug with a full glass of water and avoid lying down for at least 30 min. To facilitate esophageal passage and decrease the mucosal adherence generating of gastrointestinal injuries, some researchers developed polymeric microparticles based on pullulan and Eudragit® S100 by spray-drying technique (de Arce Velasquez et al. 2014). Only microparticles prepared with high amounts of Eudragit® S100 resulted in a satisfactory gastroresistance profile. Then, tablet formulations were prepared by direct compression of risedronate-loaded microparticles using direct tableting excipients like poly(vinylpyrrolidone), colloidal silicon dioxide, stearate magnesium, and microcrystalline cellulose. It was expected that risedronate-based tablets are prepared in this way to reduce the possibility of high local concentration of a drug near the gastrointestinal mucosa.

4.9 Conclusions and Future Remarks

Pullulan is a versatile molecule for chemical modifications made to functionalization and to increase the chance of electrostatic, hydrogen bonding type or hydrophobic interactions with a variety of other low molecular weight compounds. In some cases, pullulan itself had the role of a cellular target, e.g., liver cell receptors or cancer cells.

Most of drugs are hydrophobic molecules and poorly soluble in water. To enhance the therapeutic effects of drugs, in design of drug delivery systems, even the hydrophilic-hydrophobic balance should be considered. There are many types of carriers, micelles, nanoparticles, microparticles, conjugates, films, and hydrogels, each one with more or less favorable properties for loading and delivery of drugs.

The new trend takes into consideration a simulation stage of interactions between polymeric carrier, drug, and the rest of components from pharmaceutical formulations such as surfactants, additives, and taste modifiers.

An ideal drug delivery system should transfer a proper amount of drug to a target site in human or animal body, to keep constant the drug concentration for a desirable time, but with minimal or no side effects. Thus, in vivo and in vitro cytotoxicity tests for new systems and knowing of drug release profile are necessary conditions for optimization of pharmaceutical formulations.

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Chapter 5
Microbial Modifications of Flavonols

Prakash Parajuli, Biplav Shrestha, Jae Kyung Sohng, and Ramesh Prasad Pandey

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Abstract Development of microbial cell factories via application of synthetic biology, protein engineering for metabolic engineering has revolutionized the maximum use of microbial consortium for biosynthesis and structural alteration of valuable flavonoids. From a single enzyme expression to complex metabolic pathway, it has been possible to manipulate strains of *Escherichia coli*, *Saccharomyces cerevisiae*, *Streptomyces*, and *Bacillus* for target-based modification of compounds to industrial level in laboratory. Biotransformation, a biotechnological approach, can be applied to structurally modify and generate library of natural products such as flavonoid derivatives.

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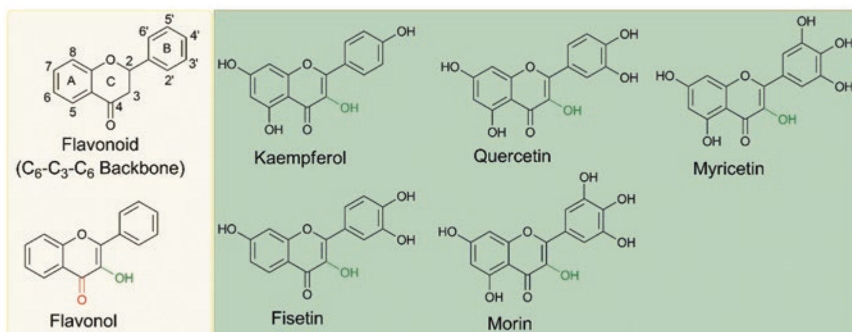
This chapter highlights the significance of engineered new molecules and biotransformation approaches used to generate flavonoids by the use of microbial platforms. Basically, *E. coli* has been engineered by expressing secondary metabolites post modifying enzymes, glycosyltransferases, *O*-methyl transferases, and prenyltransferases, in particular to generate the natural and nonnatural flavonol derivatives. Indigenously present cytoplasmic cofactors, coenzymes, and donor substrates are utilized by such enzymes for target-based chemical modifications. Engineering the central carbon flux pathway to enhance the flow of carbon toward target donor substrates and cofactors such as nucleotide diphosphate (NDP)-sugars, S-adenosyl methionine, dimethylallyl pyrophosphate, and other cofactors which enhanced the cytoplasmic pool while maximizing the biotransformation efficiency for level up production are discussed. Moreover, heterologous expression of different pathway genes from different organisms and engineering of glycosyltransferases and *O*-methyl transferases into bacterial host does help to generate nonnatural flavonol glycosides.

5.1 Introduction

Flavonols are a group of phytochemicals that are widely available in plant-based foods and vegetables such as onions, broccoli, kale, apples, and tea. Structurally they are 3-phenolic ringed compounds of a flavonoid class having 3-hydroxyflavone backbone in C_6-C_3-C_6 carbon framework. Quercetin, kaempferol, myricetin, fisetin, and morin are the most ubiquitous flavonols studied for various biological activities (Fig. 5.1a). They are derived from the phenylpropanoid pathway through a common unit of naringenin chalcone converted into naringenin and further modified to flavonol backbones by the action of flavanone 3-hydroxylase (Fig. 5.1b) (Zhang and Liu 2015). *Arabidopsis thaliana* is a model plant for flavonoid biosynthesis where 35 molecules of flavonols are found among 54 flavonoid molecules (Saito et al. 2013). The structural diversity to the flavonoid core occurs after post biosynthesis modifications such as glycosylation, hydrogenation, hydroxylation, methylation, prenylation, etc. during biosynthesis which also alters their biological significances. Recent biotechnological techniques and tools have harnessed these natural modification steps of plant secondary metabolites into microbial platforms generating various natural and nonnatural scaffolds (Pandey et al. 2016a).

5.2 Significance of Flavonol and Their Microbial Modified Derivatives

Astragalin (kaempferol 3-*O*-glucoside), afzelin (kaempferol 3-*O*-rhamnoside), kaempferol 7-*O*-rhamnoside, kaempferitrin (kaempferol 3, 7-*O*-dirhamnoside), and kaempferide (kaempferol 4′-*O*-methoxide) are the commonly known kaempferol derivatives synthesized expressing various regiospecific glycosyltransferases and *O*-methyltransferases in microbial hosts (Simkhada et al. 2010; Pei et al.



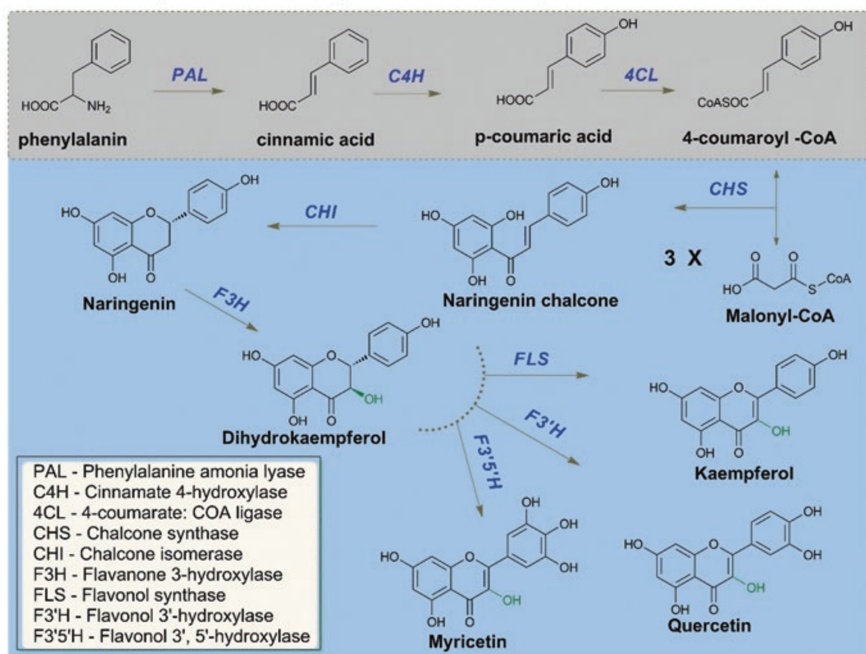


Fig. 5.1 (**a**) Figure showing the basic flavonoid backbone and flavonol representation including structures of major flavonols. (**b**) Biosynthesis pathway of flavonols via phenylpropanoid route involves series of enzyme with respective functions

2016). Various biological significances have been reported of each compound. Astragalin possesses cardioprotective (Qu et al. 2016), antiproliferative (Li et al. 2017), and anti-inflammatory (Ma et al. 2015; Zhang et al. 2017) effects. Afzelin has antibacterial effect against *Pseudomonas aeruginosa* (Lee et al. 2014) and has DNA-protective, anti-inflammatory, and UV-absorbing antioxidant activity (Shin et al. 2013). Kaempferitrin prevents bone loss (Ma et al. 2015) while exhibiting

antidepressant (Cassani et al. 2014) and antidiabetic effects (Da Silva et al. 2014; Jorge et al. 2004). Kaempferide is also a naturally occurring flavonol derivative which also has anticancer potential (Nath et al. 2015; Marnon et al. 2015). Myricetin is a flavonol having various therapeutic values (Phillips et al. 2011; Xue et al. 2015; Mondal et al. 2016; Buchter et al. 2015) including anti-HIV (Pasetto et al. 2014), mitochondrial activating agent (Jung et al. 2017), and attenuates gastric acid secretion, thereby inhibiting H^+, K^+-ATPase that functions as a proton pump in gastric parental cells (Miyazaki et al. 2018).

Quercetin (3, 3′, 4′, 5, 7-pentahydroxyflavone) has been extensively studied for its various biological significances. Due to the fact of bioavailability, poor aqueous solubility, and the rapid body clearance, several derivatives of quercetin have been proposed and used to intense research for potentially improved properties in clinical applications. Recently, some of the flavonols and their derivatives caught the research attentions due to highly promising biological activities. Quercetin 3-*O*-*β*-D-glucoside was studied against Ebola viral infection (Qiu et al. 2016); isoquercitrin was reported as a strong antiviral agent against African historical and Asian epidemic strains of Zika virus tested over human hepatoma, epithelial, and neuroblastoma cell lines (Gaudry et al. 2018). Polyhydroxy flavonols (quercetin and myricetin) were used as promising inhibitor of CatB (cathepsin B, a cysteine protease involved in tumor progression that represents a potential therapeutic target in cancer) (Ramalho et al. 2015), other flavonols are studied to have antiparasitic activity against *Trypanosoma brucei* (Borsari et al. 2016), while tamarixetin was studied as strong antioxidant (Lemmens et al. 2014; Moalin et al. 2012) and tamarixetin 3-*O*-*β*-D-glucoside as a potential anti-ulcer (Yadav et al. 2017) molecule. Other post-modified flavonols such as rhamnetin (7-*O*-methyl quercetin) and isorhamnetin (3′-*O*-methyl quercetin) are known to have better bioavailability and anti-inflammatory effect than its aglycon (Lee et al. 2011; Jnawali et al. 2014). Hyperoside (quercetin 3-*O*-galactoside) inhibits the proliferation and stimulation of osteogenic differentiation of human osteosarcoma cells (Zhang et al. 2014). It protects against hypoxia/reoxygenation during cardiomyocytes injury (Xiao et al. 2017). Isorhamnetin 3-*O*-galactoside was found to have greater anticoagulant and profibrinolytic effect compared to hyperoside (Ku et al. 2013). Quercetin (quercetin 3-*O*-rhamnoside) and guajavarin (quercetin 3-*O*-arabinoside) have shown better cytotoxic and antiviral activity compared to ribavirin (dos Santos et al. 2014). Quercetin 7-*O*-rhamnoside has shown considerable antiviral activity during early stage of porcine epidemic diarrhea virus (Choi et al. 2009; Song et al. 2011). Quercetin 3-*O*-*α*-L-rhamnoside was found to protect against snake venom isolated from a plant *Euphorbia hirta* (Gopi et al. 2016). Quercetin 3-*O*-xyloside was recently presented as a new immunostimulator agent (Lee et al. 2016). Rhamnazin (3′, 7-*O*-dimethyl quercetin) has been introduced as a novel angiogenesis inhibitor with potential antitumor efficacy (Yu et al. 2015; Philchenkov and Zavelevych 2015). Beside anticancer and antioxidant activities, a glycoside derivative isorhamnetin 3-*O*-glucuronide has been extensively studied which is suggested as a valuable therapeutic agent for inflammation-related pathological illnesses (Park et al. 2016). Morin was found to be a novel inhibitor of glycogen synthase kinase 3β (GSK3β)

by reducing tau pathology in Alzheimer's disease condition (Gong et al. 2011). Fisetin is another flavonol (5-deoxy quercetin) with potential biological activities including memory enhancer (Maher et al. 2006), neuroprotective effect (Ahmad et al. 2017), and anti-Alzheimer's (Currais et al. 2014; Kim et al. 2016). Fisetin and myricetin were studied for antimalarial activities and were found to have dual inhibition function against falcipain-2 and plasmepsin II, thereby proving chance to development as antimalarial drug (Jin et al. 2014). The significance of flavonol derivatives discussed here is tabulated in Table 5.1. Although numerous derivatives of myricetin have been reported, microbial post-modified derivatives, myricetin 3-*O*-glucoside (Parajuli et al. 2015) and myricetin 3-*O*-rhamnoside (Thuan et al. 2013; Parajuli et al. 2015), are limited. So far microbial modified flavonol derivatives are presented in Figs. 5.2 and 5.3.

5.3 Current Approaches for Microbial Flavonol Modifications

Biotransformation is an alternative and cost-effective strategy to produce various natural and nonnatural flavonoid derivatives based on the simple enzymatic modification. The most promising biotechnological technique applied nowadays is in vivo whole-cell biotransformation. The major microbial post modification platforms for glycosylation, methylation, hydroxylation, and prenylation are generated in microbial hosts (*Escherichia coli*, *Saccharomyces cerevisiae*, *Streptomyces* strains, fungal mycelia) by overexpressing various secondary metabolites post-modifying enzymes including glycosyltransferases, *O*-methyl transferases, cytochrome P450s, and prenyltransferases. Application of these enzymes to modify the structures of natural flavonoids to improve their physicochemical and biological properties has been of a great scientific and industrial interest due to their large availability, low cost, and wide substrate spectra. Besides the single genetic manipulations and one step reaction, total biosynthetic pathways of flavonoids are copied and heterologously expressed into desired host bacteria for the biosynthesis and modification from simple and low-cost precursor using various biotechnological tools (Kaneko et al. 2003; Malla et al. 2012; Stahlhut et al. 2015).

Biotransformation is considered to be the most explored techniques in flavonol modifications used by current scientists to achieve target products even in industrial scale. Since the microbial indigenous primary metabolites such as cofactors (ATP, S-adenosyl L-methionine, NDP-sugars), amino acid and coenzyme (pyridoxal-5′-phosphate), nucleotide diphosphate sugars as sugar donor substrate are utilized by post-modifying enzymes like methyltransferase and glycosyltransferases for chemical modifications in exogenously supplied flavonols as acceptor substrates (Fig. 5.3).

Table 5.1 Lists of flavonol derivatives studied for their specific significance are tabulated with references

Flavonol derivatives	Significance of flavonols	References
Quercetin 3-*O*-β-D-glucoside	Ebola viral infection	Qiu et al. (2016)
Isoquercitrin	Zika virus infection	Gaudry et al. (2018)
Quercetin	Anticancer/inhibitor of cathepsin B (CatB)	Ramalho et al. (2015)
Tamarixetin	Antioxidant	Lemmens et al. (2014) and Moalin et al. (2012)
Tamarixetin 3-*O*-β-D-glucoside	Anti-ulcer	Yadav et al. (2017)
Rhamnetin	Anti-inflammatory	Lee et al. (2011) and Jnawali et al. (2014)
Isorhamnetin	Anti-inflammatory	Lee et al. (2011) and Jnawali et al. (2014)
Hyperoside	Against hypoxia/reoxygenation	Xiao et al. (2017)
Isorhamnetin 3-*O*-galactoside	Anticoagulant and profibrinolytic effect	Ku et al. (2013)
Quercetin and guajavarin	Better cytotoxic and antiviral activity	dos Santos et al. (2014)
Quercetin 7-*O*-rhamnoside	Antiviral for porcine epidemic diarrhea virus	Choi et al. (2009) and Song et al. (2011)
Quercetin 3-*O*-α-L-rhamnoside	Antivenom	Gopi et al. (2016)
Quercetin 3-*O*-xyloside	Immunostimulator agent	Lee et al. (2016)
Rhamnazin	Angiogenesis inhibitor	Yu et al. (2015) and Philchenkov and Zavelevych (2015)
Isorhamnetin 3-*O*-glucuronide	Therapeutic agent for pathological illness	Park et al. (2016)
Astragalin	Cardioprotective, antiproliferative; anti-inflammatory	Qu et al. (2016), Li et al. (2017), Ma et al. (2015), and Zhang et al. (2017)
Afzelin	Antibacterial, DNA-protective, anti-inflammatory and UV-absorbing antioxidant	Lee et al. (2014) and Shin et al. (2013)
Kaempferitrin	Prevents bone loss, antidepressant, antidiabetic effects	Ma et al. (2015), Cassani et al. (2014), Da Silva et al. (2014), and Jorge et al. (2004)
Kaempferide	Anticancer	Nath et al. (2015)
Myricetin	Anticancer/inhibitor of cathepsin B (CatB), antimalarial; anti-HIV-1, mitochondrial activating agent	Ramalho et al. (2015), Jin et al. (2014), Pasetto et al. (2014), Jose et al. (2016), and Jung et al. (2017)
Fisetin	Memory enhancer, neuroprotective effect, anti-Alzheimer's; antimalarial	Maher et al. (2006), Ahmad et al. (2017), Currais et al. (2014), Kim et al. (2016), and Jin et al. (2014)
Morin	Inhibitor of glycogen synthase kinase 3β	Gong et al. (2011)

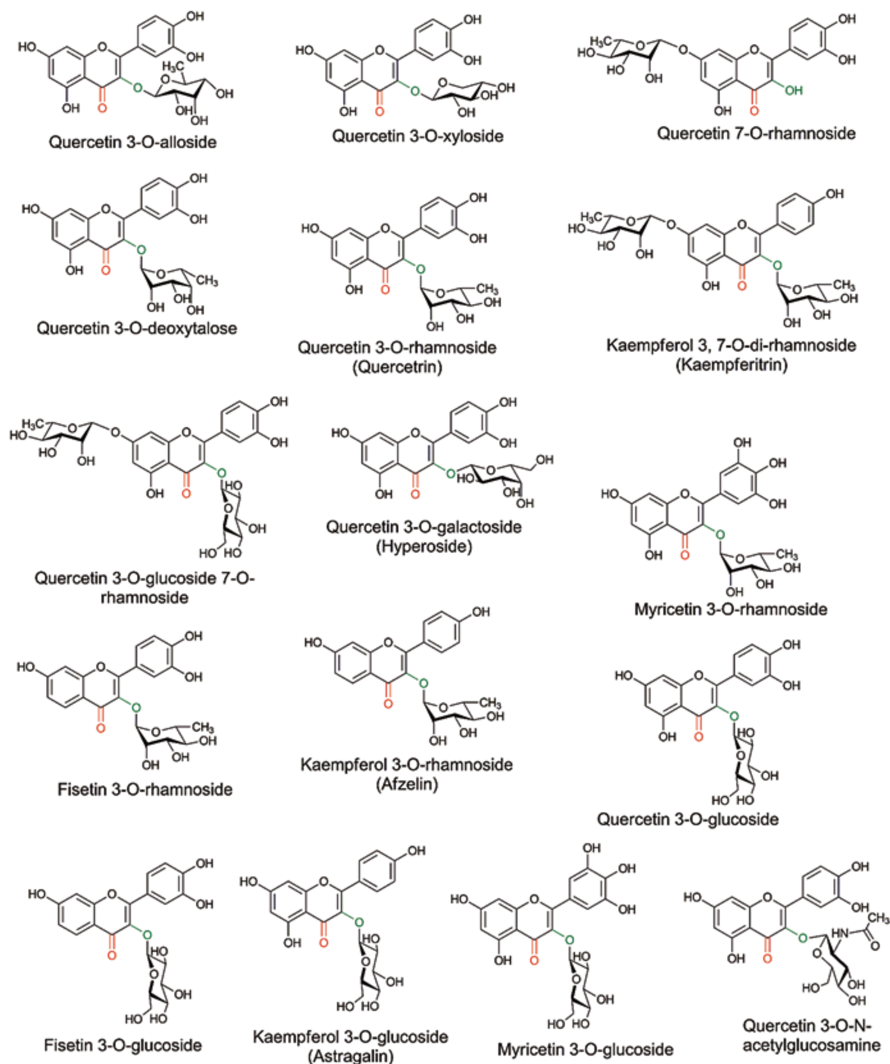


Fig. 5.2 Flavonol glycoside structures synthesized from microbial modification using various glycosyltransferases

5.3.1 Glycosylation

Glycosylation is a common post-modification step involved at the later stage during biosynthesis of natural products in plants. Glycosyltransferase mediates the biochemical reaction to form glycoside bonds via transfer of an activated nucleotide diphosphate sugar to an acceptor molecule. Flavonoids are usually present in their

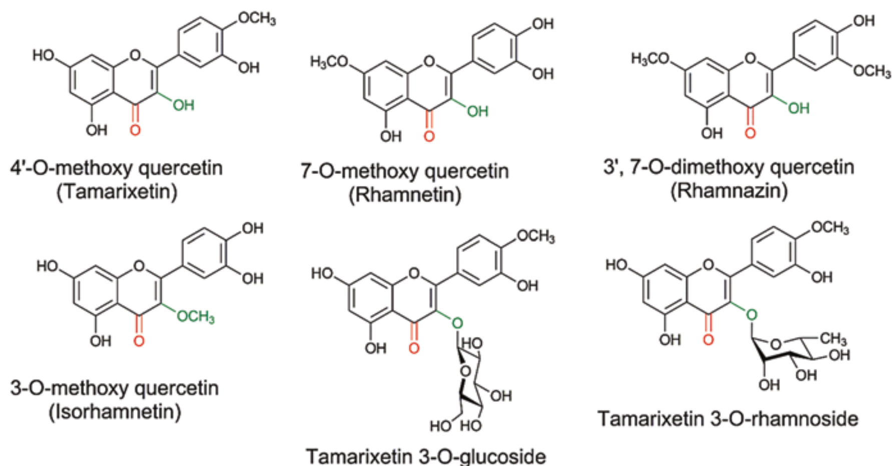


Fig. 5.3 Flavonol *O*-methoxide structures synthesized from microbial modification using various *O*-methyltransferases

O- or *C*-glycosides in plants. Various biological activities are associated with the types of sugar moieties attached to flavonoids including their physical nature like solubility and stability (Plaza et al. 2014). Most common glycosylation modification in flavonols takes place in 3-hydroxyl and 7-hydroxy position. Either simply overexpression of glycosyltransferases or nucleotide diphosphate sugar (NDP-sugar) biosynthetic pathways (Fig. 5.4) including glycosyltransferases are engineered in *E. coli* for regiospecific biotransformation of flavonols. Novel quercetin glycoside quercetin 3-*O*-(6-deoxytalose) including quercetin 3-*O*-glucoside and quercetin 3-*O*-rhamnoside was reported by engineering *E. coli* glycolysis pathway and expression of *tll* (encoding dTDP-6-deoxy-L-lyxo-hexulose reductase, i.e., dTDP-talose synthase) and AtUGT78D1 from *Arabidopsis thaliana* (Yoon et al. 2012). UDP-xylose pathway enzymes phosphoglucomutase (*nfa44530*) from *Nocardia farcinica*, glucose-1-phosphate uridylyltransferase (*galU*) from *E. coli K-12*, and UDP-glucose dehydrogenase (*calS8*) and UDP-glucuronic acid decarboxylase (*calS9*) from *Micromonospora echinospora* spp. *calichensis* were overexpressed in multiple vector along with *Arabidopsis thaliana* glycosyltransferase (*ArGt-3*) to biotransform quercetin into quercetin 3-*O*-xyloside in *E. coli* host (Pandey et al. 2013). In the same year, improved production of myricetin 3-*O*-rhamnoside was reported in *E. coli* mutant expressing *ArGt-3* (Thuan et al. 2013). The *E. coli* mutant strain was generated disrupting glucose-6-phosphate utilizing pathway genes: glucose phosphate isomerase (*pgi*), glucose-6-phosphate 1-dehydrogenase (*zwf*), and UDP-*α*-D-glucose hydrolase (*ushA*) (Pandey et al. 2013). An improved production of quercetin 3-*O*-xyloside was reported by Han et al. (2014) by overexpressing UDP-xylose synthase (*uxs*), UDP-glucose 6-dehydrogenase (*ugd*), and AtUGT78D3 from *A. thaliana* in a UDP-4-amino-4-deoxy-L-arabinose (*L-Ara4N*) formyltransferase/

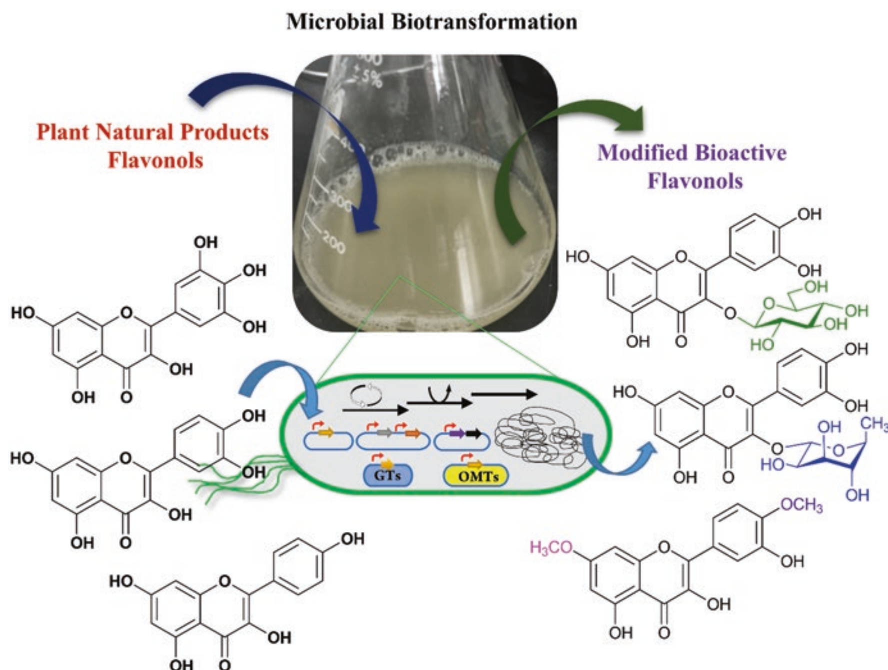


Fig. 5.4 Representation of a simple microbial biotransformation of flavonols to modified bioactive molecules in engineered *E. coli*. The modified products are glycosides and *O*-methoxides in common

UDP-glucuronic acid C-4″-decarboxylase (*arnA*) deleted *E. coli* mutant. Similarly, *ArGt-3* was also used along with TDP-glucose synthase (*Tgs*) from *Thermus caldophilus* GK24, TDP-glucose 4,6-dehydratase (*DH*) from *Salmonella typhimurium* LT2, TDP-4-keto-6-deoxyglucose 3,5-epimrase (*epi*), and TDP-glucose 4-ketoreductase (*Kr*) from *Streptomyces antibioticius*Tu99 to synthesize quercetin 3-*O*-rhamnoside and kaempferol 3-*O*-rhamnoside whereas enzymes Tgs and DH along with TDP-hexose 3-epimerase (*GerF*) and TDP-4-keto-6-deoxyglucose reductase (*GerK*) from *Streptomyces* sp. KCTC 0041BP to synthesize quercetin 3-*O*-alloside (Simkhada et al. 2010).

In our recent report, different flavonols were modified into their natural and non-natural glycosides (Parajuli et al. 2015; Pandey et al. 2015). We constructed sugar cassettes assembling UDP-glucose and TDP-rhamnose pathway-specific enzymes and inserted into *E. coli* strain to biotransform different flavonols (fisetin, quercetin, kaempferol, and myricetin) into respective glycosides efficiently (Parajuli et al. 2015). In the same year, an expanded in vivo glycosylation platform was generated in *E. coli* W for efficient galactosylation catalyzed by galactosyltransferase (F3GT) from *Petunia hybrid* and rhamnosylation catalyzed by rhamnosyltransferase (*RhaGT*) from *A. thaliana* using a cheap source of sugar as sucrose to increase the

pool of UDP-galactose and UDP-rhamnose for biosynthesis of 3-*O*-galactoside and 3-*O*-rhamnoside of various flavonols: quercetin, kaempferol, fisetin, morin, and myricetin (De Bruyn et al. 2015).

Similarly, *E. coli* BL21 (DE3)/Δ*pgi*Δ*zwf*Δ*galU* mutant was engineered expressing enzymes (*tgs*, *dh*, *epi*, and *kr*) to distract the flow of carbon flux toward thymidine diphosphate 4-keto-4,6-dideoxy-D-glucose (dTKDG) along with sugar amino-transferases: 4-aminotransferase (*gerB*) from *Streptomyces* sp. GERI-155 to generate dTDP-D-viosamine pool, 4-aminotransferase (*wecE*) from *E. coli* K-12 to generate pool of dTDP-4-amino 4,6-dideoxy-D-galactose, and two genes for dTDP-3-amino 3,6-dideoxy-D-galactose (*fdtA* and *fdtB*) from *Aneurinibacillus thermoaerophilus* L420–91 (DSM 10154). Here also an *Arabidopsis* glycosyltransferase, ArGt-3, catalyzed to transfer these unnatural sugars to generate nonnatural quercetin and kaempferol derivatives through microbial biotransformation. In the meantime, novel fisetin glycosides were also produced in engineered *E. coli* host conjugating various amino sugars at 3-hydroxyl position of fisetin (Pandey et al. 2016b). Microbial modifications of quercetin into other glycosides as quercetin 3-*O*-4-deoxy-4-formamido-L-arabnose, quercetin 3-*O*-*N*-acetylglucosamine, quercetin 3-*O*-arabinoside, quercetin 3-*O*-6-deoxytaloside, and quercetin 3-*O*-glucuronide are covered in recent review in detail (Pandey et al. 2016a). We recently testified the microbial synthesis of tamarixetin glucoside at significant yield in *E. coli* for the first time (Parajuli et al. 2018). In previous years, without engineering sugar pathways and microbes, glycosyltransferases were simply overexpressed to modify flavonols into glycosides. Quercetin 3-*O*-glucoside, quercetin 7-*O*-glucoside, quercetin 3′-*O*-glucoside, quercetin 4′-*O*-glucoside, kaempferol 3-*O*-glucoside, and isorhamnetin 3-*O*-glucoside have been reported (Lim et al. 2004; Kim et al. 2006a, 2010).

5.3.2 Methylation

Methylation is another common post-modification after the biosynthesis of secondary metabolites. Hydroxyl, carbon, or nitrogen atoms present in terminal positions are decorated by methyl groups to signify the chemical structures of secondary metabolites. Especially S-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferases catalyze methylation to plant flavonols. Very few of the SAM-dependent microbial origin *O*-methyltransferases are characterized to methylate plant flavonols. Microbial C-methyl derivatives of flavonols have not yet been reported. However, plant-originated *O*-methyltransferase has been functionally expressed in microbial hosts for the modification of different flavonols in respective hydroxyl positons. SOMT-2 originated from *Glycine max* overexpressed in *E. coli* biotransformed quercetin into 4′-*O*-methoxy quercetin (Kim et al. 2005a). The same group considered co-expression of two regiospecific *O*-methyltransferases

ROMT-9 and SOMT-2 from rice in *E. coli* and produced 3′-*O*-methylated and the 3′,4′-*O*-dimethylated quercetin derivatives (Kim et al. 2005b). In the following year, 7-*O*-methylated derivatives of quercetin, kaempferol, and isorhamnetin were produced using *Poplar*-originated *O*-methyltransferase: POMT-7 (Kim et al. 2006b). Similar co-expression method was applied to biotransform quercetin into quercetin 3′-*O*-methylquercetin, 3′,4′-*O*-dimethylquercetin, 7,3′-*O*-dimethyl quercetin, and 7,3′,4′-*O*-trimethylquercetin, respectively, using ROMT-9 and POMT-7 by Kim et al. (2008). A putative *O*-methyltransferase, SIOMT3, from tomato was isolated and overexpressed into *E. coli* and the transgenic *E. coli* efficiently modified quercetin, myricetin, and laricitrin into methoxide derivatives (Lee et al. 2017). Fusion of two regiospecific3′-*O*-methyltransferases (*SIOMT3*) from tomato and 7-*O*-methyltransferase (*OsNOMT*) from rice was reported recently to biotransform quercetin into rhamnazin efficiently (Lee et al. 2017).

Plant *O*-methyltransferases are regiospecific. However, there are few reports of using *Streptomyces*-derived *O*-methyl transferases for biotransformation of selective flavonols. SaOMT-2 from *S. avermitilis* MA-4680 and SpOMT2284 from *S. peucetius* ATCC27952 were explored for flavonoids methylation where SaOMT-2 biotransformed kaempferol, quercetin, and isorhamnetin into their methoxides regiospecifically and SpOMT2284 catalyzed *O*-methylation over quercetin and rutin non-regiospecifically (Kim et al. 2006c; Koirala et al. 2014). We have recently characterized *O*-methyltransferase (*GerMIII*) from *Streptomyces* sp. KCTC 0041BP to regioselectively produce 4′-*O*-methoxides of quercetin, myricetin, fisetin, and quercetin 3-*O*-glucoside, respectively (Darsandhari et al. 2018). Microbial and plant source *O*-methyltransferases are tabulated in Table 5.2.

5.3.3 Hydroxylation

Hydroxylation is an important post modification for the diversification of plant secondary metabolites. They are biosynthesized through phenylpropanoid metabolic pathway where flavonols, in particular quercetin, myricetin, morin, and fisetin, are different hydroxylated skeleton of kaempferol. However, very limited studies have been reported producing hydroxylated derivatives of flavonols expressing hydroxylases (CYP P450 mono-oxygenase) in microbial platform. But through microbial transformation of flavonols, hydroxylated derivatives were detected and characterized from the culture media in preparative scale. Hosny et al. (2001) reported the hydroxylation of fisetin and quercetin through the biotransformation via *S. griseus.* Those hydroxylated products were subsequently *O*-methylated into geraldol and 3, 7, 3′-trihydroxy-4′-methoxyflavone in case of fisetin and isorhamnetin and dillenetin, 3, 5, 7-trihydroxy-3′-4′-dimethoxyflavone, in case of quercetin.

Table 5.2 *O*-methyltransferases from microbial and plant sources used for post-modification of flavonols

S. No.	O-Methyltransferase (organism source)	Products catalyzed by O-methyltransferases	References
Microbial O-methyltransferase			
1	SpOMT2884 (*Streptomyces peucetius*)	*O*-Methylation on quercetin, rutin,	Koirala et al. (2014) and Chiang et al. (2015)
2	SaOMT5 (*Streptomyces avermitilis*)	*O*-Methylation of quercetin	Yoon et al. (2010)
3	ScOMT1 (*Streptomyces coelicolor* A3(2))	*O*-Methylated products were isorhamnetin, tamarixetin, fisetin methoxide, gossypetin	Yoon et al. (2005)
4	SaOMT-2 *Streptomyces avermitilis*	*O*-Methylation of kaempferol and quercetin	Kim et al. (2006c)
5	SpnK (*Saccharopolyspora spinosa*)	4′-*O*-Methoxy quercetin 3-*O*-glucoside	Parajuli et al. (2018)
Plant O-methyltransferase			
1	SOMT-2 *Glycine max*	*O*-Methylated quercetin	Kim et al. (2005a)
2	ROMT-9 and SOMT-2 *Rice* and *Glycine max*	*O*-Methylated quercetin	Kim et al. (2005b)
3	POMT-7 Poplar, *Populus deltoides*	*O*-Methylated kaempferol, quercetin	Kim et al. (2006b)
4	SIOMT3 *Tomato*	*O*-Methylated quercetin, rhamnetin	Lee et al. (2017)
5	OsNOMT *Rice*	*O*-Methylated kaempferol, quercetin, isorhamnetin	Lee et al. (2017)
6	CdFOMT5 *Citrus depressa*	*O*-Methylated quercetin	Itoh et al. (2016)

Second column shows products catalyzed by particular *O*-methyltransferase from first column

5.3.4 Prenylation

Prenylated flavonoids are uncommon, and they are characterized by the presence of lipophilic prenyl (5-carbon) chain, dimethylallyl or geranyl chain (10-carbon), or farnesyl (15-carbon). No reports have been found to generate prenylated flavonols through microbial transformation in particular although few prenylated derivatives of flavonoids reported have been reviewed in Pandey et al. 2016b (Fig. 5.5).

5.4 Conclusion

Apart from multifaceted therapeutic applications, flavonol and its derivatives have long been explored for potential nutritional values since they are particularly abundant in daily consumable vegetables, fruits, nuts, red wine, green tea, etc. Microbial modification of flavonols has been profoundly reliant on the biotransformation of

Fig. 5.5 Engineering natural and nonnatural NDP-sugar biosynthetic pathway in *E. coli* for regio-specific modification of flavonols catalyzed by specific uridine diphosphate glycosyltransferase. Red cross indicates the blocked pathway. Single black arrow is one step reaction while double black arrow indicates two steps reaction. *glk*: hexokinase, *pgm* phosphoglucomutase, *galU* UDP-glucose synthase, *ugd* dehydrogenase, *uxs* decarboxylase, *RHM1* UDP-rhamnose synthase, *tgs* TDP-D-glucose synthase

aglycon into their analogous glycosides or *O*-methoxides using post-modifying enzymes. Utilizing primary metabolites of *E. coli* including cofactors, donor substrates, or coenzymes for modification of flavonols has been cheap and simple, since they are indigenously present in bacterial cell. These natural commodities have been valued by engineering strategy to enhance the production of target compounds. A range of molecular biology tools, including metabolic engineering and synthetic biology, has been used to achieve significant bioconversion in host cells.

Engineering *E. coli* either by deletion or extra copy overexpression of glycolysis pathway genes enabled the carbon flux toward target NDP-sugar/s accumulation, where glycosyltransferase expression facilitates regiospecific modification of flavonols to their natural and nonnatural glycoside analogues (Simkhada et al. 2010; Yoon et al. 2012; Parajuli et al. 2015; Pandey et al. 2016b). However, for flavonol *O*-methoxides, few engineering approaches have been reported to increase production from microbial cell factories beside protein fusion for double modification and the simple expression of *O*-methyltransferases. Thus, expression of glycosyltransferases and *O*-methyltransferases from plant and microbial sources and rewiring native pathway via diversion of carbon flux toward primary precursor were more efficient to modify and synthesize target-based flavonol derivatives rather than anonymous microbial whole-cell biotransformation. Even through the biotransformation, modern microbial engineering approaches have helped to program and control bacterial robustness in production.

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Chapter 6
Downstream Processing for Biopharmaceuticals Recovery

Anu Mehta

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Abstract The invention of genetic engineering tools has given birth to a new type of pharmaceuticals known as biopharmaceuticals. These are the drug molecules that have therapeutic effects and are synthesised in biological cell systems. Drug like recombinant insulin is a prominent prototype example of biopharmaceutical which is commonly available in the market at cheap prices for diabetic patients, globally. The production of these therapeutic molecules differs from chemically synthesised low molecular weight drugs. Upstream and downstream processes altogether comprise the production process of biopharmaceuticals. The downstream processing costs 70% of the total production cost of a particular biopharmaceutical, largely contributed by expensive chromatographic techniques such as affinity, hydrophobic interaction, ion exchange and size exclusion. Although chromatography is a reliable and conventional approach to carry out single step purification of biopharmaceuticals, the columns are run in a series to increase the purification fold. This makes the process tedious, and problems like diffusional spreading and resolution are also

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observed with chromatography procedures. The concern is important as we aim to bring various biopharmaceuticals into market that can treat innumerable diseases at a cheap price.

The current chapter emphasises the process and technology related to the upstream process and the three chronological steps – initial recovery, purification and polishing – involved in downstream processing of biopharmaceuticals. The chapter encompasses the hurdles encountered in the downstream processing in particular with chromatography process that makes high-quality production of biopharmaceuticals an expensive affair thus making it difficult to reach the public. New technologies designed to offer faster and cheaper purification such as aqueous two-phase extraction system, and nano-magnetic-based antibodies separation system have been discussed further. Moreover, we have reviewed and emphasised the requirement of using combination of physical, mathematical, biological and computational approaches, which can help to design efficient production and purification systems for the ample, cheap and continuous market supply of this new category of drugs.

6.1 Introduction

Biopharmaceuticals are pharmaceutical products which are obtained by biotechnological processes. The term 'biopharmaceutical' was used for the first time in the 1980s to address and describe those therapeutic proteins which are produced by genetic engineering and/or hybridoma technology (Gary 2000, 2003). Most pharmaceutical drugs are chemically synthesised low molecular weight (LMW) molecules, whereas biopharmaceuticals are high molecular weight (HMW) molecules which include glycoproteins (sugar-attached polymers of L-amino acids) and nucleic acids (polymers of nucleotides), in which the former category predominates the share (Jozala et al. 2016). These biopharmaceuticals possess three-dimensional (secondary, tertiary, quaternary) structures which are absent in LMW pharmaceutical drug molecules (Crommelin et al. 2003). The three-dimensional structure of molecules determines their biological activity; a small error in it can make that molecule lose its functional capability. Moreover the glyco-conjugated proteins cannot be chemically synthesised and hence requires the need of using biological cell machinery in combination with genetic engineering, for their production.

The advent of genetic engineering facilitated the way for the production of a protein say of known sequence and hybridoma technology paved the way for its large-scale production. The cells in vitro are cultured and genetically modified for the production of these therapeutic molecules. It includes the use of bacterial cells and filamentous fungi to different eukaryotic cell systems, e.g. yeast (*Pichia pastoris*), transgenic animals and transgenic plants. Production of therapeutic proteins using transgenic plants is referred to as *molecular pharming*. Table 6.1 list some of the commercially available biopharmaceuticals showing different cell systems used for their production.

Drug development is a lengthy procedure, and to launch a novel drug in the market costs approx. 15 years of extensive in vitro and in vivo research. In the last

Table 6.1 The table enlists some of the commercially available biopharmaceuticals using bacteria, yeast, plant and animal cell expression system

Biopharmaceutical	Route of administration	Commercial name	Clinical use
Biopharmaceuticals produced using bacteria cell expression system			
Insulin (fast acting)	Subcutaneous	Lispro (Humalog)	Diabetes
Glucagon	Parenteral	Glucagon	Hypoglycaemia
Parathyroid hormone	Subcutaneous	Preotact	Osteoporosis
Somatotrophin	Subcutaneous	Humatrope	Growth hormone deficiency
Somatostatin	Subcutaneous, intramuscular, intravenous	Octreotide (Sandostatin)	Tumours, acromegaly, GI fistulae
G-CSF	Subcutaneous	Neulasta	Tumour-related infections
Anti-VEGF antibody	Intravitreal	Lucentis	Macular degeneration
Biopharmaceuticals produced using yeast cell expression system			
HPV vaccine	Intramuscular	Gardasil	Treating infections against HPV
Serum albumins	–	Recombumin	Manufacture of other human therapeutics
Platelet-derived growth factor	Topical application	Regranex	Wound repair, neuropathic and diabetic ulcers
Somatotropin	Subcutaneous	Valtropin	Growth hormone deficiency
Biopharmaceuticals produced using plant cell expression system			
β-Glucocerebrosidase	Intravenous	Imiglucerase	Gaucher's disease
Biopharmaceuticals produced using mammalian cell expression system			
IgG-MAB specific for TNFα	Subcutaneous	HUMIRA	Rheumatoid arthritis
Urokinase	Intravenous	Kinlytic	Dissolves blood clots
IgG-MAB specific for TNFα	-	Enbrel	Inflammatory diseases (rheumatoid arthritis, ankylosis spondylitis, juvenile idiopathic arthritis
Interferon β-1a	Subcutaneous	Rebif	Multiple sclerosis

Abbreviations: *GI* gastrointestinal, *G-CSF* granulocyte colony-stimulating factor, *VGEF* vascular endothelial growth factor, *HPV* human papilloma virus, *IgG-MAB* immunoglobulin G monoclonal antibody, *TNFα* tumour necrosis factor α
#The data for the table shown is procured from manufacturers' pages

5 years, 73 biopharmaceuticals have got approval for use in humans ("Biopharmaceutical Products in the US and European Markets" 2002–present), and recently in 2015, amongst top 10 drugs with sales of USD 59 billion, 6 were recombinant protein biopharmaceuticals ("Top drugs by sales revenue in 2015: Who sold the biggest blockbuster drugs?" 2016). In 2004, US biopharmaceuticals had 60% share in global pharmaceutical market (Parmar 2006). India has always been a hot spot of these US- and Europe-based companies to establish their markets. A web database BioPharma details the information that 1126 US and European companies established across the globe are involved in the biopharmaceuticals' production. However, India's biopharmaceuticals demand is met by its own established

companies which are approximately 30 in number (Varma 2009). Amongst them, Biocon Ltd. has been Asia's premier biopharmaceutical company. Many of its biopharmaceutical products are commercially available in the market, e.g. INSUGEN (rh-insulin) and other insulin analogues (https://www.biocon.com/biocon_aboutus.asp).

Cost-effective large-scale production of biopharmaceuticals is an important deciding factor for their commercial launch in the health-based market. The manufactured biopharmaceuticals must be economical to benefit masses over the globe or at least a nation. Over the past three decades, biopharmaceutical industry has undergone a rapid growth from both medical and economical viewpoints. Cost-utility analyses (CUA) of biopharmaceuticals ease the decision to launch them in the market. Studies have shown that CUA of biopharmaceuticals is comparable to that of classic pharmaceutical drugs; thus they also provide value for the money invested. However, their effectiveness is reported comparatively lesser in cancer and neurological diseases relative to those which work against infectious diseases (Wilson and Neumann 2012). This makes us to conclude that the cost-effectiveness of these classes of drugs varies depending upon their effectiveness upon the kind of pathophysiological conditions that they target.

It is worth mentioning that genetic engineering-based production of a biopharmaceutical by different pharmaceutical companies is derived from the similar gene yet they differ a little in their physical characteristics. This is because of the differences in the posttranslational modifications and variations in the manufacturing protocols. To differentiate between them, different terminology is used. The pioneering version of the biopharmaceutical of a kind is termed as *reference medicine*, and the following versions manufactured by other companies are termed as *biosimilars* (Jozala et al. 2016). The mode of administration of biopharmaceuticals in a patient, initially, was conventional, e.g. oral, dermatological, etc. However, to retain the biological activity of biopharmaceuticals during drug delivery, different approaches such as microsphere-based controlled release approach, etc. have been used (Mitragotri et al. 2014). Such engineered biopharmaceuticals with enhanced stability and more efficient drug delivery system are referred to as *biobetters/biosuperiors* (Mitragotri et al. 2014; Beck 2011; Strohl 2015). Thus, biosimilars are considered generic versions of biopharmaceuticals.

The production of biopharmaceuticals is a multistep process. The procedure initiates from the development of the protocol for in vitro production of pharmaceuticals using living cells to study the efficacy in clinical studies and then to scale up the economic production by maintaining the product stability with the same clinical efficacy. Using cells as reactor, variations due to scaling up bring environment variations that a cell experiences. Thus the production kinetics and manufactured product characteristics also show a change with the process of scaling up, and hence large-scale production of biopharmaceuticals of a constant good quality is in itself a hard task to accomplish. Understanding and managing the biomanufacturing are complex; however, aiming the scalability in the early development and tailoring the generic process with keeping things as simple as possible are the key ways that help the scaling up – rapid and a low-risk process (Fish and Williams 2007).

6.2 Outline Scheme of Biopharmaceuticals Production

Biopharmaceutical production is broadly divided into two major processes: *upstream and downstream*, each including various steps. Upstream processes aim to transform the substrate into desired metabolic products, whereas downstream processes aim to purify the desired metabolic product with good yield (Gronemeyer et al. 2014). The upstream process includes series of steps:

1. Identification of altered protein expression responsible for the cause of a pathophysiological condition/disease.
2. Identification, characterisation and isolation of the gene responsible for the same protein expression in healthy organism.
3. Selection of suitable and efficient host cell type to carry out genetic engineering.
4. Identification and selection of transformed cell(s)/line.
5. Scale up the transformed cells with process monitoring and parameter optimizations to achieve maximal biopharmaceutical production.

One of the major steps in the upstream process that must be most sincerely carried out is the selection of a cell line. Commercially, cloning and subcloning of 'n' chosen cell types/lines starts from the master cell bank (MCB). The clones are selected which have robust growth profiles and cell stability (phenotypic and genotypic) ("Considerations for Successful Upstream Process Development" 2012). Expression of human insulin by genetic engineering of *E. coli* cells is an elementary and one of the best examples till date to understand the upstream processing. However, Chinese hamster ovary (CHO) cells, baby hamster kidney (BHK) cells, mouse myeloma (NS0) and hybridoma (SP2-0) are the most common expression system for the production of monoclonal antibodies and cytokines (Jayapal et al. 2007). It's been recorded that in 2010, 70% of recombinant proteins for therapeutic purpose are being expression using CHO cell lines (Li et al. 2010).

There are many parameters for the selection of transformed cell(s)/cell line(s), but amongst them the two major criteria are titre and posttranslational processing profiles of the biopharmaceutical produced (Gronemeyer et al. 2014). These two parameters ensure the quantitative and qualitative assessment of the product in question. *Titre* is defined as the amount of biomolecule produced and released in extracellular aqueous solution compared to the total volume of bioreactor used in the upstream process. The titre and hence productivity of a transformed cell line are assessed in a large-scale bioreactor system, whereas reproducible glycosylation profiles in particular determines the quality of biopharmaceutical (Durocher and Butler 2009). The ability of a cell line to show reproducible glycosylation patterns or other posttranslational modifications depends upon the kind of cell/cell line used and physical and chemical conditions provided for its growth and cultivation (Hossler et al. 2009; Zhu 2012). These physical and chemical conditions are addressed using variable composition of the cell culture media. The culture medium acts a buffer system for the growth of in vitro cell culture systems; it also acts as a

portal to supplement the nutrients to the growing cells. All cell culture media serves these two functions; however, optimal medium must be selected for the best functioning of the cells under consideration and also to ensure that the media constituents do not act as stumbling block in the further downstream processes ("Considerations for Successful Upstream Process Development" 2012).

The aim of carrying out downstream processing remains the same whether the biomolecule of interest is produced by the transformed cells intra-/extracellularly; however the steps to carry out the procedure albeit show differences. In case of extracellular production of target biomolecule, the culture medium is concentrated, and then purification is carried out. But in case the target biomolecule is produced intracellularly, it becomes important to harvest and lyse the cells, followed by debris removal. Then the biomolecule of interest is purified from the cell homogenate by the similar procedures, and mostly intracellular synthesised biomolecules are available and stored as inclusion bodies. Since here, in specific reference to proteins, aggregation causes change in tertiary and quaternary structures; an additional step of refolding is required to make the molecule of interest for commercial therapeutic use. Thus the downstream process includes following series of steps:

1. *Extraction/isolation*: Initial recovery of the product from the bioreactor.
2. *Purification*: Procure purified product by the removal of contaminants from the recovered product.
3. *Polishing*: Removing the contaminants, unwanted forms of biomolecule of interest which is formed due to its misfolding during previous steps of the downstream processing (Jozala et al. 2016) and formulation.

The downstream processing can be carried out either in batch or continuous mode. The more the number of steps involved, it will add to the cost of production of the biomolecule in terms of time and money. It has been noticed that purification efficiency does not improve with continuous downstream processing; rather the process becomes more complex. This disadvantage is outweighed by gain in high yield in continuous operating units (Jungbauer 2013). So, almost equivalent number of operation units work irrespective of continuous or batch mode. However, when the product has less stability, less number of operation units is required in continuous mode (Warikoo et al. 2012). In general, a minimum of three operation units for the above-mentioned three steps are found in any downstream processing unit.

The *yield* of biopharmaceutical obtained is the final parameter used to assess the efficiency of the downstream processes carried out. It is calculated as ratio of the mass of final purified biomolecule and mass of the biomolecule taken at the start of downstream process. Often 99% or more purity is required along with good yield. Different forms of chromatography are traditionally carried out for this purpose. It is necessary to mention that all the steps mentioned above needed to be carried out at a good pace and include research for the development of technologies which can speed up these steps. With the advancement of technology, nowadays, new methods of process development have been incorporated to ease the control and speed up the process. This includes platform technologies, quality by design and design of experiment – experimental optimisations based on high-throughput screening methods,

simulation-based operation units, etc. (Bhambure et al. 2011; del Val et al. 2010; Shukla and Thömmes 2010). It is important to have a robust and reliable scale-up and purification system before release of any biopharmaceutical in the consumer market (Jozala et al. 2016). New approaches including the combination of mechanistic models with artificial neural networks (ANNs) are also in progress. These novel approaches are expected to provide yield with a minimum purity of 99.9% (Pirrung et al. 2017). Figure 6.1 displays the steps of upstream and downstream processing units involved in production of biopharmaceuticals.

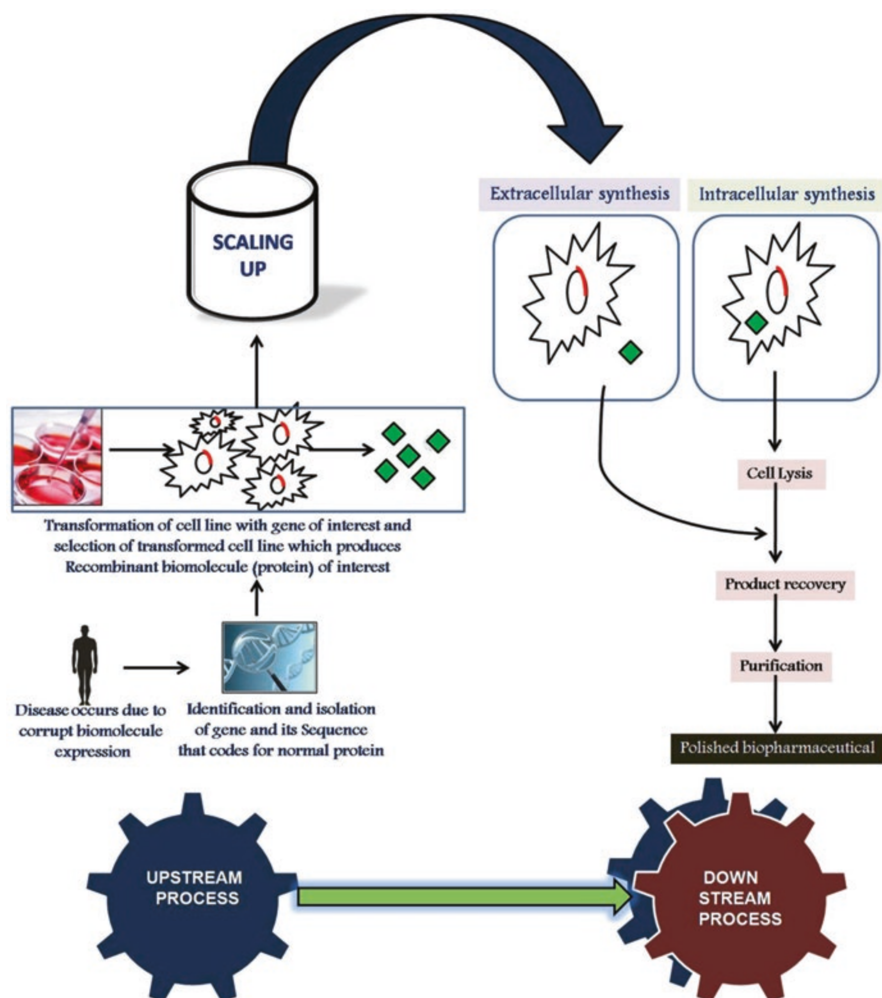

Fig. 6.1 Illustration of steps involved in upstream and downstream processing of biopharmaceuticals. The upstream process includes series of steps that aim to transform the substrate into desired metabolic products, whereas downstream processes aim to purify the desired metabolic product with good yield

6.3 Focus on Downstream Processing

6.3.1 Initial Recovery: Process and Technologies

As mentioned earlier, initial recovery refers to the procedures by which broth clarification is carried out, so that the product in consideration can be extracted from extracellular medium or from the separated cells by their lysis. This involves the use of methods like centrifugation, filtration, sedimentation and flotation. With time, the biopharmaceutical industry over the globe has shifted from batch to continuous mode of operation in downstream processing. Here, we will highlight the technological insights of currently adopted continuous mode of operations used in downstream processing.

(a) **Continuous centrifuges:** The initial step of downstream processing, i.e. product recovery, aims to recover (a) the product from the extracellular medium, if the synthesised product is released extracellularly, or (b) the biomass which is followed by clarification of cell homogenates, washing and recovering of the inclusion bodies and harvesting precipitated protein or biomolecule of interest, if the product is stored intracellularly. These are usually achieved with the help of centrifugation. The centrifuges operating in pseudo-continuous manner so that the suspension fed and clarified liquid are removed in continuous fashion are known as continuous centrifuges (Jungbauer 2013; Langer 2011). There are various types of centrifuge configurations used: hybrid centrifuge rotor (Ivory et al. 1995), continuous tubular bowl centrifuge (Lander et al. 2005), combined centrifugation and cell culture (Brouns et al. 1990), tube centrifuge (Jungbauer 2013; Rathore et al. 2015), chamber centrifuge (Jungbauer 2013; Rathore et al. 2015) and disc stack centrifuge (Jungbauer 2013; Rathore et al. 2015). Amongst these, only disc stack centrifuge can be operated in a pseudo-continuous manner (Erikson 1984). The centrifuge has a vertical rotor with numerous conical discs attached radially and all along the rotor length. These discs provide increase in the sedimentation area (Fig. 6.2). This type of centrifuge is further available in two designs: split bowl and disc nozzle (Jungbauer 2013). Split bowl design is used when the feed has high solid content, for example, in case of yeast fermentation biomass content is very high, whereas for low solid content, feed disc nozzle design is used. Different capacities of disc continuous centrifuges are deployed depending on small-scale, pilot-scale or large-scale production. The smallest commercially continuous disc centrifuge must be fed at 1l/min, which is a too high for a laboratory-scale experiment. But, for initial studies and procurement of biopharmaceutical, it is important to carry out the experimental studies at laboratory scale. For this purpose, small-scale decanters are used which are fed at 30 ml/min (Jungbauer 2013). These centrifuges with different capacities cannot be interchanged for use because the separation efficiency differs in each case which can affect downstream yield of the purified product of interest.

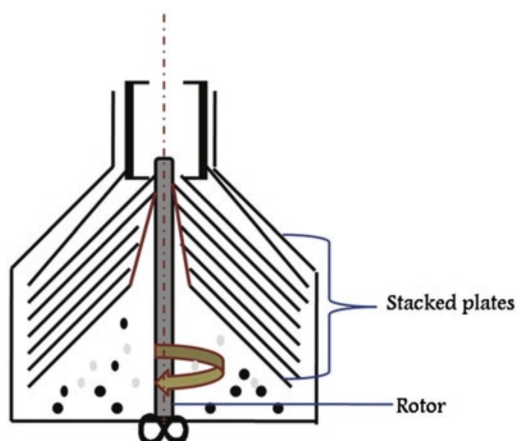

Fig. 6.2 Diagrammatic representation of disc stack centrifuge architecture. The radially attached stacked plates all along the rotor provide an increase in surface area

(b) **Continuous filtration**: Filtration is another way to separate the solid-liquid components of the culture established in the upstream process. Thus, by filtration, cells can be harvested, by-products can be removed and culture medium containing the expressed biomolecules of interest can be concentrated. This is achieved by using different pore-sized filters (Cheryan 1986). There are broadly three types of filtration: (a) microfiltration (used for cell harvesting by retaining suspended particles in the broth and allowing water, salts and macromolecules in the filtrate), (b) ultrafiltration (used for concentrating biopharmaceutical product) and (c) nanofiltration (used for the removal of viruses). For microfiltration, filters of pore size ranging between 0.1 and 10 μm are used, whereas filters with pore size in the range of 0.01–0.1 μm are used for ultrafiltration, and filters with pore size of 0.001 μm (1 nm) carry out nanoscale level filtration. Much better version of filtration complemented with electrophoretic technique known as electrofiltration is also used which reduces cost and time of operation (Gözke et al. 2012; Wellhoefer et al. 2013).

Continuous/cascade filtration refers to the filtration process where one filtration unit is fed into next consecutive filtration unit which varies in size from each other (Siew et al. 2013). There are major two ways to carry out continuous filtration: dead end/normal flow and tangential flow (Rathore et al. 2015). Dead-end filtration is not efficient, as it involves the passage of solution from a single filter upon which with time collection of particulate matter occurs gradually, which leads to the formation of a filter cake. The cake provides increased resistance to the flow of matter across filter and reduces the efficiency of filtration process. However, this filter cake is removed via reverse flushing to keep the process going on (Bailey and Ollis 1986). This system of filtration is applicable for separation of inclusion bodies (Wellhoefer et al. 2013). Tangential flow filtration is more commonly used as it has higher efficiency in comparison to the former one mentioned. This is achieved by allowing the sample to run parallel to the filter (Fig. 6.3); the filtrate is recycled numerous times, and hence

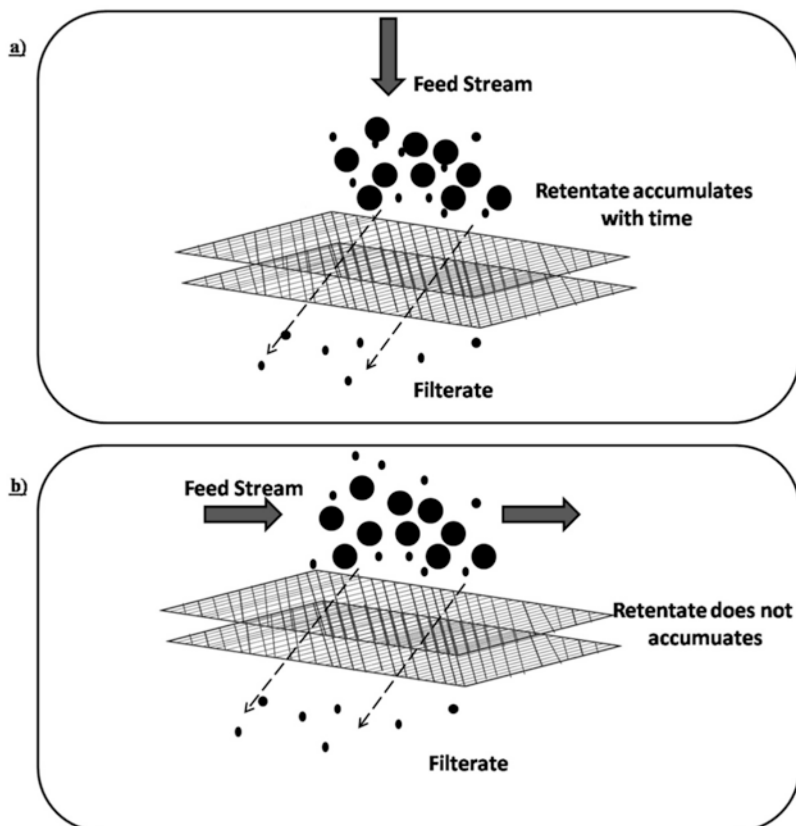

Fig. 6.3 Diagram showing the difference in the functioning of (**a**) dead-end filtration and (**b**) tangential flow filtration. In dead-end filtration, feed stream runs perpendicular to the plane of the filters, whereas in tangential flow filtration, the feed stream runs parallel to the filters

large volumes are concentrated to smaller volumes. This is achieved using any of the two different kinds of designs: single-pass tangential flow filtration (SPTFF) and alternating tangential flow filtration (ATF) (Alford et al. 2008; Bonham-Carter and Shevitz 2011).

(c) **Cell lysis**: The cell lysis is an additional step in case the biopharmaceutical is produced inside the cell as inclusion bodies. Many recombinant proteins have been found to be aggregated as inclusion bodies, e.g. interferon-β, interleukin-2, protein C, proinsulin, etc. A list of a few of the well-known biopharmaceuticals expressed and stored as inclusion bodies is mentioned in Table 6.2.

Before proceeding to cell lysis, it is important to confirm the presence of inclusion bodies by microscopic visualisation. Phase-contrast microscopy and electron microscopy are used for this purpose. In phase-contrast microscope, inclusion bodies appear as highly refractile bodies, whereas in electron

Table 6.2 List of some commercially available biopharmaceuticals which are expressed as inclusion bodies

Biomolecule expressed	Recovered after cell lysis and centrifugation in:	References
Human growth hormone	Supernatant	Courtney et al. (1984)
Insulin A chain	Pellet	Goeddel et al. (1979a, b)
Insulin B chain	Pellet	Szoka et al. (1986)
α_1-Antitrypsin	Supernatant	Winkler et al. (1985)
Urokinase	Pellet	Bennett et al. (1984)
Calcitonin	Pellet	Pennica et al. (1985)
Human TNF	Supernatant	Itakura et al. (1977)
Somatostatin	Pellet	Dunnill and Lilly (1974)

Abbreviation: *TNF* tumour necrosis factor

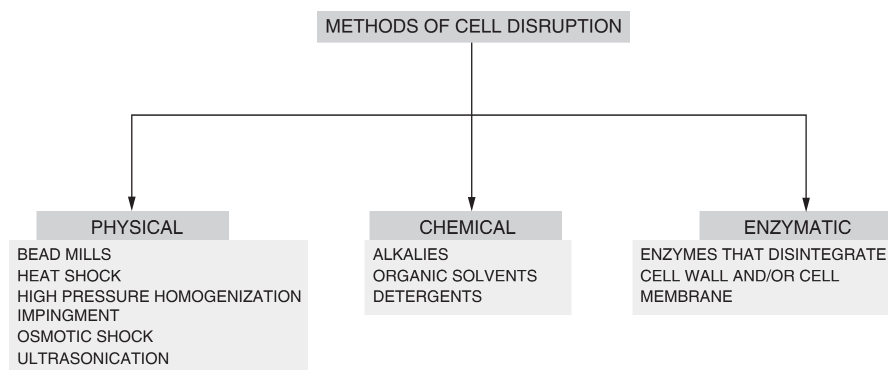

Fig. 6.4 Cell disruption to extract intracellular synthesised target biomolecule includes primarily physical, chemical and enzymatic methods

microscope, they appear as free amorphous aggregates in cytosol or in contact with a membrane which can be located distinctly (Schoemaker et al. 1985; Schoner et al. 1985). Once presence of inclusion bodies is confirmed, then sample can be preceded for lysis.

Cell disruption can be achieved by physical, chemical or enzymatic methods alone or in combination with each other. Figure 6.4 shows different types of cell lysis methods.

(1) **Physical methods:** This includes application of physical forces for cell lysis. There are several ways to achieve it: bead mill (which uses glass beads for grinding), heat shock, high-pressure homogenisation, impingement, osmotic shock and ultrasonication.

Bead Mills involve the use of glass beads which are placed in a jacketed high-speed reaction vessel. The reaction vessel in its centre has a rotating shaft. Agitators fitted with shaft provide the kinetic energy to beads present inside. The cells to be lysed are placed in the reaction vessel, and as a result, the force experienced by cells via glass beads results in cell rupture. The reaction vessel is jacketed to keep the vessel at an optimum temperature as temperature is increased while the process operates.

There are different types of designs available for reaction vessels; however the basic principle of operation remains the same. The size of glass beads used depends upon the type of cells to be lysed. Glass beads with diameter <0.5 mm are used for bacterial cells, whereas those with diameter >0.5 mm are used for yeast cells (Chisti and Moo-Young 1986).

Heat Shock based cell lysis is generally used in large-scale production systems. Certain transformed Gram-negative bacteria release intracellular expressed biomolecules, when heated up to 50 °C; similarly from *E. coli*, these can be released by heating them to 90 °C for 10 min. Better results are seen when short-term heat shocks followed by prolonged low temperatures are given to the bacterial cells (Middelberg 1995).

High-pressure Homogenisation is based to carry out cell lysis by forcing them to pass through a narrow orifice which is under high pressure. This instrument contains two components: a piston pump and a valve. The piston pump is in the form of a triplex, and the valve has a slit of ~100 mm which generates a pressure of ~1500 bar. After high-pressure application, when the cells experience sudden release of pressure at the valve, it causes their lysis. Modern homogenisers work in continuous mode (Saboya et al. 2003).

Impingement refers to a mode of cell lysis by making them to strike on a stationary surface at high velocity. The instrument used to carry out the process is known as micro-fluidiser. This method brings 90% lysis efficiency for *E. coli* cells (Shao et al. 2015).

Osmotic Shock is another way for carrying out cell lysis. The sample is placed in a hypertonic or hypotonic medium which can lead to cell shrinkage or swelling and ultimately bursting, respectively. However, this method is not commonly used because of its low efficiency. Also high amount of salt usage leads to further complications in downstream processing of the target biomolecule.

Ultrasonication involves the use of ultrasonic waves for cell disruption, but this method is employed for lab-scale experiments only.

(2) **Chemical methods:** It involves the use of alkalis, organic solvents or detergents. The use of alkali in intracellular product recovery is applicable if

the expressed molecule of interest is alkali stable. For example, recombinant growth hormone is recovered from transformed *E. coli* cells by treating it with NaOH (pH 11). Similarly various organic solvents are used for the same purpose, e.g. toluene; alcohols such as methanol, ethanol, butanol; and DMSO. These organic solvents have the capacity to extract phospholipids from the cell membrane, hence destabilising the integrity of the membrane causing cell lysis. Ionic detergent such as cetyl trimethyl ammonium bromide (CTAB) and sodium lauryl sulphate and non-ionic detergents such as Tween 20 and Triton X-100 are also used for lysing the cells. They act by denaturing membrane proteins and ease their solubilisation and extraction from the membrane (Harrison 2011). However, in ionic detergent-based cell lysis, proteins recovered show change in their ability to undergo salt precipitation; thus before proceeding to the next step, recovered protein can be ultrafiltrated or subjected to ion-exchange chromatography.

(3) **Enzymatic methods:** Different set of enzymes are used which can disintegrate the cell membrane and/or cell wall. Most commonly used enzymes are lysozyme, glucanase, mannose and proteases. The kind of enzyme used depends upon the type of cell to be lysed. In case of prokaryotic bacterial cells, the cell wall composition differs. Lysozyme acts on β-(1→4) glycosidic linkages present between N-acetyl muramic acid (NAM) and N-acetyl glucosamine (NAG) of peptidoglycan (Crapisi et al. 1993). Peptidoglycan layer is easily accessible in case of Gram-positive bacteria; thus lysozyme is more effective on Gram-positive cells in comparison to Gram-negative cells in carrying out the lysis. Anionic sugar residues comprising lipopolysaccharide of Gram-positive bacterial cell wall are stabilised by cations like calcium and magnesium. EDTA chelates with these cations and destabilises the cell wall integrity. Thus, lysozyme is used in general in combination with EDTA (Dean and Ward 1992). Glucanases are used in combination with proteases for lysis of yeast cells (Scott and Schekman 1980).

(d) **Precipitation and crystallisation**: Once the biomolecule of interest is available in the medium (with/without cell lysis), the next step is to capture that target molecule. Precipitation and crystallisation are the methods that serve this purpose. Selection of the batch and continuous modes for precipitation/crystallisation depends upon the kind of biomolecule to be recovered. For example, blood plasma proteins are separated by carrying out precipitation in batch mode (Buchacher and Iberer 2006). The continuous mode of precipitation/crystallisation offers advantages over batch mode in terms of consistency and reproducibility; production of precipitates with optimal size, strength and density; minimising supersaturation and avoiding particle break-up. However, literature study shows that despite the above-mentioned advantages, batch mode is practically used in the field (Watt 1970).

6.3.2 Purification: Process and Technologies

Chromatography is the most widely used technique for the purification of the extracted recombinant protein or other target biomolecule. It works on the principle of differential partitioning of the molecule of interest between two phases, which is a function of the type of interactions that exist between the molecule of interest and the solvent in which it preferentially partitions. The target molecule to be purified from a mixture is placed in suitable mobile phase and allowed to interact with a solid stationary phase. The stationary phase is chosen and provided in the column on the basis of affinity interactions of molecule of interest with it. These interactions are then destabilised with the help of eluent such that the target molecule which is the most tightly bound molecule will be released at the end of the run of column and least bound which are preferably unwanted molecules will be released from the column at the earliest. Elution can be carried out in an isocratic or continuous/discontinuous gradient manner.

On the basis of the types of interactions existing between target molecule and stationary phase, there are different types of chromatography: affinity, hydrophobic interaction, ion exchange and size exclusion (Saraswat et al. 2013). Protein A affinity chromatography in specific is used for purification of MABs (Liu et al. 2010; Shukla and Hinckley 2008). Different kinds of ligands are used in affinity chromatography, e.g. polyhistidine-tagged resin and protein L (Saraswat et al. 2013). The method provides high flow rate and capacity in addition with high specificity for the IgG antibodies. The method also provides high degree of purity (>95%) by removal of host cell proteins (HCPs), viruses, DNA and media components (Liu et al. 2010; Gagnon 2012; Lain et al. 2009). But, problems are faced using this method in MABs purification because of leaching of protein A/L/the tags, which causes non-specific binding of HCPs and DNA, thus decreasing the degree of purification obtained (Ghose et al. 2006; Tarrant et al. 2012; Lowe 2001). For MABs having a basic isoelectric point, cation-exchange chromatography can work as a better alternative method for their purification as compared to protein A affinity chromatography (Liu et al. 2010; Lain et al. 2009; Chon and Zarbis-Papastoitsis 2011). Humira (anti-TNFα MAB) and Synagis (MAB targeting RSV F protein) are examples of commercially available MABs purified using cation-exchange chromatography (Chon and Zarbis-Papastoitsis 2011). The cost of purification of MABs using cation-exchange chromatography is almost 1/5 compared to protein A chromatography (Lain et al. 2009; Chon and Zarbis-Papastoitsis 2011). However, other chromatography types are used in next downstream process, i.e. polishing, which will be discussed later.

Carrying out the process of chromatography is also an important factor that decides the % purity of the target molecule. Traditional methods include batch mode operation and packed column operation; recently chromatography carried out in continuous modes is in use. In traditional operation methods, the column capacity is not completely utilised which limits its efficiency at preparative scale (Gueorguieva et al. 2011). The feed solution is continuously applied at the top, and fractions are

separated at the bottom of the column in case of continuous system. This system is suited for multicomponent fractionation. The continuous mode of operation helps to overcome the limitations encountered in batch mode of operation. It can further be operated in different modes – annular, countercurrent, concurrent and carrousel. A continuous chromatography method where the chromatography bed is packed into a rotating annulus is annular continuous chromatography (Jungbauer 2013). The method came into existence in the early 1990s (Bloomingburg et al. 1991). It has been used for purification of recombinant blood clotting factor IX by removal of impurity vitronectin (Iberer et al. 2002), virus vaccines (Ozdural et al. 2007) and recombinant antibodies from cell culture supernatant (Giovannini and Freitag 2001). In countercurrent loading, at least three columns are required; the first column is loaded to saturation, and the breakthrough is loaded onto the second column. The saturated column is washed and eluted as in a batch operation (Jungbauer 2013). Multi-column countercurrent chromatography is useful for resolving various protein variants and gives high yield and purity of the target product (Aumann and Morbidelli 2007). This method is used for the purification of commercially available recombinant streptokinase (Martinez Cristancho et al. 2013) and single-chain Ab fragments (Mahajan et al. 2012) and various MABs (Gueorguieva et al. 2011; Mahajan et al. 2012).

Recently efforts have been done to develop alternative and an easy way of carrying out chromatography. Membrane chromatography is one of the alternative formats which combines the advantage of membrane filtration with liquid chromatography. This is known to be a cost-effective strategy to purify large volume of feed streams (Rathore et al. 2018). Further attempts have been made to increase the binding capacity of membrane using hydrogels and nanofibre-based matrices. These types of materials offer high specific area and higher ligand density which help in efficient purification of biopharmaceuticals (Liu et al. 2017).

6.3.3 Polishing: Process and Technologies

The final step in downstream processing includes final removal of specific impurities, misfolded forms of biomolecule of interest and charge variants, concentrating the product and final formulation (Kramberger et al. 2015). Again like the previous step, chromatography-based methods are predominantly used in this, e.g. size-exclusion chromatography and hydrophobic interaction chromatography; however other methods like ultrafiltration are also used. In addition, process like crystallisation is also used in certain cases like in the final purification of small protein insulin. So here below are described a few of the most commonly used methods for polishing:

(a) **Hydrophobic interaction chromatography (HIC)**: HIC is the most commonly used polishing method for MABs as aggregates are easily retained on it (Ghose et al. 2009). This is carried out with the help of kosmotropic salts, e.g.

$(NH_4)_2SO_4$, KH_2PO_4 and $Na_3C_6H_5O_7$, which interact with water molecules to reduce solvation of protein molecules in water. This further causes exposure of hydrophobic patches on the proteins to promote their binding (Liu et al. 2010). Such misfolded or aggregated proteins are eluted from the HIC column by using organic mobile phases. However, large amount of salt usage in it has two major implications: (a) the sample after purification has to be repurified using ultrafiltration to remove excess of salts which can affect the target protein stability and (b) high salt usage has corrosive effects on stainless steel tanks which can further cause economy issues related to the process (Chen et al. 2008; Gagnon 2006). Thus, advances have been made to carry out polishing using HIC in no-salt conditions to get rid of the problems faced in the former case (Gagnon 2012; Arakawa et al. 2007; Tsumoto et al. 2007; Kato et al. 2004).

(b) **Simulating moving bed (SMB) chromatography:** This method is effective for the separation of binary mixtures, which rarely exist in the product mixtures obtained from a biotechnological process. So, they are used in modified version, where the procedure is repeated to separate a kind of impurity in one cycle of operation (Mun et al. 2003). There are certain cases where a biomolecule or target molecule exists in different enantiomers; however only a particular conformation is known to be biologically important. This method is used to separate the biologically relevant enantiomers from the feed/pool (Rajendran et al. 2009).

(c) **Crystallisation:** The procedure is used for final purification and formulation of the biological molecule of therapeutic importance. The technique was used recently to obtain purified single-chain antibody using aqueous two-phase system (Huettmann et al. 2014). The method brings high purification fold as molecules folded and arranged in a regular lattice structure are selected from the misfolded aggregates of the target biomolecule (Zang et al. 2011). Commercially available biopharmaceutical recombinant protein insulin is produced and purified using crystallisation procedures. Just like other techniques, this method can also be carried out in batch or continuous mode; however, like always the latter mode of operation provides better control of crystal morphological characteristics and enhanced quality (Kwon et al. 2014).

(d) **Single-pass** *and high-performance tangential flow filtration (SPTFF and HPTFF)*: As mentioned earlier about the tangential flow filtration, it is preferred over dead-end mode. The conventional tangential flow filtration has limited use for the separation of solutes from the produce that differs in their size by a factor of ~ 10-fold. However, the tangential flow filtration applications have broad scope in the polishing process as well. Here, it is used for final purification step that involves the separation of virus from target protein or buffer-protein or misfolded protein-protein separations (van Reis et al. 1997). Comparative studies between conventional and SPTFF have shown that the latter one gives higher reproducible results for protein concentration with 98.9% product recovery. SPTFF is applicable for final step purification of IgG molecules (Casey et al. 2011). HPTFF has further superior features, as it provides high-resolution protein separation. It involves the use of size difference and charge difference as

well (Zydney and van Reis 2001). The optimal conditions for HPTFF working are pH that is close to pI of the lower molecular weight protein and low salt concentrations (~10 mM ionic strength). These conditions exclude the proteins which have extra charged residues that lead to misfolding (Zydney and van Reis 2001). HPTFF also has application in separation of monomers from oligomers (Lebreton et al. 2008), singly pegylated protein from highly pegylated species of the same protein (Ruanjaikaen and Zydney 2011) and MABs from mammalian HCPs (van Reis and Zydney 2007).

(e) **Continuous refolding:** In case, the target protein is synthesised by the transformed cells as inclusion bodies, the procured protein must be refolded. This is because inclusion bodies are recovered after treating with chaotropic agents and detergents, which cause protein denaturation. The in vitro protein folding is a slow and costly process (Eiberle and Jungbauer 2010; Jungbauer and Kaar 2007). One of the ways is to carry out refolding using refolding buffer. The buffer dilutes the concentration of chaotropes and detergents associated with the recovered protein in a single step, which allows intramolecular interaction and prevents intermolecular aggregation, promoting the refolding. The method is simple and has been used for decades at industrial scale preferably (Jungbauer and Kaar 2007). Another method is expanded bed chromatography which has been used for refolding of α-lactalbumin (Machold et al. 2005). In this case, the chromatography bed/stationary phase is expanded using upward flow of the equilibration buffer. The crude mixture is fed into the expanded bed in upward motion, where the target molecules are captured as in case of conventional chromatography. The elution buffer is also allowed to run in the upward direction for the release of the target molecules (Kennedy 2005).

(f) **Cascade diafiltration:** The final polishing and formulation process is important as in this step complete removal of salts is done to make them compatible for the biological use. For this, diafiltration is used which reduces the salt concentration in the recovered protein by a factor of 10^4 after 9.2 diavolumes, such that one million-fold salt reduction would require ~14 diavolumes (van Reis and Zydney 2007). Diafiltration carried out in continuous mode is performed by using a cascade of membrane set-ups which are arranged in countercurrent manner. This is called as cascade diafiltration. In this, the feed is allowed to enter continuously up to the assembled final membrane set-up, in a way that the retentate from each set-up stage is recycled to the previous stage of the cascade (Peeva et al. 2014). The following mathematical equation defines the fractional removal of a small impurity in cascade diafiltration:

$$f = (1-\beta)/1-\beta^{N+1}$$

where f is the fraction removed and β is the ratio of diafiltration buffer flow rate to the feed flow rate.

In the simplest case when the system is inclusive of 3 set-ups, $\beta = 10$, thus achieved f value is 9×10^{-4}; this can be increased using higher number of setups in the system (Zydney 2015).

6.4 Current Issues in Downstream Processing of Biopharmaceuticals

Advances in the field of molecular biology and biotechnology have contributed a lot to improve the upstream processes like host cell engineering, cell line improvement, optimisation of media formulations, enhanced bioreactor designing for better production of target molecules and improved controlled conditions for in vitro large-scale culture of cells (Hodge 2005). Research advances in genetic engineering have been made to control posttranslational modifications, e.g. glycosylation of glycoproteins. Sialic acid content in recombinant glycoproteins can be increased in the target proteins by overexpression of sialyltransferase (Lin et al. 2015) and down-regulation of sialidase expression using siRNA technology (Ngantung et al. 2006). In certain cases, fut8 gene knockout strategy has been used to produce defucosylated antibodies (Yamane-Ohnuki et al. 2004). A recent advancement has been made in the manufacture of genetically engineered T cells carrying T cell receptors or chimeric antigen receptor (CAR) in T cell-based cancer immunotherapy. It involves the use of single-use Cellbag bioreactor, bearing capacity up to 25 L in which cells can expand to >10^7 cells/ml (Wang and Rivière 2016). These efforts, in recent years, have improved upstream processes undoubtedly by increasing overall production of MABs from few mg/L to now reaching about 27 g/L (http://www.dsm.com/en_US/html/dpp/news_items/16_06_08_perc6.htm).

However, it is noteworthy to mention that very few efforts have been done to improve the downstream processing. This has resulted in increased cost of production due to the use of costly traditional downstream processes. It has been seen that downstream processing accounts for 80% of the total manufacturing cost of a biopharmaceutical (Frost and Sullivan 2004; Roque et al. 2004). Thus, high cost of downstream processes is one of the major issues associated with biopharmaceutical production. Another issue associated with downstream processing of biopharmaceutical is the never-ending requirement of quality product, for which we need to make novel advancements that can help to achieve it easily and again in a cost-effective manner.

6.5 Alternatives and Advancements Made to Address the Issues

Chromatography is the most conventional and widely used method for purification of proteins at large scale with high resolution in single-step protocol. In certain cases, two or three chromatography systems are run in series that brings higher

purification fold. For example, platform approach is used for purification of MABs in which first chromatography column is used to capture the target protein from the feed, and the latter two are involved in polishing of the product where DNA, leached protein A, LMW clipped species, HMW aggregates and HCPs are removed (Shukla et al. 2007). However, chromatography-based purification offers disadvantages such as high cost of operation, has limited capacity and offers diffusional spreading that may affect the resolution of the process. To address the issues mentioned, efforts have been made to move from traditional, costlier methods of operation to advanced technologies which can cut the cost of downstream processing. Some of the promising alternatives that have been brought into use are mentioned here below:

6.5.1 Aqueous Two-Phase Extraction (ATPE)

This is a kind of liquid-liquid extraction method developed by Albertsson (1970) and is not novel in origin; rather the idea of its application as a replacement for chromatography is novel. However, the designing and implementation of ATPE system process at a large scale are limited because of the poor understanding of the molecular mechanisms operating for the solute partitioning. Various factors determine the differential partitioning of the target molecule and contaminating molecules in aqueous two-phase system, for example, physico-chemical properties, e.g. charge, size and hydrophobicity, and system composition. Thus the driving forces responsible for the differential partitioning of the molecules are van der Waals forces, electrostatic interactions, hydrogen bonding, hydrophobic interactions and steric effects (Huddleston et al. 1991). And, it is possible to manipulate the operating forces by changing – pH, phase components and ionic strength of the phase or adding a ligand in it, with the aim to target the biomolecule of interest and get it separated from the contaminating population. Therefore, in order to use this technology at industrial scale, it is important to understand and predict the partitioning behaviour of the target molecule and the contaminants as well, which is a tedious task in itself, thus limiting its use. In addition, the procedure involves the requirement of large amount of pure water, which can pose another limitation to the access of the method. However, practically speaking, for the large biopharmaceutical companies, the estimated cost of pure water can be as low as US$ 0.2/L and thus can be easily overcome (Zhou and Tressel 2006). Although both phases used are water based, the system displays high viscosity of the coexisting phases which lowers the recovery speed of target biomolecule and usage of polymers like dextran, further making the technique costly (Ferreira et al. 2016). In lieu of the problems mentioned, recent attempts have been made on the protocol modification from polymer/polymer to polymer/salt and salt/salt combinations, to reduce the cost (Azevedo et al. 2007). These modifications: (a) use cheaper salts and hence reduce costs and (b) show lower viscosity contrary to conventional systems and hence provide faster separation rates.

6.5.2 Nano-magnetic Separation of Antibodies

Cheap and easily synthesised magnetic nanoparticles can be used at industrial scale by coating with different polymeric substances that enhance their stability in colloidal solutions, morphology and functionality as well. These nano-magnetic particles can be used in combination with ATP extraction system which enhances the speedy recovery of target molecule (by a factor of 10 w.r.t. ATP extraction system) from the feed (Azevedo and Aires-Barros 2011; Wikstrom et al. 1987; Larsson 1994). After washing out the contaminants, the target molecule can be eluted and formulated for biological delivery. The method is advantageous over classical chromatography-based separation and purification method, as it is simple to carry out, does not require expensive liquid chromatography systems and hence is economical. Moreover, it can be integrated with cell disintegration steps for procuring intracellular proteins and hence decreases the processing time to a greater extent (Schuster et al. 2000). The method enhances the recovery of the peptides in intact form which get otherwise broken in traditional chromatography systems (Hofmann et al. 2002). A list of commercially available peptides and proteins of therapeutic importance purified by this method is enlisted in Table 6.3. In the near future, we expect the increased usage of this technology at laboratories and industries for biopharmaceutical downstream processing.

Table 6.3 List of some commercially available biopharmaceuticals purified by non-magnetic particle-based separation method

Biopharmaceutical	Source	Magnetic carrier	References
β-Galactosidase	*E. coli* homogenate	Silanised magnetite	Dunnill and Lilly (1974)
Lysozyme	Hen egg white	Magnetic chitin	Safarik and Safarikova (1993)
Caspase	Human cells	Magnetic agarose	Himeji et al. (2002)
Albumin	Human plasma	Magnetic poly(2-hydroxyethyl methacrylate) beads	Odabasi and Denizli (2004)
MABs	Mouse hybridoma culture broth	Magnetite particles	Shinkai et al. (1992)
Thioredoxin (histidine tagged)	*E. coli*	Magnetic agarose	Schafer et al. (2002)
Uricase (histidine tagged)	*Bacillus* sp.	Ion-chelating magnetic agarose beads	Nishiya et al. (2002)
Aldolase (histidine tagged)	*E. coli* homogenate	Magnetic core and nickel-silica composite matrix	Frenzel et al. (2003)

6.6 Challenges and Future Trends

Although the advancements have been done as mentioned above in the downstream processing of biopharmaceuticals, yet the cost of production and the need of continuous ample supply of the biopharmaceutical in the market are still big challenges to be resolved. The developed conventional platform technologies and methods are used at industrial scale and are still not completely replaced by new methods as it would require skilled labour to troubleshoot the problems faced in the procedure, which is not easily available and hence for them is a risk-taking challenge. However research and development of the concerned firms is working to get advanced ways to improve the downstream processing of biopharmaceuticals. The upcoming future trends are in silico-based methods: algorithmic (super structure optimisation, model-based hybrid approach) and non-algorithmic (heuristic approach based, experimental and platform approach) methods. For this, different databases need to establish, for the designing of programmes that can help the purification and polishing process with ease. Also, hybrid process development approaches are in pipeline that include combination of high-throughput experimentation and mathematical models (Hanke and Ottens 2014). These methods are expected to offer greater sensitivity to minute changes in the downstream processes, and hence a better control strategy could be devised that would lead to better recovery of the product in the process and can cut down the cost.

6.7 Conclusion

The therapeutic benefits offered by biopharmaceuticals have allured the pharmaceutical company to increase the ways and strategies for their cost-effective production. Major costs are involved in their downstream processing which offers a potential area to carry out further research and development. Combinatorial sciences can offer a lucrative solution to the problem; however till then conventional chromatography for purification will hold promise for quality production of these biopharmaceuticals.

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Chapter 7
Engineering *Streptomyces peucetius* for Doxorubicin and Daunorubicin Biosynthesis

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Abstract Doxorubicin and daunorubicin are notable members of the type II polyketide synthase family and clinically important cancer chemotherapeutic agents and are produced by a mutant strain *S. peucetius* ATCC 27952. They belong to the anthracycline-type antitumor drugs. Doxorubicin remains one of the most widely used antitumor drugs for the treatment of various cancers because of its broad spectrum of activity. As a result, numerous works have been carried to unravel the biosynthetic pathway and the underlying regulatory mechanisms to gain insight into the mechanisms of the genes involved. Consenquently, there is a need to develop an overproducing strain at the industrial scale, to produce doxorubicin as an anticancer drug. Therefore a significant amount of progress has been made in unraveling the bottlenecks in the pathway, manipulating the biosynthesis, improving production, and generating novel derivatives by engineering *S. peucetius* strain.

Here we review in depth, various pathway engineering approaches and strategies that have been applied during these courses of time, since the discovery of these compounds, for the efficient production of daunorubicin and doxorubicin. The major pathway engineering approaches discussed in this chapter are divided into three parts: the first part includes the engineering of the thymidine diphosphate-L-daunosamine biosynthesis pathway genes which is important for the enhanced production of the glycone which in turn is used for the glycosylation reaction. Similarly the second part includes the engineering of the polyketide genes responsible for the production of the aglycone moiety that undergoes several modifications to generate the important compounds doxorubicin and daunorubicin. Lastly, we discuss the engineering of the several regulatory genes involved either directly or indirectly in regulation and control of the production of daunorubicin and doxorubicin.

7.1 Introduction

Streptomyces belonging to the group of actinomycetes are filamentous gram-positive bacteria that undergo morphological and physiological differences to produce a wide range of secondary natural products. The soil-dwelling *Streptomyces* are the key producers of numerous molecules including antibiotics, antivirals, anticancer, and other bioactive molecules, owing to their secondary metabolism. These products may include useful therapeutic agents, such as antibiotics, antifungals, and antitumor, and thus a better understanding of the biosynthetic pathway and the regulatory mechanism of genes at the molecular level would provide useful insights into the fundamental issue of secondary metabolism in *Streptomyces* spp., ultimately helping to engineer strains for overproduction of these useful metabolites (Hao and Hutchinson 2006). Polyketide secondary metabolites in microbes are mainly produced via various polyketide synthases (type I, type II, type III) and non-ribosomal polyketide synthases (NRPKS) enzymes. Among these, type II polyketide synthases consist of a significant and chemically diverse group of bacterial secondary metabolites, such as tetracyclines and actinorhodin produced by *S. coelicolor*; rhodomycinone, doxorubicin, and daunorubicin produced by *S. peucetius*; jadomycin A

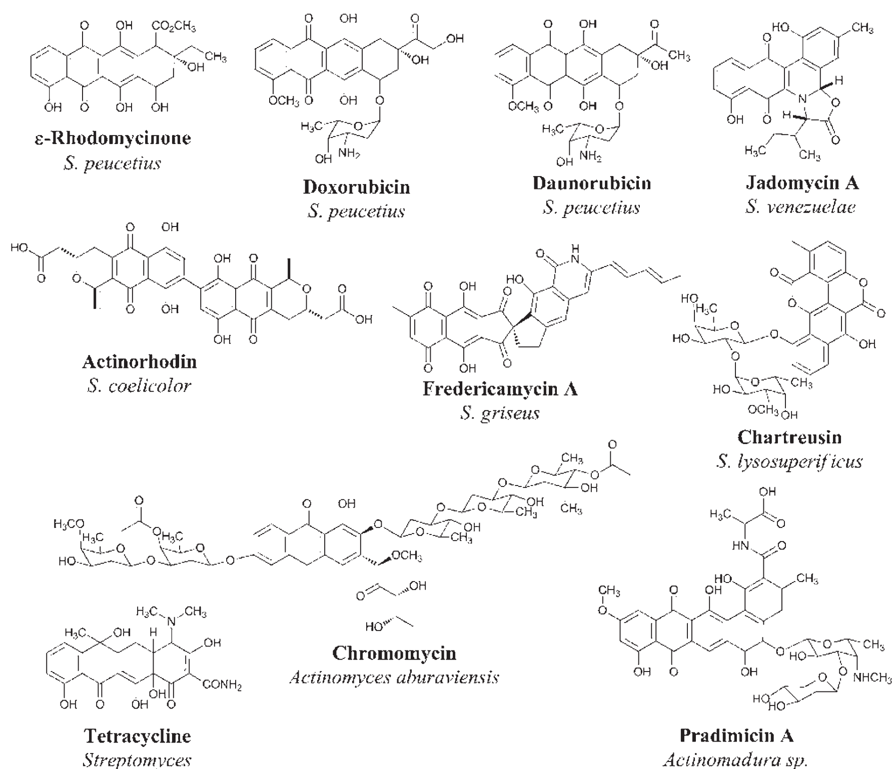

Fig. 7.1 Structures of different type II polyketides synthases (PKSs) produced by various actinomycetes including *S. peucetius* major products ε-rhodomycinone, daunorubicin, and doxorubicin

produced by *S. venezuelae*; pradimicins produced by *Actinomadura* sp.; charteusin produced by *S. lysosuperificus*; chromomycin produced by *Actinomyces aburaviensis var. verrucosus*; and many more (Fig. 7.1).

Doxorubicin and daunorubicin are notable members of the type II polyketide synthase family, and clinically important cancer chemotherapeutic agents. These molecules are produced by *Streptomyces peucetius* ATCC 27952 which is a mutant strain of *S. peucetius* 29050 (Arcamone et al. 1969). Daunorubicin was discovered in 1962, when it was first isolated from *Streptomyces caeroleorubidus* in France; however, it was also isolated from *S. peucetius* in Italy and the Soviet Union with different names (Aubel-Sadron and Londos-Gagliardi 1984), such as rubidomycin, daunomycin, and rubomycin. Daunorubicin is reported to be produced from a number of organisms; however, doxorubicin was exclusively produced by *S. peucetius subsp. caesius* (Grein 1987). Doxorubicin is a chemotherapy medication belonging to the anthracycline and antitumor drug family and is also known by its trade name Adriamycin. It is routinely used in the treatment of numerous human cancers, including breast, ovarian, liver, lung, bladder, gastric, and thyroid cancers, multiple myeloma, non-Hodgkin's and Hodgkin's lymphoma, Kaposi's sarcoma, neuroblastoma, soft tissue sarcoma, and pediatric cancers (Cortes-Funes and Coronado 2007;

Thorn et al. 2011). Because of its broad spectrum of activity, doxorubicin remains one of the most widely used antitumor drugs for the treatment of various cancers (Allwood et al. 2002).

The biosynthesis of both daunorubicin and doxorubicin is initiated by a type II polyketide synthase starting from one propionyl-CoA starter unit and extended by nine malonyl-CoA units, to produce a decaketide that is converted to aklanonic acid that leads to the formation of an aglycone ε-rhodomycinone (Hutchinson 1997). The aglycone of daunorubicin, ε-rhodomycinone, is a tetracyclic ring consisting of quinone-hydroquinone groups lying adjacent to each other, along with a methoxy group, a short carbonyl side chain. The sugar is attached to aglycone by a glycosidic bond and is known as L-daunosamine, which consists of 3-amino-2, 3, 6-trideoxy-L-fucosyl moiety, and is synthesized from D-glucose 1-phosphate using a variety of genes. Finally, series of post-modifications, like methylation, decarboxylation, and hydroxylation, leads to the formation of daunorubicin and ultimately doxorubicin. Doxorubicin is the C-14 hydroxylated form of its immediate precursor, daunorubicin. The only difference between these two molecules is the side chain of doxorubicin, which terminates with a primary alcohol, whereas daunorubicin terminates with a methyl group (Fig. 7.1) (Minotti et al. 2004).

S. peucetius has a self-resistance system that helps it to overcome the toxicity of the antibiotic daunorubicin and doxorubicin inside the cell. The four genes, namely, *drrA*, *drrB*, *drrC*, and *drrD*, present in the doxorubicin biosynthetic gene cluster of *S. peucetius* mediate the self-resistance. The first two genes *drrA* and *drrB* belong to ABC transporter type I and together form an ATP-dependent efflux pump to remove daunorubicin out of the cell (Brown et al. 2017; Guilfoile and Hutchinson 1991; Kaur and Russell 1998), whereas the third gene, *drrC*, imparts resistance through excisional repair by binding to DNA at regions intercalated by daunorubicin and then removing it (Prija and Prasad 2017). DrrD is a flavin-binding protein involved in the self-resistance mechanism, and DrrD devoid mutant exhibits partial loss of self-resistance to daunorubicin (Karuppasamy et al. 2015).

7.1.1 Objective

Doxorubicin and daunorubicin have been of interest since their discovery in 1962 and their use as a potent anticancer drug in various forms of cancer. There has been significant work involving the enhanced production of these important metabolites from *S. peucetius* using various approaches. The drive to develop a recombinant strain of industrial importance for mass production of this anticancer drug has been addressed in this chapter. Using the fermentation process combined with pathway engineering strategies and engineering the regulatory genes with modifications at the molecular level have been discussed and explained in this chapter thus providing an overview of the doxorubicin and daunorubicin biosynthesis in *S. peucetius.*

7.1.2 Mode of Action of Doxorubicin

Doxorubicin is one of the most potent US Food and Drug Administration-approved anthracycline classes of anticancer agents. It exerts its antiproliferative activity on tumor cells via three proposed mechanisms: (1) DNA binding by intercalation between DNA double helix and disrupt DNA replication and transcription process, (2) disruption of topoisomerase-II-dependent DNA repair, and (3) production of free radicals ultimately damaging cell components such as cell membranes, nucleic acids, and proteins (Fig. 7.2). Collectively, these modes of actions result in DNA disruption and loss of DNA mismatch repair function that ultimately leads to cell death (Gewirtz 1999; Thorn et al. 2011). However, cardiotoxicity is the major factor limiting its medicinal use as it alters iron and calcium regulations in mitochondria (Swain et al. 2003; Carvalho et al. 2009).

Doxorubicin and most of the anthracycline class of compounds intercalate between deoxyribonucleic acid (DNA) base pairs and bind with DNA associated

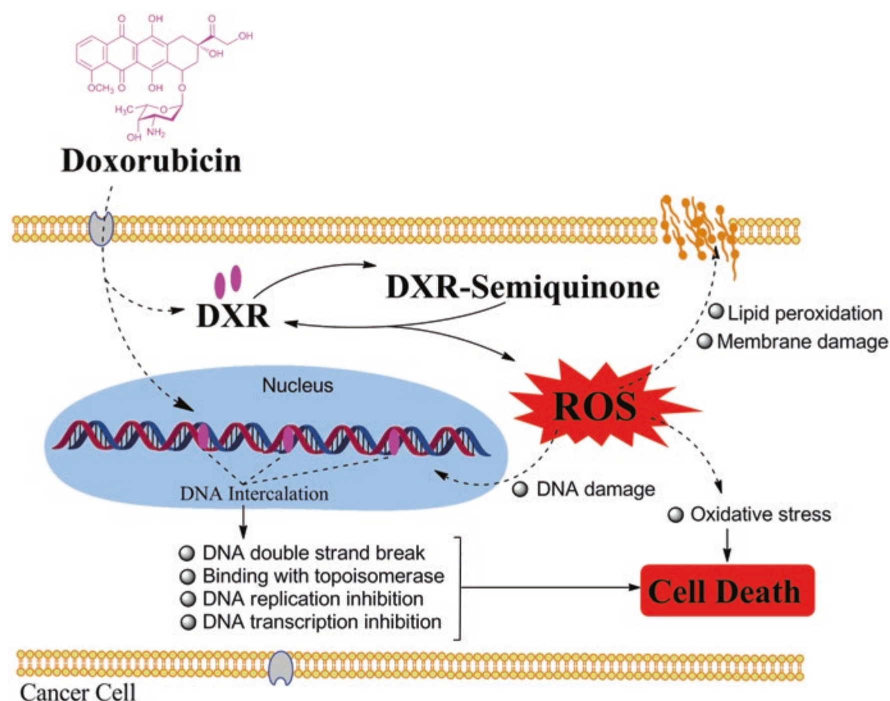

Fig. 7.2 Mode of action of doxorubicin (DXR) inside the cancer cell. Doxorubicin interacts with the DNA by intercalation, disruption of topoisomerase-II-dependent DNA repair, and inhibition of the replication and transcription process. Doxorubicin is oxidized to doxorubicin semiquinone which is unstable intermediate and converted back to DXR-releasing reactive oxygen species (ROS) that causes oxidative stress and induces damage to cell membrane, lipid peroxidation, and DNA damage leading to cell death

enzymes (topoisomerase II), inhibiting DNA replication and ribonucleic acid (RNA) transcription. The aglycone molecule intercalates in between DNA strands, whereas the sugar unit binds to the minor groove in the DNA by displacing water molecules and ions. The amino sugar (L-daunosamine) attached at the seventh hydroxyl position of the ring sits in the minor groove and intercalates the flanking base pairs immediately adjacent to the flanking site, which stabilizes the binding complex of doxorubicin and the DNA molecule. The amino group and hydroxyl group of the sugar facing outside the minor groove interact with the polymerase enzymes, thus inhibiting their function in DNA replication (Pigram et al. 1972; Frederick et al. 1990). Alternatively inside the cell, doxorubicin undergoes one electron reduction to form a DOX-semiquinone radical, an unstable metabolite. It is reoxidized back to doxorubicin and leads to the formation of reactive oxygen species and hydrogen peroxide. These reactive radicals cause oxidative stress to the cell that destructs multiple cell components such as cell membrane, nucleic acids, and ultimately trigger apoptotic pathways of cell death (Fig. 7.2) (Doroshow 1986; Thorn et al. 2011).

7.2 Biosynthesis of Daunorubicin and Doxorubicin

The gene cluster of *S. peucetius* has been studied extensively, because of its ability to produce doxorubicin and daunorubicin, which have broad clinical applications. *S. peucetius* 29050 complete genetic map was first published in 1999 by Lomovskaya et al. The *S. peucetius* ATCC 27952 strain was sequenced, and its genome was analyzed in 2004 (Parajuli et al. 2004). The 8.7-Mb genomic sequence analysis of *S. peucetius* ATCC 27952 identified a 40 kb daunorubicin and doxorubicin biosynthetic gene cluster, which is 5.3 % of the total genome consisting of 37 open reading frames (Fig. 7.3). Biosynthesis of doxorubicin is completed in three stages: (A) formation of the aglycone, ε-rhodomycinone through condensation of one propionyl-coenzyme A and nine malonyl coenzyme A precursor units; (B) formation of activated amino sugar moiety, thymidine diphosphate (TDP)-L-daunosamine from D-glucose-1-phosphate; and finally (C) glycosylation of ε-rhodomycinone by glycosyltransferase, followed by different post-modifications like decarboxylation, methylation, and hydroxylation to produce the final compound (Hutchinson and Colombo 1999). In recent years, detail study of the daunorubicin and doxorubicin biosynthetic gene cluster that comprises catalytic enzymes, transcription factors,

Fig. 7.3 Physical and functional map of the daunorubicin and doxorubicin gene cluster. The relative sizes of the open reading frames and the direction of gene transcription are designated by pointed boxes, which are colored according to the types of functions that are indicated below

and resistance gene has improved the understanding of the biosynthesis machinery and regulatory mechanisms that control the doxorubicin biosynthesis.

7.2.1 Biosynthesis of ε-Rhodomycinone

Doxorubicin biosynthesis starts with the formation of an important intermediate ε-rhodomycinone (Dickens et al. 1995), whose entire carbon backbone is synthesized by a type II polyketide synthase (PKS) enzyme that is encoded by the *dpsABCDGEFY* genes (Fig. 7.4). A 21-carbon decaketide is initially formed by serial condensation of 9 malonyl-CoA units to 1 propionyl-CoA starter unit, and this multistep reaction involves enzymes from the polyketide synthase family, like 3-oxoacyl ACP synthase (*dpsA*) (Meurer and Hutchinson 1995), ketosynthases (*dpsB* and *dpsC*) (Grimm et al. 1994; Bao et al. 1999), acyltransferase (*dpsD*), and an acyl carrier protein (*dpsG*) (Lomovskaya et al. 1999). The ketoreductase (*dpsE*) carries out reduction of the decaketide, followed by aldol condensation, and then three steps of ring cyclization catalyzed by DpsF and DpsY, to form 12-deoxyalkanoic acid (Lomovskaya et al. 1998). A keto group is introduced into this intermediate by monooxygenase (*dnrG*) to form alkalonic acid, which is subsequently converted to aklaviketone by alkalonic acid-S-adenosyl-L-methionine methyl ester transferase, encoded by a homodimeric protein *dnrC* (Madduri and Hutchinson 1995). Further, alkalonic acid methyl ester cyclase, encoded by *dnrD*, carries out the cyclation reaction. Finally, the 7-oxo moiety of aklaviketone is reduced to a hydroxyl group, to form ε-rhodomycinone in two sequential steps executed by the enzymes aklaviketone reductase, encoded by *dnrH*, and a hydroxylase, encoded by *dnrF* (Filippini et al. 1995).

7.2.2 Biosynthesis of Thymidine Diphosphate-L-Daunosamine

The biosynthesis of thymidine diphosphate-L-daunosamine involves a seven gene cluster, namely, *dnmL*, *dnmM*, *dnmU*, *dnmT*, *dnmJ*, and *dnmV*. The biosynthesis begins from D-glucose-1-phosphate. The sequential action of two enzymes glucose-1-phosphate thymidylyl transferase and thymidine diphosphate-D-glucose 4, 6-dehydratase, encoded by *dnmL* and *dnmM*, respectively, catalyzes the first two enzymatic reactions to generate the intermediate thymidine diphosphate-4-keto-6-deoxy-D-glucose (TKDG) (Gallo et al. 1996). DnmU, an epimerase, carries out the epimerization of thymidine diphosphate-4-keto-6-deoxy-D-glucose to thymidine diphosphate-4-keto-6-deoxy-L-glucose, to which a keto group and an amino group is added at the C-3 position, followed by the enzyme hydratase and aminotransferase, which are encoded by *dnmT* and *dnmJ*, respectively. Finally, *dnmV*, a ketoreductase, reduces the C-4 ketone to a hydroxyl group, to generate thymidine diphosphate-D-daunosamine (Fig. 7.4) (Otten et al. 1997).

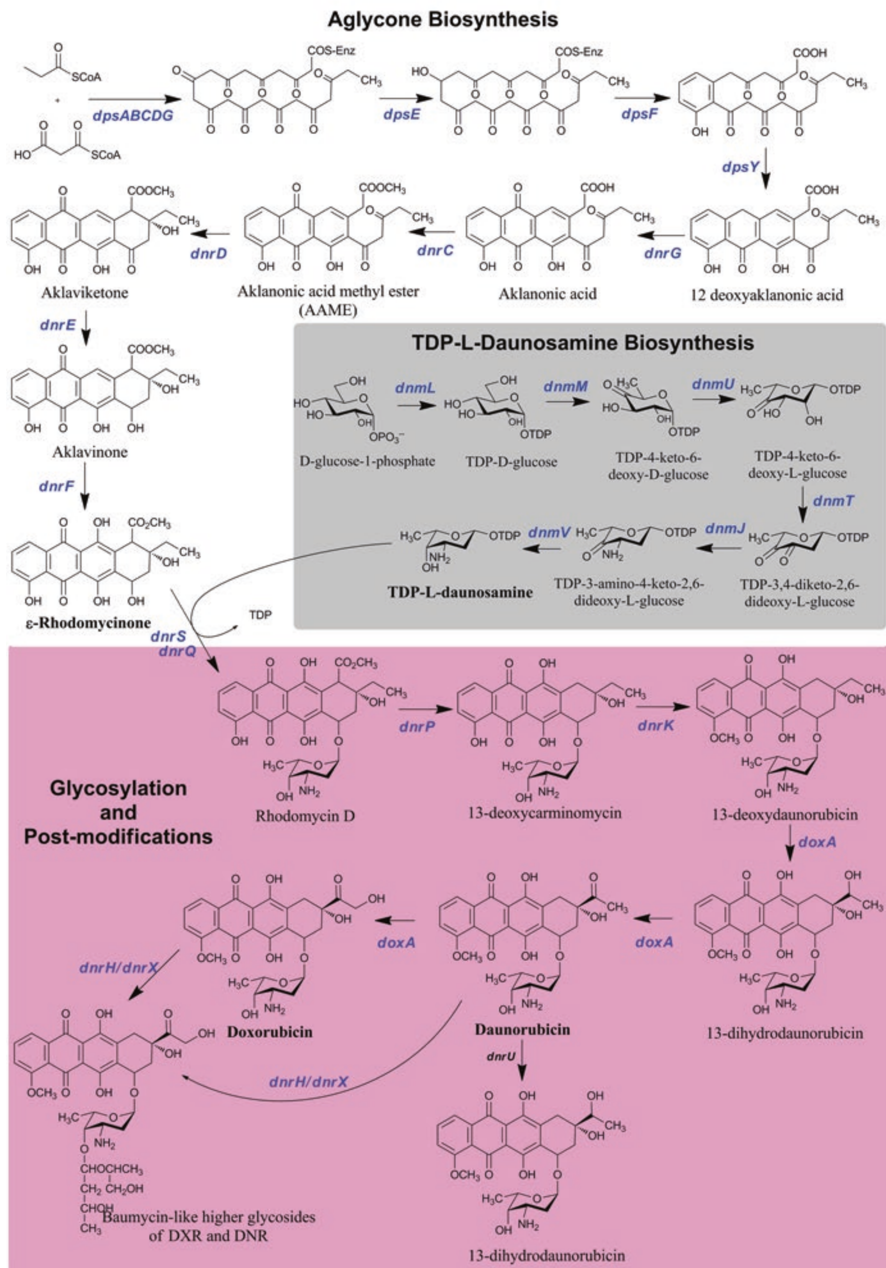

Fig. 7.4 Biosynthetic pathways of doxorubicin (DXR), daunorubicin (DNR), and ε-rhodomycinone from propionyl-CoA and malonyl-CoA along with biosynthetic pathway for thymidine diphosphate-L-daunosamine starting from D-glucose-1-phosphate. Also shown in the figure is the pathway for the glycosylation and post-modification of the final compound daunorubicin and doxorubicin into 13-dihydrodaunorubicin and baumycin-like higher glycosides

7.2.3 Glycosylation and Post-modifications

After the completion of the polyketide stage, ε-rhodomycinone is converted to rhodomycin D, a daunosamine conjugated derivative by the enzyme *DnrS*. Rhodomycin D is then converted to 13-deoxycarminomycin (Furuya and Hutchinson 1998) by the *DnrP* esterase, and this is followed by *O*-methylation by the methyltransferase encoded by *DnrK*, to produce 13-deoxydaunorubicin (Dickens et al. 1997). The latter metabolite undergoes C-13 oxidation by the cytochrome P450 enzyme in two stages, first forming an intermediate 13-dihydrodaunorubicin, and then daunorubicin. *DoxA* is responsible for both steps (Walczak et al. 1999). Daunorubicin is eventually hydroxylated by the same *DoxA* enzyme at the C-14 position, to generate doxorubicin (Fig. 7.4).

7.2.4 Regulation of Daunorubicin and Doxorubicin Biosynthesis

S. peucetius has various types of regulatory genes that control the production of daunorubicin/doxorubicin, which include transcription factors *dnrO*, *dnrN*, and *dnrI*, transcriptional repressor *drrD*/*dnrW*, transcriptional control by a coherent feed forward loop, self-resistance, and feedback regulation (Jiang and Hutchinson 2006). The *dnrO* is the major transcriptional regulator located adjacent to the *dnrN* gene. It encodes a protein that has a helix-turn-helix DNA binding domain close to its *N*-terminal region and belongs to a member of the TetR family of transcriptional regulators. The inactivation of *dnrO* leads to the complete loss of anthracycline antibiotics biosynthesis in *S. peucetius.* DnrO is essential for the expression of the pathway-specific *dnrN* transcriptional activator, and this in turn activates *dnrI* (Otten et al. 2000). DnrI, being the master regulator, binds to the several regions of polyketide synthases and activate the efflux regulatory genes (Madduri and Hutchinson 1995; Tang et al. 1996). In contrast, DnrO negatively regulates biosynthesis pathway genes due to self-repression phenomena (Lei and Parekh 2005). The self-repression of *dnrO* is an important event, as it is the key factor for the feedback regulation of daunorubicin biosynthesis, and this activates the transcription of *dnrN* and *dnrI*, which in turn leads to the activation of daunorubicin biosynthesis in a sequential manner (Ajithkumar and Prasad 2010). Thus, the existence of tightly regulated antibiotic biosynthesis machinery has been explained and extensively studied by making use of *dnrO*, *dnrN*, and *dnrI* transcription regulator mutants, leading to the better understanding of the doxorubicin biosynthesis in *S. peucetius* (Vasanthakumar et al. 2013).

7.3 Pathway Engineering and Production of Daunorubicin and Doxorubicin

The production of daunorubicin and doxorubicin from *S. peucetius* is hindered by several factors such as (1) the low availability of thymidine diphosphate-L-daunosamine sugar, (2) low efficiency of glycosylation reaction, (3) cytotoxicity, and (4) regulatory mechanisms. This could be overcome by generating a robust *S. peucetius* strain capable of producing practical amount of target molecules using recent biotechnological tools. Till date, several studies have been performed to enhance the production of daunorubicin and doxorubicin from this strain. The basic approaches used to enhance the production of daunorubicin and doxorubicin are summarized in Fig. 7.5.

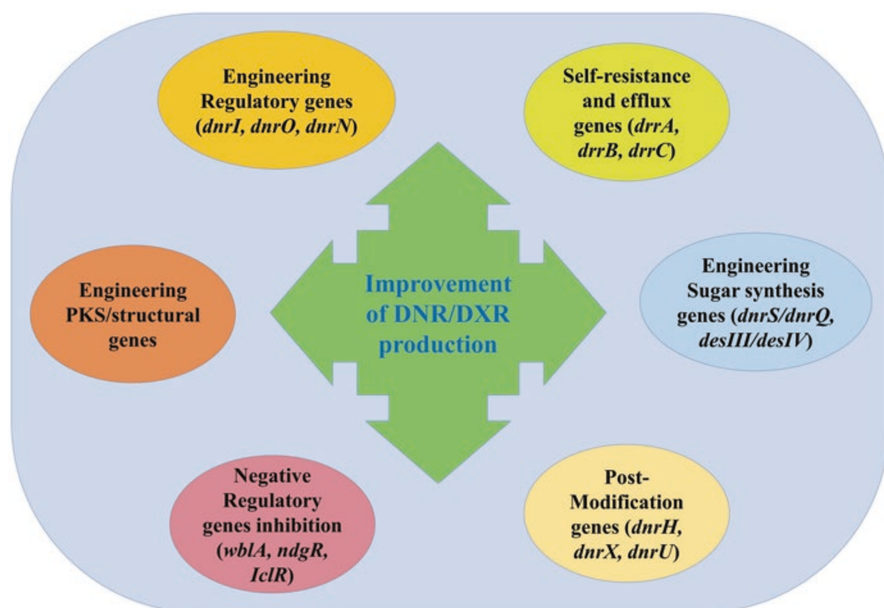

Fig. 7.5 Overall scheme used for enhanced production of daunorubicin and doxorubicin (DNR/DXR) from *S. peucetius*. The scheme shows the pathway engineering strategy and modification of regulatory genes; structural genes; sugar synthesis genes, overexpressing the positive regulators; and the resistance genes followed by inactivation of the post-modification genes thereby improving the yield of both daunorubicin and doxorubicin

7.3.1 Engineering of Thymidine Diphosphate-L-Daunosamine Biosynthesis Pathway Genes

The deoxysugar moieties constitute a very important role in the production of daunorubicin and doxorubicin. Thymidine diphosphate-L-daunosamine sugar formation and its glycosylation by the enzyme DnrS along with DnrQ are considered the rate-limiting step in the biosynthesis of doxorubicin (Dekleva et al. 1985). During daunorubicin and doxorubicin biosynthesis, there is a limited production of glycosylated intermediates because of the low glycosylation efficiency of DnrS/DnrQ glycosyltransferases. Therefore, Malla et al. (2009) explored the overall effects of glycosyltransferase expression for the efficient glycosylation of ε-rhodomycinone and expression of sugar genes to increase the thymidine diphosphate-L-daunosamine pool. Homologous TDP-sugar biosynthesis genes from *S. venezuelae* ATCC 15439, *desIII* (glucose-1-phosphate thymidylyltransferase), and *desIV* (thymidine diphosphate-D-glucose 4, 6-dehydratase) were cloned and overexpressed. Additionally, *dnrS*, along with *dnrQ*, which codes for the activator protein DnrQ, were also overexpressed, and their effects were analyzed in *S. peucetius* ATCC 27952. Introduction of multicopies of *dnrS*/*dnrQ* produced noticeable 2.8-fold enhancement over the parental strain. Furthermore, co-overexpression of *dnrS*/*dnrQ* along with *desIII*/*desIV* increased the doxorubicin production by a 5.6-fold more than the *S. peucetius* parental strain.

DnrH encodes a glycosyl transferase involved in the post-modification stages of the daunorubicin and doxorubicin biosynthesis. Studies involving the *dnrH* mutant produced by inactivation of this gene led to an eightfold increase in daunorubicin production and twofold decrease in ε-rhodomycinone accumulation. Introduction of *dnmT* mutant into the *dnrH* mutant, daunorubicin production increased ninefold compared to the wild-type *S. peucetius*. Doxorubicin production was also improved approximately threefold in the *dnrH* mutant in comparison to the wild-type strain (Scotti and Hutchinson 1996). This is due to the fact that daunorubicin and doxorubicin are further modified into baumycin, like higher glycoside, by these post-modification enzymes, and thus their deletions lead to high production titers of both daunorubicin and doxorubicin. It has also been reported that the DnmT enzyme is present in limiting amounts in the *S. peucetius* (Dickens et al. 1996).

7.3.2 Engineering of the Polyketide Synthase Genes

Earlier studies carried out by Ye et al. (1994), Gerlitz et al. (1997), Bao et al. (1999), Lomovskaya et al. (1999), and Strohl et al. (1998) have shown the importance of the early polyketide synthase genes, like dpsABCDFGY, to the doxorubicin pathway, as their inactivation leads to complete or partial loss in daunorubicin and doxorubicin production. Thus there is a strong possibility that the overexpression of these genes may lead to a significant increase in the production of these anthracyclines in

S. peucetius. Studies carried out by the disruption of the late modifying genes of the doxorubicin biosynthetic pathway, such as *dnrU*, *dnrV*, and *dnrX*, found that individual *dnrX* or *dnrU* mutants produced more doxorubicin than their parental strains, whereas the production of daunorubicin and ε-rhodomycinone decreased (Lomovskaya et al. 1998; Lomovskaya et al. 1999). Doxorubicin production increased approximately twofold in the double *dnrX* and *dnrU* mutant when compared with only *dnrX* mutant, which was an approximate sevenfold increase, when compared with the wild-type strain.

This increase in production is accredited to daunorubicin not being able to be converted to 13-dihydrodaunorubicin, and neither daunorubicin and doxorubicin being able to be further modified to acid-sensitive metabolites, due to the deletion of these modifying enzymes (Walczak et al. 1999). Additionally, when the *dnrV* and *doxA* genes were introduced and overexpressed in the above *dnrX*, *dnrU*, and *dnrH* mutants, a smaller increase in doxorubicin production was observed (Lomovskaya et al. 1999), possibly due to the fact that the oxidation rate of daunorubicin to doxorubicin is 170-fold less efficient than the conversion rate of 13-dihydrodaunorubicin to daunorubicin. Hence, the increased levels of DoxA are less likely to change the extent of doxorubicin production in *S. peucetius*.

As mentioned earlier, *S. peucetius* ATCC 27952 self-resistance system imparts resistance against the toxicity of the antibiotic daunorubicin and doxorubicin inside the cell, and helping in this endeavor are the four resistance genes *drrA*, *drrB*, *drrC*, and *drrD*. Owing to this fact, when three of these resistance genes *dnrABC* were cloned under strong *ermE** promoter into the pIBR25 expression vector, the recombinant expression strains, pDrrAB25, pDrrC25, and pDrrABC25, produced more doxorubicin than the parental strain, with a 2.2-fold increase in pDrrAB25, a 5.1-fold increase in pDrrC25, and a 2.4-fold increase in pDrrABC25. Thus, doxorubicin production is positively affected when the resistance genes are introduced in multiple copies (Malla et al. 2010a).

7.3.3 Engineering of the Regulatory Genes

Secondary metabolite production in *Streptomyces* spp. is regulated by two different classes of regulatory genes: cluster-situated regulators and global regulators or pleiotropic regulatory genes. Most of these cluster-situated regulators control the biosynthesis of a particular antibiotic and are also known as pathway-specific regulators. On the other hand, the global regulatory genes may not always be present in biosynthetic gene cluster but regulate morphological and physiological differentiation and secondary metabolite biosynthesis in *Streptomyces* (Umeyama et al. 2002). The study of these regulatory genes provides a theoretical basis for antibiotic biosynthesis in *Streptomyces* and also helps to increase the yield of antibiotics by the use of pathway engineering and manipulation of these regulatory genes at molecular level.

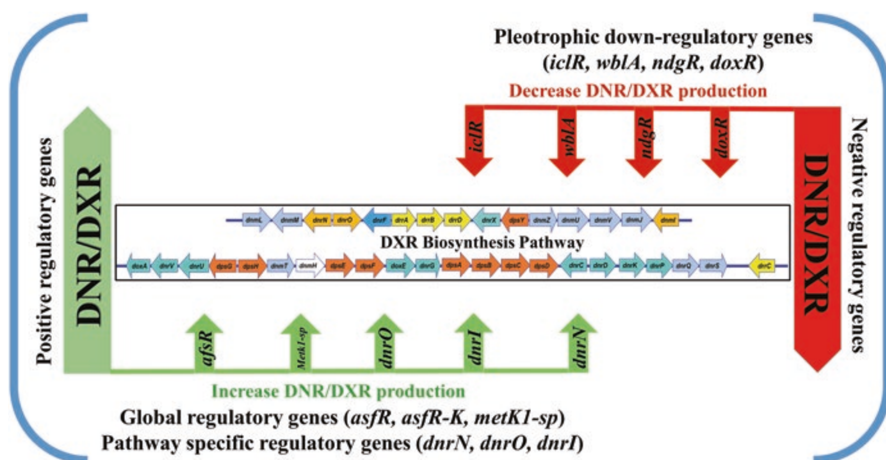

Fig. 7.6 Schematic representation of genes involved in regulation of daunorubicin and doxorubicin (DNR/DXR) production in *S. peucetius*. Global regulatory genes like *asfR* and *Metk1*-sp and pathway-specific regulatory genes like *dnrO*, *dnrI*, and *dnrN* act as positive regulatory genes and thus have positive effect in daunorubicin and doxorubicin production, whereas the pleiotropic downregulatory genes such as *iclR*, *wblA*, *ndgR*, and *doxR* decrease the daunorubicin and doxorubicin production and hence the negative regulatory genes. Overexpression or inhibition of the regulatory genes has a profound effect in the production of the final compound doxorubicin

Similarly in *S. peucetius*, as discussed earlier, DnrI, DnrN, and DnrO act as transcriptional regulator and control production of daunorubicin and doxorubicin (Fig. 7.6). The DnrO is the major transcription regulator, and its inactivation leads to complete loss of antibiotic production. In *S. peucetius*, DnrI is required for the transcription of biosynthetic and resistance genes of the daunorubicin and doxorubicin gene cluster and thus controls the expression of almost all of the biosynthetic and resistance genes (Madduri and Hutchinson 1995), while DnrN controls the expression of DnrI (Otten et al. 1995). Consequently, the introduction of positively acting regulatory genes like DnrI and DnrN has profound effects on the production of antibiotics like daunorubicin. In *S. peucetius*, the production of daunorubicin was increased 2.5-fold, whereas the ε-rhodomycinone yield was raised to nearly 10-fold (Otten et al. 1995; Stutzman-Engwall et al. 1992).

Introduction of regulatory genes such as *dnrN*, *dnrI*, *afsR*, and *metK1*-sp under strong *ermE** promoter increased doxorubicin production by 1.2-fold in recombinant strains NI (with dnrN-dnrI), 1.4-fold in NIS (with *dnrN*-*dnrI*-*metK1*-sp), and 4.3-fold in NIR (with *dnrN*-*dnrI*-*afsR*) (Malla et al. 2010b). AfsR is a global regulator which constitutes the AfsK-AfsR system. The expression of *afsR* from both *S. peucetius* ATCC 27952 and *S. venezuelae* in *S. peucetius* enhanced production of doxorubicin by fourfold and eightfold, respectively (Parajuli et al. 2005). Furthermore the overexpression of this pleiotropic activator *afsR* enhanced other antibiotics such as actinorhodin in *S. lividans*, clavulanic acid in *S. clavuligerus*, and streptomycin in *S. griseus* (Maharjan et al. 2009).

S. peucetius does not contain a functional copy of bldA-tRNA. Although *bldA* is non-essential for the survival of the *Streptomyce*s species, it plays an important role in secondary metabolism. When the regulatory gene *dnrO* codon was thoroughly examined, a TTA codon was found which is hardly encoded by bldA-tRNA. Multiple engineered strains of *S. peucetius* were generated by heterologously expressing *bldA* and *dnrO* individually and a combination of both *bldA* and *dnrO*. Overexpression of these pathway-specific negative regulators enhanced the production of daunorubicin 1.25-fold, as compared to the parental strain (Pokhrel et al. 2016). Likewise these genes, engineering of genes, like dephosphocoenzyme A (*coaE*), which catalyzes the last step in the biosynthesis of the cofactor coenzyme A, has been shown to have positive increase in doxorubicin production. When these two genes *coaA* and *coaE* were overexpressed independently in the doxorubicin-producing wild-type strain, there was 1.4- and 1.5-fold increase in doxorubicin production, respectively. Both genes in combination exhibited 2.1-fold enhancement in doxorubicin production (Lee et al. 2014).

Besides overexpressing positive regulators to enhance production titer, as mentioned above, there are a few negative regulators present in *S. peucetius*, whose overexpression or inactivation may have a negative or positive effect on daunorubicin and doxorubicin production (Fig. 7.6). One such negative regulator is *wblA*, which controls antibiotic production and morphological differentiation in actinomyces, and when this *wblA* regulator from *S. coelicolor* was introduced into the doxorubicin-overproducing strain, it led to significant decrease in the production of doxorubicin (Kang et al. 2007).

The *dox R* regulator belonging to the *IclR* type family of transcription regulator was found in the genome of *S. peucetius*, and when overexpressed in *S. peucetius* strain, it strongly repressed the production of antibiotics. Furthermore, it exerted an adverse consequence on the regulatory system of doxorubicin, wherein the binding of DoxR inhibited the *dnrI* expression, leading to the blockade of doxorubicin production (Chaudhary et al. 2014). Another regulatory gene *ndgR* which is a regulator for nitrogen source-dependent growth and antibiotic production, similar to an *IclR*-like regulator from *S. coelicolor*, can bind to the promoters in the doxorubicin biosynthetic gene cluster in *S. peucetius* (Yang et al. 2009), and its inactivation in *S. coelicolor* leads to increased actinorhodin production. Thus, the deletion of the *doxR* and the *ndgR* regulatory genes may also have a positive effect on daunorubicin and doxorubicin production.

7.4 Conclusion

Because of low production yield of doxorubicin and high market demand, engineering of *S. peucetius* strain is a beneficial goal. Until the late 1990s, the annual production of doxorubicin was over 225 kg, and it was the most widely used anticancer drug. Moreover, doxorubicin is also considered as lead molecule to generate other value-added derivatives by enzymatic and chemical modifications with improved

pharmacological properties for clinical cancer treatment (Arcamone et al. 1997; Allwood et al. 2002). Although doxorubicin can be produced semi-synthetically from its precursor daunorubicin, the process is tiresome, and the yield is quite low. Thus, sustainable fermentation technology combined with pathway engineering approaches is currently needed to enhance the production of these drugs (Hutchinson and Colombo 1999; Malla et al. 2010c).

In summary, we conclude that by identification of the key steps in *S. peucetius* that hinder daunorubicin and doxorubicin production, like the low availability of thymidine diphosphate-L-daunosamine sugar and the low efficiency of glycosylation, cytotoxicity, and the regulatory mechanisms, daunorubicin and doxorubicin production can be raised significantly in the wild-type strain of *S. peucetius* by genetic engineering. This would involve overexpression of the genes regulating doxorubicin production and also the genes in the biosynthetic pathway, along with the deletion of negative regulators and inhibiting the post-modification steps of daunorubicin and doxorubicin into other metabolites (Table 7.1). Additionally, high-daunorubicin-and doxorubicin-producing strains can be generated by overexpression of the genes in the sugar pathway of thymidine diphosphate-L-daunosamine, a very

Table 7.1 Increase in daunorubicin and doxorubicin production by the engineering of the PKS pathway, sugar pathway, and regulatory pathway genes in *S. peucetius*

Gene overexpression/ inactivated	Effect in production	References
dnrI + dnrN overexpression	2.5-fold increase in daunorubicin	Otten et al. (1995) and Stutzman-Engwall et al. (1992)
dnrH inactivation	Eightfold increase in daunorubicin and threefold increase in doxorubicin	Scotti and Hutchinson (1996)
dnmT + *dnrH* inactivation	Ninefold increase in daunorubicin	Scotti and Hutchinson (1996)
dnrS/*dnrQ* overexpression	2.8-fold increase in doxorubicin	Malla et al. (2009)
dnrS/*dnrQ* + *desIII*/*desIV* overexpression	5.6-fold increase in doxorubicin	Malla et al. (2009)
dnrX + *dnrU* inactivation	Sevenfold increase in doxorubicin	Lomovskaya et al. (1998) and Lomovskaya et al. (1999)
drab overexpression	2.2-fold increase in doxorubicin	Malla et al. (2010a)
drrC overexpression	5.1-fold increase in doxorubicin	Malla et al. (2010a)
drab Coverexpression	2.4-fold increase in doxorubicin	Malla et al. (2010a)
dnrN-dnrI overexpression	1.2-fold increase in doxorubicin	Malla et al. (2010b)
dnrN-dnrI-metK1-sp overexpression	1.4-fold increase in doxorubicin	Malla et al. (2010b)
dnrN-dnrI-afsR overexpression	4.3-fold increase in doxorubicin	Malla et al. (2010b)
afsR overexpression	Fourfold increase in doxorubicin	Parajuli et al. (2005)
bldA-dnrO overexpression	1.25-fold increase in doxorubicin	Pokhrel et al. (2016)
coaA overexpression	1.4-fold increase in doxorubicin	Lee et al. (2014)
coaE overexpression	1.5-fold increase in doxorubicin	Lee et al. (2014)

important step in the production of daunorubicin and doxorubicin, along with the engineering of the polyketide synthase genes. Engineering of wild-type strain using combined effect of regulatory genes and other biosynthesis genes along with self-resistance and cofactors limiting genes using state-of-the-art systems/synthetic biology and metabolic engineering tools could certainly generate a high-doxorubicin-producing strain for commercial production of these valuable anticancer drugs.

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