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TABLE OF CONTENTS

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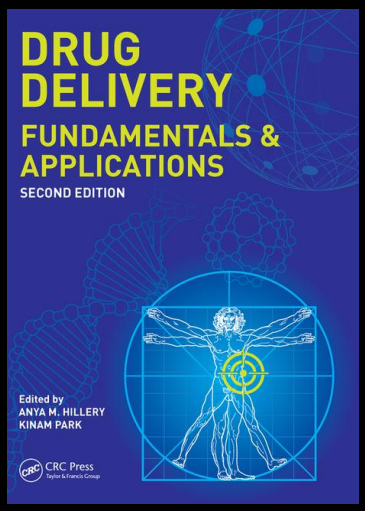

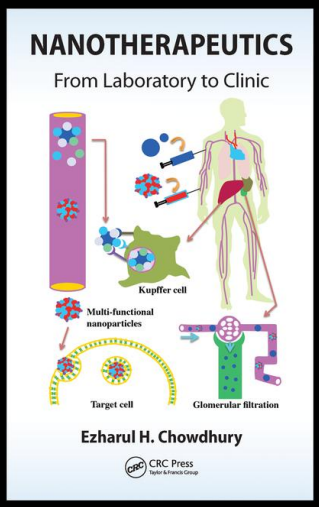

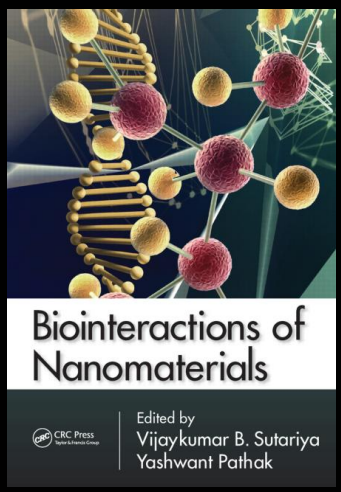

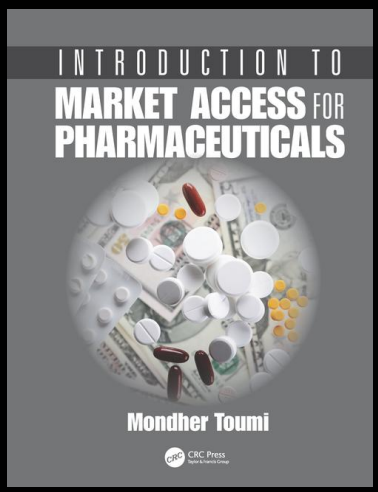

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Introduction

About this Free Book

Explore Essential Principles for Pharmaceutical Science, Biomedicine and Biotechnology is a FreeBook brought to you by CRC Press - Taylor and Francis Group. It contains a collection of curated content from some of our bestselling books and leading experts. The T&F/CRC Press book list has been expanding to keep up with demands in the pharmaceutical, biomedical and biotechnological industries. We have a wide range of references, monographs, textbooks and practical guidebooks to serve the needs of professors, students and professionals. Researchers and teachers in a variety of related areas including advanced drug delivery and targeting will find resources here that will be useful as required texts and supplementary reading. Here is a selection of representative chapters from just four of our recent publications to give you an idea of the quality and variety of subject matter addressed in our portfolio:

- *Drug Delivery: Fundamentals and Applications, Second Edition*; Chapter 5: An Overview of Epithelial Barriers by Anya M Hillery and Kinam Park
- *Nanotherapeutics: From Laboratory to Clinic*; Chapter 5: Protein-Based Macromolecular Drugs by Ezharul Hoque Chowdhury
- *Biointeractions of Nanomaterials*; Chapter 5: Biosensing Devices for Toxicity Assessment of Nanomaterials by Vijaykumar B. Sutariya, Yashwant Pathak
- *Introduction to Market Access for Pharmaceuticals*; Chapter 5: Early HTA Advice by Mondher Toumi

The chapters presented here cover fundamental principles and cutting edge topics such as nanocarriers, sustained release, pharmacokinetics of small drugs, food, consumer products, pharmaceuticals, medicine, agriculture, nutraceuticals, dietary supplements, cosmetics, flavors, and fragrances.

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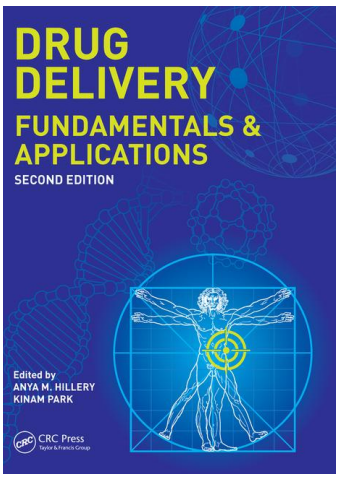
CHAPTER 1

An Overview of Epithelial Barriers



5 Nanotechnologies for Drug Delivery and Targeting
Opportunities and Obstacles

Alexander T. Florence and Daan J.A. Crommelin

CONTENTS

5.1 INTRODUCTION

Routine parenteral administration by injection serves to deliver drugs to body tissues. The most important routes of injection of these sterile products are intramuscular (i.m.), intravenous (i.v.), and subcutaneous (s.c.). The process of developing conventional parenteral formulations involves the selection of appropriate vehicles (e.g., aqueous, oily, and emulsions) to achieve the desired bioavailability following injection, as described in Chapter 3. The present chapter focuses on advanced drug delivery and targeting systems (DDTS), in the nanometer range, administered via the parenteral route and serves to provide the reader with a basic understanding of the principle approaches to drug targeting.

5.1.1 Rationale for the Development of Parenteral Drug Delivery and Targeting Systems

As introduced in Chapter 2, there are many limitations associated with conventional drug therapy. An i.v.-administered drug is subject to a number of pharmacokinetic processes in vivo, which can decrease the drug's therapeutic index, including the following:

- *Distribution*: Drugs administered i.v. distribute throughout the body and reach nontarget organs and tissues, resulting in drug wastage and consequently side effects and possibly toxicity.
- *Metabolism*: The drug may be rapidly metabolized in the liver or other organs.
- *Excretion*: The drug may be cleared rapidly from the body through the kidneys.

As a result of these processes, only a small fraction of the drug will reach the target tissue. Moreover, it may be cleared rapidly from this site and, therefore, not be available long enough to induce the desired effect. Reaching the target cell is often not the ultimate goal; in many cases, the drug has to enter the target cell to reach an intracellular target site. As discussed in Chapter 4, many drugs do not possess the required physicochemical properties to enter target cells; they may be too hydrophilic, too large, or labile or not transportable by the available active transport systems.

DDTS aim to overcome the limitations of conventional drugs and thus improve drug performance. An ideal DDTS should

- Specifically target the drug to target cells or target tissue
- Prevent the drug reaching nontarget organs, cells, or tissues
- Ensure minimal drug leakage during transit to target
- Protect the associated drug from metabolism
- Protect the associated drug from premature clearance
- Retain the drug at the target site for the desired period of time
- Facilitate transport of the drug into the cell
- Deliver the drug to the appropriate intracellular target site
- Be biocompatible, biodegradable, and nonantigenic

In certain situations, some of these requirements may be inappropriate. For example, the drug may work outside the cell; thus, cell penetration may not be necessary. In this chapter, there are also examples mentioned of passive targeting approaches (see Section 5.3.1), where the drug does not require to be specifically targeted to the cell or tissue.

The parenteral route of administration is associated with several major disadvantages. Parenteral administration is invasive and may require the intervention of trained medical professionals. Parenteral formulations often require refrigeration for storage. Strict regulations govern their use, which generally dictate that they are as simple as possible. The inclusion of excipients

in the formulation is kept to an absolute minimum. Developing a DDTS requires an enormous amount of R&D investment in terms of cost, effort, and time, which can cause a significant delay in the development and marketing of a system and thus result in the final product being relatively expensive. Parenteral DDTS must, therefore, offer real therapeutic advantages to justify their use. Box 5.1 lists a number of pharmacokinetic considerations to decide if the use of DDTS is indicated for a particular drug.

BOX 5.1 PHARMACOKINETIC CONSIDERATIONS RELATED TO DRUG TARGETING

- Drugs with high total clearance are good candidates for targeted delivery.
- Carrier-mediated transport is suitable for response sites with a relatively small blood flow.
- The higher the rate of elimination of free drug from either central or response compartments, the greater the need for targeted drug delivery; this also implies a higher input rate of the drug carrier combination to maintain the therapeutic effect.
- For maximizing the targeting effect, the release of drug from the carrier should be restricted to the response compartment.
- The rate of release and quantity of drug released should be at the appropriate therapeutic level.

Drugs used in the treatment of diseases that are life threatening or that dramatically affect the quality of life of the patient are prime candidates for inclusion in a DDTS. Such drugs include those used in the treatment of cancer, as well as life-threatening microbial, viral, and fungal diseases. Chronic diseases such as arthritis can also be found on the priority list.

5.1.2 Generalized Description of Parenteral Drug Delivery and Targeting Systems

The technology used for targeted drug delivery with carrier systems differs from the technology to achieve prolonged release profiles for a drug. If prolonged release of a drug via the parenteral route is required, s.c. or i.m. injection of a controlled release system is the first option to consider. The relevant technology is already available and validated for many years. Long-acting injections and implants are the subject of Chapter 6; some examples include

- The long-, medium-, and short-acting insulin formulations, prepared by crystal manipulation or physical complex formation
- Depot injections (aqueous suspensions, oily injections) of contraceptives and psychotropic drugs (such as fluphenazine esters)
- Polymeric implants, for example, Zoladex®, a biodegradable matrix implant of the luteinizing hormone-releasing hormone (LHRH) agonist goserelin, for s.c. administration
- Infusion pumps

In contrast, a DDTS generally comprises three functionally specific units, as listed in Table 5.1: the drug or therapeutic agent, the carrier system, and a "homing" ligand.

A "homing" ligand is a target-specific recognition moiety. For example, galactose receptors are present on liver parenchymal cells; thus, the inclusion of galactose residues on a drug carrier can allow the interaction of the carrier to the target cells, once the two are in close proximity. A number of different target-specific recognition moieties are available and discussed further in the succeeding texts. However, an important point to note here is that target-specific recognition moieties are not idealized "magic bullets," capable of selectively directing the drug to the appropriate target and

TABLE 5.1

Components of Drug Delivery and Targeting Systems

Drug Delivery and Targeting Systems Component	Purpose
The active moiety	To achieve the therapeutic effect
The carrier system, which can be either soluble or particulate	To effect a favorable distribution of the drug To protect the drug from metabolism To protect the drug from early clearance
A "homing" ligand[a]	To specifically target the drug to the target cells or target tissue

[a] Not necessary when "passive" targeting approaches are used.

ignoring all other nontarget sites. Although the so-called homing ligand can increase the *specific- ity* of the drug for its target site, the process must rely on the (random) encounter of the homing ligand with its appropriate receptor during its circulation lifetime. This is a stochastic process, that is, one involving elements of chance rather than certainty; for example, particle escape from the circulation (extravasation) or a ligand–receptor interaction leading to cellular uptake. Box 5.2 lists the main relevant processes that have an element of uncertainty in terms of outcome.

The carrier systems that are presently on the market or under development can be classified into two groups based on their size:

1. Soluble macromolecular carriers
2. Particulate carrier systems

This classification is sometimes rather arbitrary, as some soluble carriers are large enough to enter the colloidal size range. A useful distinction is that with macromolecular carrier systems, the drug is "covalently attached" to the carrier and has to be released through a chemical reaction. In contrast, with colloidal carriers, the drug is generally "physically associated" and does not need a chemical reaction to be released. Here, diffusion barriers in the carrier comprise the major barriers for pre- mature release from the system. Stimulus-sensitive systems are also being devised to ensure that

BOX 5.2 STOCHASTIC PROCESSES IN VECTOR-MEDIATED DRUG TARGETING

Definition: A stochastic process involves some random variable or variables, that is, it involves chance events or a probability, rather than a certainty, of an event occurring. Four examples are as follows:

1. Aggregation of nanoparticles under shear flow in the blood circulation or in static conditions, e.g., in cell culture. Aggregation can cause jamming of particles and would change the apparent size—biological action paradigm.
2. Extravasation. The escape from the circulation after i.v. administration.
3. Nanoparticle ligand–receptor interactions. Requiring both close approach (to within several nm) of particles and receptors and the correct orientation of vectors toward the receptor, both being random events.
4. Diffusion of vectors in tissues, which are complex environments involving iterative approaches to reach destinations.

drug is only released once the systems have reached the target. Release in this case is instigated by an external source, such as heat or magnetic force, the subject of Chapter 14.

Soluble carriers include antibodies and soluble synthetic polymers such as poly(hydroxypropyl methacrylate), poly(lysine), poly(aspartic acid), poly(vinylpyrrolidone), poly(*N*-vinyl-2-pyrrolidone-*co*-vinylamide), and poly(styrene-*co*-maleic acid anhydride).

Many particulate carriers have been designed for drug delivery and targeting purposes for i.v. administration. They usually share three characteristics:

1. Their size range: the minimum size is approximately 10 nm and the maximum size relevant for drug targeting is approximately 10–300 nm.
2. They are all biodegradable, but at different rates.
3. The drug is physically associated with the carrier, and in general, drug release kinetics are controlled by either diffusional transport or matrix degradation, or both.

Figure 5.1 shows a selection of some of the wide variety of both soluble and particulate DDTS that are currently under development or commercially available.

A full appreciation of the respective advantages and disadvantages of soluble and particulate carriers cannot be gained without first taking into account the relevant anatomical, physiological, and pathological considerations in vivo, described next.

5.2 ANATOMICAL, PHYSIOLOGICAL, AND PATHOLOGICAL CONSIDERATIONS

The body is highly compartmentalized and should not be considered as a large pool without internal barriers for transport. A schematic figure (Figure 5.2) illustrates the organs involved. The degree of body compartmentalization or, in other words, the ability of a macromolecule or particulate to move around, depends on its physicochemical properties, in particular

- Molecular weight/size
- Charge
- Surface hydrophobicity
- Surface attached ligands for interaction with surface receptors.

The smaller its size, the easier a drug carrier can passively move from one compartment to another. An important question is whether, and where, the carriers can pass through the endothelial lining of the blood circulation. Under physiological conditions, the situation exists as depicted in Figure 5.3. A continuous endothelium, as found in most parts of the body, comprises a continuous tube of endothelial cells, separated by intercellular clefts, i.e., narrow gaps between neighboring endothelial cells (Figure 5.3a). The cells are positioned on a continuous basal membrane. The exact characteristics of this barrier are still under investigation, but it is clear that particulate systems greater than 10 nm cannot pass through. Fenestrated capillaries, as found, for example, in the kidney and most endocrine glands, have many small pores (fenestrations) in the plasma membrane of the endothelial cells, ranging from 70 to 100 nm diameter (Figure 5.3b). Again, the subendothelial basement membrane is continuous. Sinusoidal capillaries (Figure 5.3c) are found in the liver, spleen, and bone marrow. The endothelial cells have large fenestrations, as well as large intercellular clefts, that allow the movement of molecules such as proteins through the capillary walls. The subendothelial basement membrane is either absent (as in the liver) or present as a fragmented interrupted structure (as in the spleen and bone marrow). Sinusoids in the liver contain phagocytic Kupffer cells, which remove bacteria and other particulates from the blood.

This anatomical information has important implications for the rational design of targeted carrier systems. If a therapeutic target is located outside the blood circulation and if normal anatomical

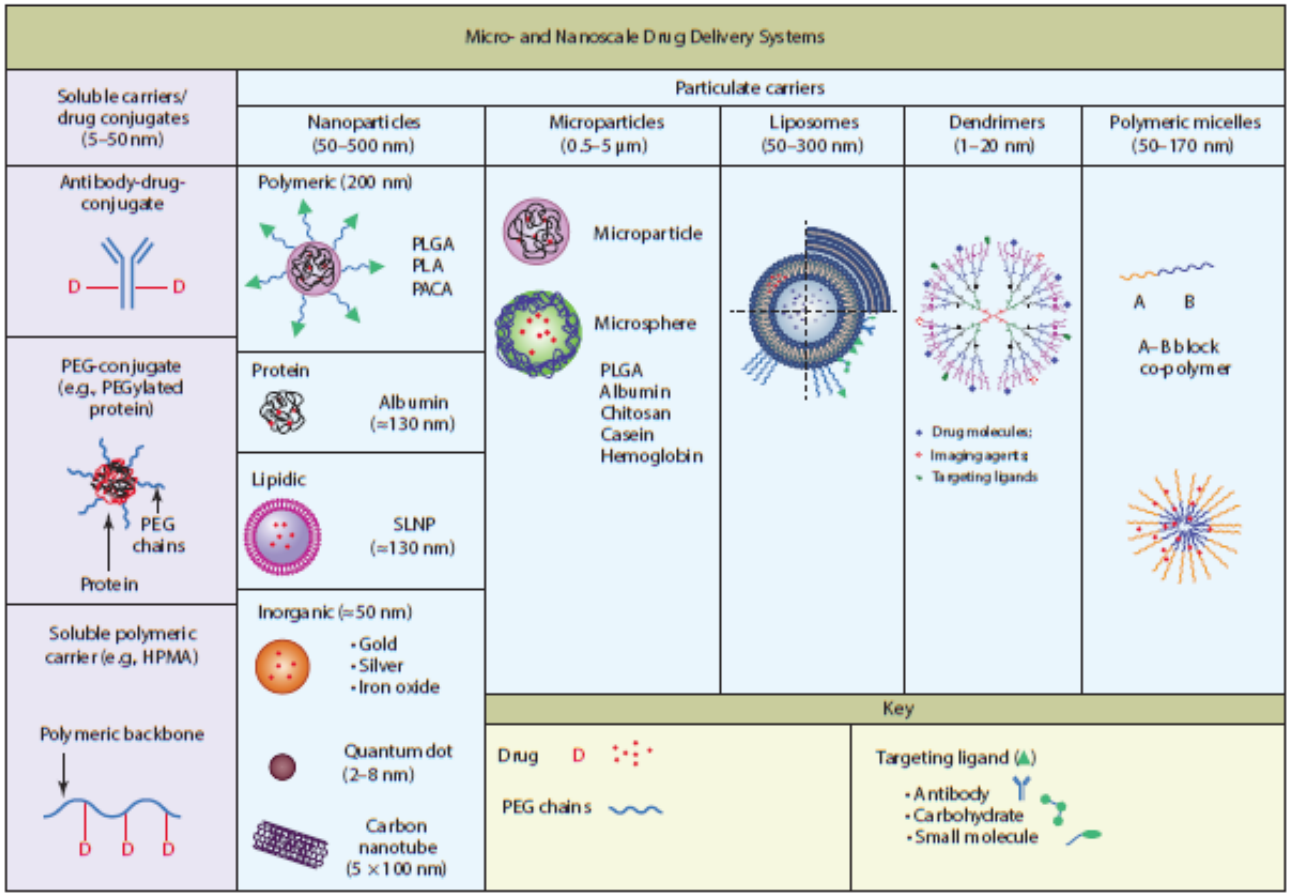


FIGURE 5.1 Schematic illustration of various types of nano-/micro-DDTS. Soluble carriers include antibodies, hydrophilic polymers such as PEG, and soluble synthetic polymers such as poly(hydroxypropyl methacrylate). Particulate carriers include nanoparticles, microparticles, liposomes, dendrimers, and micelles. Particulate carriers can also have covalently attached PEG chains (e.g., PEGylated liposomes, PEGylated microparticles) and contain targeting ligands or a targeting ligand–PEG conjugate. PLGA, poly(lactic-*co*-glycolic acid); PLA, poly(lactic acid); PACA, poly(alkyl cyanoacrylate); SLNP, solid–lipid nanoparticles. (*Note:* This figure illustrates some generalized drug carrier systems. Not all of these examples are necessarily injected intravenously and/or are intended for drug-targeting purposes. Many of the larger constructs are injected subcutaneously or intramuscularly for sustained release. Also, the size values quoted are indicative only; a wide variation in these values is possible).

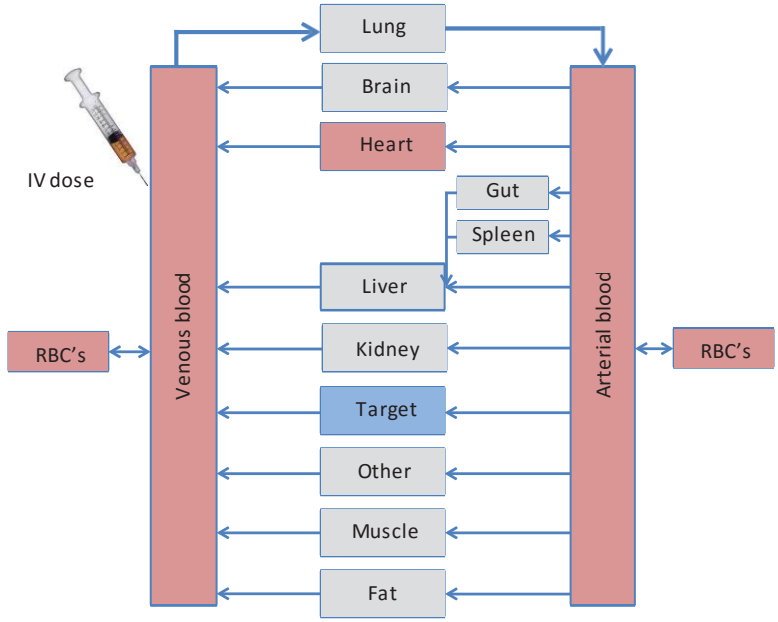


FIGURE 5.2 Simplified diagram showing the flow of the blood through the different organs of the body.

conditions exist around the target site, a small-sized macromolecular carrier must be selected, in order to achieve sufficient "escaping tendency" from the blood circulation. Particulate carriers will generally fail to extravasate, simply because there is no possibility for endothelium penetration in normal circumstances.

In addition to the issue of endothelial permeability, the effect of macrophages in direct contact with the blood circulation (e.g., Kupffer cells in the liver) on the disposition of carrier systems must be considered. Unless precautions are taken, particulate carrier systems are readily phagocytized by these macrophages and tend to accumulate in these cells. Phagocytic uptake by the cells of the mononuclear phagocyte systems (MPS), also known as the reticuloendothelial system (RES), has been introduced in Chapter 4 (Section 4.3.3). The MPS constitutes an important part of the body's immune system and comprises both fixed cells, such as macrophages in the liver (also known as Kupffer cells), spleen, lungs, bone marrow, and lymph nodes, and mobile cells such as blood monocytes and tissue macrophages. Its functions include

- The removal and destruction of bacteria
- The removal and metabolism of denatured proteins
- Antigen processing and presentation
- Storage of inert colloids
- Assisting in cellular toxicity

The cells of the MPS are always on the alert to phagocytize "foreign body–like material." Thus, aside from being responsible for the removal of particulate antigens such as microbes, other foreign particulates, such as microspheres, liposomes, and other particulate carriers, are also susceptible to MPS clearance.

Clearance kinetics by the MPS are highly dependent on the physicochemical properties of the "foreign" particulate; on particle size, charge, and surface hydrophobicity; and perhaps, on shape. These properties are considered in turn.

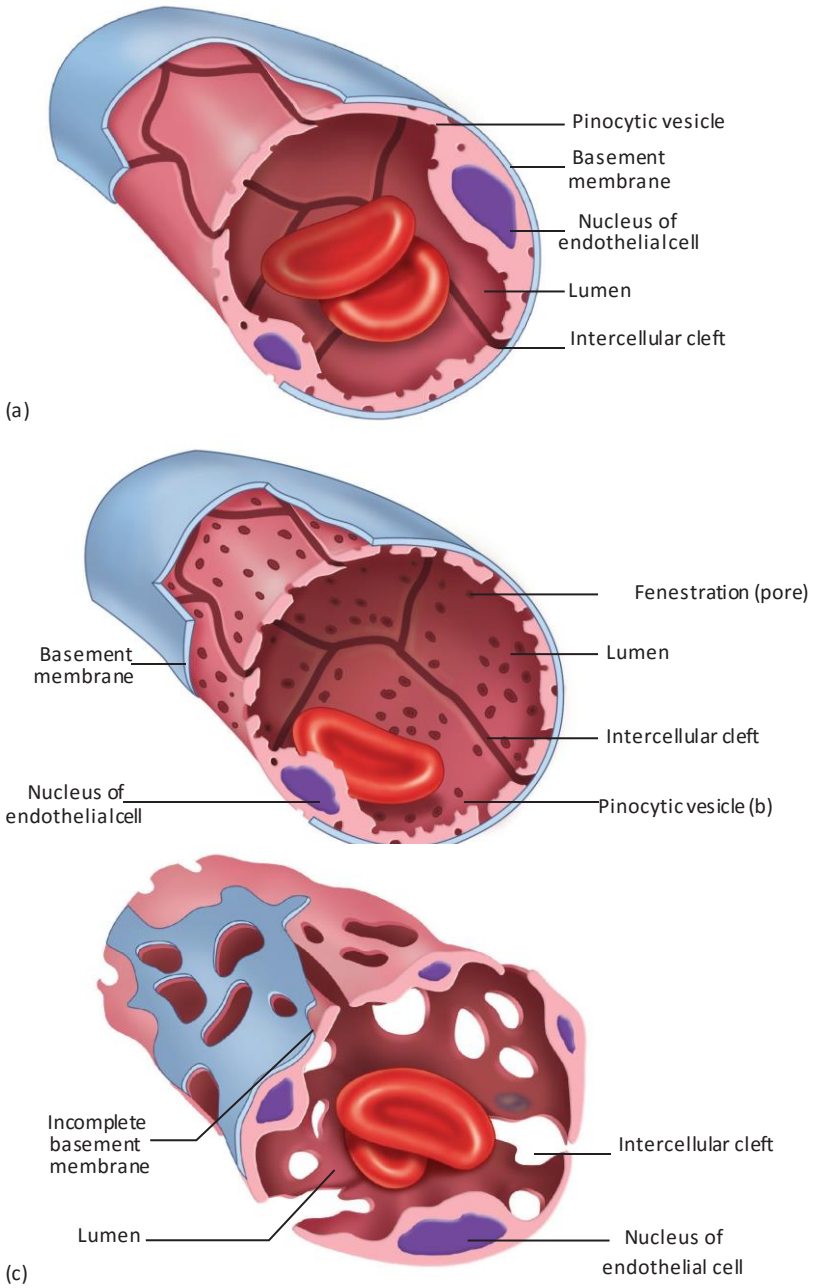


FIGURE 5.3 Schematic illustration of the structure of the three different classes of blood capillaries: (a) continuous, (b) fenestrated, and (c) discontinuous (sinusoidal) capillaries.

Particle size: Particulates in the size range of 0.1–7 μm tend to be cleared by the MPS, localizing predominantly in the Kupffer cells of the liver.

Particle charge: For liposomes, it has been shown that negatively charged vesicles tend to be removed relatively rapidly from the circulation, whereas neutral vesicles tend to remain in the circulation for longer periods.

Surface hydrophobicity: Hydrophobic particles are immediately recognized as "foreign" and are generally rapidly covered by plasma proteins known to function as opsonins, which facilitate phagocytosis. The extent and pattern of opsonin adsorption depends highly on surface characteristics such as charge and hydrophilicity. Strategies to decrease MPS clearance, by increasing the hydrophilicity of the particle surface, are described in Section 5.3.1.

A further consideration is that in some "pathological" conditions, the endothelium exhibits modified characteristics. In general, the permeability is enhanced; the phenomenon is called the "enhanced permeability and retention" (EPR) effect, coined by Hiroshi Maeda, as will be discussed in Section 5.2.1. For example, the endothelial fenestrations in inflammation sites can be as large as 20 nm. In tumor tissue, even larger fenestrations can be found. However, in this case, the pattern is not uniform and depends on the tumor type and stage of development. Even within one tumor, highly permeable sites can be identified in close proximity to sites of low permeability. Also, necrotic tissue affects tumor permeability. Because of the EPR effect, particles in the colloidal size range can "theoretically" enter tumor tissue or sites of inflammation. This phenomenon can be exploited for drug delivery. However, while the EPR effect has been demonstrated in animals, there has been no clear evidence of the effect in human tumors. The EPR effect has been invoked in many publications as a means of access of carrier systems to such sites, but the evidence for the mechanism, or mechanisms, of accumulation is often not to be found. Extrapolation of animal data to humans is less than clear, as it is with other phenomena too. The status of this putative mechanism and some of these questions are discussed further for example in Sections 5.2.1, 5.5.1.1 and 5.5.1.2.

Shape: The development of asymmetric carbon nanotubes has given the opportunity to investigate the influence of shape on many processes. Most current vector systems are spherical or quasi-spherical, with different degrees of flexibility. But shape clearly affects the movement and behavior of the systems, for example, their transport through pores (see Figure 5.4) and their interaction with biomembranes. Shape can affect endocytosis: elongated particles have been found to be more readily endocytosed than spherical equivalents. Cytotoxicity is also influenced by shape, as asbestos has taught us. Carbon nanotubes are said to have a propensity for membrane interactions, but more work is required to elucidate all the shape-related factors in attachment and uptake.

5.2.1 Enhanced Permeation and Retention Effect: Concept or Reality?

Much of the research carried out on vector-mediated targeting is performed in small animal models, usually rodents. In these animal models, a certain degree of accumulation of vectors has been observed, for example, at tumor sites, with both active and passive carrier systems, a feature habitually ascribed to the EPR effect. A certain accumulation indeed occurs in rodents, but this has rarely exceeded about 5% of the vector. Imaging techniques show this modest accumulation but unless the drug itself can be visualized, the data are optimistic, as the drug may be trapped within the carrier particles. In addition, the literature is difficult to interpret as it has been found that different imaging techniques can lead to different data. The EPR effect has been considered recently by a number of experts in a consensus paper that points out the heterogeneity of the EPR effect and the limited evidence for the effectiveness of the mechanism, which was originally proposed, as stated in this chapter, for (flexible) macromolecules rather than (rigid) nanoparticles.

Nanoparticle uptake is often determined by radiolabeling of the vector and not the drug. The only sensible approach is to measure the drug in the tumor and to distinguish active free drug, from the drug that is still bound to the carrier. One problem in assessing true targeting is that the capacity of a vector system for the drug is crucial, and hence, the same vector carrying different drugs might

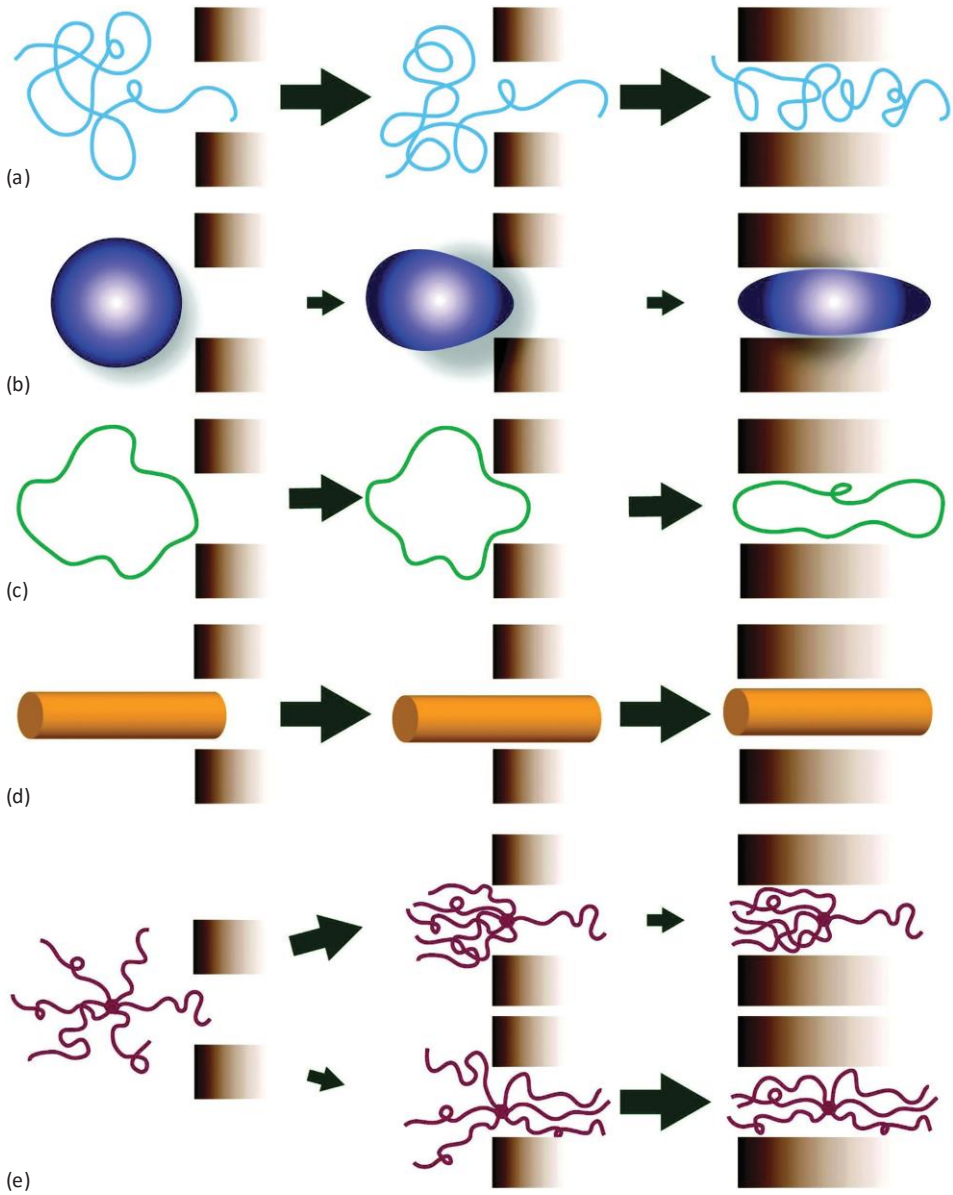


FIGURE 5.4 Polymer architecture and the passage of a polymer through a pore, for polymers with approximately equivalent hydrodynamic volume; (a) linear random coil polymer readily penetrates and reptates through a pore; (b) polymer with a rigid, globular conformation must deform to pass through. Beyond a threshold MW, both entry and passage through the pore could be difficult; (c) a cyclic polymer lacks a chain end for entering the pore and must deform to enter and pass through it; (d) polymer with a rigid elongated or tubular conformation easily enters and passes through; and (e) arm orientation and distance between chain ends of branched polymers impact the rate of entry and passage through a pore. While initial entry of only one chain end (top) may occur rapidly, passage of the entire polymer through the pore is sterically hindered, because the remaining arms must deform for the polymer to pass through; a symmetric conformation (bottom) is less likely since multiple chain ends must penetrate the pore at the same time. Once entry has been achieved, passage of the polymer would be less hindered than for the asymmetric distribution.

produce quite different results. Drug load can be high, as in many liposomes, but in some nanoparticles, it can be as low as 5% w/w. Should 10% of such a carrier reach the target, and the available drug is only 5% of the vector mass, the so-called drug targeting represents only 0.5% of the administered dose. Many studies suggest that maximal vector uptake is in the vicinity of 5%: arithmetic matters! The rate of release and diffusion of drug from the carrier is clearly another issue in effective therapeutic action. Distribution of drug throughout tumor tissue is not, unsurprisingly, homogeneous since tumors are not themselves homogeneous. In tumor tissue extracellular matrix barriers, necrotic tissue and pressure differences may form barriers for access to parts of the diseased tissue in spite of pore formation in the endothelium. Generally speaking, the larger the carrier, the more its penetration is hindered. And drugs such as doxorubicin (although a small molecule) do not necessarily diffuse freely through tumor tissue, not only because of the complex diffusion pathways but also as they can bind to cellular and tissue components.

And then the following question has to be answered: how predictive are animal models for the therapeutic effects in the human patient? As seen in a few observations, the tumor cell lines used in the most "popular" animals (rodents) are fast growing (days, weeks). These tumor cell lines are rather homogeneous compared to human tumors; furthermore, the immune systems in rodents differs from man. Not only that, but adsorption of proteins onto the surface of nanoparticles in the blood varies with residence time in the circulation. Both the plasma proteins in rodents and the dynamics of circulation are different from those in man. Therefore, direct extrapolations from findings in animals to humans can only be made after careful validation studies. In some patients, indeed a degree of accumulation in the primary tumor and metastases has been demonstrated by imaging techniques, but the same imaging techniques showed no EPR effect in the majority of other patients.

5.3 PASSIVE AND ACTIVE TARGETING

5.3.1 Passive Targeting

Passive targeting exploits the "natural" (passive) distribution pattern of a drug carrier in vivo and no homing ligand is attached to the carrier. For example, as described earlier, particulate carriers tend to be phagocytized by cells of the MPS. Consequently, the major organs of accumulation are the liver and the spleen, both in terms of total uptake and uptake per gram of tissue. An abundance of MPS macrophages and a rich blood supply are the primary reasons for the preponderance of particles in these sites. After phagocytosis, the carrier and the associated drug are transported to lysosomes, and the drug is released upon disintegration of the carrier in this cellular compartment. This passive targeting to the MPS (and particularly to the liver) is advantageous in a number of situations, including

- The treatment of macrophage-associated microbial, viral, or bacterial diseases (e.g., leishmaniasis)
- The treatment of certain lysosomal enzyme deficiencies
- The immunopotentiation of vaccines (further information on the use of nanoparticles for vaccines is given in Chapter 17)
- The activation of macrophages, by loading the carrier system with macrophage-activating agents such as interferon γ, to fight infections or tumors

If the drug is not broken down by the lytic enzymes of the lysosomes, it may be released in its active form from the lysosomal compartment into the cytoplasm and may even escape from the phagocyte, causing a prolonged release systemic effect.

Technology is available to reduce the tendency of macrophages to rapidly phagocytize colloidal drug carrier complexes. The process of "steric stabilization" involves the coating of the delivery system with synthetic or biological materials, which make it energetically unfavorable for other macromolecules to approach the surfaces carrying these chains. A standard approach is to graft

hydrophilic, flexible polyethylene glycol (PEG) chains to the surface of the particulate carrier (termed "PEGylation"). This highly hydrated PEG layer reduces the adsorption of opsonins from the plasma and consequently slows down phagocytosis. The net effect of PEG attachment is that macrophage/liver uptake of the particles is delayed or reduced, thus increasing the circulation time.

In order to avail of the EPR effect, two conditions should be satisfied:

1. The size of the drug carrier system should exceed the size of normal endothelial fenestrations to ensure that the carrier system only crosses inflamed/tumor endothelium; a certain size range is preferred as there is an upper limit to the endothelial fenestration dimensions under pathological conditions.
2. The circulation time in the blood compartment should be long enough to allow the carrier systems to "escape" from the circulation at the pathological site, though not all will.

As described earlier, the circulation time of a particulate carrier in the blood can be prolonged using "stealth" technology to enhance particle hydrophilicity. If the circulation time is sufficiently prolonged and the particle size does not exceed, say, 200 nm, then accumulation in tumor and inflammation sites can be observed. The goal today is to enhance accumulation in targets, which in tumors in man has rarely exceeded around 5% of the vector.

5.3.2 Active Targeting

In active-targeting strategies, a specific ligand is attached to the carrier system, to improve delivery to a specific cell, tissue, or organ. Thus, delivery systems designed for active-targeting are usually composed of three parts: the carrier, the ligand, and the drug. Preferably, the ligand is covalently attached to the carrier, although successful targeting attempts of noncovalently attached ligand–carrier combinations have also been described.

Target sites for active-targeting strategies can differ widely. A list of cell-specific receptors and their corresponding ligands, expressed under physiological conditions, is presented in Table 5.2. Thus, for example, galactose can be used to target a drug carrier to parenchymal liver cells. Receptors are not always exposed directly to the vector, which might thus have to access the target and then interact. In the future, it is expected that the rapidly growing field of genomics will be used to identify specific receptors for targeting purposes, which might enhance the effectiveness of targeting.

Other receptors may become available under pathological conditions. Such receptors include

- Antigenic sites on pathogens (bacteria, viruses, parasites)
- Infected host cells expressing specific antigenic structures
- Tumor-associated antigens (TAAs) (i.e., antigenic structures specifically occurring at the surface of tumor cells)
- Substances such as fibrin in blood clots (i.e., potential ligands for targeting of fibrinolytics)

TABLE 5.2

Examples of Cell-Specific Ligands/Carriers In Vivo

Cell	Cell-Specific Ligands/Carriers
Parenchymal liver cells	Galactose, polymeric IgA, cholesterol ester—VLDL, LDL
Kupffer cells	Mannose/fucose, galactose (particles), (oxidized) LDL Liver
endothelial cells	Mannose, acetylated LDL
Leucocytes	Chemotactic peptide, complement C3b

Abbreviations: VLDL, very-low-density lipoproteins; LDL, low-density lipoproteins.

Sometimes it is necessary for the carrier-bound drug to reach all target cells to be clinically successful, as is the case with antitumor therapy. The so-called "bystander" effects can help to achieve fully effective therapy. Bystander effects occur when the targeted drug carrier reaches its target site, and released drug molecules also act on surrounding nontarget cells. In other cases, not all target cells have to be reached, as is the case, for example, for targeted gene delivery for the local production of a therapeutic protein. However, the cell nucleus is a target that is difficult to reach, as several barriers inside the cell have to be overcome (e.g., cytoplasmic membrane, endosomal wall, and the nuclear membrane).

Antibodies raised against a selected receptor are extensively used as homing ligands, as described in the succeeding texts. Modern molecular biotechnology permits the production of large amounts of tailor-made material. Other potential candidates are also emerging, in the cytokine and the growth hormone family and, finally, among the adhesion molecules that play a role in the homing of inflammatory cells to inflammation sites.

5.4 SOLUBLE CARRIERS FOR TARGETED DRUG DELIVERY

As described earlier, the major advantage of soluble carriers over particulate carriers is their greater ability to extravasate; also, being soluble, they can be taken into cells via the process of pinocytosis. Active-targeting strategies for soluble carriers include attaching rather simple ligands such as galactose, for targeting to liver parenchymal cells (see Table 5.2); alternatively, more complicated structures, such as antibodies, or antibody fragments (Fab or single-chain antibodies [SCA]) can be used as targeting ligands.

However, a number of disadvantages are also associated with the use of soluble carriers:

- Limited drug-loading capacity: a low ratio of drug to carrier limits the mass transport mediated by the drug carrier.
- The drug is covalently bound to the carrier: this can mask the active site of the drug and the conjugation reaction may damage a labile drug moiety.
- The carrier confers limited protection on the drug.
- The rate of cleavage of the drug from the carrier may not be optimal for activity.

5.4.1 Monoclonal Antibodies

The therapeutic antibodies that are on the market all come from the IgG family, with the IgG1 isotype being the most commonly used. Monoclonal antibodies (MAbs) belong to the largest molecules in our therapeutic arsenal with a molecular weight (MW) of 150,000 (1000 times the MW of paracetamol/acetaminophen). As shown in Figure 5.5, the structure consists of two heavy and two light chains. A sugar chain is connected to each heavy chain. The antigen-binding site of IgG molecules represents the homing part, which specifically interacts with the target (cells, pathogens, tissue). These antigen-binding sites are located at both tips of the Y-shaped molecules. The sites that are responsible for the pharmacological effects of IgG, such as complement activation and macrophage interaction, are located at the stem part of the Y. The rest of the molecule forms the connection between the homing ligand and the pharmacologically active sites, and also contributes to the long blood circulation characteristics of the IgG molecule, which has an elimination half-life much greater than 24 hours.

Humanized or human antibodies have replaced earlier generations of murine antibodies. There are four options in basic structure. The earliest generation was fully based on mouse hybridoma technology; then, the Fc part was mouse derived, with the Fab fragment comprising a human sequence (chimeric MAb), followed by humanized antibodies, where only the complementarity-determining region was mouse derived. Nowadays, fully human MAbs have entered the field. Mouse-derived antibodies can induce human antimouse antibodies (HAMA) in the patient. In multiple injection schemes,

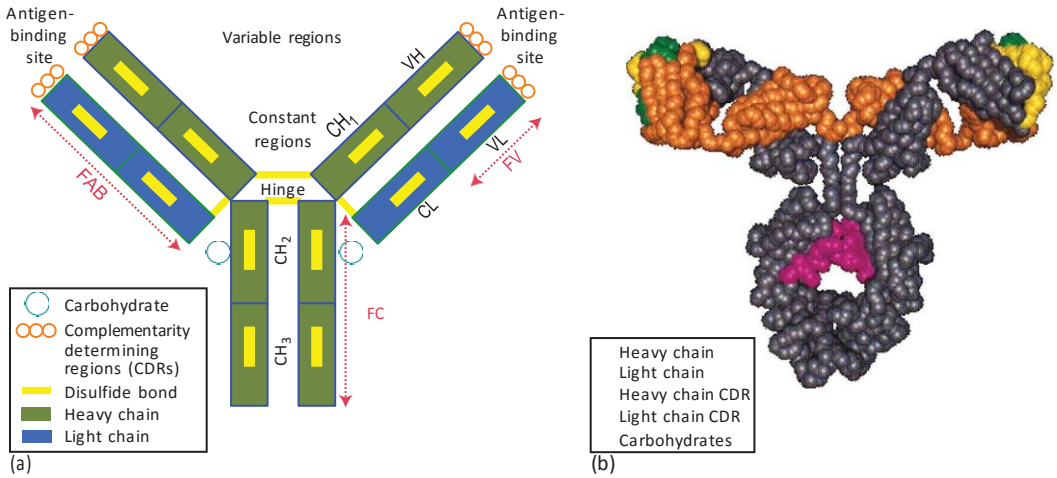


FIGURE 5.5 Molecular structure of IgG, Fab, and single-chain antibody: (a) cartoon and (b) a 3D representation.

this HAMA reaction can cause neutralization of the homing capacity of the homing ligand, loss of activity, and anaphylactic reactions. Chimeric, humanized, and human antibodies induce HAMA (much) less frequently but, for instance, can still raise anti-idiotypic antibodies against the binding site structure. These anti-idiotypic antibodies can also interfere with the targeting performance.

MAbs have rather complicated names as shown in Figure 5.6. The WHO International Nonproprietary Name (INN) system provides the logic behind these names (see WHO 2008). Murine MAb names end with *–momab*, chimeric with *–ximab*, humanized with *–zumab* and, finally, fully

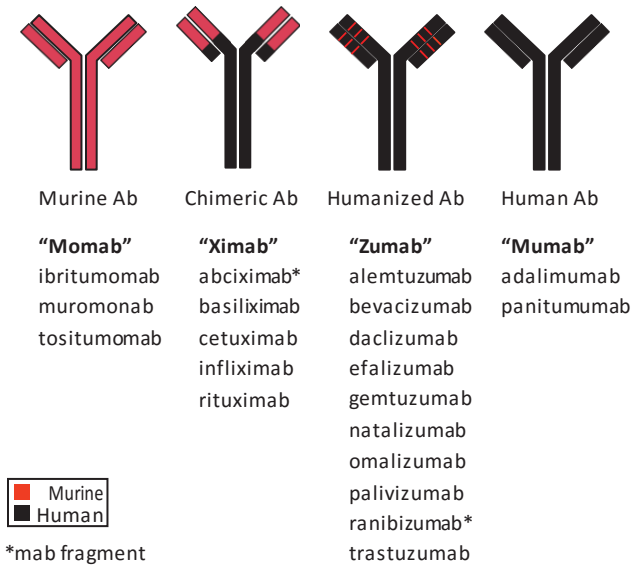


FIGURE 5.6 Monoclonal antibody (Ab) nomenclature.

human MAb with *–mumab*. The therapeutic indication is also referred to, e.g., *–li-* for an immunomodulating and *–tu-* for an antitumor effect. This helps to understand INN such as trastuzumab and infliximab.

The large size of MAbs (around 15 × 8 × 5 nm) and their hydrophilicity have some disadvantages. Penetration through endothelial and epithelial barriers and transport through extracellular spaces are slow. This leads to low uptake percentages at the target site if the target is located beyond the vascular endothelium (extravascular space). For instance, in cancer patients, the uptake of MAb in solid tumors is around 3% of the dose, which means that 97% ends up somewhere else in the body. Therefore, to facilitate (diffusional) transport, often the full antibody molecule (MW 150 kDa) is not utilized for target ligand (i.e., specific receptor) binding, but only the antigen-binding domain carrying the Fab (MW 50 kDa) fragment or even smaller fragments (SCA, MW 25 kDa).

In 1986, the first marketed MAb for therapeutic use was the mouse anti-CD3 antibody muromonab (OKT3), for the prevention of rejection of kidney transplants. Since then, a long list of MAb has been approved mainly for oncology, autoimmune diseases, and transplant rejection. In 2012, 5 out of 10 on the list of the globally most successful medicines (in terms of revenue) were MAbs, a clear indication of the fast-growing success of this family of medicines.

MAbs against TAAs have been developed to assist in tumor imaging. The MAb is conjugated with a diagnostic imaging agent (e.g., In). Commercial products include

- Satumomab pendetide (Oncoscint® CR/OV), for colorectal and ovarian adenocarcinomas
- Capromab pendetide (Prostascint®), for prostate cancer
- Arcitumomab (CeaScan®), for a number of carcinoembryonic antigen (CEA)-rich tumors

The therapeutic use potential of MAb as an "all-in-one" bioactive and targeting ligand molecule per se may be limited because of the limited immunological activity on the Fc part. Therefore, antibody–drug conjugates (ADC) have been developed. An example of an ADC is the MAb–drug conjugate, Adcetris (brentuximab vedotin). This ADC is used for the treatment of Hodgkin lymphoma patients. Adcetris® consists of three components:

1. The chimeric IgG1 antibody cAC10, specific for human CD30 (exposed on lymphoma cells), which binds (random collision events, as well as access issues permitting, cf. stochastic paradigm, Box 5.2) to the tumor.
2. Monomethyl auristatin E (MMAE), a microtubule-disrupting agent (anticancer agent).
3. A protease-cleavable linker that covalently attaches MMAE to cAC10. It is stable outside the cell, and upon cell internalization, it is cleaved, releasing MMAE.

Bispecific antibodies are manufactured from two separate antibodies to create a molecule with two different binding sites. One binding site links the MAb to the target cell. The other site is chosen to bring T-lymphocytes or natural killer cells in close contact with the target site, in order to exert a pharmacological effect, for example, to kill the target cell. This approach is now in early stage clinical trials.

5.4.2 Soluble Polymeric Carriers

Over the years, different soluble polymeric systems have been developed in attempts to enhance drug performance. Here again, the emphasis is on the improvement in drug disposition conferred by the carrier and ligand, as well as the protection offered by the system against premature inactivation. The strategy, as shown in Figure 5.7, involves the use of a soluble macromolecule, the molecular weight of which ensures access to the target tissue. The drug moiety can be bound via either a direct linkage or a short chain "spacer." The spacer overcomes problems associated with the shielding of the drug moiety by the polymer backbone and allows cleavage of the drug from the polymer.

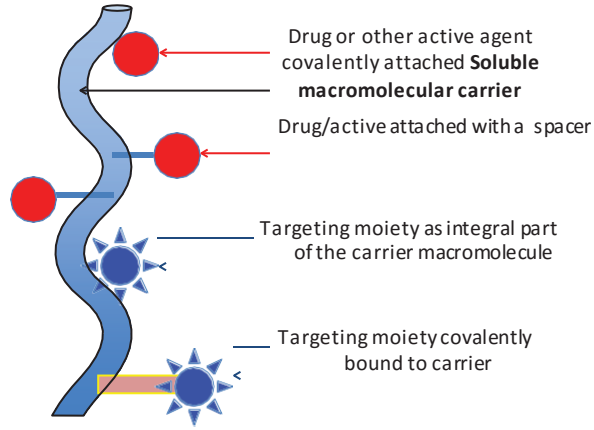


FIGURE 5.7 Components of a soluble macromolecular site-specific delivery system.

The spacer allows greater exposure of the drug to the biological milieu thereby facilitating drug release. A targeting moiety, which can be either an integral part of the polymer backbone or covalently bound, may also be incorporated into the system.

A crucial feature of such carrier systems is their solubility, which enables them to be taken up into target cells by the process of pinocytosis (see Chapter 4, Section 4.3.3.1). The intact carrier enters the target cell through pinocytotic capture. Through an endosomal sorting step, the carrier reaches the lysosomes where it is exposed to the actions of a battery of degradative enzymes. The drug–carrier linkage is designed to be cleaved by these enzymes, liberating free, active drug that can leave the lysosome by passage through its membrane, reaching the cytoplasm and other parts of the cell. Intralysosomal release of the drug from the carrier can also be achieved by making the drug–carrier linkage acid labile, as the lysosomal interior has a pH of approximately 4.5–5.5.

5.4.2.1 HPMA Derivatives

Poly(*N*-(2-hydroxypropyl)methacrylamide) has been investigated as a soluble macromolecular carrier system, using doxorubicin as the active drug. The bulk of the conjugate consists of unmodified HPMA units, which comprise about 90% of the carrier; the remaining units are derivatized with doxorubicin (see also Chapter 1, Figure 1.10). A tetrapeptide spacer (sequence Gly-Phe-Leu-Gly) connecting doxorubicin to the HPMA units proved to be cleavable by lysosomal thiol proteinases. Enzymatic cleavage breaks the peptide bond between the terminal glycogen and the daunosamine ring, liberating free doxorubicin, which can diffuse to the cytoplasm and nucleus where it (presumably) exerts its action.

Targeting moieties can also be incorporated into this delivery system. Targeting systems that have been investigated include

- *Galactose*: for targeting to parenchymal liver cells
- *Melanocyte-stimulating growth factor*: for targeting to melanocytes
- *MAbs*: for targeting to tumors

Interestingly, the doxorubicin–polymer conjugate alone, without a target-specific ligand, showed an enhanced therapeutic index in animal models and considerable accumulation of the drug in tumor tissue. The EPR effect, as discussed earlier, is held responsible for this phenomenon. After optimizing conjugate performance in terms of doxorubicin "payload" and desired molecular weight range of the polymer backbone, clinical grade material is now available and clinical trials are in progress to evaluate the potential of this concept.

5.4.2.2 SMANCS

The cytotoxic neocarzinostatin (NCS) is a small protein (MW 12 kDa) associated with a low-molecular-weight chromophore. NCS is rapidly cleared by the kidney and its cytotoxicity is non–cell specific. To modify its disposition, two poly(styrene-*co*-maleic acid anhydride) copolymers (MW 1500) have been coupled to one molecule of NCS, to give styrene-maleic-anhydride-neocarzinostatin (SMANCS) systems (see also Chapter 1, Figure 1.11). It was the work with SMANCS that led Hiroshi Maeda to develop the concept of the EPR effect.

SMANCS has been shown to retain nearly all the in vitro activity of NCS, with much improved pharmacokinetic properties. Tumor uptake has been shown to increase in animal models. Clinical successes have been reported with SMANCS formulated in Lipiodol® (a lymphographic vehicle) after intra-arterial administration in patients with unresectable hepatocellular carcinomas. The fact that the drug is administered in this lipid vehicle perhaps complicates interpretation, but clinical trials, mainly in Japan, are still being conducted some decades after the compound's first discovery.

5.4.2.3 Drug-Lipid Conjugates

One of the issues already touched upon is the need for optimization of drug loading and (too) early release from carrier systems. This can be an issue of polymer–drug compatibility in polymeric systems, although in liposomal systems interactions between drug and lipid are more conducive to high loadings (e.g., for doxorubicin liposomes discussed in Section 5.5.1). An alternative is to prepare drug–lipid conjugates, where the hydrophobic component is a compound such as squalene, which aggregates to form micelle-like structures with around 50% of drug content.

Coupling drugs to relatively low-molecular-weight insoluble or poorly soluble lipids, such as squalene—a natural lipid—has been proposed as a means of changing the biodistribution of the drug and to enhance activity. Using low-molecular-weight "carriers" means that the drug content is a significant element of the system. Gemcitabine, an anticancer nucleoside that is rapidly deactivated in vivo, displays an enhanced half-life and residence time when coupled to squalene. The gemcitabine–squalene compound (Figure 5.8) of which 40% is the drug payload, associates in aqueous solution because of its dual hydrophile–lipophile (surfactant) nature, forming aggregates of around 100–130 nm in diameter. These aggregates have a greater cytotoxic activity and accumulate to a higher extent in the liver and spleen than the drug itself. Other drugs have been coupled to squalene and clinical trials are in progress. Because of their structure, these squalene derivatives can also be readily incorporated into liposomes.

5.5 PARTICULATE CARRIERS FOR DRUG TARGETING

Advantages of particulate carriers include the following:

- The high drug loading that is possible with some systems.
- The drug does not have to be chemically attached to the carrier.
- A considerable degree of protection from degradation in vivo and control of drug release may be conferred on drug molecules encapsulated within the carrier.

The carrying capacity of a particulate is determined by the affinity of the drug for the carrier material: large proteins and peptides may have problems in mixing isotropically with polymeric core materials. This can lead to premature release of the drug.

However, a major limitation of these systems is their inability to cross continuous endothelial barriers (Figure 5.3) and leave the general circulation. In general, microparticulate carriers are phagocytized by the macrophages of the MPS, thereby rapidly localizing predominantly in the liver and spleen. However, sterically stabilized particulate carriers have extended circulation times and can remain in the blood; they either act as circulating drug reservoirs, or may slowly escape from the blood pool at pathological sites which exhibit increased vascular permeability.

Intravenously administered particles with dimensions exceeding a few μm (the diameter of the smallest capillaries) will be filtered by the first capillary bed they encounter, usually the lungs, leading

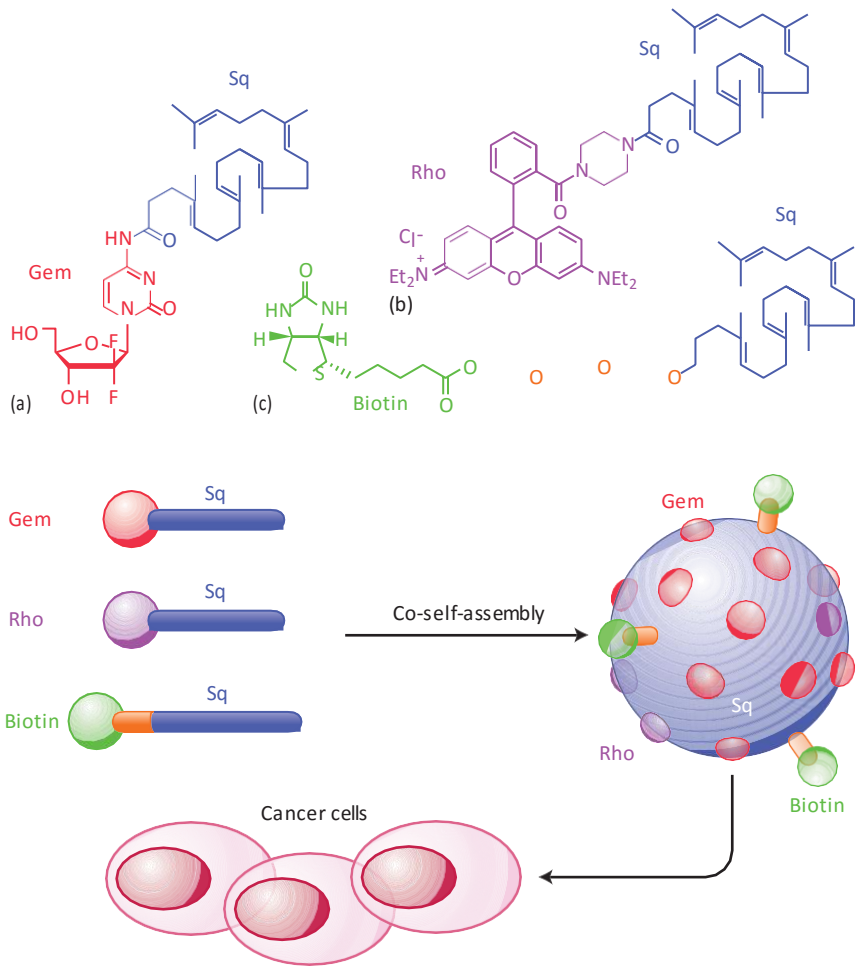


FIGURE 5.8 The structures (above) of (a) of gemcitabine-squalene (Gem-Sq), (b) the marker rhodamine- squalene (Rhod-Sq), and (c) targeting molecule biotin-squalene (Biotin-Sq) and (below) their assembly into multifunctional particles for cancer cell delivery.

to embolism. Intra-arterially administered particles with dimensions exceeding the diameter of the smallest capillaries (around 7 μm) will be trapped in the closest organ located upstream; for example, administration into the mesenteric artery leads to entrapment in the gut and into the renal artery leads to entrapment in the kidney. This approach is under investigation to improve the treatment of diseases in the liver. Arterial chemoembolization involves the use of drug-eluting microspheres, which combine the action of blocking the arterial blood supply to hepatic tumors, causing ischemia, and delivering drugs such as doxorubicin and irinotecan, which then have prolonged contact with the tumor.

Active-targeting strategies for particulate systems are similar to those discussed for soluble macromolecular systems (see Table 5.2 and Section 5.4.1 on antibodies).

5.5.1 LIPOSOMES

Liposomes are vesicular structures based on one or more lipid bilayer(s) encapsulating an aqueous core (Figure 5.9). The lipid molecules are usually phospholipids, amphipathic moieties with a hydrophilic head group, and two hydrophobic chains ("tails"). Such moieties spontaneously orientate in water to give the most thermodynamically stable conformation, in which the hydrophilic head group faces out

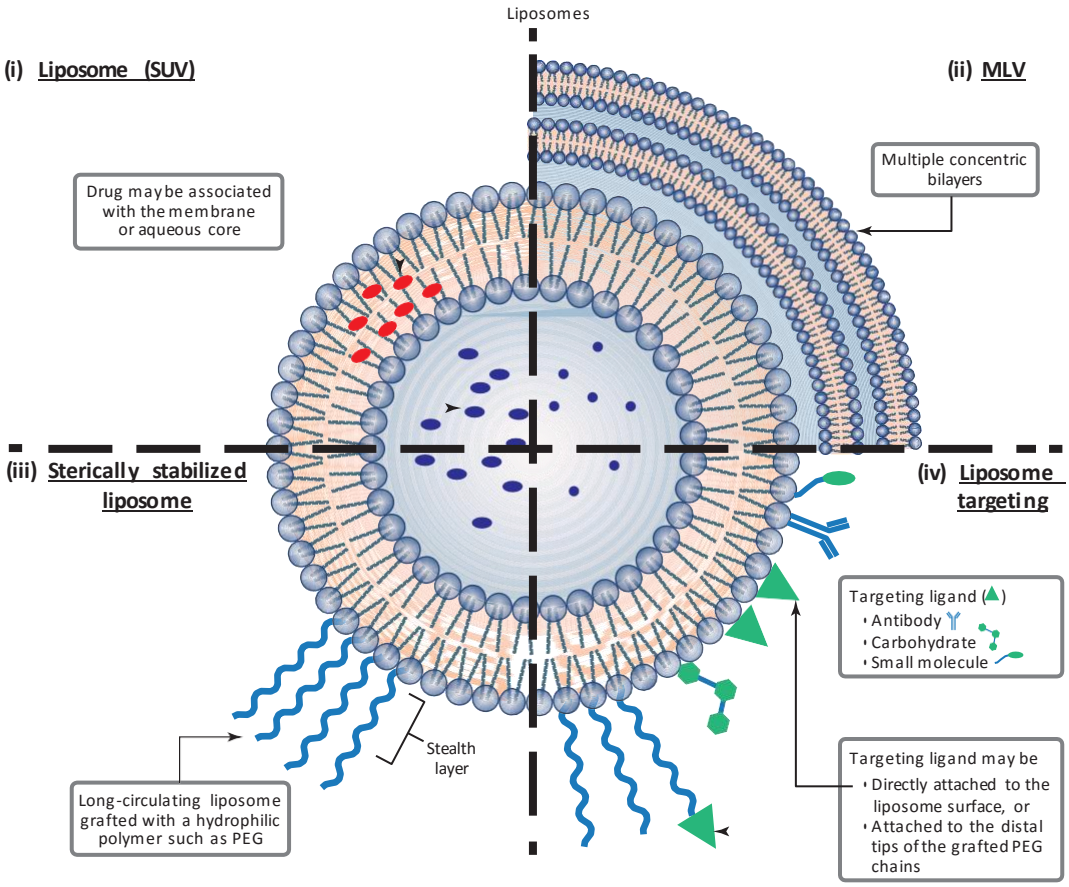


FIGURE 5.9 Schematic illustration of four possible different types of liposome: (i) small unilamellar vesicle (SUV), showing a water-soluble drug entrapped in aqueous interior and a water-insoluble drug incorporated into the liposomal membrane; (ii) multilamellar vesicle (MLV), with three concentric lipid bilayers; (iii) sterically stabilized liposomes, containing a "stealth" layer of polyethylene glycol (PEG), which shields the lipo- some from opsonization and uptake by the reticuloendothelial system; (iv) target-specific recognition ligands (including antibodies, sugars, and various other molecules) that can be coupled to liposomes, either to the liposome surface directly, or to the ends of grafted PEG chains.

into the aqueous environment and the lipidic chains orientate inward avoiding the water phase; this gives rise to bilayer structures. In order to reduce exposure at the edges, the bilayers self-close into one or more concentric compartments around a central discrete aqueous phase. Dependent on the preparation protocol used, liposome diameters can vary between 20 nm and 20 μm, thus encompassing the nano- and microrange of diameters. In general, they can be multilamellar or unilamellar, i.e., a multitude of concentrically orientated bilayers surrounds the aqueous core or only one bilayer surrounds an aqueous core, respectively. However, other structures have also been described.

If multilamellar structures are formed, water is present in the core of the liposome and also entrapped between the bilayers. Depending on the physicochemical nature of the drug, it can either

- Be captured in the encapsulated aqueous phase (i.e., the aqueous core and the aqueous compartments between the bilayers) (hydrophilic drugs)
  or
- Interact with the bilayer surface (e.g., through electrostatic interactions) or be taken up in the bilayer structure (lipophilic drugs)

Thus, liposomes can serve as carriers for both water-soluble and lipid-soluble drugs. The liposomal encapsulation of a wide variety of drugs, including antitumor and antimicrobial agents, chelating agents, peptides, proteins, and genetic material, have all been described.

Bilayer composition can be almost infinitely varied by choice of the constituent lipids. Phosphatidylcholine, a neutral phospholipid, has emerged as the major lipid component used in the preparation of pharmaceutical liposomes. Phosphatidylglycerol and phosphatidylethanolamine are also widely used. Liposomal bilayers may also accommodate sterols, glycolipids, organic acids and bases, hydrophilic polymers, antibodies, and other agents, depending on the type of vesicle required. The rigidity and permeability of the bilayer strongly depend on the type and quality of lipids used. The alkyl-chain length and degree of unsaturation play a major role. For example, a C18 saturated alkyl chain produces rigid bilayers with low permeability at room temperature. The presence of cholesterol also tends to rigidify the bilayers. Such systems are more stable and can retain the entrapped drug for relatively longer periods, whereas more "fluid" bilayer systems can be prepared if a more rapid release is required.

Liposomes can be classified on the basis of their composition and in vivo applications:

- *Conventional liposomes*, which are neutral or negatively charged, are generally used for passive targeting to the cells of the MPS.
- *Sterically stabilized ("stealth") liposomes*, which carry hydrophilic coatings, are used to obtain prolonged circulation times.
- *Immunoliposomes ("antibody targeted")*, which can be either conventional or sterically stabilized, are used for active-targeting purposes.
- *Cationic liposomes*, which are positively charged, are used for the delivery of genetic material.

As phospholipid bilayers form spontaneously when water is added, the important challenge in liposome preparation is not the assembly of simple bilayers (which happens automatically), but in causing the bilayers to form stable vesicles of the desired size, structure, and physicochemical properties, with a high drug encapsulation efficiency. There are many different approaches to the preparation of liposomes; however, what they all have in common is that they are based on the hydration of lipids.

Liposomes represent highly versatile drug carriers, offering almost infinite possibilities to alter structural and physicochemical characteristics. This feature of versatility enables the formulation scientist to modify liposomal behavior in vivo and to tailor liposomal formulations to specific therapeutic needs. It took decades to develop the liposome carrier concept to a pharmaceutical product level, but a number of commercial preparations are now available in important disease areas and many more formulations are currently undergoing clinical trials. Examples of the different applications and commercial products of various types of liposomal systems are given in the following texts.

5.5.1.1 Conventional Liposomes

These can be defined as liposomes that are typically composed of only phospholipids (neutral and/or negatively charged) and/or cholesterol. Most of the early work on liposomes as a drug carrier system employed this liposomal type. These systems are rapidly taken up by the phagocytic cells of the MPS, localizing predominantly in the liver and spleen, and are therefore used when targeting to the MPS is the therapeutic goal. Conventional liposomes have also been used for antigen delivery and a liposomal hepatitis A vaccine (Epaxal) has received marketing approval in Switzerland.

A commercial product based on conventional liposomes has been introduced for the parenteral delivery of the antifungal drug, amphotericin B, which is associated with a dose-limiting nephrotoxicity in conventional formulations. AmBisome, a liposomal formulation of amphotericin B,

comprises small unilamellar vesicles with diameters between 50 and 100 nm. Two other lipid-based formulations of amphotericin B are used in the clinic:

- Abelcet consists of ribbonlike structures having a diameter in the 2–5 μm range.
- Amphocil comprises a colloidal dispersion of disk-shaped particles with a diameter of 122 nm and a thickness of 4 nm.

In spite of the large differences in structural features (a further example of "liposomal" versatility), all formulations have been shown to greatly reduce the kidney toxicity of amphotericin B, allowing higher doses to be given and thereby improving clinical efficacy.

5.5.1.2 Long-Circulating Liposomes

At present, the most popular way to produce long-circulating liposomes is to covalently attach the hydrophilic polymer, PEG, to the liposome bilayers. As discussed in Section 5.3.1, the highly hydrated PEG groups create a steric barrier against interactions with molecular and cellular components in the biological environment. Figure 5.10 shows how "PEGylation" of liposomes can extend their blood circulation profile.

Long-circulating liposomes can enhance their chances of extravasation and thus accumulate at sites where pathological reactions occur. For example, the commercial product Doxil (marketed as Caelyx in Europe) consists of small-sized PEGylated liposomes, encapsulating the cytostatic doxorubicin. The resulting long-circulation times and small size of the vesicles facilitate their accumulation in tumor tissue

DaunoXome liposomes are also long-circulating liposomes, in this case encapsulating the cytostatic daunorubicin. Although a nonstealth system, long-circulation times are attained by using a particularly rigid bilayer composition, in combination with a relatively small liposome size. The encapsulation of these anthracycline cytostatics in liposomes affects a modified biodistribution of the drug; the drug is distributed away from the heart, where in free form it can exert considerable toxic effects, and is preferentially taken up by solid tumor tissue.

5.5.1.3 Immunoliposomes

Immunoliposomes have specific antibodies or antibody fragments on their surface to enhance target site binding. The primary focus of their use has been in the targeted delivery of anticancer agents.

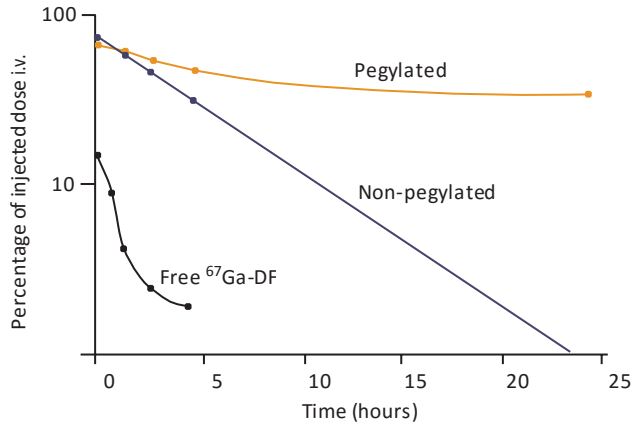


FIGURE 5.10 Comparison of the blood levels of free gallium desferal (^{67}Ga-DF), with ^{67}Ga-DF encapsulated in polyethylene glycol stabilized liposomes, and nonstabilized liposomes, upon intravenous administration in rats.

Long-circulating immunoliposomes can also be prepared. The antibody can be coupled directly to the liposomal surface; however, in this case, the PEG chains may provide steric hindrance to antigen binding. Alternatively, a bifunctional PEG linker can be used, to couple liposomes to one end of PEG chains and antibodies to the other end of these chains (Figure 5.9). Steric hindrance is not a problem in the latter approach.

5.5.1.4 Cationic Liposomes

Cationic liposomes demonstrate considerable potential for improving the delivery of genetic material. The cationic lipid components of the liposomes interact with, and neutralize, negatively charged DNA, thereby condensing the DNA into a more compact structure. Depending on the preparation method used, the complex may not be a simple aggregate, but an intricate structure in which the condensed DNA is surrounded by a lipid bilayer. These systems are discussed further in Chapter 16.

5.5.2 Niosomes

An alternative to phospholipid-based liposomes can be found in niosomes (nonionic surfactant vesicles) developed by L'Oreal for cosmetic use. These systems, which can be uni- or multilamellar, are based on several different families of synthetic, nonionic low hydrophilic-lipophilic balance (HLB) amphipathic molecules, such as sorbitan monolaurate and others of this series, or alkyl glucosides, all formed with cholesterol. While there is considerable experimental experience with niosomes as parenteral delivery systems, there are no clear advantages over liposomal systems: although composed of nonionic surfactants/cholesterol mixtures rather than phospholipid/cholesterol mixtures and thus having a potential for greater variation in their composition, they turn out to have very similar physical and biological properties.

5.5.3 Polymeric Micelles

As described in Chapter 3 (Section 3.8), when low MW amphipathic (surfactant) molecules with distinct hydrophilic and hydrophobic sections are dispersed in water, spherical micelles in the nanometer size range are formed above a certain concentration, the critical micelle concentration (CMC). These micelles can solubilize drug molecules in their hydrophobic core, a useful formulation technique that has been used for the preparation of injections of paclitaxel, for example. There is a constant exchange between the surfactant monomers and their micelles. The more hydrophobic the surfactant, the lower the CMC. Nonionic surfactant systems generally have very low CMCs so that the micellar phase can exist even after dilution. However, micelles used as carriers for drug-targeting purposes must be stable in the blood circulation and should not disintegrate upon contact with blood components so that the drug load can be kept on board. In spite of the low CMCs of surfactant molecules, they are fragile. Using polymeric amphipathic materials to form the so-called polymeric micelles allows the formation of more stable systems suitable for targeting. The rate of exchange between the micellar and monomeric forms is slow because of the strong interaction forces between the molecules in the polymeric micelle.

There are several types of polymeric surfactant. Micellar systems based on amphipathic block copolymers have been explored as i.v.-administered drug carrier systems. These block copolymers can be, for example, composed of a hydrophilic PEG block (A) and a hydrophobic block (B) based on poly(aspartic acid) or poly(β-benzyl-l-aspartate). These form micelles in aqueous solution with spherical core/shell structures having diameters around 20–40 nm (see also Chapter 1, Figure 1.14). The hydrophobic core of these micelles can be loaded with lipophilic drugs such as doxorubicin. After i.v. administration, the micelles may accumulate at tumor sites. Some of the characteristics of these micellar systems are listed in Box 5.3. A subset of polymeric micelles is formed by lipid-core micelles, which comprise systems formed from conjugates of soluble copolymers with lipids. One example is the PEG–phosphatidylethanolamine conjugate.

BOX 5.3 POLYMERIC MICELLES AS DRUG CARRIERS

- Critical micelle concentration of the amphipathic copolymers is low; interaction between polymer units is strong, so that blood components cannot disrupt the aggregates.
- Molecular weight of the polymeric unit is small enough to allow clearance through glomerular filtration.
- Diameter is large enough to prevent penetration through intact endothelium.
- Diameter can be chosen in the range where the EPR effect is observed (<200 nm).
- Release kinetics of the drug depend on the selected polymer structure (hydrophilicity/hydrophobicity balance).
- Drug is in the hydrophobic core of the micelle and is protected from exposure to aqueous degradation processes.
- They have been shown to have a high drug–carrying capacity ("payload").

These systems are chemically versatile (using different AB or ABA copolymers), as it is possible by varying the copolymer characteristics to enhance payload and by using covalent binding strategies to further optimize their performance.

5.5.4 Lipoprotein Carriers

Lipoproteins are nature's template for targeting growing cells, as they transport cholesterol to such cells by receptor-mediated endocytosis. These endogenous lipid carrier systems comprise a lipid core and a coat where the ligand apolipoproteins can be found. The lipid core material consists of cholesterol and other lipids (cholesterol esters, triacylglycerols and phospholipids), transported in plasma and other body fluids as lipoproteins, i.e., complexes of the lipid material bearing apolipoproteins as targeting moieties. This has given rise to their exploration as systems aimed at cancer cells. There are different types of lipoproteins: (1) high-density lipoproteins, size about 10 nm; (2) low-density lipoproteins (LDL), size about 25 nm; (3) very-low-density lipoproteins (VLDL), size about 30–90 nm; and (4) chylomicrons, size about 10–90 nm. Most studies have focused on LDL, which has a plasma half-life of 3–4 days. On some tumor cells, LDL receptor density is increased, making cytostatic-loaded LDL an interesting potential drug delivery system. With the Kupffer cells playing a major role in the uptake process, 90% of the LDL receptor activity is concentrated in the liver.

The obstacle to any clinical use is that LDL has to be extracted from patients, the cholesteryl esters in the interior of the particle removed, and the system processed to replace the interior with drug molecules. An alternative is to covalently attach drugs to the exterior, but this and other processing techniques can disrupt the conformation of the apolipoprotein and, hence, diminish or eradicate targeting properties. Some years ago, synthetic cholesterol ester–rich microemulsion systems were used to mimic LDL, but this work does not seem to have progressed.

5.5.5 Polymeric Micro- and Nanoparticles

Many polymers can be used in the preparation of micro- and nanoparticles (Figure 5.1). The choice of polymer is often based on the biodegradability of the polymer, the ease with which the polymer can be prepared as nanoparticles, and the loading capacity of the system for the chosen drug. The surface properties of the nanoparticles and the presence of reactive groups on the surface are clearly important in the ability to alter the behavior of these systems. The loading capacity of polymeric nanoparticles is determined by the affinity of the drug for the polymer: it cannot be assumed that all drugs will be freely miscible with the polymer in question, as mentioned earlier. The difficulty

in the formulation of poly(lactic-*co*-glycolic acid) (PLGA) implants of LHRH is a case in point. Smaller molecules may mix (or dissolve) more readily, but any incompatibility will affect stability, capacity, and release rates. The nature of the drug dispersion within nanoparticles is thus crucial in terms of the subsequent rate of release of the active drug.

The polymers used for the formulation of nanoparticles include synthetic polymers such as poly(alkyl cyanoacrylates) (PACAs), PLGAs, and poly(caprolactones), as well as natural polymers such as albumin, gelatin, alginate, collagen, and chitosan. We discuss some of these in the following texts. Although we can generically refer to "polymeric nanoparticles," their surface properties and the nature of their interior structure will depend on the polymer employed. Particle interiors may be porous or solid and surfaces will have different functionalities and contours that will influence both drug release and the ability to functionalize the particles.

5.5.5.1 Poly(Alkyl Cyanoacrylates)

The PACAs, used widely as tissue adhesives, are biodegradable and well tolerated. PACA nanoparticles can be produced by an emulsion polymerization technique or by precipitation from the monomer in the presence of surfactants. The alkyl cyanoacrylate monomer polymerizes rapidly in the presence of water to form nanoparticles in vigorously stirred oil-in-water dispersion, the process of polymerization being initiated by anions (OH^-) at the oil–water interface. The drug to be encapsulated is incorporated in the particles as the oil phase is evaporated.

Degradation kinetics in vivo are controlled by the alkyl-chain length. Poly(butyl cyanoacry- late) nanoparticles are degraded fairly rapidly (1 day), while poly(hexyl cyanoacrylate) nanoparticles take a number of days to degrade in vivo. PACA nanoparticles accumulate in the liver (60%–90% of the injected dose) and the spleen upon i.v. injection. The macrophages in the liver are their major target. PACA nanoparticles loaded with doxorubicin have shown an increased therapeutic index in a number of animal tumor models, due to a reduction of the peak drug con- centration in cardiac tissue, the organ most severely affected by doxorubicin upon injection. This is a common finding with doxorubicin delivery through liposomes, niosomes, and other carriers, so is not unique to PACA systems. The release of drug from the Kupffer cells upon breakdown of the nanoparticles in the lysosomal system may induce a slow release pattern that is still tumoricidal but lacks the cardiotoxic effects.

Sterically stabilized PACA nanoparticles can be prepared by adsorbing polyoxyethylene polymers of the poloxamer or poloxamine class. In addition, PEGylated nanoparticles can be prepared using block copolymers such as poly[methoxy-polyethylene glycol-cyanoacrylate-*co*-hexadecyl cyanoacrylate].

5.5.5.2 Poly(Lactic-co-Glycolic Acid)

The versatility of most polymers in the formation of nanoparticles lies in the ability to control the molecular weight of the polymer and also the ratio of the comonomers. This has been demonstrated strikingly with PLGA polymers. Figure 5.11 shows the relevant structures.

PLGAs have been a prominent material in the fabrication of experimental nanosystems. Changing the ratio of the monomers (lactic acid/glycolic acid) leads to different affinities for encapsulated drugs. The rate of release of drugs is also modulated by the molecular weight. One advantage of PLGA systems is that they degrade in vivo by hydrolysis into lactic acid and glycolic acid. The preparation, properties, and degradation of PLGA microspheres for use as long-acting injections are further discussed in Chapter 6.

5.5.5.3 Albumin and Other Protein Microspheres and Nanoparticles

In 2005, the FDA approved an albumin–paclitaxel formulation, Abraxane. This formulation showed an improved therapeutic index in a number of solid tumors in the clinic. The exact nature of these albumin-based nanoparticles (size around 300 nm) is still under investigation,

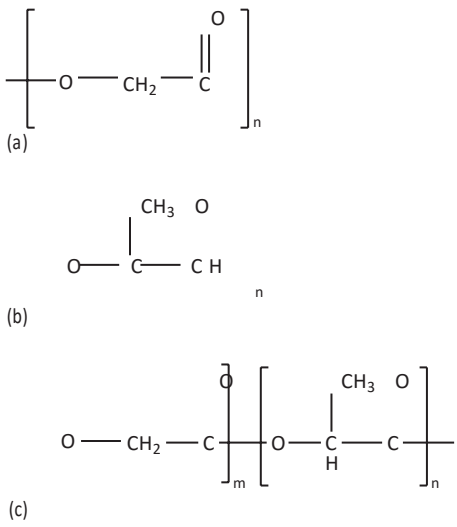


FIGURE 5.11 Structure of (a) poly(glycolic acid), (b) poly(lactic acid), and (c) poly(lactic-*co*-glycolic acid).

and several mechanisms have been suggested for the improved performance compared to conventional paclitaxel therapy. Among those is the possibility of increased uptake of albumin by fast growing solid tumors/tumor endothelia, as tumor cells need albumin-transported nutrients for their growth.

Albumin particles may also be prepared by stabilizing the structure by cross-linking the monomers. In the preparation of such albumin microspheres, an aqueous solution of albumin and the drug moiety is initially emulsified in oil, forming a water-in-oil emulsion. The protein can be chemically cross-linked by the addition of a cross-linking agent such as glutaraldehyde or butadione or thermally cross-linked. Either method produces stabilized particles for possible use in drug delivery. The size of the particles is based on the droplet size of the initial emulsion and can range from 15 nm to 150 μm. The rate of drug release has been shown in some cases to be dependent on the degree of cross-linking achieved. Hemoglobin, casein, and ferritin have been used as the basis for protein microspheres; there is no reason why such materials cannot be used as nanoparticles also.

5.5.6 DENDRIMERS

Dendrimers (treelike-branched polymers) are still a promising class of polymeric drug carrier system, some 30 years after their conception. They have several potential advantages over many other polymers in that they are synthesized in such a manner that each dendrimer molecule has exactly the same chemical composition and thus diameter, which can range from several nanometers upward. They are formed from a core multivalent molecule to which several dendrons (partial dendrimers) may be covalently attached, or other reactive groups, layer by layer or "generation" by "generation" (Figure 5.12). Research into all aspects of dendrimer chemistry and technology has escalated since the original work by Tomalia and others in the 1980s. The wide variety of chemical architectures of these spherical or quasi-spherical structures allows the construction of monodisperse particles in the 1–20 nm diameter range.

Dendrons are usually asymmetric partial dendrimers, which can be either used as such or formed covalently or noncovalently into complete dendrimers. This allows the formation of "Janus" forms with both a hydrophilic and hydrophobic surface. Because many such amphipathic dendrons and lipophilic dendrimers associate in solution, a range of supramolecular structures can be formed. These themselves may have potential as novel delivery systems. Drugs may be situated in the

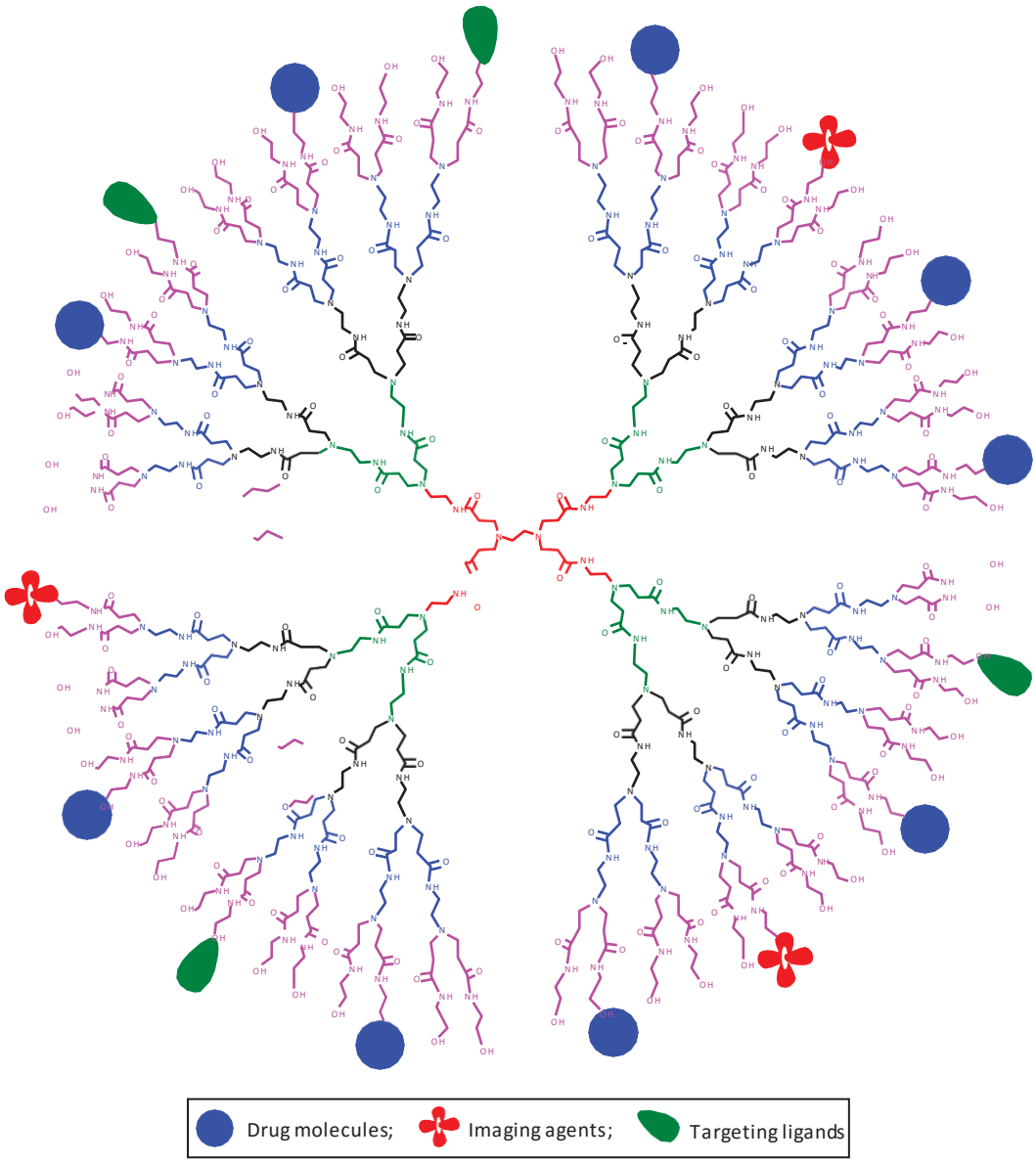


FIGURE 5.12 Polyamidoamine (PAMAM) dendrimer structure. Drug molecules may also be encapsulated in the interior of the structure.

interior of the dendrimer, on the dendrimer surface, perhaps covalently attached, or in intermediate positions. Hydrophobic dendrimers can associate into dendrimer aggregates 100–200 nm in diameter; hydrophilic dendrons can form vesicles—dendrisomes—so the family of systems based on these structures is immensely varied.

5.5.7 Other Particulate Carrier Systems

A variety of other nanoparticulate DDTS have been studied, including metallic (gold, silver, and iron oxide) nanoparticles, mesoporous silica nanoparticles, quantum dots, and carbon-based nanomaterials, including nanotubes, nanorods, and nanocages (see also Chapter 18). Many new DDTS

systems leverage techniques from the microelectronics industry, to precisely fabricate DDTS in the nanometer range; these nanofabrication techniques are described in Chapter 19.

5.6 PHARMACEUTICAL ASPECTS OF CARRIER SYSTEMS

In order for parenteral DDTS to become commercial products, certain pharmaceutical issues need to be addressed, including

- Purity of the carrier material
- Reproducibility of the characteristics of the drug carrier system
- Drug carrier–related safety aspects, including immunological responses
- Scaling-up possibilities
- Shelf life

Historically, DDTS were developed in environments where the primary goal was "proof of concept," rather than developing a commercial product. The typical pharmaceutical considerations described earlier were not dealt with seriously in the early days of drug carrier research; thus, early drug carrier systems were associated with long gestation periods from product development to product marketing.

The time frame associated with the development of a drug-targeting concept to a targeted drug product can be illustrated by the "liposome story." Liposomes were originally used as biochemical tools for the study of cell membrane behavior in the 1960s; the idea to use them as drug carriers was subsequently developed in the early 1970s. It took more than 20 years to develop the system from a concept to the first commercial parenteral liposome preparation carrying a drug (amphotericin B). Although this may seem like quite a long gestational period, it must be remembered that liposomes were one of the first colloidal carrier systems designed for targeted drug delivery. Comparatively, little was known about such systems and many technological and biopharmaceutical hurdles had to be overcome before marketing authorization for the first product could be obtained. Some of the hurdles encountered and solved over the years while developing liposomes as drug carriers are outlined here.

Poor quality of the raw material: In the early 1980s, the quality of lipids of several suppliers could vary considerably. Nowadays, a few suppliers provide the global market with high-quality products. Interestingly, over the years, the price per unit has dropped considerably while the quality has improved.

Poor characterization of the physicochemical properties of liposomes: Liposome behavior in vitro and in vivo is critically dependent on their physicochemical properties. Therefore, a full physicochemical characterization of pharmaceutical liposomes is required in early stages of a development program (Table 5.3). In later development stages, these quality control assays can be used to obtain regulatory approval and to ensure batch-to-batch consistency.

Shelf life: Shelf-life issues that need to be addressed include avoidance of preadministration leakage of the liposome-associated drug (retention loss), size stability (occurrence of fusion or aggregation), and phospholipid degradation (occurrence of peroxidation and hydrolysis).

Scaling-up problems: Several of the laboratory-scale liposome preparation methods were difficult to scale up to industrial scale.

Safety data: As these carriers are novel delivery systems, there initially existed a paucity of data on their safety during chronic use. However, their present safety record and the experience with marketed parenteral liposome preparations (e.g., amphotericin B, doxorubicin and daunorubicin) indicate that the safety of these systems is not a major limiting factor.

TABLE 5.3

Quality Control Assays of Liposomal Formulations

Assay	Methodology/Analytical Target
Characterization	
pH	pH meter
Osmolarity	Osmometer
Phospholipid concentration	Lipid phosphorus content/HPLC
Phospholipid composition	TLC, HPLC
Cholesterol concentration	Cholesterol oxidase assay, HPLC
Drug concentration	Appropriate compendial method
Chemical stability	
pH	pH meter
Phospholipid peroxidation	Conjugated dienes, lipid peroxides, FA composition (GLC)
Phospholipid hydrolysis	HPLC, TLC, FA concentration
Cholesterol autoxidation	HPLC, TLC
Antioxidant degradation	HPLC, TLC
Physical stability	
Vesicle size distribution	
Submicron range	DLS
Micron range	Coulter Counter, light microscopy, laser diffraction, GEC
Electrical surface potential	Zeta-potential measurements
Surface pH	pH sensitive probes
Number of bilayers	SAXS, NMR
Percentage of free drugs	GEC, IEC, protamine precipitation
Dilution-dependent drug release (simulating release upon injection)	Measure the extent of drug release from the drug–liposome product following dilution
Relevant body fluid–induced leakage	GEC, IEC, protamine precipitation
Biological characterization	
Sterility	Aerobic and anaerobic cultures
Pyrogenicity	Rabbit or LAL test
Animal toxicity	Monitor survival, histology, pathology

Abbreviations: FA, fatty acids; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; DLS, dynamic light scattering; GEC, gel exclusion chromatography; SAXS, small-angle x-ray scattering; IEC, ion exchange chromatography; LAL, limulus amebocyte lysate.

Biochemists, who worked with drug-loaded liposomes in the early days, had a completely different perception of "stability," reproducibility, upscaling, and toxicity than pharmaceutical scientists, who are familiar with the development of pharmaceutical formulations. For example, for a biochemist, a shelf life of a week at −70°C may be acceptable, whereas a pharmaceutical product would be expected to have a minimum shelf life of 2 years, preferably without refrigerator cooling. It took several years and considerable "mental adaptation" to bridge this cultural gap. Currently, quality is ensured by improved purification schemes, the introduction of validated analytical techniques, and a better insight into lipid degradation mechanisms, leading to better shelf-life conditions (Table 5.3). These quality control considerations are discussed further in Chapter 21.

The development of liposomal systems has thus contributed greatly to the development of drug carrier systems in general and has highlighted the various pharmaceutical hurdles that must be overcome before a DDTS can reach the marketplace. In addition, liposomal development has

provided fundamental knowledge on the fate of particulate systems in vivo and how this fate can be manipulated for therapeutic gain.

5.7 OBSTACLES TO SUCCESSFUL TARGETING

We have discussed earlier in this chapter some of the exciting developments in pharmaceutical nanotechnology for drug delivery and targeting. For balance, it is essential to recount some of the features of nanosystems and their interaction with the biological environment that often impede *quantitative* delivery of drugs and other therapeutic agents to target organs, tissues, cells, or cell compartments. It is widely recognized that only maximally around 5% of the administered dose of drug in carrier systems reaches the desired target. Quantitative targeting signifies that losses to nontarget tissues and organs will be minimized. When this is realized, it will transform the field. Drug targeting through the use of nanotechnology is at the same time simple in concept but complex in reality. The route from the point of injection to the site of action in a target is not a straightforward one, as Figure 5.13 attempts to illustrate, recounting the possibility of particle aggregation, jamming in narrow pores and vessels, the lack of uptake into the target, the premature release of the drug, and other factors that are listed in Box 5.4. What cannot be shown in the figure are the dynamics of the processes involved. One reason that we need to consider the delivery of drugs within nanoparticles in a different light from the delivery of free molecules is that once particulates are involved, we introduce the element of chance into the equation. If a small molecule drug is stable and soluble in biological fluids such as the blood, it will diffuse readily by way of the circulation throughout the body, not, of course, equally

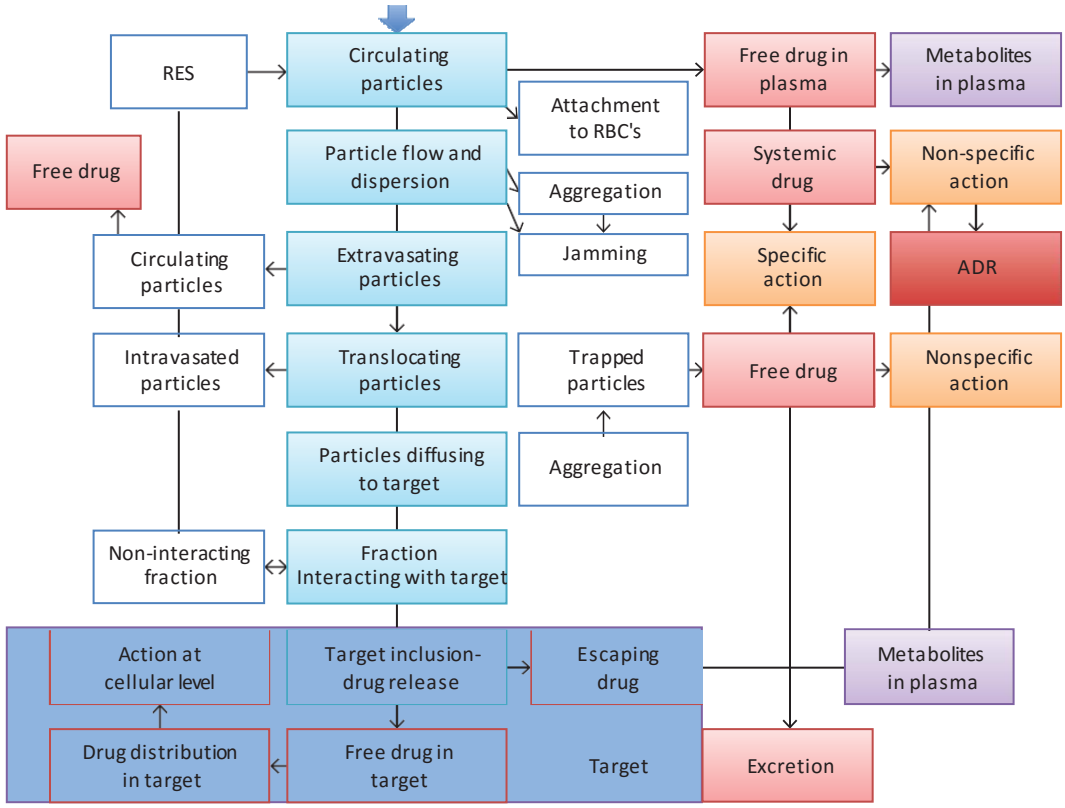


FIGURE 5.13 Diagrammatic (and simplified) illustration of the complex route from site of intravenous injection to target, with each potential stage labeled. Note the events that occur in the target as well as en route to the target tissue. RBC's, red blood cells; ADR, adverse drug reaction.

BOX 5.4 FACTORS REDUCING THE EFFECTIVENESS OF TARGETED SYSTEMS

- Premature release of drug load before the target is reached
- Problems in accessing the target site (size, charge, hydrophobicity)
- Adsorption of nanosystems to erythrocytes
- Complex flow patterns in the blood, which discourage epithelial contact
- Ligand loss during circulation
- Incomplete ligand–receptor interaction
- Poor diffusion of released drug into the target tissues
- Suboptimal levels of drug attained in the target
- Leakage of drug molecules from the target site
- Design of systems based on inappropriate cell choice (activity) or species (distribution)

to each site in the body, but sufficient at appropriate doses to affect specific receptors. The rates of metabolism and clearance are issues discussed elsewhere. The interaction of free drug molecules with their receptors is an equilibrium process, but because of the high number of free molecules, the probability of interaction is very high at the correct dose. One question to be posed is how we estimate the dose of nanoparticles to be delivered to, say, a tumor, as the delivered dose depends on the release of drug from the carrier as well as the degree of penetration of the particles into the target. With nanosystems, their small size is both vital for access to targets and for their accumulation in specific sites, say, through the EPR effect, but their trajectories are often complex. Fundamental to nanoparticle targeting is the behavior of the systems in vivo. Box 5.4 summarizes some of the issues that require control and further study to ensure that quantitative targeting is achieved. The use of external stimuli to activate the carriers to release their content is increasingly being studied, as described in Chapter 14. While much effort is concentrated on the uptake of drug into target cells, consideration also has to be given to the loss of drug by leakage back into the circulation. There is, as discussed, no true "homing" device as biological interactions with nanoparticulates (ligand–receptor interactions) occur over very small distances (of the order of <10 nm). Even then, particles must be oriented in the optimum configuration and external forces can, of course, reduce the extent of interaction.

5.7.1 Challenges

It is not only the properties of the carrier system that determine success. The nature of the drug itself and, of course, drug carrier interactions are also of great importance. If we consider the case of paclitaxel (Figure 5.14), once released from the vector, it has several characteristics that can influence the outcome: it has a low water solubility, which can lead to precipitation, it is a medium-sized molecule (MW 853.9 Da) with thus a lower diffusion coefficient than smaller drugs, it binds to albumin and is a P-gp activator. While the drug is in the carrier, the effects of efflux pumps are avoided, but once released, the drug, of course, can be acted on by cellular efflux systems.

There are pharmaceutical challenges to secure the production and design of the "perfect" or optimized nanosystem for targeting, which occur at several stages, including small-scale preparation in the laboratory (crucial for successful experiments), later production on a larger scale, instability during in vitro testing in cell culture systems, or during storage, and there are all the issues we have discussed that occur after administration. Different routes present different challenges. Intravenously, the flow of nanoparticles in the blood, their entrapment by the Kupffer cells, their interaction with red blood cells, their escape from the circulation, their diffusion in the extracellular matrix, their stability both physical and chemical, the potential loss of surface ligands, and the camouflage by PEG chains of the ligand, are indeed the gamut and mélange of chemical and biological problems which must be overcome.

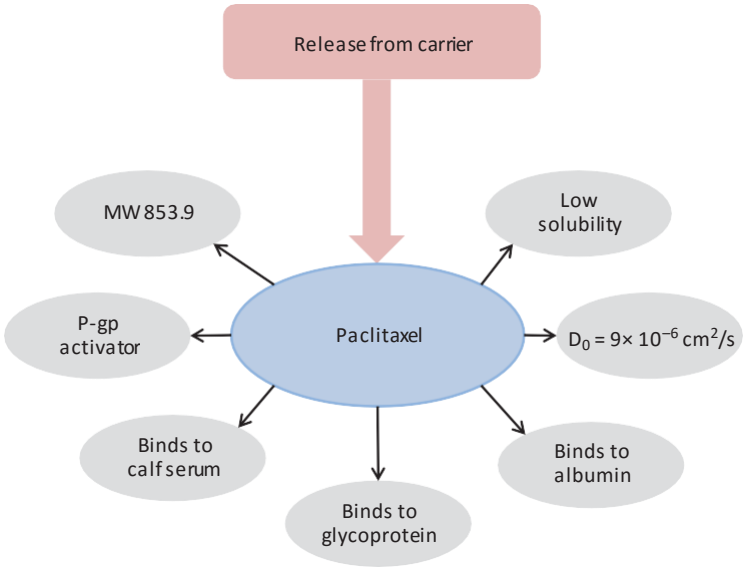


FIGURE 5.14 Paclitaxel properties that impact on its efficacy after its release from a carrier system. This illustrates the point that drug targeting needs not only appropriate delivery systems but also (more) appropriate drugs.

5.7.1.1 Particle Size and Particle Size Distribution

It is important to have systems with as narrow a size range as possible, as size is a parameter that determines biological activity and the fate of the particles. It is possible to prepare nanoparticles that are monosized, but often particles have a range of initial diameters. The size is then reported as a mean or median diameter by weight or number. The correct reporting of diameters and size distribution is crucial for comparison of data obtained in different laboratories. Both are valuable to explain outcomes. A system with 10% of the particles above the desired range may indeed have 90% of the drug load in these particles. The initial size of systems may not be the size once the system has "aged" or has been added to cell culture media or injected. Nanoparticles are colloids and the factors affecting colloid stability, of course, apply to them. Aggregation of particles through flocculation or coalescence results in an increase in the effective diameter of the product. Systems must be designed to prevent aggregation without affecting action. The adsorption of PEG chains by providing a hydrophilic barrier on particle surfaces achieves a degree of stability through steric or enthalpic stabilization but may also reduce the interaction of the particles with surfaces of cells by the same token or may mask the smaller surface ligands.

5.8 CONCLUSIONS AND PROSPECTS

Progress has been made toward the delivery of drugs more precisely in the body, but much remains to be done. Several targeted drugs have entered the marketplace successfully. They have in common that they are indicated for the treatment of life-threatening diseases like cancer, severe immunological diseases, and fungal infections and, therefore, contribute considerably to our therapeutic armamentarium. Targeted drug delivery concepts are still in some regard in statu nascendi some 40 years after Speiser advocated the use of nanoparticles and nanocapsules in therapy. Much has been learned along the way. Multidisciplinary approaches where different disciplines join forces (molecular biology, biotechnology, pathology, pharmacology, immunology, pharmaceutical sciences, engineering, clinical sciences, etc.) turned out to be the key to future success. In particular, our insights into the anatomical, physiological, and pathological constraints to the targeting concept have been growing fast over the last two decades. That know-how will help us to speed up new developments on

a rational basis. Moreover, progress in molecular biology and biotechnology, enabling scientists to engineer protein structures and to produce them on a large scale, will have a great impact on drug-targeting concepts and the actual production of targeted drug delivery systems. Box 5.5 lists several attributes of ideal systems, indicating some of the complex sequence of events required to initiate a therapeutic response. In reading about the new developments in drug targeting, it would be wise to measure the claims against this list, which, although not exhaustive, is a guide. Doubt about any one of these attributes should be taken into account in evaluating the approach.

BOX 5.5 PRIMARY ATTRIBUTES OF TARGETED SYSTEMS

1. Appropriate chemical architecture: size, shape, and flexibility
2. Acceptability in terms of safety, compatibility, and biodegradability
3. Access to tissues and receptors, which might sometimes be hidden
4. Affinity to receptors sufficient to engage for sequential effects, such as cellular uptake
5. Accumulation in target tissues[a]
6. Absorption into target cells[a]
7. Activation and/or release of the drug at an appropriate rate[a]
8. Action of the drug at the cellular level and sometimes at the level of the nucleus
9. Adaptability in terms of facile modification to accommodate different surface ligands or more than one active agent to optimize efficacy

[a] This list refers mainly to targeting to extravascular sites. Of course, sometimes the target may be a circulating molecule (such as with anti-TNF antibodies) or a biological structure (such as a thrombus in the targeted delivery of antithrombotics); hence, some of the aforesaid attributes such as 5, 6, and 7 would need modification.

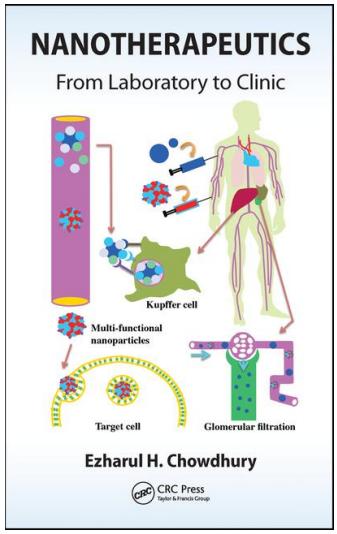
The existing generation of targeted drug delivery systems contains active compounds that often have been used for many years in their "free" form before they were associated with a carrier. Examples are amphotericin B, doxorubicin, and daunorubicin. These drugs were chosen on the basis of their pharmacokinetic and pharmacodynamic profile while administered in the "free" form. It is expected that in the near future, "carrier-dependent drugs"—drugs that have not been used before in "free form" in therapy and indeed molecules that have been designed purposely for encapsulation—will enter the market.

In spite of thousands of papers in the field, there are still relatively few quantitative studies examining each part of the procession from the point of administration to the point of delivery of the payload at the target site. Systems have often been tested in cell culture systems and not in test animals. When the latter has occurred, the choice of animal model (mouse, rat, guinea pig, or other species), has not always been chosen well. It is well known that an individual delivery vector can induce markedly different effects in different cell lines. Of great importance too is the extrapolation of data obtained in small animals to the human situation, the ultimate test of most delivery systems. Scaling factors are bound to influence outcomes: if nanoparticle size is important, then the manner in which these nanoparticles circulate in vivo is crucial, as we have discussed earlier. Factors such as the time taken for systems to circulate, the different concentrations of nanoparticles used—measured in terms of mg/kg to achieve equal dosage and whether or not this is appropriate—will cause differences in flow and accumulation of carriers, shear forces acting on particles and receptors, and the nature of tumor models. As dynamic processes are involved, all these factors come into play in the ability to effectively extrapolate data.

Nonetheless, it is the complexity of the field that is the driving force for further research and it is imperative that such work continues, however, with greater attention to the factors discussed in this chapter.

CHAPTER 2

Protein-Based Macromolecular Drugs



chapter five

Interactions and orientation of therapeutic drugs in the vicinity of nanoparticles

5.1 Dendrimer–drug interactions

The existence of large numbers of charged primary amine groups on the surface of dendrimers assists in electrostatic binding with ionizable drugs. In addition, several classes of dendrimers have also ionizable tertiary amine groups available for ionic complexation with the drugs at the branching points in the core. Since the pK_a values of the primary and tertiary amines are, respectively, 10.7 and 6.5, the solvent pH has an important role in drug binding in addition to the influences of dendrimer size, surface structure, and functionality of the drug molecules.

Although the interior structures of polyamidoamine and poly-propyleneimine, the most widely used dendrimers, can promote both hydrophobic encapsulation and hydrogen bonding of a drug with the tertiary amines, the primary mechanism of drug complexation within the dendrimer structure is via electrostatic interactions with the surface primary and inner tertiary amines. The larger void volume avail- able for drug accommodation and the stronger electrostatic affinity of higher-generation dendrimers toward the drug contribute generally to the higher drug loading into their structures (Figure 5.1).

The attachment of polyethylene glycol (PEG) chains to the dendrimer surface could increase the encapsulation efficiency of hydrophobic drugs by increasing the overall volume of the drugs for complexation through hydrogen bonding and electrostatic interactions. While the longer PEG chains in general accelerate more drug loading, very long chains (larger than approximately 5000 Da) can also reduce the encapsulation efficacy because of the formation of large PEG structures, thus filling the interior space of the dendrimer and reducing the volume for drug encapsulation. A greater control over drug release can be achieved by covalently linking a drug through an amide or ester linkage with the surface of a dendrimer (Figure 5.1). The insolubility of a drug–dendrimer complex that happens because of coupling or complexation of a large number of drugs to the dendrimer surface could be addressed through the attachment of PEG chains on the dendrimer surface.

The primary amines of a dendrimer can also be used to electrostatically complex on its surface with the negatively charged phosphate back- bone of DNA, oligodeoxyribonucleotide (ODN), or small interfering RNA (siRNA) for cellular delivery (Figure 5.1).

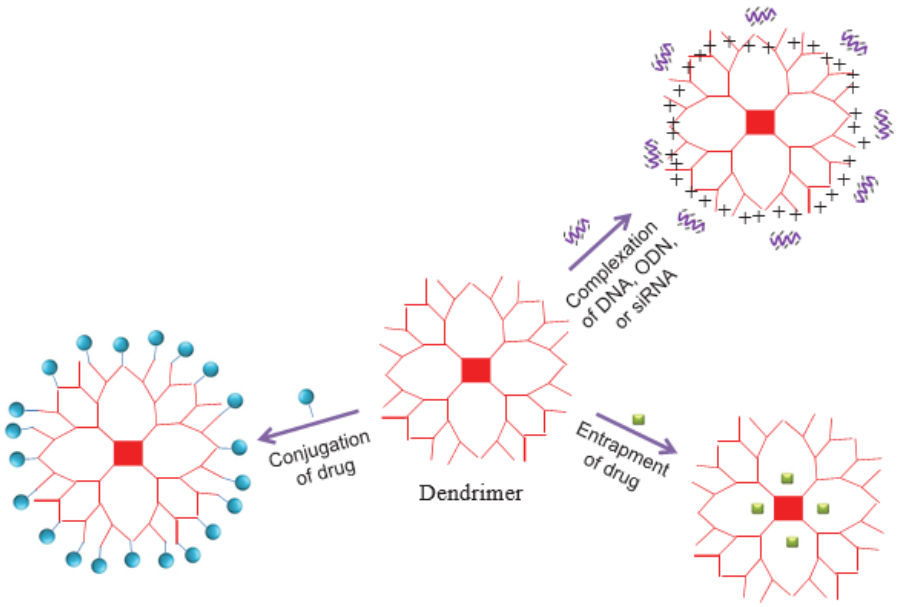


Figure 5.1 Dendrimer–drug interactions.

5.2 *Amphiphilic block copolymer– drug interactions*

Amphiphilic block copolymers (ABCs) have been extensively used in solubilization of hydrophobic drugs, sustained-release formulation, and nucleic acid delivery. Among the different ABC-based nanodelivery systems, the polymeric micelle has attracted much attention because of its unique spherical core/shell structure characterized by a hydrophilic block (shell) interfacing the surrounding aqueous environment and a hydrophobic block (core) that is chemically tethered to the former and serves as a nano-depot for accommodation of hydrophobic drugs or nucleic acids (DNA, ODN, or siRNA) through hydrophobic and electrostatic forces.

5.2.1 *Drug loading into polymeric micelles*

Drug entrapment into polymeric micelles depends on the miscibility between polymers and drugs with an increase in the miscibility accelerating drug accommodation into the micelles and the extent of hydrophobic interaction between drug and the micellar core. Introduction of hydro- tropes, which are small molecules with both hydrophobic and hydrophilic moieties into the micelle core, could increase the miscibility between the core and the hydrophobic drugs (Figure 5.1). The length of the hydro- phobic block and the type and degree of substitution on it differentially affect the loading

efficiency in the polymeric micelles depending on the (hydrophobic) drug molecules. However, more drug loading as a result of stronger hydrophobic interactions is often accompanied by slower drug release.

5.2.2 Polymeric micellar drug conjugate

Early efforts were directed to reduce premature drug release by preparing drug–polymer conjugates through relatively stable linkers (e.g., amide and ester bond). In this case, the excessive stability of the polymeric prodrug may lead to the inactivity of the final product. The design of pH-responsive polymeric drug conjugate micelles has provided an exciting opportunity to achieve the site-specific release of incorporated drug from its carrier. This method involves formation of an acid-labile linkage between the therapeutic agent and the micelle-forming copolymer, a linkage that is stable at physiological pH, but which will be cleaved at the acidic pH of a tumor extracellular space or its endosomes, leading to the site-specific release of the parent chemotherapeutic agents from their micellar nano- conjugates (Figure 5.2).

5.2.3 Electrostatic complexation with DNA/siRNA

A polymeric micelle can be formed to act as a nonviral vector by chemically linking a hydrophilic block to serve as the shell, with a polycationic segment to form the core by neutralizing the negative charges present on DNA, ODN, or siRNA (Figure 5.2). An extensively used model of this type is poly(ethylene oxide)–poly(L-lysine) [PEO–P(Lys)], the stability of which can be improved by replacing some of the lysine residues in the core with thiol groups that can readily form disulfide linkages through cross-links with other neighboring thiol groups, thereby developing a network in the core after DNA complexation. Since the cross-linked core is cleavable inside the cell that possesses an increased level of glutathione, DNA can be selectively released only within a cell, but not in blood or any other extracellular space. Copolymers of PEO–poly(ε-caprolactone) (PEO–PCL) with the grafted polyamine on the PCL block can similarly be used to form a stable micelle core encapsulating siRNA with a high affinity. It is noteworthy that siRNA or ODN, being much shorter and thus fewer negative charges than plasmid DNA, has less affinity for ionic interactions with a vector.

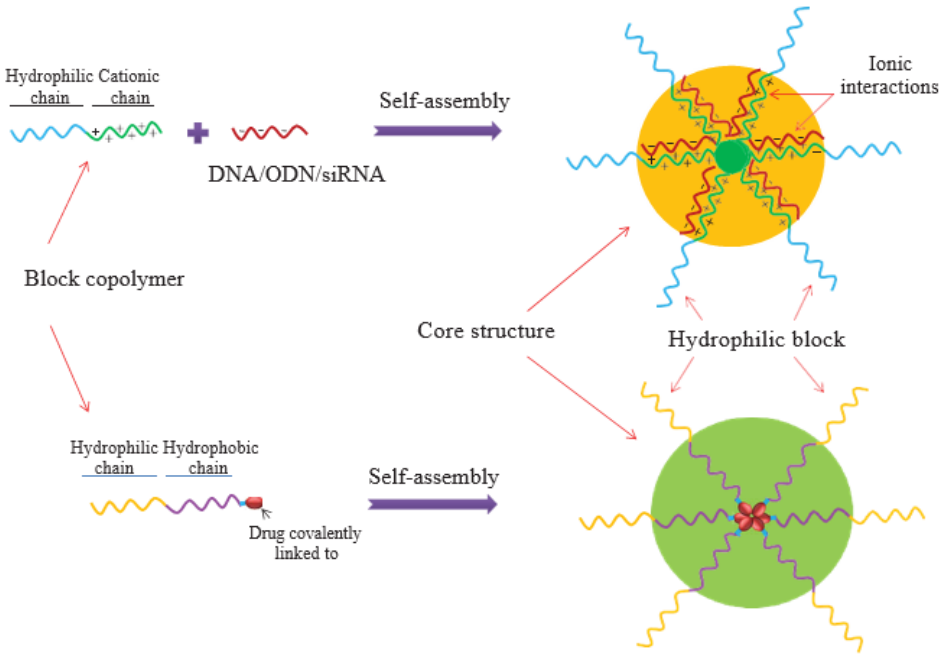


Figure 5.2 Polymeric micelle–drug interactions.

5.3 *Liposome–drug interactions*

Liposome with a lipid bilayer can accommodate hydrophilic drugs into its aqueous interior and hydrophobic drugs inside the lipid bilayer. Electrostatic interaction could enhance hydrophilic drug encapsulation through the use of anionic or cationic lipids, enabling the charged liposome to interact with drugs having the opposite charge. The incorporation of cholesterol could contribute to the encapsulation efficiency of hydrophilic drugs by reducing the rotational freedom of the phospholipid hydrocar- bon chains and thus decreasing the loss of the entrapped hydrophilic compounds. Liposomal membrane composition affects both partitioning and encapsulation efficiency of the lipophilic drugs, since these drugs are encapsulated within the lipid membrane depending on their solubility in the phospholipid bilayer. Being hydrophilic in nature, a protein usually resides in the aqueous compartment. However, if a protein can transform into a "molten globule" state with an unfolded intermediate conformation exposing the hydrophobic part, it may exist either in the aqueous interior or in the lipophilic bilayer. Additionally, protein incorporation in a lipo- some might depend on the liposomal charge states and the pH and ionic strength of the hydration medium. Cationic liposomes are widely used as a carrier of anionic nucleic acids, which can associate by ionic interactions with the cationic outer or

inner surface of the liposomes (Figure 5.3).

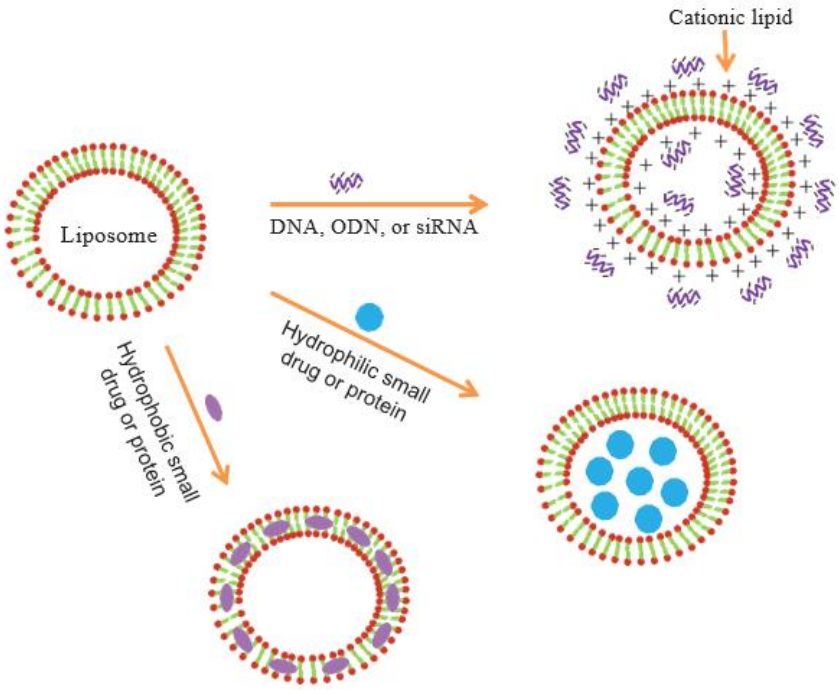


Figure 5.3 Drug–liposome interactions.

5.4 *Inorganic nanoparticle–drug interactions*

Three main strategies undertaken for drug loading into inorganic nano-particles include direct attachment of a drug to the nanoparticles via covalent linkage, adsorption of the drug onto the nanoparticle surface by electrostatic interactions, and drug entrapment or encapsulation. Existence of a suitable functional group in a drug molecule could offer an opportunity for covalently linking the drug to the nanoparticle through a biodegradable linker. On the other hand, ionic interactions of a drug with the nanoparticle are commonly based on the particle surface charge that, in turn, can be tuned by either surface enrichment with metal ions or surface stabilization (or modification) with functional groups, such as amine or thiol ligands (Figure 5.4). Thus, Au and SiO2 nanoparticles modified with NH_2-terminal ligands or CdS nanoparticles enriched with metal ions could electrostatically bind with the negatively charged DNA molecules. The carbonate apatite nanoparticle that has been developed in our laboratory is another interesting candidate as a potential drug carrier, since its surface, which carries both positive and negative charges, allows adsorbing a drug (small drug, DNA, siRNA, or protein) regardless of whether it possesses positive or negative charge(s). Finally, for effective drug entrapment, a hydrophobic drug could be loaded onto the nanoparticle while an associated outer hydrophilic shell would avoid drug leaking and stabilize the

particles in the surrounding aqueous environment. Inorganic nanoparticles with hollow interiors and porous shells are promising for encapsulation and eventually sustained release of small drugs (Figure 5.4).

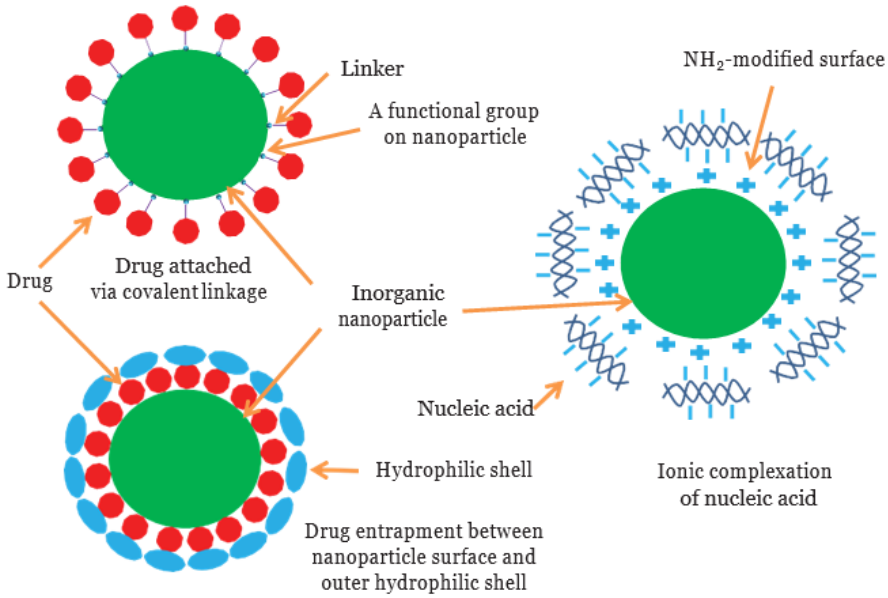
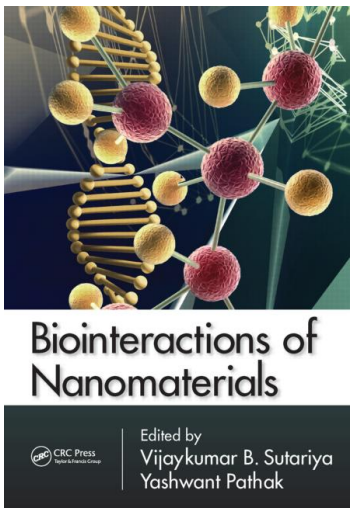


Figure 5.4 Inorganic nanoparticle–drug interactions.

CHAPTER 3

Biosensing Devices for Toxicity Assessment of Nanomaterials



5 Biosensing Devices for Toxicity Assessment of Nanomaterials

5.1 INTRODUCTION

5.1.1 BIOSENSORS

Living cells are associated with electrical characteristics and are thus responsive to, and even generate, electric fields and currents. Knowledge of these electrical properties of cells has led to the development of the field of bioelectronics. Bioelectronics is the application of electronics to biology and medicine and can be broken down into two categories. Physically interfacing electronic devices with biological systems have led to technologies such as the cardiac pacemaker, implantable electrical bone growth simulators, deep brain simulators, and electrical nerve simulation. The other aspect of bioelectronics is electronics for both the detection and characterization of biological materials, such as on the cellular and subcellular level. This can be seen in the example of cell-based biosensors that use live cells as sensing elements to monitor the physiological changes induced by internal aberrations or external stimuli.

Biosensors are becoming valuable tools for analyzing various physical, chemical, and biological processes. Since 1956, when Professor Leland C. Clark Jr. first published a paper on the oxy- gen electrode, researchers have incorporated and enhanced biosensing technologies in fields such as health care, the food industry, and environmental monitoring. The attraction to biosensors stems from their accurate, precise, and reproducible measurements in a cheap, small, and portable manner.

A biosensor is commonly defined as a device that detects, records, and transmits information regarding a physiological change or process. Biologically derived recognition entities (enzymes, antibodies, microorganisms, cell receptors, cells, etc.) are coupled to a transducer that detects the biological reaction and converts it into a signal, which can be physicochemical, optical, electro- chemical, thermometric, or magnetic (Figure 5.1).

Biosensing technology has spread throughout many disciplines due to its great specificity, sensi- tivity, and diversity in uses. Molecular and enzymatic biosensors were among the first to be intro- duced in the late 1960s with thermal, optical, and electrochemical biosensors following shortly thereafter.

Planar microelectrode biosensors, used to monitor cellular behavior, were first introduced by Thomas in 1972 to monitor the electrical activity of contracting embryonic, chick heart cells. Since then, microelectrode biosensors have been used to study cell cultures *in vitro* under different conditions. For instance, Gross et al. used a microelectrode biosensor to monitor and eventually stimulate neuronal cell activity *in vitro* from the brain and spine. Other uses include monitoring metabolism, fluorescent probes and reporter, and electrophysiology.

Whole-cell impedance-based biosensors, pioneered by Giaever and Keese were developed to monitor the proliferation and motion of a population of anchorage-dependent cell cultures. By monitoring whole-cell activity, one can monitor changes in membrane receptors, channels, and enzymes that may be expressed by the cell. Morphological changes can also be monitored using electrical impedance sensing (EIS) biosensors, since cellular membranes exhibit dielectric proper- ties. EIS biosensors are especially beneficial for monitoring the behavior of the whole cell because they provide information about the total physiological responses of cells to external stimuli. Biosensors that incorporate whole cells can have an advantage over other biosensors for certain applications because they can provide functional information without damaging the cells. Most current biosensors are used to detect enzymes, DNA/RNA (deoxyribonucleic acid), and immunological components, converting the biological phenomena into electrical signals and allowing for specifically

targeted results.

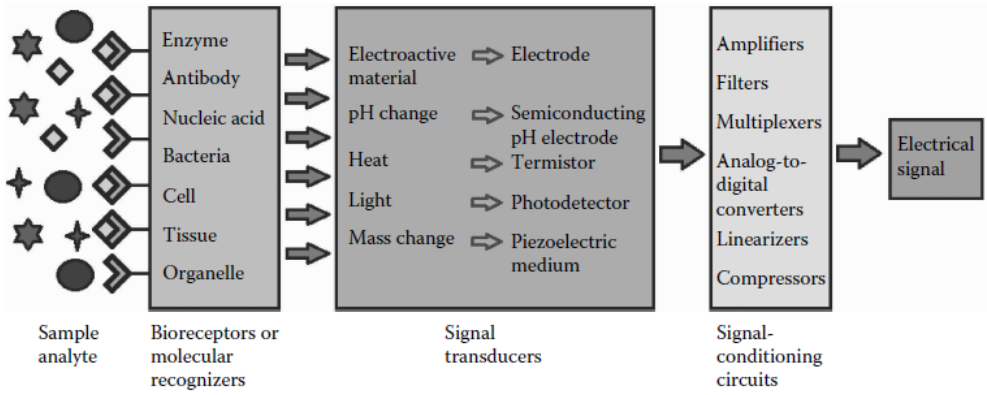


FIGURE 5.1 Schematic of the biosensor.

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5.1.2 NANOTOXICITY

Biomedical engineering, drug delivery, environmental health, pharmaceutical industries, and even electronics and communication technologies, all incorporate nanotechnology, leading to greater potentials for advancements in current research. For example, in the health-care field, nanomaterials are being considered in the development of new drugs and new therapies for disease control and improving the quality of life. More recently, nanomaterials have been used in tissue engineering and medical imaging, leading to improved diagnostics and new therapeutic treatments. However, due to their novel stature, nanoscale materials (including nanotubes, nanowires, nanowhiskers, fullerenes or buckyballs, and quantum dots) have to be tested for unintended hazards for human health and the environment.

To ensure the compatibility of nanomaterials for medical applications and for the safety of the environment, testing for toxicological parameters is a necessary first step in nanotechnology research. The bulk material properties of metals change when they are in the nanoscale, and they may pose certain threats to biological systems that their bulk counterparts may not. To date, a number of studies are addressing nanotoxicity; however, there is a variability of methods, materials, and cell lines used, leading to the need for a standard testing method, or methods, in nanotoxicity testing, which is becoming increasingly important to validate these novel techniques.

The use of nanomaterials in biomedical sciences has placed nanomaterials directly in contact with biological materials, and, thus, it is necessary to observe their interaction closely.

Another risk to be considered is the emission of hazardous air pollutants associated with the use and manufacture of nanomaterials that contain particulate matter on the order of 1–100 nm in size. Any material in the respirable size range, <100 nm in diameter, may have toxic effects on lung fibroblasts after inhalation. In particular, nanoparticles with sizes <20 nm affect the alveolar region of the lung.

Methods such as the mitochondrial reduction of tetrazolium salts into an insoluble dye (the MTT test) and enzyme lactate dehydrogenase (LDH) release tests are traditional *in vitro* biological methods used in the current nanotoxicity studies. These measure cellular viability and proliferation and, thus, are used as markers for cell viability. They consist of procedures that provide a general sense of cytotoxicity, as they show results only at a final time point. As a result, the kinetic model (absorption, distribution, metabolism, and excretion) of the nanoparticle uptake is not usually observed with these conventional methods. Following biological exposure, the particles may transport across cell membranes, especially into the mitochondria, causing internal damage that may affect cell behavior and, over time, may lead to cell death.

In this chapter, the various types of biosensors used to detect nanotoxicity will be explored. We will explore biosensing methods measuring nanotoxicity toward cell monolayers, single cells, and individual components of the cell. The integration of biomolecules with nanotechnology has great

future perspectives in the rapidly developing fields of environmental (pollution control and monitoring) and biomedical research, drug delivery, electronics, and communication technologies. With the increasing number of nanomaterial applications, assessing their toxicity should be the first important step toward creating safety guidelines for their handling and disposal. Studies of the biological effects of nanoscale materials that might answer these questions have lagged behind other aspects of nanotechnology development. Biosensing technology is shown to be sensitive enough to measure the micromotions of a cell and, is therefore able to monitor the progression of the cytotoxicity with a rapid, real-time, and multi-sample analysis, creating a versatile, noninvasive tool that is able to provide quantitative information with respect to alterations in cellular function under various nanomaterial exposures.

5.2 CELLULAR-BASED BIOSENSORS FOR NANOTOXICITY

Whole-cell EIS-based sensors, pioneered by Giaever and Keese, were the first demonstration of a system capable of monitoring the proliferation and motion of a population of anchorage-dependent cell cultures *in vitro*. Giaever and Keese cultured human lung fibro- blast cells on modified cell culture dishes consisting of a large reference electrode (2 cm^2) and four smaller electrodes (3×10^{-4} cm^2). They applied an alternating current (AC) voltage, through a resistor, to a single small electrode in the dish, resulting in a constant current source, which enabled the impedance to be determined by the measurement of the resulting voltage. They were able to observe the effects of cell proliferation (impedance increase) as well as the micromotion of the cells (fluctuations in observed impedance).

Giaever and Keese then used their biosensor to examine the effects of different proteins on cell adhesion, spreading, and motility, to create a mathematical model of cell motion, and the use of this impedance method in cell-based sensor applications. Connelly modified Giaever and Keese's biosensor design by adding a glass ring around the electrode area, to contain the cell culture media, and inserting a permeable, cellulose nitrate membrane to separate the culture dish into two sides, each with two measurement electrodes, creating a control and a test electrode.

EIS technology is shown to be sensitive enough to measure the micromotion of a cell and, therefore, be able to monitor the progression of cytotoxicity with rapid, real-time, and multi-sample analysis, creating a versatile, noninvasive tool that is able to provide quantitative information with respect to alterations in cellular function under various nanomaterial exposures. Thus, EIS biosensors are ideal for detecting toxic nanomaterials in industrial products, chemical substances, environmental samples (e.g., air, soil, and water), or biological systems (e.g., bacteria, viruses, or tissue components), as they are able to monitor the progression of the cytotoxicity in real time, demonstrating the kinetic effects of the nanomaterials toward whole cells.

Chip-based biosensors show a promising future for monitoring cellular nanotoxicity as they allow rapid, real-time, and multi-sample analysis creating a versatile, noninvasive tool that is able to provide quantitative information with respect to alterations in cellular function under various nanomaterial exposures. A different chip-based approach to evaluate nanotoxicity was experimented by Kim. They fabricated a chip using a lithography technique where gold was the sensing electrode and was modified with RGD-MAP-C to enhance cell (SH-SY5Y) adhesion on the chip. Silica nanoparticles of various sizes and surface chemistries were examined to understand the effects of induced nanotoxicity on SH-SY5Y cells by studying the cell viability at different concentrations of nanoparticles ranging from 50 to 400 μg/mL and at various time points. Electrochemical measurements of nanotoxicity were recorded using differential pulse voltammetry and were compared to absorption- and fluorescence-based techniques to evaluate the benefits of electrochemical measurements in assessing nanotoxicity.

5.3 TECHNIQUES AND DEVICES FOR NANOTOXICITY TESTING

Nanoparticles with many novel properties are used in various applications and come in contact with complex and dynamic biological systems. It is challenging to characterize nanoparticles throughout their biological interaction and to quantify the uptake rate and localization inside cells. Cells under nanotoxicity may undergo necrosis or repairable, oxidative DNA damage, recovering from it eventually or resulting in apoptosis. Nanotoxicity may alter cell differentiation, proliferation, morphology, or cell–cell communication. Traditional methods for evaluating nanotoxicity consist of bulk

analysis, where the resultant is assumed to be the average of the whole cell population. Cell heterogeneity has been recently studied by researchers, suggesting that the cells in a subpopulation may exhibit different behavior from the general population. This means that the cell population study in aggregation may hinder some very important cell mechanisms, understating that is only visible at single-cell level. Single-cell nanotoxicity studies are believed to provide more realistic cell behavior under nanoparticle interactions.

5.3.1 Carbon Fiber Microelectrode

Carbon fiber microelectrodes have been widely used for single-cell analysis due to their ability to detect diffusion-limited current at very high scan rates, allowing quick measurements of fast tempo- ral events of the cells (Bard 2001). Carbon fiber microelectrodes have high sensitivities due to their very small tip (5–10 μm) and low noise for very sensitive detections of changes in cell behavior. The carbon fiber microelectrode amperometry technique has the potential to reveal the biophysics of exocytosis and, thus, can be an important tool in understanding cellular communication under the influence of nanoparticles.

Marquis conducted carbon fiber microelectrode amperometry to characterize serotonin exocytosis from murine peritoneal mast cells cocultured with fibroblasts in the presence of Au nanoparticles. The interaction between various concentrations of serum-coated Au nanoparticles sized between 12 and 46 nm after coculturing with mast cells for 48 h, suggested an altered exocytosis mechanism. The study reported decreases in granule transport and fusion events, and increased intracellular matrix expansions and a higher number of serotonin exocytosis per granule. A further expansion of these studies showed the effects on cell viability when the nanoparticle exposure was extended for 48 and 72 h (Marquis et al. 2009). Love and Haynes (2010) carried out a study to evaluate the effect of citrate-reduced noble nanoparticles, Au (28 nm), and Ag (61 nm), on neuroendocrine cells. An inductively coupled plasma–atomic emission spectroscopy (ICP–AES) measurement was carried out for the uptake quantification; cells were lysed and the total metal content was a measure of the nanoparticle uptake. The rate of uptake for 1 nM of Ag and Au nanoparticles was found to be different for each type after 24 h of exposure, 3.4×10^4 versus 7.5×10^5 nanoparticles per cell, respectively, suggesting a higher internalization of the Au nanoparticles. The differences in the rates of nanoparticle internalization were assumed to be affected by several factors such as size, surface charge, and functionalization. Transmission electron microscopy (TEM) was used for the localization of nanoparticle assessment and to verify the internalization of nanoparticles in cellular granules and not only on the cell membrane. Carbon fiber microelectrode amperometry revealed the changing exocytosis behavior of chromaffin cells in this study. Metal oxides are commonly used in consumer products. The carbon fiber microelectrode amperometry technique has been used to understand the nanotoxicity of metal oxide (nonporous SiO_2, porous SiO_2, and nonporous TiO_2) nanoparticles on immune cells by Maurer-Jones et al. (2010). The results revealed functional changes in chemical messenger secretions from mast cell granules. Nanoparticle surface properties are known to play a major role in deciding their interactions with biological systems. Marques et al. (2011) executed a study on noble nanoparticles, Au (~26.5 nm), and Ag (~33.3 nm), with different zeta potentials (surface charge), by modifying the nanoparticle surface using cationic or anionic thiols. It was noted that positive surface-charged nanoparticles (Au+ and Ag+) were more susceptible to internalization by the mast cells compared to their negatively charged counterparts (Au– and Ag–). Carbon fiber microelectrode amperometry was further utilized by Love et al. (2012) to evaluate the changes in cellular communication in neuroendocrine cells after size-dependent Ag nanoparticle- and surface-functionalized Au nanoparticle exposure for 24 h. The study revealed that even if the Ag nanoparticles (15–60 nm) did not alter cell viability, they showed size-dependent cellular uptake and an increase in the speed of exocytosis- release kinetics. On the other hand, polyethylene glycol (PEG)-functionalized Au nanoparticles did not change cell viability. However, they decreased the number of molecules released from each vesicle.

5.3.2 Atomic Force Microscopy

Atomic force microscopy is a powerful, force-sensitive technique and has been successfully applied in single-cell studies to gather the information on cell structure, topography, membrane nanostructures, and the mechanics (e.g., adhesion force, elasticity) of mammalian cells at a nanoscale

resolution under physiological or near-physiological conditions. An atomic force microscope can be used to study the mechanics of cells under the influence of nanoparticles. Wu applied atomic force microscopy to reveal insights on the toxic effects of diesel exhaust particles on vascular endothelial cells at the single-cell level to understand the biophysical properties of the cells. Atomic force microscopy was utilized in two different strategies in this experiment: (1) to measure the mechanical properties of the cell, such as Young's modulus and adhesion force in the growth medium and (2) for topography and membrane visualization, cells were fixed and imaged.

5.4 BIOSENSORS FOR NANOTOXICITY BIOMARKER DETECTION

5.4.1 BACKGROUND

The rapid growth of the nanotechnology industry has led to a large-scale production and application of engineered nanomaterials, which are used not only in medicine and industry, but also in various consumer products such as food products, textiles, sunscreens, and cosmetics. While, on the other hand, the increased utilization of nanomaterials could affect human health and the environment due to increased exposures. The current knowledge is limited to the potential health effects caused by nanomaterials; however, it shows that they may cause adverse effects at the routes of exposure such as the skin, gastrointestinal tract, and lungs. Furthermore, some nanomaterials made of certain metals may have genotoxic or carcinogenic effects. One of the most discussed mechanisms behind the health effects induced by nanomaterials is their ability to enhance the generation of reactive oxygen species (ROS), causing oxidative stress, DNA damage, and unregulated cell signaling, which eventually leads to changes in cell motility, apoptosis, and carcinogenesis. Therefore, there is a great need for setting up reliable methods to assess the potential toxicity of the nanomaterials with short deadlines and reasonable costs, ensuring their compatibility for medical applications and for the safety of the environment. While most reliable methods for toxicity evaluation rely on costly, *in vivo* experiments, *in vitro* assays present a promising screening method. Cell cultures offer a useful prescreening method to assess the toxicity of various external agents on cells.

5.4.2 COMMON METHODS FOR NANOTOXICITY ASSESSMENT

An assortment of assays is used to assess the toxic effects of nanomaterials *in vitro*. These assays can be generally classified into three groups based on their objects of measurement, that is, cell viability/proliferation, direct/indirect intracellular ROS levels, and genomic markers.

5.4.2.1 Cell Viability/proliferation assay

Traditional *in vitro* cell viability/proliferation or cytotoxicity assays, for the measurement of cellular viability and proliferation, are used in the current nanotoxicity studies. These assays include the Alamar Blue assay, which incorporates a fluorometric/colorimetric growth indicator based on

the detection of metabolic; the Trypan Blue assay, where cells with an intact membrane are able to exclude the Trypan Blue dye; the Neutral Red assay, based on the ability of viable cells to incorporate and bind the supravital dye, neutral red, in the lysosomes; formazan-based assays (MTT, MTS, and WST), which are used for the detection of various stages in the apoptosis process of cells and the clonogenic assay, based on the ability of a single cell to grow into a colony.

5.4.2.2 DIRECT/INDIRECT INTRACELLULAR ROS MEASUREMENT

The effects of ROS on cell metabolism typically involve the mechanisms in the apoptosis process. Direct/indirect intracellular ROS measurement assays include the glutathione (GSH) assay, a luminescent-based assay for the detection and quantification of GSH in cells; lipid peroxidation measurement, which measures increased concentrations of end products of lipid peroxidation, indicating increased oxidative damages in the cells; 2-, 7-dichlorofluorescein (DCFH) assay, which detects intracellular DCFH oxidation due to the presence of hydrogen peroxides; and electroparamagnetic resonance (EPR) assay to directly measure free radicals in cells and tissues.

5.4.2.3 ASSAYS ON THE GENOMIC LEVEL

Assays on the genomic level measure any damage that occurs to the DNA of the cells. Examples of this type of assay include the comet assay, also known as single-cell gel electrophoresis assay, a technique for the detection of DNA damage at the level of the individual eukaryotic cell; and DNA damage biomarker assay, as it is well known that excessive generation of ROS can oxidize cellular biomolecules and the resulting free radicals also lead to oxidative modifications in DNA, including strand breaks and base oxidations.

5.4.3 BIOSENSING APPROACHES FOR INFLAMMATORY BIOMARKERS DETECTION

Most current *in vitro* cytotoxicity assays rely on the evaluation of cell viability as discussed previously. However, the cellular stress and inflammatory responses can also be used to evaluate cellular toxicity by the detection of specific biomarkers. Cytokines are of particular interest as one group of such biomarkers, and their release by cells has been studied as a marker of a cellular immune response. Cytokines regulate the growth and function of immune cells during inflammation and in later immune responses. Therefore, the concentration of cytokines in the medium, typically below 10 ng/mL, will increase in response to inflammation.

Immunoassays are often used for the *in vitro* detection of secreted cytokines. Their principles are based on capturing cytokines by a specific antibody and then measuring their levels. A sandwich immunoassay format has been used for the detection of interleukin 6 (IL-6), interleukin 8 (IL-8), or monocyte chemotactic protein-1 (MCP-1). In particular, enzyme-linked immunosorbent assay (ELISA) is a simple and sensitive method for monitoring the effects of nanoparticles on immune cells by assessing the levels of cytokines that are released into the cell culture supernatant after the addition of nanoparticles to a cell culture. The ELISA method was first described in 1971, and enables the simple and accurate quantification of inflammatory markers in cell culture supernatants through antibodies and enzymatic detection reactions. ELISA results have been reported for nanoparticles of different compositions and origins, for example, for titanium dioxide, iron oxide, zinc oxide, carbon, carbon nanotubes, fullerenes, silica, and quantum dots. The most commonly tested human and murine inflammatory markers are the chemokine IL-8, followed by tumor necrosis factor-a (TNF-a), and IL-6.

However, ELISA tests are usually time consuming and require multiple operations. Therefore, the real-time detection of cytokines has been investigated with surface-immobilized immunoassay detection on optical sensing instruments, such as the surface plasmon resonance (SPR) or waveguide-grating sensors. These techniques are sensitive to changes in the refractive index at the liquid–solid interface and, thus, allow the adsorption of monitoring biomolecules at the sensor surface with high sensitivity. Combined with immunoassays, they allow the sensitive detection of cytokines.

5.4.4 PAPER-BASED BIOSENSOR FOR ROS-INDUCED DNA OXIDATIVE DAMAGE BIOMARKERS DETECTION

As mentioned in the previous section, one of the most discussed mechanisms behind the health effects induced by nanomaterials is their ability to enhance ROS generation. An excessive generation of ROS can oxidize cellular biomolecules (i.e., DNA), leading to oxidative modifications in DNA. 8-Hydroxyguanine and its nucleoside, 8-hydroxy-2′-deoxyguanosine (8-OHdG), are the most studied DNA damage products due to the relative ease of their measurement and pre- mutagenic potential. Elevated 8-OHdG levels have been noted in numerous tumors and, are thus widely used as a biomarker for oxidative stress and carcinogenesis.

A novel lateral flow immunoassay (LFIA) has been developed to measure the concentration of 8-OHdG and, thus, reveals the nanotoxicity at the genomic level. LFIA, also known as the immunochromatographic test strip, has been widely used as an in-field and point- of-care diagnostic tool for testing cancer biomarkers, proteins, drugs, hormones, and metabolites in biomedical, food, and environmental settings.

The principle of the immunostrip is mainly based on a competitive-type immunoreaction in the lateral flow strip. In a typical assay, a sample solution containing a desired concentration of 8-OHdG is applied to the sample application pad. The sample moves along the strip, due to capillary force, and is

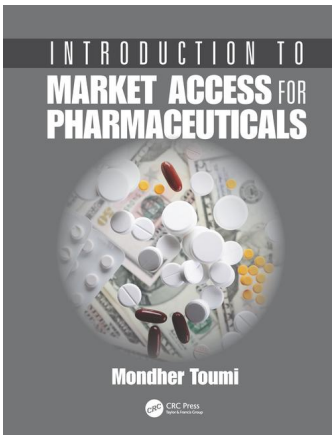
finally captured by specific antibodies through immunoreactions. The accumulation of gold nanoparticles on the test zone induces a characteristic red band that is visible to the naked eye. This color change indicates the colorimetric detection of 8-OHdG in a sample and the color intensity is inversely proportional to the concentration. The LFIA strip provides a simple approach for a rapid nanotoxicity assessment.

5.5 CONCLUSION

With the increasing number of nanomaterial applications, assessing their toxicity should be the first important step toward creating safety guidelines for their handling and disposal. Studies of the biological effects of nanoscale materials that might answer these questions have lagged behind other aspects of nanotechnology development. In this chapter, we have discussed various biosensing methods for monitoring nanotoxicity, including techniques for single-cell nanotoxicity testing, such as carbon fiber microelectrodes and atomic force microscopy, chip-based sensors for nanotoxicity, and biosensors for nanotoxicity biomarker detections, such as biosensing approaches for inflammatory biomarker detections and paper-based biosensors for ROS-induced DNA oxidative damage biomarker detections.

CHAPTER 4

Early HTA Advice



5 Early HTA Advice

5.1 OVERVIEW OF THE EARLY HTA ADVICE PATHWAYS

Medicine developers have an opportunity to gain feedback from regulators and HTA bodies, early in the development process of a medicine. The authorities concerned with these schemes use various terms, such as *early dialogue* or *scientific advice*; however, these are often specific to an organization and there is no consensus on terminology. In this chapter, we use the general term *early HTA advice* or the organization- specific terms when relevant.

Such advice can help pharmaceutical companies establish what evidence the HTA authorities will need in order to determine a medicine's benefit-risk balance (in the marketing authorization process) and its *value-for-money* in real-life use (in the HTA process). Companies may seek several kinds of early HTA advice in different stages of the drug development process, as described in the following sections and summarized in Table 5.1.

5.1.1 HTA-EMA Parallel Scientific Advice

Manufacturers can apply for parallel scientific advice from European Medicines Agency (EMA) and national HTA bodies at any stage of development of a medicine, whether the medicine is eligible or not for the centralized authorization procedure in the European Union.

In the process, EMA and HTA bodies are equal partners. Manufacturers can be flexible in the choice of HTA bodies and the EMA can facilitate the contacts. If considering more than five HTA bodies, additional discussion with an EMA scientific advice officer is recommended. Typically, companies engage early in informal discussions with HTA bodies and EMA announcing intention for procedure, product, and timescale, and which HTA bodies will participate. More details on how to initiate the various early advice pathway is presented below.

5.1.2 MultiHTA Advice

The SEED consortium (Shaping European Early Dialogues) was a European commission project led by the *French Haute Autorité de Santé* (HAS) in years 2013–2015. It consisted of 14 national and regional HTA bodies. The SEED consortium developed a proposal for a permanent model for early dialogue that would govern the process from 2016. The permanent process has not been established at the time of writing this publication.

5.1.3 EUnetHTA Pilot Assessment of Relative Effectiveness

Pilot assessment of relative effectiveness was a project of the European network for Health Technology Assessment (EUnetHTA) which aimed to test methodology, procedures, and national or local implementation of joint rapid relative effectiveness assessments (Rapid REA), and to test the capacity of national HTA bodies to collaborate and produce structured rapid core HTA information on relative effectiveness. As a result of the project, methodological guidelines for Rapid REA of pharmaceuticals were published by EUnetHTA in March 2013. The SEED project described above builds on the results of the EUnetHTA pilot project.

TABLE 5.1
Types of Early HTA Advice Offered by EMA and HTA Bodies

Type of Advice (Organization)	Prerequirements	Time Frame (Days)	Procedure Details Including Timelines (and Required Documents)	Fees	Specificities of the Process (Choice of HTA Bodies, Nonbinding, Key Focus of Questions, etc.)
HTA-EMA parallel scientific advice (EMA and chosen national HTA bodies)	At any stage of development of a medicine, whether the medicine is eligible for the centralized authorization procedure or not. This may include postauthorization safety and efficacy studies and risk management planning incorporating risk minimization measures Very early with nonclinical proof of concept and no clinical data When exploratory clinical data are available	45–80	Companies engage early in informal discussions with HTA bodies and EMA announcing intention for procedure, product and timescale, and which HTA bodies will participate. **Premeeting. Day 0–D59** • Assessment of the briefing package (book) by EMA (list of issues sent to applicants) and HTA bodies • Presentation for the face-to-face meeting to be sent by the applicant within 2 weeks of receipt of the list of issues to EMA and HTA bodies, with written responses, if requested • EMA will arrange a closed preparatory teleconference with HTAs after responses to the list of issues and presentation, to identify critical divergences between EMA/HTAs (communicated to the applicant in advance) **Meeting. Day 60–62** • A face-to-face meeting between all stakeholders, lasting approximately 4 hours (EMA premises) **Outcome. Day 63—70** • Minutes of the meeting are circulated by the company within 5 days to all participants • EMA final advice letter contains Committee for Medicinal Products for Human Use (CHMP) regulatory advice only • HTA bodies feedback is provided directly to company during the face-to-face meeting, or by annotating the applicant's minutes, or by providing written answers	EMA charges €63,000–84,000 for initial request and €32,000–42,000 for follow-up to the initial request. Fee reduction for small and medium-sized enterprises and for orphan drugs. Participating HTA agencies may also charge a fee.	• EMA and HTA bodies are equal partners • Flexible in choice of HTA bodies • EMA can facilitate contacts • HTA bodies are chosen by the applicants • Not legally binding • Confidential

(*Continued*)

TABLE 5.1 (*Continued*)
Types of Early HTA Advice Offered by EMA and HTA Bodies

Type of Advice (Organization)	Prerequirements	Time Frame (Days)	Procedure Details Including Timelines (and Required Documents)	Fees	Specificities of the Process (Choice of HTA Bodies, Nonbinding, Key Focus of Questions, etc.)
Priority medicines scheme *PRIME* (EMA and chosen national HTA bodies)	• Medicines that may offer new therapeutic options to patients who currently have no treatment options, or a major therapeutic advantage over existing treatments. • The medicines would have to show preliminary clinical evidence indicating that it has the potential to bring significant benefits to patients with unmet medical needs and hence be of major interest from a public health and therapeutic innovation perspective. • EMA proposes earlier entry into the scheme for micro-, small-, and medium-sized enterprises (SMEs) and applicants from the academic sector on the basis of compelling nonclinical data and tolerability data in initial clinical trials.	40	The applicant should submit a request for PRIME support electronically to EMA including a justification and summary of available data. Eligibility submissions are accepted according to a schedule published at http://www.ema.europa.eu/ema/index.jsp%3Fcurl%3Dpages/regulation/general/general_content_000660.jsp%26mid%3DWC0b01ac058096f643 Upon receipt of the request, one Scientific Advice Working Party (SAWP) reviewer and one EMA scientific officer will be appointed for the procedure to start in accordance with published timetables, as follows: Day 1 Start of procedure (SAWP 1 meeting). Day 30 Discussion and recommendation during SAWP plenary (SAWP 2 meeting). Day 40 The CHMP final recommendation is adopted during the plenary meeting. Of note, requests related to Advanced therapy medicinal products (ATMPs) will also be circulated, after the SAWP, to the Committee for Advanced Therapies (CAT) for review and recommendation prior to finalisation and adoption by CHMP. The outcome, including the reasons that led to the CHMP's decision, will be sent by EMA to the applicant. An appeal mechanism is not foreseen.	EMA charges €63,000–84,000 for initial request and €32,000–42,000 for follow-up to the initial request. Fee reduction for small and medium-sized enterprises and for orphan drugs. Participating HTA agencies may also charge a fee.	Can include: • Scientific advice on key decision points/issues for the preparation of MAA with the potential to involve multiple stakeholders (e.g., HTA bodies, patients), when relevant. • Early appointment of CHMP/CAT Rapporteur (in line with current process, objective criteria, and methodology) • An initial kick-off meeting with multidisciplinary participation from the European network.

(*Continued*

TABLE 5.1 (*Continued*)
Types of Early HTA Advice Offered by EMA and HTA Bodies

Type of Advice (Organization)	Prerequirements	Time Frame (Days)	Procedure Details Including Timelines (and Required Documents)	Fees	Specificities of the Process (Choice of HTA Bodies, Nonbinding, Key Focus of Questions, etc.)
MultiHTA advice (A consortium of national HTA bodies led by the French health agency HAS)	• Generic or biosimilar products are out of scope • It focuses on development • strategies and not on preassessment of data. • The advice is prospective; advice on on-going pivotal trials will not be accepted. • For drugs, it should ideally be requested during the phase II to discuss the content of the planned phase III, that is, planned confirmatory trial(s) and the economic rationale.	110	Intent letter. 4 months before meeting Briefing book submission. Day -90 Upgradation of the briefing book. Days -75 to -7 exchanges via e-mail between applicant and HTA bodies HTA Bodies release written positions. Day -7 Early advice meeting. Day 0 1. Preliminary discussion among HTA bodies only 2. Face-to-face meeting of HTA bodies with the company 3. Conclusions among HTA bodies only **Minutes. Day 10** The company provides the draft detailed minutes of the meeting. **Minutes revision. Day 20** The draft minutes are revised in writing by participating HTA bodies each correcting only the position of their agency and commonly agreed statements.	To be announced in 2016	• Early advices are restricted to one indication; however one or more lines of treatment may be discussed within this indication. • Questions should be related to HTA in the view of reimbursement and pertaining mainly to relative effectiveness, economic aspects, and other areas relevant for reimbursement. • The company can choose areas to be discussed • Not legally binding • Confidential
EUnetHTA pilot assessment of relative effectiveness (EUnetHTA and member HTA bodies)	Not taking new applications. To be replaced by the SEED permanent process from 2016.				

(*Continued*)

TABLE 5.1 (*Continued*)
Types of Early HTA Advice Offered by EMA and HTA Bodies

Type of Advice (Organization)	Prerequirements	Time Frame (Days)	Procedure Details Including Timelines (and Required Documents)	Fees	Specificities of the Process (Choice of HTA Bodies, Nonbinding, Key Focus of Questions, etc.)
Adaptive pathway (EMA and chosen national HTA bodies)	• Treatments in areas of high medical need where it is difficult to collect data via traditional routes and where large clinical trials would unnecessarily expose patients who are unlikely to benefit from the medicine. In other cases EMA scientific advice should be pursued. • Conventional development pathway must not be decided • There must be iterative aspects of the development (conditional market authorization or expansion) • There must be a need to discuss development with HTA bodies • Real-world data must be considered for regulatory purpose	~50 minimum	• Timelines are flexible as this is a pilot project. • Applicants submit with EMA a proposal containing the main elements of the proposal: iteration, real-world data and HTA/patient interaction. Following EMA's reply, they submit a final Powerpoint presentation. • EMA has 14 days to set a date of a teleconference or a face-to-face meeting, which should happen not earlier than 28 days from the reception of the final presentation. • The teleconference can last 1.5–2.5h • One week after the teleconference the company should send minutes for record keeping. Minutes are not commented by EMA or HTA bodies.	n/a	• The teleconference cannot be considered a formal advice: there is no in-depth discussion of scientific aspects, that is, within the remit of a formal scientific advice procedure • Applicant can choose the HTA bodies that will participate • Confidential
National HTA advice (A national HTA body)	Country-specific procedures are described in the text in Section 5.2.				

5.1.4 Adaptive Pathway

This is an accelerated scientific advice pathway of EMA for therapies indicated for serious conditions with high unmet needs. It requires that there is an iterative development with use of real-life data. It provides the possibility to engage various stakeholders including regulators, HTA bodies, and patient representatives in multiple discussions along the development pathway.

5.1.5 Priority medicines Scheme

EMA has developed a priority medicine scheme (PRIME) to optimize the development and accelerated assessment of medicines of major public health interests. PRIME reinforces early dialogue and builds on regulatory processes such as scientific advice to optimize the generation of robust data and the accelerated assessment procedure to improve timely access for patients to priority medicines. This kind of early advice focuses on key development milestones, with the potential involvement of multiple stakeholders including HTA bodies and payers, as well as patient organizations, where relevant.

5.2 NATIONAL EARLY HTA ADVICE PROGRAMS

Several countries have put in place early HTA advice programs for the clinical development plan of medicines. These programs are described below.

5.2.1 France

HAS offers prospective advice for companies. It can be con- ducted in English. It is confidential, not legally binding, and there are no fees involved.

In order to prepare for the HAS early meeting, manufacturers should consider three conditions for eligibility as follows:

- Ongoing clinical development: results available for phase II studies and phase III study plan not yet initiated
- New therapeutic strategy (e.g., new mode of action)
- Unmet medical need

5.2.1.1 Questions to Focus on

Manufacturers should focus on questions related to the development of the drug (i.e., comparator choice, modalities of administration, endpoints), but can also inquire about the therapeutic strategy of the disease in France, endpoint validity, quality of life assessment modalities, and so on.

For questions related to pharmaco-economic studies, manufacturers should focus on methodology choices such as types of analyses, included/excluded comparators, modeling perspective, population, time horizon, but also model choice, type of costs, and so on.

5.2.1.2 Process

Manufacturers willing to engage with this agency should follow the steps below:

- The company sends to HAS evidence to justify that the product meets the early advice eligibility criteria
- If the criteria are valid, the company receives meeting date suggestions from HAS
- First draft of the briefing package is then sent to HAS. A briefing book should include early stage data and methodology of planned phase III trials (if applicable, also pharmaco-economic study objectives and design)
- At this stage HAS may request questions or additional data
- Final briefing package should be sent at least 2 weeks before the planned meeting date

As an outcome of the meeting, the company should send to HAS the minutes of the meeting within 1 month. Minutes should include a summary of the context, background information on the disease, and target population, justification for seeking advice, company's question/position, HAS's answers to questions, comments, and conclusions. Finally, the minutes are validated by HAS.

Timelines of the early HTA advice with the French HTA agency HAS.

Steps of the Procedure	Timelines
• The company sends to HAS evidence to justify that the product meets the early advice eligibility criteria	–
• If the criteria are valid, the company receives meeting date suggestions from HAS	–
• First draft of the briefing package is then sent to HAS	–
• HAS may request questions or additional data	–
• Final briefing package is sent by company to HAS	2 weeks before planned meeting date
• The company sends to HAS the minutes of the meeting	1 month after the meeting
• HAS validates the minutes of the meeting	–

5.2.1.1 Content of the Dossier

The Dossier should have the following structure:

- Background information on the disease to be treated including current management
- Background information on the product including product positioning and potential assessment from other agencies
- Efficacy and safety data from phase I and phase II clinical trials with levels of evidence
- Details of phase III clinical trial plan
- Protocol synopsis (at minimum)
- If applicable, the description of the pharmaco- economic study with expected effects in terms of health outcomes and costs
- If the economic assessment relies on an existing model, to provide publications presenting the economic model
- Questions and the company's position
- Questions related to study design, comparator, and endpoint choice (at minimum)

5.2.2 The United Kingdom

NICE offers prospective and confidential early scientific advice. Advice can be sought at any time. However, a useful time for requesting scientific advice could be during phase II studies before the planning of phase III studies. Only a limited number of advice slots are available.

Fees for the procedure vary, depending on the number and complexity of questions asked in the

company briefing book (around £49,000 [+VAT] maximum).

NICE also offers a possible joint scientific advice meeting with the UK Medicines and Healthcare Products Regulatory Agency (MHRA). However, MHRA and NICE produce separate advice documents.

Manufacturers also have an option to request an advisory input from the clinical practice research datalink (CPRD), in order to gain knowledge of what real world data might be available either observationally or interventionally.

Remaining aspects of the application are described below.

5.2.2.1 Questions to Focus on

Manufacturers should focus on development strategies rather than pre-evaluation of data to support a submission to NICE. They can also inquire about the interpretation of NICE technology appraisal methods guidance and its relevance for the product's evidence development plans, research design considerations or preferences to support each proposed indication, economic evaluation design considerations or preferences, and about considerations and insights from existing models.

5.2.2.2 Process

Manufacturers willing to engage with this agency should follow the following steps below:

- The company requests a slot and should contact NICE at least 25 weeks before they wish to receive the final advice report
- NICE will send a copy of the standard contract to be agreed and signed by both parties at least 1 month before the briefing book submission
- Company sends the briefing book to NICE
- NICE confirms project size and total cost of the project
- Clarification questions are sent from NICE within 7 weeks of receiving the briefing book
- Company responds to NICE clarification questions within 2 weeks
- Face-to-face meeting with the company and NICE takes place approximately 11 weeks after the briefing book submission (at NICE premises) and will last 3 hours

As an outcome of the meeting, NICE sends a written advice report to the company approximately 7 weeks afterwards for a medium project or 9 weeks for a large project. Any possible clarification questions requested by NICE should be answered by the company within 15 working days. Finally, NICE should answer further company questions within 20 working days.

Timelines of the early HTA advice with the UK HTA agency NICE.

5.2.2.3 Content of the Dossier

The Dossier should have the following structure:

- Background information
- Disease and unmet needs
- Proposed product indication
- Treatment guidelines or recommendations
- Value proposition(s) for the product
- Data currently available on the product
  - A Brief description of the mode of action and pharmacological class
  - Proposed dosing regimen and route of administration
  - Data from completed clinical studies
- Proposed evidence plans for the product
  - Clinical trial designs, study population, comparator, endpoints, and the duration of observation
  - If available, a plan for the economic evaluation can be presented
- Questions and company's position

- Key references

Other key points to be considered when drafting the dossier are that any relevant information should be labeled as *commercial-in-confidence* (not as *confidential*), should not exceed 50 pages, including appendices, and should not include preclinical data.

5.2.2.4 Light Scientific Advice

Another type of advice offered by NICE is the so called *Light scientific advice* that is designed for small to medium enterprises. The advice is a concise and quicker version of the standard scientific advice. Its key features are as follows:

- Provides answers to each of the company's key questions
- 12 week process from project start (additional 3 weeks for optional clarifications)

Steps of the Procedure	Timelines
• The company requests a meeting slot at NICE	At least 25 weeks before the company wishes to receive the final advice report
• NICE sends a copy of the standard contract to be agreed and signed by both parties	At least 1 month before the briefing book submission
• Company sends the briefing book to NICE	–
• NICE confirms project size and total cost of the project	7 weeks of reception of the briefing book by NICE
• Clarification questions are sent by NICE to the company	
• Company responds to NICE clarification questions	Within 2 weeks of receiving by the company NICE's briefing book comments
• Face-to-face meeting with company and NICE	11 weeks after the briefing book submission
• NICE sends a written advice report to the company	7 weeks for a medium project or 9 weeks for a large project
• Possible clarification questions requested by NICE are answered by the company	Within 15 working days
• NICE answers further company questions	Within 20 working days

Other requirements and documents needed are similar as those for the full scientific advice from NICE described above.

5.2.3 Germany

G-B A consultation is possible as per the SGB (German Social Code) V, section 35a, paragraph 7 in the following scope:

- On documents and studies to be submitted
- On the appropriate comparator

Additionally, Federal Institute for Drugs and Medical Devices or the Paul Ehrlich Institute is involved if the product is evaluated before starting phase III studies.

Companies have to submit their request using a specific form, along with a cover letter. Timelines for the procedure range from 4 to 5 months. Fees are between €5,000 and €10,000.

5.3 STRATEGIC CONSIDERATIONS

5.3.1 Multidisciplinary Approach

Early HTA advice procedures require collaboration of multiple departments in the company, for example, preclinical development, regulatory, market access, internal representatives of the countries where participating agencies are located, medical affairs, health economics and outcomes research. They need to achieve clear strategic positioning, internal alignment, and maintain a proactive engagement to maximize the outcome of the advice.

5.3.2 Briefing Book Is the Cornerstone of Early HTA Advice

The briefing book is a document required in all types of early HTA advice. It gives a unique opportunity for the company to bring authorities' awareness to the disease and its related burden. It is crucial in order to develop a comprehensive background on the drug and its development plan. It is used to support discussions in advice meetings. Multidisciplinary approach will be required to draft the book.

The briefing book needs to incorporate accurate and specific questions (but not too narrow) so that detailed and clear answers can be obtained from the HTA bodies or regulators. It is also important to identify sensitive questions and review all questions or company's positions by external experts.

5.3.3 How to Choose the Right Option for Early HTA Advice

The company needs to identify the appropriate timing to seek advice. Broadly, this can be done in the following phases of product development:

- Very early in the drug development (nonclinical or proof of concept)

At this stage, the company may seek clarifications or adjustments of general clinical trial design but with limited patient data. The company is likely to obtain a general response with a less specific advice.

- Later in the drug development (prior to phase III)

Here, the company can obtain more precise responses regarding clinical trial design and pharmaco-economic questions. When phase III plans have been finalized, advice can still help to adjust design or statistical analysis plan of phase IIIb or IV studies.

The decision of when to consider the early HTA advice will depend on the following four main factors, assuming the product fulfils the criteria for the advice requested:

1. The development type envisaged: (i) traditional linear from phase I to III, (ii) medicine adaptive pathway to patients (MAPP), or (iii) a combined phase II or III development
2. The disease landscape
3. The product profile
4. The objective of the advice

Finally the risk related to involving in early HTA advice also depends on the type of advice chosen: (i) parallel, (ii) multi- HTA, (iii) national and on the time of advice in development such as (a) end of phase IIa, (b) end of phase IIB, (c) end of phase III, and (d) pre-submission.

Although all those risks are interrelated, the risk analysis can be done in two steps: first, the time of advice, and second the type of advice. It is an artificial separation that allows us to conduct a stepwise analysis and therefore simplify the process. In the second stage when deciding on the type of advice, this may obviously have backward consequences on the time for advice.

This is a multidimensional decision process, where all dimensions are interrelated and impact each other. Except for the type of development chosen (traditional, MAPP, or combined phase II or III development) for which the options are independent and mutually exclusive, all others may be linked and coexist. Therefore, it is important to ensure that they are not double counted.

Overall, the advice should be sought early enough to ensure that the company can appreciate the advice from HTA bodies and integrate them in all phases of the development. If the advice is performed too early, population(s) and indication(s) may be dramatically affected by the requests of the HTA agencies and end of phase IIb should be a reasonable time to request for an advice. If the company has actual questions or misalignment on the population and/or indication, the end of phase IIa is recommended to seek for an advice.

End of phase III appears too late for an early HTA advice as most questions are already addressed in the phase III study. HTA bodies may feel bounded and unable to impact development decision because a large volume of evidence is already gathered and the room for influencing the product

positioning in the development is minimal. However, early HTA advice at the end of phase III may support designing the phase IIIb study program.

In case of combined phase II or phase III, and in case of MAPP there is no option for end of phase IIb study HTA advice; therefore, the end of the proof of concept study is the only reasonable early option for a fruitful dialogue that may inform the company's decision process and optimize the product's value through the development phase.

5.3.4 Types of Risk for the Company When Considering Early HTA Advice

When a company involves in an early HTA advice there are two potential risks involved: (i) target population related risks—the risk of the HTA and/or regulatory authorities asking to adjust the targeted population and eventually also the indication, as a result of the procedure and (ii) development plan related risk—the risk that the authorities request a major revision of the phase III trial or the overall development plan, as a result of the procedure. Even though the advice is nonbinding, the company will likely need to comply with the requests, in order to achieve unrestricted HTA recommendation once the drug is approved for use. Since such compliance can result in, for example, increased development costs and time, these outcomes should be considered as risks by the company. These risks are further discussed below.

5.3.4.1 Target Population Related Risk

HTA agencies may request to narrow the population indication because at the time of a future HTA review the evidence will be primarily gathered from the population they consider for reimbursement. This will put them in a more comfortable position to appreciate the product's value versus the appropriate comparator. Also, by avoiding situations where they need to restrict the population, they protect themselves from negative media coverage of the decision and from discontent of the patient groups and prescribers.

HTA agencies may consider restricting the product to more severe patient groups, to second or third line, to add on therapy, to patients with poor prognosis, or those at risk of safety issues that the product may avoid compared to its comparator. HTA agencies may also be willing to enlarge the population to prevent niching a product in a small group with high unmet need, thus leading to a high differential value. If the product is developed in a wider population, the added benefit may be more diluted although still present.

The risks increase when no development guidelines exist or when they are too vague, not specific enough, and when there are no historical products that may have paved the way and rendered a specific requirement for a new product.

Such risks are not constant over the product development stages. However, the more advanced the development is, the risk is less, but also the benefit from HTA consultation can be smaller. Delaying the consultation reduces the risk, but also reduces the benefit. This is a trade-off to consider.

The risk is very high at the end of the phase IIa study because it is affecting the target population. As the phase IIb has not yet been performed, there is still room to revisit the target population and the indication. At that stage, all decisions are still possible. So HTA agencies may be more assertive at that stage to impact the population or indication.

After the end of the phase IIb study, the risks diminish dramatically because in order to achieve a marketing authorization approval, usually two studies are needed in the same population. The phase III is expected to replicate the result of the phase IIb in a reasonably similar population. Even if some adjustments are possible they should not be dramatic. If one significantly changes the population, one may be requested by regulators to redo their phase IIb study. Therefore, the risk of a dramatic change of the population targeted for the indication is very small.

At that stage, HTA agencies can push to enlarge the included population in the phase III by introducing wider inclusion criteria and less strict exclusion criteria. For example, include older patients, more severe patients, accept more comorbidity, or accept more comedications in order to be closer to the population that will be treated in real life.

At the end of phase III, the HTA consultation is unlikely to be a multiHTA advice as this scheme assumes that HTA agencies can influence the phase III trial design. However, it may be possible in theory to apply for a parallel advice.

The expected impact on the population and/or indication at the time of pre-submission is null.

5.3.4.2 Development Plan Related Risk

HTA agency consultation may lead to dramatic changes in the development plan. This may affect the phase III study only, but it may also affect the overall development plan, leading to additional unexpected studies that otherwise would not be requested by the regulatory agency.

HTA may request a broader phase III study, but also a broader development plan. This risk is similar at the end of proof-of-concept and at the end of phase IIb, although smaller at the end of phase IIb.

Such adjustments may delay registration and/or reimbursement.

Also, when undergoing a scientific advice, there is some risk that the HTA agencies require substantial changes either in the phase III study or in the development plan as a whole. Of course, this risk diminishes as the development is more advanced.

The HTA body may request substantial changes in the development plan and the phase IIb and phase III design. Among the HTA request that may influence the phase IIb and/or phase III trial, we can consider the following:

Introduce an active comparator, introduce a second comparator, introduce an additional arm(s) with different treatment regimen(s), enlarge or narrow the inclusion or exclusion criteria, additional end point, change the calculation method of the primary or secondary end points, lengthen the duration of the study, lengthen the post dosing follow-up, request a non-inclusion patients' registry with a baseline characteristic and so on.

Such risks are difficult to appreciate, and their appreciation is based on the company expertise and experience. It has to be confronted with external experts' opinion.

Such advise could be an opportunity to design a phase IIIb study that would specifically address the HTA agencies' expectations.

One possible reason to seek advice at the end of phase III is, when the phase III revealed an unexpected benefit. Then, the company may need advice for optimizing the value of this benefit. Three types of advises are possible:

- How to design an acceptable study for HTA to optimize the appreciation of the benefit
- Which observational study will allow to quantify the magnitude and the scope of the problem addressed through this benefit
- How this benefit could be translated in a cost-effectiveness advantage

At that stage, HTA agencies requirements may impact the reimbursement but not the registration. It can be seen as a way of show a goodwill with HTA agencies although not affecting the registration process.

Finally, seeking an advice at the time of pre-submission to HTA agencies can help to raise awareness about the condition, understand the resistance of HTA agencies to the way the evidence is presented and overcome the resistance by addressing them, or adjusting the evidence package.

It may also be an opportunity to collect information through database analysis, chart abstraction, or cross-sectional design to bring evidence that will address the resistance while the dossier is under review.

5.4 CONCLUSIONS

The main goal of the early HTA advice is to achieve consensus between HTA bodies and the EMA (when relevant) on the global drug clinical development plan. Simultaneous feedback from HTA bodies and regulators can help companies to identify key areas of consensus and divergence between these different stakeholders.