Chapter 4
Application of Nanotechnology in Drug Delivery and Targeting

Abstract Lipidic nanoparticulate self-assembled structures are effective carriers for drug delivery. This chapter describes the most famous nanotechnological drug delivery systems that are already used in clinical practice and clinical evaluation or in academic research. Liposomes are nanocolloidal lyotropic liquid crystals that are able to deliver bioactive molecules. Their membrane biophysics and thermodynamic properties reflect to the creation of metastable phases that affect their functionality and physicochemical behavior. Thermo- and pH-responsive liposomes are innovative nanotechnological platforms for drug delivery and targeting. Polymeric micelles and polymersomes are nanostructures that are promising drug carriers, while dendrimeric structures are considered as real nanoparticulate systems that are used in drug delivery and as nonviral vectors as well as in prevention of serious infections leading to diseases. Vaccines based on nanoparticles such as liposomes are an emerging technology and liposomes seem to meet the requirement criteria of adjuvanicity.

Keywords Packing parameter • Liposomes • polymersomes • micelles • Dendrimers • Vaccines

4.1 Pharmaceutical Nanotechnology

Nanotechnological systems are used as bioactive molecules' delivery systems for therapeutic purposes and for tissue imaging. Furthermore, new scientific studies combine nanosystem application for simultaneous disease treatment and monitoring disease progression, helping the clinical doctor to get the most accurate diagnosis. These systems are known as theranostics (diagnostic-therapeutic products) (see Chap. 3). In this chapter, the most important nanotechnological drug delivery systems will be studied, starting from their technological development in the laboratory to their clinical use regarding their biophysics, therapeutic efficacy, and safety. The pharmaceutical scientists have a great advantage when they try to understand the behavior of these nanosystems in a technological point of view and by correlating their functionality with the living organisms as well. Pharmaceutical nanotechnology research aims in the development of new medicines based on bioactive molecule

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delivery with nanosystems and in geometric increase of medicines that are currently in clinical studies. Pharmaceutical industry follows the same path, investing in the bioactive molecule development, in pharmaceutical nanotechnology point of view.

4.1.1 Lipidic Nanocarriers

Lipidic nanocarriers [4] are considered the most beneficial and promising technological platforms for encapsulating bioactive molecules. The biocompatibility and biodegradability due to their lipidic nature are advantageous properties, among others, like high ability entrapment of bioactive molecules with different solubilities. However, they have emerged as interesting nanosystems for delivering bioactive molecules to the target tissues. Their ability to improve the active and passive targeting mechanisms is of importance for exploring new disease targets. The most common and interesting lipidic carriers are liposomes. Commercially available lipidic nanocarriers that contain amphotericin B in the market are Abelcet® (Sigma-Tau PharmaSource Inc.) which is a lipidic complex suspension for intravenous infusion. It consists of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) in a 1:1 drug to lipid ratio. It forms ribbonlike particles with a size of 1.6–11 μm. Amphotec® (Ben Venue Laboratories, Inc, Bedford, OH) consists of amphotericin B in a complex with cholesteryl sulfate at a 1:1 drug to lipid molar ratio. The shape of the particles is determined as disk with a size of 120–140 nm. Two more lipidic nanocarriers are also referred in the literature. These lipidic nanosystems are the archaeosomes and nanocochleates.

Archaeosomes are lipidic bilayer vesicles composed of ether lipids that are obtained from the bacteria *Archaea* (Archaebacteria). They are more stable than liposomes to the changes of temperature, pH, oxidation, and hydrolysis. Omri and coworkers have reported the i.v. and oral delivery of archaeosomes in mice [62].

The name cochleates first presented in the pioneer work by Papahadjopoulos and coworkers [64]. The addition of divalent cation such as Ca-2+ (10 mM) to the lipidic preparation composed of phosphatidylserine in aqueous NaCl buffer following incubation for 1 h at 37 °C leads to the production of multilamellar structures which present cylindrical morphology. The spiral configuration was studied by using freeze fracture electron microscopy. Papahadjopoulos and coworkers proposed the name cochleate lipid cylinders. Nanocochleates are lipidic nanocarriers composed mainly of charged lipids and a divalent cation. They are applied for delivery of DNA, proteins, and peptides. Their structure (cigar-shaped MLV structure) is the reason for considering them as candidates for oral and systemic delivery of bioactive molecules [4].

4.1.1.1 Liposomes

Just before mentioning lipid nanosystems for therapeutic and diagnostic purposes, like liposomes, a very important step is the short description of the lipid bilayers that consisted of. Liposomal lipid bilayer is a study subject of many

scientists like physicists, chemists, biologists, biophysics, etc., while its thermodynamic behavior is also an important study subject that relates with their stability and functionality. Its physicochemical characteristics and thermodynamic phase transition (see Chap. 2) that characterize the lipid system thermotropic behavior and, therefore, its effectiveness are related to the geometry of its structural materials. The question arising during lipid membrane study is: "which is the driving force that causes the self-assembly of biomaterials, e.g., lipids – and in the case of liposomes, mostly phospholipids – in structures, e.g., spherical structure instead of flat (foliate) structure?" or even better "which is the driving force creating the specific nanostructure type?" The answer is based on the physicochemical and thermodynamic characteristics of the medium where a nanostructure is self-assembled.

The geometric characteristic determination of nanoparticulate systems containing amphiphilic surfactants (e.g., phospholipids) such as liposomes is based on the packing parameter S_p. This parameter is symbolized in this book as S_p so there will be no confusion with parameter S that will be mentioned later on and relates to the biomolecule orientation in a nanosystem (Figs. 4.1 and 4.2) [39]:

$$S_p = \frac{V}{a_0 l_c}$$

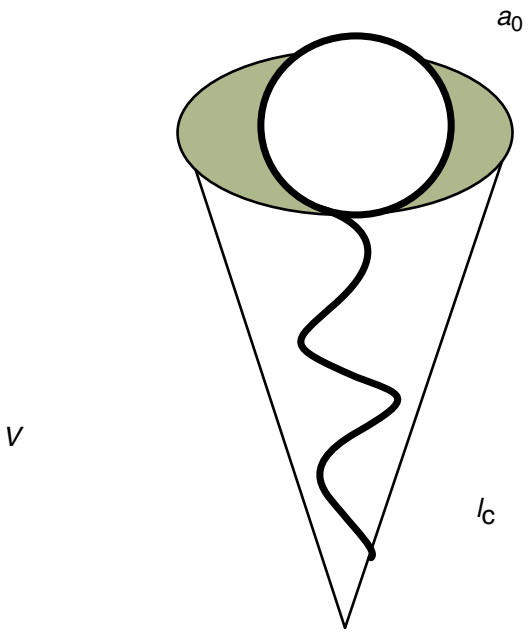

Fig. 4.1 The geometric characteristics of surfactant amphiphilic

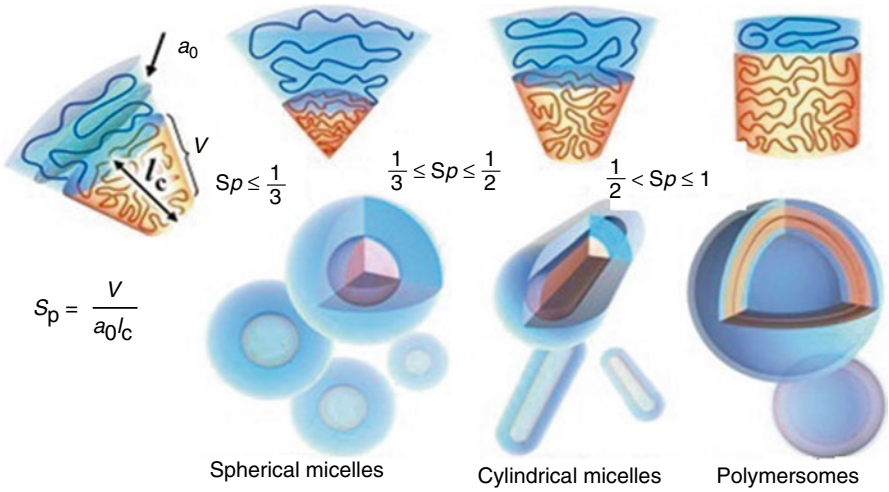

Fig. 4.2 Nanostructures of self-assembly of biomolecules resulting in a different shape. Calculation of packing parameter, S_p

$$S_p = \frac{V}{a_0 l_c} \tag{4.1}$$

where V is the volume of the hydrophobic chain(s), l_c is the length of the hydrophobic chain(s), and a_0 is the surface area of the hydrophilic polar group/head of the amphiphilic (surfactant) molecule, e.g., phospholipids. The geometrical characteristics of the structural units of nanosystem affect through their self-assembly process, its organization, shape, and structural characteristics. Another important parameter is the one referring to the biomolecule orientation regarding the vertical settlement axis. This parameter is characterized with the letter S and can have values 1 and 0.3–0.9 that have been emerged from the experimental data.

The mathematical type that calculates the S and based on its value classifies the system in crystalline form ($S=1$) in isotropic liquid ($S=0$) and in liquid crystal ($S=0.3$–0.9) is the following:

$$S = \frac{3\cos^2\theta - 1}{2} \tag{4.2}$$

$S=1$ (crystal), $S=0$ (liquid), and $S=0.3$–0.9 (liquid crystal). Where $\cos^2$ is the settlement axis angle of the nanosystems' biomolecule to the vertical axis. Important categories of self-assembled nanosystems with variations regarding the biomolecule chemical structure will be presented in detail in the following pages.

Liposomes were firstly described by Alec Bangham in 1965 [5]. In reality, the simple phospholipid stirring (Fig. 4.3) in water has led to the development of a phospholipid dispersion that aimed in cell membrane study regarding its biophysical

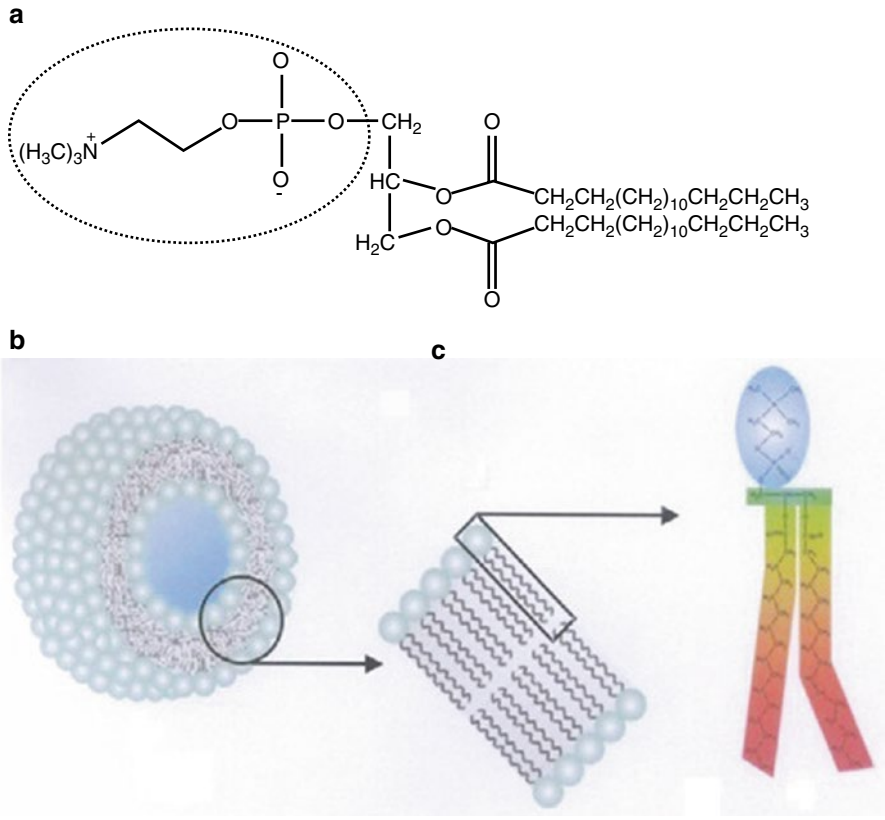

Fig. 4.3 (**a**) Structure of phospholipid. In cycle, the polar group (choline). (**b**) Liposomes (**c**) lipidic bilayer phospholipid

behavior. Liposomes belong to the self-assembled colloidal nanodispersion systems composed of lipidic bilayers that are able to incorporate lipophilic bioactive molecules or to entrap them into their aqueous core [5, 65]. This phospholipid dispersion was basically related to large phospholipid structures of irregular shape and micrometer size with multiple lipid layers. The lipid layers' polar heads were oriented toward the water molecules where the lipid chains have created the lipid bilayer through hydrophobic interactions. This effort was the beginning of the nanosystem development, liposomes that delivered bioactive molecules. During the past decades, liposomes are one of the most promising bioactive molecule delivery nanosystems. Liposomal technology is a developing research field, while scientific reports in this field are hundreds per year. Liposomes are closed *pseudo-spherical* structures that consisted of one or more lipid bilayers where inside them entrap water media and are characterized as thermodynamically unstable colloidal dispersions. Lipid bilayers consisted mainly of phospholipids and cholesterol without excluding the use of other biomaterials, e.g., polymers as liposomal structural units. Phospholipids, due to their

amphiphilic character, in water environment orient so their polar heads are toward the water medium, while lipophilic hydrocarbon chains are protected from the water molecule connection, developing hydrophobic interactions. On liposomal surface, small molecules or macromolecules can be connected; these include antibodies that change the physicochemical properties of their surface. This fact is very important and depends upon the nanosystem functionality and its physical stability on time of production and over time.

The discovery of multilayer's irregular structures that lipids form where in water medium could be of use as a simulation model for cell membrane is a historical observation that enriched the biophysical and colloidal dispersion system science. In the early 1960s, there was a great interest in studies related to lipid colloidal system properties that could be used as design standards of cell membrane behavior. Initially they were called bangosomes, since AD Bangham was the first one that noticed them, but later on they were called liposomes from the Greek words *λίπος* (fat) and *σώμα* (body) [5]. Bangham had suggested the term amphisomes as more appropriate since the cell membrane structural parts were amphiphilic molecules. Studying the cell membranes' dynamic properties like fluidity and lipid layers' mesophase change was a challenging field when studying biophysics and thermotropic behavior of lipid membrane and, therefore, liposomal dispersions. Lipid vesicles in their initial form (simple dispersion in water environment) had no thermodynamically organized structures and were characterized as multilamellar vesicles (MLVs).

In 1967 Demetrios Papahadjopoulos and coworkers [63] described the structure of sonicated microvesicles that later on were known as small unilamellar vesicles (SUVs). These researches offered the biomembrane potential structure, explaining lipid layer fluidity and diffusion properties. The evolutions in this scientific field have created a fertile ground in scientific groups that started studying the possibility of applying lipid dispersions as vehicles for bioactive molecule transfer and delivery. During the past years, research related to liposomes as transport and delivery vehicles for therapeutic purposes has been developed [41]. The scientific interest in this area included the interaction of encapsulated biomolecules (nucleic acids, proteins, bioactive molecules) with the liposomes' structure units, their in vivo administration, the mechanisms when liposomes enter the cell, and their immunological properties. Changing the lipid composition (phospholipids and generally lipids of different structure), ζ-potential, liposomes' size, and size distribution were characterized as important properties that could define liposome behavior in vivo and in vitro. Phospholipids' distribution in water medium resulted in spontaneous development of lipid vesicles, and not liposomes, since liposome development needed a specific process (see Appendix). Lipidic vesicle structures (e.g., double layer, cubic structures, hexagonal structures, etc.) depend upon the phospholipid or lipid concentration that takes part in the process and their geometry that is related to their chemical structure. If the conditions mentioned before, i.e., phospholipid/lipid concentration and chemical structure, lead to lipid bilayer development, then the hydrophobic parts come in contact with water environment in a non-favorable energy way. This results to a bending so they will interact through hydrophobic interactions minimizing lipid chain exposition in water medium. The energy required for lipid bilayer bending is covered from the

energy increase due to lipid bilayer's hydrophobic section exposition to the water environment. The result of hydrophobic lipid chains' bending and assemble is the development of multilayer lipid vesicles. Their description was reported for the first time in 1965 from Bangham and his coworkers [5]. So, lipid vesicles are the closed *pseudo-spherical* structures of one or more lipid bilayers that entrap the dispersion medium where they are. This formation offers lipid vesicles the property of being consisted of both the hydrophilic dispersion medium and the lipid chain hydrophobic section, respectively. For this reason, they can entrap hydrophobic, amphiphilic, or even hydrophilic bioactive molecules. Liposomal production with a specific process, mentioned in the Appendix – i.e., the lipid vesicle production with structural organization depended on the biomaterials' structural and geometrical characteristics, physicochemical characteristics, and their energy content – is an important fact directly related to their physical stability, the bioactive molecule entrapment ability, their release rate, and their efficacy according to their ADME (absorption, distribution, metabolism, and excretion) profile that will develop the final pharmaceutical product. During the past decades, liposomes are one of the most promising bioactive molecule carriers and delivery systems to the damaged tissues, and liposomal technology is a potential research development field while the scientific publications in this field are hundreds per year. According to the above, it can be said that liposomes are closed *pseudo-spherical* thermodynamically unstable lipid structures of dispersed lyotropic liquid crystals that are composed of one or more lipid bilayers that can bind on the inside of the water medium where they are. Lipid bilayers are composed of phospholipids, cholesterol, or other lipid molecules. Cholesterol is an important biomolecule that was chosen among the others through centuries in natural evolution process, since the system demanded thermodynamic *self-sufficiency*, structural stability, survival time ability, and life perpetuation through multivariate and multilevel cell organization. Cholesterol has been chosen as the most appropriate biomolecule that can sufficiently regulate the lipid bilayer fluidity that is necessary for its functions [38, 53]. Also, on liposomal surface, small molecules or macromolecules can be attached and modify their surface properties, for example, targeting antibodies through antigen connection. Nowadays, liposomes are considered as nanoparticles that develop liposomal colloidal nanosystems, extremely useful and highly promising for new pharmaceutical product development [28].

The target of each pharmaceutical formulation is the distribution optimization of the bioactive molecule in the human organism, the control over its release rate, and the maximization of the therapeutic result. Liposomes have the ability to entrap and transfer a wide range of bioactive molecules and have the following advantages:

- Liposomes can direct the bioactive molecule in specific parts of the organism (damaged tissues) enhancing the therapeutic effect while minimizing its accumulation in healthy tissues and, therefore, limiting toxicity and side effects. Targeted therapeutics is a continuously developing research activity.
- Liposomes can act as transport and delivery vehicles and as bioactive molecule accumulation tanks. Liposomes release the bioactive molecule in a controlled rate, modifying the bioactive molecule's pharmacokinetics.

- Protection of the entrapped bioactive molecule physicochemical integrity. Bioactive molecules entrapped in liposomes are protected since there are no enzymatic degradation processes taking place.
- Liposomes have the ability of transferring bioactive molecules inside the cell through different mechanisms, like fusion, phagocytosis, etc.

Moreover, the physicochemical properties and their behavior in biological media promote advantages over other delivery nanosystems. We have to refer that thermodynamics which relates to the stability profile of liposomes should be kept into consideration. It is worthy to note (see Chap. 2) that liposomes are not at thermodynamic equilibrium, but they behave as a kinetically trapped nanosystem, contrary to those nanosystems (e.g., polymeric nanosystems or microemulsions) that they are affected when changes in their environment occurred. However, liposomes preserve their physicochemical properties that promote their usefulness as drug delivery nanosystems [42].

Nowadays liposomal products are in the market for therapeutic purposes. Due to their double nature (lipidic bilayer, hydrophilic inside), liposomes can be used as carriers for both lipophilic and hydrophilic bioactive molecules. Depending on their nature, various bioactive molecules can be placed into the lipid bilayer or in the hydrophilic section of the liposome. In the first case, the bioactive molecule is incorporated into the bilayers of liposome, while in the second case, the bioactive molecule is encapsulated into the aqueous interior of liposome. Bioactive molecules entrapped in liposomes seem to be more effective to treat diseases and to protect healthy tissues from their exposure in toxic biomolecules. Liposomes are able to transfer infected organism antigens, malaria antigens, and bacterial toxins, and these have been successfully used for the development of chemical or cellular immunity in test animals. All of the above show that liposomes can be protein and peptide transferring systems but more research is needed for vaccine development.

Liposomes could be considered as artificial biomembranes, and their relationship regarding the biophysical behavior between biomembranes and cell biology has promoted them as leading nanosystems to deliver drugs to the target tissues. Liposomes have been successfully used in the field of cell physiology, and the pioneer work of Gregoriadis and coworkers in the 1970s provided evidences regarding the use of liposomes in enzyme replacement therapy [25–27]. They can be developed having a specific composition simulating cell membrane functionality, offering a model system for their study. Due to their relevant structure with the basic cell membrane components, they have been used to both stimulate and study them. These studies are on their thermotropic behavior, i.e., on the phase transitions, due to heat absorption or elimination. This thermotropic behavior can be related with the cell behavior and many assumptions can be concluded on their functionality. Also, this relation can highlight the *disease factors* and the biophysical changes due to *mesophases* resulting from phase transitions. The term *disease factor* can be related to the cell biophysical and thermodynamic behavior, e.g., a microbial infection can cause a cell membrane biophysical functionality change and, therefore, act as a *disease factor*. It is obvious that the more cell membranes and other cell organelles, even genetic

material's phase transitions, affect the living functions, the more dangerous the *biophysical disease factor* becomes for the organs' function and for life itself. Liposomes have been used successfully for bioactive molecule action mechanism investigation, i.e., aminoglycoside antibiotics' cytotoxicity mechanism. Also, they have been used to understand the local anesthetic mode of action and as models for studying the phototoxic active oxygen-mediated incidences. Table 4.1 presents liposomal applications in various scientific fields. It should be mentioned that liposomal applications in medicine and pharmaceutics are due to the need of new medicine development, especially new bioactive molecule delivery moieties with specific physicochemical characteristics. Liposomal properties related to their biocompatibility with cell membranes, their ability to transfer and deliver bioactive molecules and biological products, and their ability to target damaged tissues connecting with macromolecules on their surface (e.g., antibodies) are the basic reasons for health science applications.

Most important parameters studied in liposomal technology are:

- Liposome lipid composition. The predominant lipids are phospholipids and cholesterol.
- Temperature, pressure, ionic strength, and presence of ions or macromolecules, e.g., proteins, enzymes, and bioactive molecules in water environment where liposomes are.
- Lipid membrane permeability, elasticity, width and shape, and its ability to interact with other cell membranes.
- Lipid concentration in the final dispersion system.

The classification of various liposomal types can be done according to size and number of bilayers. According to these criteria, they can be distinguished in:

- Multilamellar vesicles. They consisted of many concentric bilayers of 500–5000 nm size. These can be also divided in large multilamellar vesicles (MLVs) that have up to 20 bilayers and in oligolamellar vesicles (OLVs) that usually consisted of 5 lipid bilayers.
- Large unilamellar vesicles (LUVs). They have only one bilayer and their size is between 200 and 800 nm.

Table 4.1 Applications of liposomal technology

Science	Application
Mathematics	Topological study of space of two- or three-dimensional surfaces. Fractal geometry
Physics	Physical properties of biomaterials
Biophysics	Permeability, transitions of liquid crystalline phases of materials
Physical chemistry	Study of physicochemical characteristics of colloidal systems
Biology and biochemistry	Biological membrane models. Studies on the interactions between cells
Pharmaceutics	Drug delivery and targeting
Medicine	Diagnostics, pharmacological effectiveness of liposomal products

- Small unilamellar vesicles (SUVs). They have only one bilayer and their size is 100 nm.
- Multivesicular vesicles (MVVs). They include many sections in their interior, but contrary to MLVs, they are not concentric.

An additional liposomal category can be according to the lipid bilayer composition, and therefore, there are the following:

- Conventional iposomes. Conventional are called the liposomes that consisted of neutral phospholipids (neutral phospholipids of ionic nature – their total charge is zero as they have equal number of positive and negative charges).
- pH-sensitive liposomes. These liposomes mainly consisted of phospholipids, e.g., DPPE 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine. According to Connor and coworkers [16], pH-sensitive liposomes composed of DPPE promote fusion, while the presence of phosphatidylcholine inhibits the fusion process according to water environment pH that it is exposed to, they present a different charge [46].
- Cationic liposomes. Cationic liposomes are those with a positive charge [e.g., DOTAP 1,2 dioleoyl-3-(trimethylammonium)-propane, DOTMA chloride N-{1-(2,3-dioleoyloxy)-propyl}-N,N,N-trimethylammonium]. They are used exclusively in intracellular transfer of negatively charged macromolecules like nucleic acids (DNA and RNA) and other oligonucleotides.
- Immunoliposomes or antibody-targeted liposomes [33]. This category relates in liposomes of targeted delivery that have on their surface an antibody that functions as a detection center for surface antigens of target cells. Usually, the antibody is covalently bonded with the active group on the liposome bilayer surface, usually a maleimide molecule.
- Long-circulating or sterically stabilized liposomes. These liposomes include phospholipids in their bilayer. These phospholipids are covalently bonded with polyethylene glycol (PEG). PEG offers a kind of steric stabilization, a barrier for interactions between liposomal surfaces and biological environment's components. These interactions usually include plasma protein penetration into the bilayer that can destabilize the liposome structure or have opsonization properties, whose interference lowers the immune system phagocytes' recognition ability. So, these liposomes stay for a long time in plasma circulation without having a small size.
- Stealth liposomes. These liposomes' structure is modified in order to lower their destruction rate from macrophage cells and to maximize their stay in the organism. Usually, they have a small size and on their surface they have polymers like polyethylene glycols (PEG), carbopol, and polyvinyl alcohol. Liposomes can act as bioactive molecule "tanks" for their extended release.
- Liposomes with macromolecules on their surface. These liposomes, known as ligand-targeted liposomes (LTLs), can have on their surface monoclonal antibodies, peptides, polysaccharides, receptors, hormones, vitamins, growth factors, etc. Macromolecule connection on liposomal surface can be either directly or through

a "ligand." In the case of ligand-targeted liposomes, bioactive molecules are connected on polymer chains (e.g., PEG).

Depending on macromolecule type on the liposome surface, these can be classified into the following:

- Liposomes with peptides on their surface. In this category, there are liposomes that have plasminogen on their surface and they target fibrin clots. Also, there are peptide liposomes that block angiogenesis in endothelial cell targets.
- Liposomes with polysaccharides on their surface. These liposomes through their surface polysaccharides are directed to specific cell targets.

Moreover, liposomes can be used in imaging when loaded with the appropriate contrast agent. Several efforts have been made for the accumulation of contrast agents in the required area by using carriers of the agent. Liposomes were found to be the most relevant nanoparticulate nanosystems because of their release of the agent profile [85]. Their applications in imaging and as diagnostic agents are mainly based on their biocompatibility and on their entrapment efficiency of diagnostic agents [29, 39]. Liposomes are attractive carriers that are able to overcome skin barrier and depending on their composition can behave as penetration enhancers mainly that with high values of hydrophilic-lipophilic balance. Transfersomes are liposomal formulations that have been introduced by Cevc [12] and can promote great fluxes of active ingredients through the skin. More innovative products are produced for cosmetics based on liposomal concept delivering compounds such as antioxidant, hydrated agents, etc. and even proteins [88]. It should be mentioned that, not surprisingly, liposomal technology accommodated nonmedicinal and pharmaceutical areas such as catalysis, cosmetics, ecology, etc. [40].

Initially, the bioactive molecule is dispersed in the multilayer lipid vesicle environment that is developed during lipid dispersion in aqueous medium [51, 52]. This dispersion depends on the physicochemical characteristics of the molecule and the vesicles formed. The presence or absence of charged groups within the bioactive molecule that are created depending on the pH of the dispersion medium is a crucial parameter in bioactive molecule encapsulation process in liposomes. Liposomes will be developed according to the multilayer lipid vesicles and their membrane physicochemical characteristics. Regardless on the liposome production method that follows the initial lipid dispersion in water medium and the development of multilayer vesicle structures, the bioactive molecule encapsulation into the liposomes that will be developed later on is based on three different techniques. The technique to be used is chosen according to the bioactive molecule physicochemical properties that can be either hydrophilic, hydrophobic, amphiphilic, or amphoteric (weak base or acid).

- Encapsulation. Encapsulation as a term is used basically in case of hydrophilic bioactive molecules. The process involves the bioactive molecule phospholipid or other lipid hydration with water medium. During the multilayer lipid vesicle development, the hydrophilic molecule is passively encapsulated into the inner water environment and between the bilayers. Following, liposomes with the

bioactive molecule encapsulated on the water environment are developed according to process described in the Appendix of this chapter.

- Incorporation. A hydrophobic or amphiphilic molecule is dissolved in organic solution and then mixed with phospholipids or lipids. Evaporation follows on rotation device in vacuum and a thin lipid film is produced. The thin lipid film, phospholipid and/or lipid, bioactive molecule mixture hydration with the water solution results in multilayer lipid vesicle formation where hydrophobic or amphiphilic bioactive molecules are encapsulated in their lipid bilayer due to hydrophobic interactions with its lipids. Liposomes are then developed according to methods described in the Appendix of this chapter.
- Active loading. It is based on bioactive molecule passive diffusion through liposome lipid bilayer. Molecules like weak acids are in neutral or ionic form depending on the pH of the dissolution media. A bioactive molecule like that can penetrate the liposomal bilayer. The pH inside the liposomes is regulated so the bioactive molecule can be in its ionic form and due to charged groups cannot penetrate the lipid bilayer barrier and, therefore, accumulates inside the liposomes. This method includes initially, the liposome development through multilayer lipid vesicle development and then bioactive molecule addition through encapsulation in liposome water environment as mentioned earlier. An example of bioactive molecule active loading in liposomes is the encapsulation of the anticancer bioactive molecule, doxorubicin.

According to Sidone and coworkers [73], the pharmacokinetic variability of liposomal agents was 2.7-fold and 16.7-fold greater than non-liposomal agents as measured by ratio of AUC CV% and ratio of AUC_{max} to AUC_{min}, respectively. It is of importance to figure out that the incorporated bioactive molecule into liposomes does not interact with the site tissue until it releases from the liposomal formulation. It is obvious that the pharmacokinetic profile of the liposomal product combines the pharmacokinetics of the liposomal delivery system as well as the pharmacokinetics of the incorporated bioactive molecule after it releases from the carrier. However, the total pharmacokinetic profile of a liposomal product still remains a scientific area to be developed, because the rate of the release affects the overall pharmacokinetics of the liposomal product [1]. Today many anticancer liposomal formulations are available in clinical practice, while a huge number are in clinical trials. The first one which received approval in 1995 was doxorubicin HCl as liposomal injection (Caelyx^R in Europe and Doxil^R in the United States), to treat HIV-associated Kaposi sarcoma and ovarian and breast cancer [75]. The published data by Allen and Stuart [1] showed that the pharmacokinetic data between the two well-known anthracyclines, i.e., doxorubicin and daunorubicin incorporated into different composition liposomal formulations with those of the free form of the two anthracyclines, showed that the clearance rates of the liposomal formulations are lower than that of the free form of the drugs. It is of importance that the sterically stabilized (surface PEG grafted) liposomal formulation that corresponds to Doxil/Caelyx has shown a decrease clearance rate, in comparison with that of DaunoXome® which is the liposomal formulation of daunorubicin, even though the liposomal vehicles of DaunoXome have larger hydrodynamic diameter (size).

Thermo- and pH-Responsive Liposomes

The development of stimuli, dual- and multi-stimuli-responsive nanosystems, follows the same approach for improving the pharmacotherapy as stealth and *chimeric* liposomes (see Chap. 5) (combining two different or same biomaterials). These systems use intrinsic or extrinsic (external) stimuli as triggers in order to succeed in site-specific drug delivery and to improve the safety and efficacy of bioactive molecules. pH-, thermo-, redox-, enzyme-, magnetic field-, and light-responsive nanopreparations have been already studied extensively [13, 20]. Temperature-sensitive drug delivery systems offer great potential over their counterparts due to their versatility in design, tunability of phase transition temperatures, passive targeting ability, and in situ phase transitions [37]. Thermosensitive liposomes are developed in order to improve tumor accumulation, trigger liposomal drug bioavailability, enhance drug delivery specificity (i.e., *drug painting dosing* and *chemodosing*) and drug internalization, and personalize the treatment [77, 91]. Thermosensitive liposomal formulations minimize the toxicity of the encapsulated active substance/anticancer agent, control the release rate, and enhance the long-circulating properties by modulating the composition and the "smartness" "decision making" of the nanocarriers [44]. ThermoDox® (Celsion) is a nanoengineered drug delivery system in clinical trials (phase III) and is a low-temperature-sensitive liposomal formulation incorporating doxorubicin (anthracycline) for the treatment of metastatic malignant melanoma and liver cancer. ThermoDox is the first heat-activated liposomal formulation, which consisted of three synthetic low phase transition temperature phospholipids and releases the anticancer agent at 39.5 °C [22]. In the past decades, there have been reported and developed many strategies for formulating pH-sensitive liposomes. The main categories based on the components and the mechanisms of triggering pH sensitivity are listed below. The first way is to combine polymorphic lipids such as phosphatidylethanolamine (PE), diacetylenic phosphatidylethanolamine (DAPE), dioleoylphosphatidylethanolamine (DOPE), and palmitoyl-oleoyl-phosphatidylethanolamine (POPE) [74]. Secondly, the majority of these systems may contain "cage" lipid derivatives, such as N-citraconyl-dioleoylphosphatidylethanolamine (C-DOPE) and N-Citraconyl-dioleoyl-phosphatidylserine (C-DOPS) [46]. The mechanism of action of pH-sensitive liposomes is presented in the literature [47, 74]. Since pH-sensitive liposomes cannot always sustain a slow and steady release, especially in a physiological pH solution, thus resulting in cytotoxicity for normal tissue, more sophisticated structures are being investigated, in order to overcome this possible instability of pH-sensitive liposomes. The investigation in the area of pH-sensitive liposomal vehicles for drug delivery is still an interest approach, and because of the new intracellular targets that are recognized, the efforts in this scientific field should be expanded [15].

Immunoliposomes

Immunoliposomes belong to the lipidic class of carriers that are able to reach to the target tissue via the active targeting process. They possess macromolecules on their

surface such as antibodies, carbohydrates, and hormones that act as detection centers from surface antigens that are on cell targets. During the 1980s, various techniques connecting monoclonal antibodies on liposomal surface have been developed. The preferable strategies by which the macromolecules attached on the outer surface of the liposomal membrane are adsorption to the outer surface of liposomal vehicles, insertion into the lipid bilayers, via biotin-avidin pair, and finally covalent binding [39]. The macromolecules that they are attached on the surface of liposomes have complementary ligands on the target cells, and consequently when they arrive close to the target cell, liposome binds specifically to the target site. Bioactive molecules encapsulated in liposomes that have monoclonal antibodies on their surface include anticancer agents like doxorubicin (anthracyclines), vinca alkaloids, and taxol (taxanes) that are released in the surrounding area of the target cell, reducing the adverse drug reactions and the toxicity and improving the therapeutic effect. The research on this medicines aims to the pharmacokinetic parameter improvement and the increased concentration of the bioactive molecule on target cells using immunoliposomes [33].

Mitochondriotropic Liposomes

Particulate delivery systems of bioactive molecules in highly specific targets such as subcellular organelles represent a great challenge in drug targeting. The mitochondrion is an organelle composed of two membranes which create two separate compartments and is responsible for the energy metabolism. They are unique organelles as they contain their own genome mitochondrial DNA (mtDNA). It is well known that possible drug targets are located inside the mitochondrial matrix and it is of particular interest to design and develop carriers that are able to move through the mitochondrial matrix to release drug to the target. *Mitochondrial Medicine* is a new field of biomedical research. Mitochondriotropic liposomal approach is considered as an effort for producing mitochondria-targeted particulate drug and DNA delivery systems. The use of reconstructed proteoliposomes containing mitochondrial membrane components was an effort to prove the hypothesis that liposomes by modifying their surface with a mitochondriotropic residue could be rendered as mitochondriotropic [19].

Analyzing Liposomes

Liposomes can be used as carriers for bioactive molecule (hydrophilic, hydrophobic, amphiphilic macromolecules and genetic material) encapsulation/ incorporation or transport. This property seems to be due to two different areas: a polar interior cavity and a lipophilic bilayer. Hydrophilic groups in the outside liposomal surface develop macromolecular attachment places like antibodies, peptides, etc. These properties have led scientists in the use of liposomes in bioactive molecule-targeted delivery research and in the field of diagnostics. The basic liposomal

application as analytical tools is related to the immobilization of the label molecule on the liposome surface and to the entrapment of the marker molecule inside the cavity. The marker allows liposome detection and quantitative determination and, therefore, detection and quantitative determination of the molecule under assay, using the appropriate analytical method (optical or electrochemical).

Liposomes as analytical diagnostic tools have the following advantages:

- Great outside surface, where various biological macromolecules can be attached, e.g., antibodies and peptides.
- Flexibility when choosing the individual components. This fact allows the production of liposomes with desired properties, required stability, and connection ability with various targeted/detection molecules.
- Great internal volume that allows the entrapment of a huge number of hydrophilic indicator molecules like dyes.
- Time-independent signal production.

The use of liposome flow injection systems is mentioned since 1988. Flow injection systems offer advantages like greater accuracy and possibility of automotive process. This method has been used in theophylline, estrogen, fumonisin B1, *E. coli*, etc. detection.

Biosensors According to Liposome Technology

Bioanalytical assays related to quality and quantity detection of various substances into biological fluids (e.g., blood) can be classified into two broad groups: those depending on protein detection and those depending on nucleic acid detection. Examples of assays depending on protein detection are enzyme-linked immunoassay (ELISA), radioimmunoassay (RIA), and immunoblotting. These assays depend on antibody-antigen interaction. The second assay group that depends on nucleic acid detection is applied for special DNA and RNA sequence detection.

In these assays, the detection sequence is multiplied for DNA by polymerase chain reaction (PCR) method. For RNA detection, RNA is firstly converted to complementary DNA through real-time (RT) method and then follows PCR (RT-PCR) enrichment or nucleic acid sequence-based amplification (NASBA). After multiplication, the enriched molecules (amplicons) are determined according to their size with electrophoresis in agarose gel (they have been previously died with ethidium bromide), and their possible functional identity is certified through hybridization with specialized probe molecules. Finally, the safest procedure is to find primary structure molecules with base sequencing. Nowadays, many specialized analytical assays for substance determination are available but require special equipment, analytical labs, and trained personnel. For this reason, there is a continuous effort to find simple, fast, and low-cost methods to selectively detect the quantity of substances of interest. This determination should be available without the use of expensive equipment and from nonspecialists. The speed has great interest, especially when controlling production in industrial scale. For example, if scientists are able to detect a great

concentration of an infectious factor in food, they will be able to stop production and eliminate the damages. In this case, the use of biosensors, devices that convert the biological receptor interaction with the molecule (substance) that needs determination into an analytical signal (optical, electronical, etc.). Chemical sensors are composed of two basic elements: the chemical (molecular) recognition system and the physicochemical converter. The biochemical sensors are chemical sensors where the recognition system is based on a biochemical mechanism. During the past years, there is an intense research activity in the field of liposomes used to develop biosensors. The liposomal size and the number of bilayers can be adapted depending on the production method, so small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), or multilamellar vesicles (MLVs) can be created.

Various phospholipid polar heads offer connection ability of various molecules on liposome surface, while the different chain length and their saturation degree allow liposome production of various properties. Additionally, other molecules or lipids can be integrated on lipid bilayer and offer desired abilities to liposomes. For example, the addition of phosphatidylglycerol creates negatively charged liposomes, while the addition of cholesterol lowers the membrane permeability. Due to their great outside surface and their ability to connect to other molecule lipid bilayers, liposomes can be applied in analytical determinations. Liposome advantages include their great inner volume that allows a large amount of dye to be encapsulated (or another appropriate indicator), enhancing the received signal. The analytical determinations are distinguished as homogeneous and heterogeneous. Homogeneous are the determinations where all substances are mixed together in a container and all the reactions take place without any separation step. Heterogeneous determination needs one or more separation stages to achieve excess reagent withdrawal.

The basic factors affecting liposome physicochemical stability are their composition, i.e., the kind of lipids that they are composed of, the number of the layers (production method), their charge, their water-binding ability, and the lipid concentration. Modifying these factors, liposomes that are produced have the desired properties and physicochemical characteristics.

Liposome Physicochemical Characterization and Their Physical Stability

The characterization of liposome physical properties involves techniques and measurements for size, size distribution, surface charge, and ζ-potential determination. Liposome dispersion system stability relates to its physical, chemical, and biological stability. Liposome size and size distribution measurements around the average value are important parameters to calculate the liposome dispersion physical stability. More in detail, we can mention the following: liposomes developed and dispersed in chosen water medium are in constant movement (Brown movement). The particle movement speed depends on their size, solution temperature, and viscosity. Liposome size distribution is a measure of their stability. When size distribution is stable in time, the liposome dispersion is characterized as stable and suitable for pharmaceutical use. The liposome nanosystem stability is time dependent since

according to the second thermodynamic law, it is leading to collapse. In case where liposome size distribution increases with time, the liposome dispersion system is characterized as unstable and unsuitable for pharmaceutical use. With dynamic light scattering (DLS) technique (see Chap. 2), the Brown movement calculation is possible through data collected from the light scattering. Brown movement calculation will help to determine diffusion coefficient and, therefore, liposome size and size distribution around the average value. Diffusion coefficient (D) and particle size (characterized from their hydrodynamic radius, R_h) are related mathematically with Stokes-Einstein equation (see Chap. 2).

Dynamic light scattering (DLS) technique measures particle size in relation to particle movement in a liquid, Brown movement. The results being received are the diameter of an equivalent sphere with the same particle diffusion coefficient (liposomes) in the specimen. The hydrodynamic diameter is a little larger than the actual specimen liposome diameter due to solvation phenomena and interactions between particles.

Liposomes belong to colloidal dispersion systems and are characterized as lyotropic liquid crystals (see Chap. 1). These particular liposomal lyotropic states are responsible for the *mesophases* taking place in phase transitions and are related to their thermal stress during phase transitions. Their thermal stress takes place during liposome dispersion system storage or during administration in humans. The thermodynamic parameters that affect and participate in physical stability and, therefore, in pharmaceutical effectiveness of the liposomal product are the following:

- T_m: temperature of basic transition from liquid crystalline state to isotropic fluid.
- $\Delta T_{1/2}$: width of the transition at half peak height. Range in the middle of the peak. This temperature range is related to the cooperativity of system phospholipids or phospholipids and enclosed bioactive molecule.
- ΔH: system enthalpy change.
- $C_{p\,max}$: maximum systems' heat capacity under constant pressure.

The identification and study of the phase transitions of liposomal dispersion systems lipid bilayers allow the control over the thermodynamic parameters mentioned above, in order to rationally design the liposomal system with the most satisfactory physical and thermal stability. The design of liposomal nanosystem and their evaluation in technology level and in vivo behavior are linked with their thermodynamic response. At the same time, thermal analysis and more in particular differential scanning calorimetry (DSC) (see Chap. 2) are valuable tools for liposomes' physical stability prediction and physicochemical property interpretation [51] (Fig. 4.4).

The basic electron microscopy methods used in liposomal physical characteristics study are non-flame atomic spectroscopy (NFAS), transmission electron microscopy (TEM), cryogenic TEM (Cryo-TEM), scanning electron microscopy (SEM), freeze fracture electron microscopy (FFEM), scanning tunneling microscopy (STM), scanning force microscopy (SFM), atomic force microscopy (AFM), lateral force microscopy (LFM), cryogenic atomic force microscopy (Cryo-AFM), near-field scanning optical microscopy (NSOM), and magnetic resonance force microscopy (MRFM).

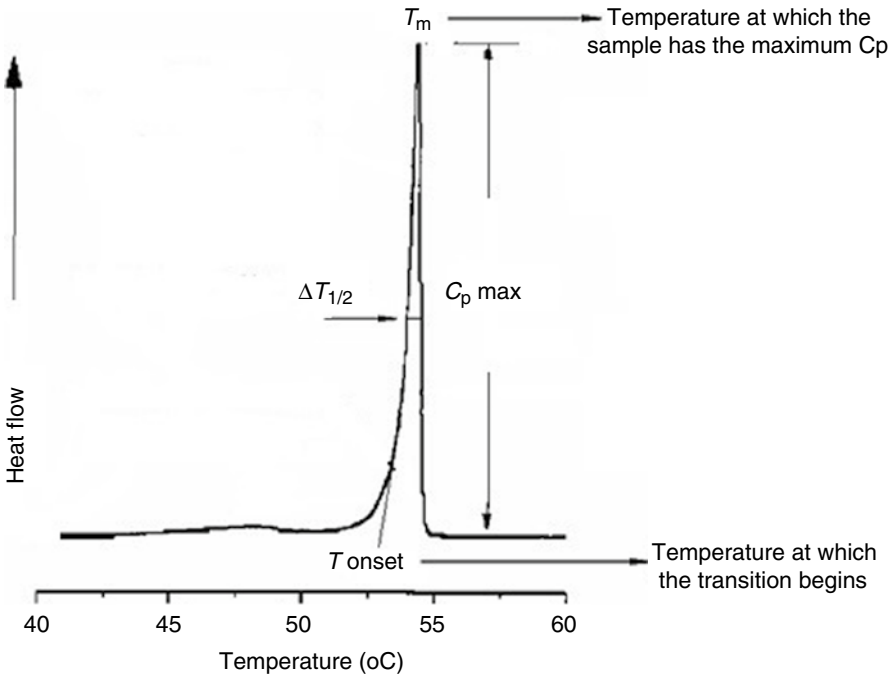

Fig. 4.4 A classical graph of differential scanning calorimetry (DSC) shows the T_m, $\Delta T_{½}$, C_{pmax}, and T_{onset} during the phase transitions of the phospholipid dipalmitoyl phosphatidylcholine (DPPC)

Atomic force microscopy (AFM) is an electron microscopy technique (see Chap. 2) used in the study of the structure of liposomal bilayers, their dynamics, and stability. The latter is studied by the use of AFM, and useful calculations concerning the liposomal morphology [57] can be extracted (Fig. 4.5).

Until recently pharmaceutical formulations containing bioactive molecules of anthracyclines, a class of known anticancer antibiotics, have been approved and used in clinical practice. The great interest in anthracycline entrapment in liposomes came up since during their administration they can cause acute and most importantly accumulative cardiotoxicity in patients. To overcome this problem of cardiotoxicity, the following strategies were followed:

- Extended anthracycline administration in order to avoid acute concentration increase in plasma
- Coadministration of substances that prevent free radical formation
- Development of new anthracyclines
- Anthracycline entrapment in liposomes in order to modify their pharmacokinetics and improve their therapeutic index

The formulations already in the market contain doxorubicin in sterically stabilized liposomes (Doxil®/Caelyx®), doxorubicin in conventional liposomes (Myocet) and daunorubicin in conventional liposomes (DaunoXome®) (Table 4.2).

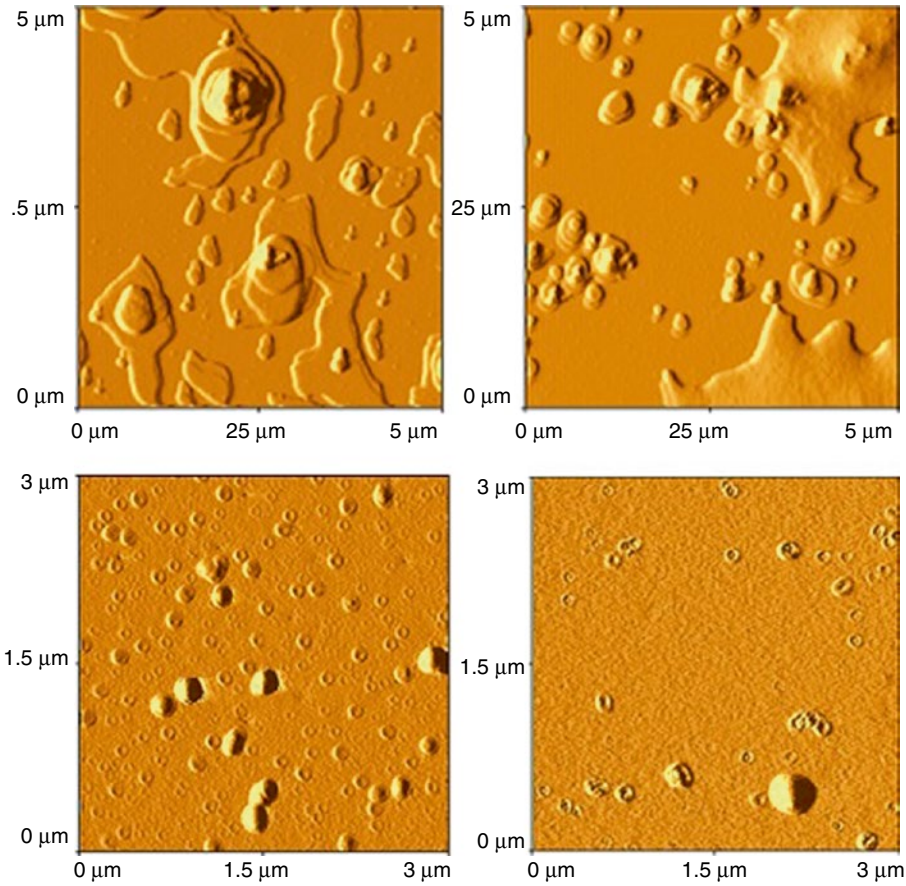

Fig. 4.5 AFM images of lipid bilayers (*top images*) and of liposomes (*down images*) which are composed of egg phosphatidylcholine/dipalmitoyl phosphatidylglycerol (EPC/DPPG) (Adapted from [57]; Cooperation of Laboratory of Pharmaceutical Nanotechnology, Faculty of Pharmacy, University of Athens and the National Technical University of Athens)

These formulations differ in indications, liposome size and lipid composition, and drug release rate. The reason why there are such a small number of liposomal formulations is related to stability and sterility problems as well as final product lifetime. AmBisome® (Gilead Science Inc.) is a liposomal formulation of the polyenic antibiotic amphotericin B. It is a freeze-dried product for intravenous infusion. It consists of hydrogenated soy phosphatidylcholine (HSPC), cholesterol, distearoyl phosphatidylglycerol (DSPG), and α-tocopherol. It is considered as a real liposomal product that consists of unilamellar liposomes with a size of 80 nm. It must also be noted that liposomal product development in large-scale production must follow Good Manufacturing Practices (GMP) as defined from international pharmaceutical control agencies in the United States (Food and Drug administration, FDA) and in Europe (European Medicines Agency, EMA).

Table 4.2 Liposomal medicines in the market

Encapsulated drug	Trade name	Company	Indication	Approval	Innovator company
Amphotericin B	Abelcet	Sigma-Tau PharmaSource, Inc., Indianapolis, IN	Severe fungal infections	1995	The Liposome Company
Amphotericin B	AmBisome	Gilead Sciences, Inc., San Dimas, CA	Severe fungal infections	1997	Vestar
Amphotericin B	Amphotec	Ben Venue Laboratories, Inc., Bedford, OH	Severe fungal infections	1996	Sequus, Pharmaceuticals Inc.
Cytarabine	DepoCyt	Enzon/SkyePharma	Lymphomatous meningitis (intrathecal administration)	1999	Chiron Corporation and SkyePharma
Daunorubicin	DaunoXome	Gilead Sciences, Inc.	Kaposi sarcoma	1996	Gilead
Doxorubicin	Lipodox (generic of Doxil)	TTY Biopharm Company Ltd., Taipei, Taiwan	Kaposi sarcoma, ovarian/breast cancer	2013 (FDA approved; USA)	Sun Pharma
Doxorubicin	Doxil (USA), Caelyx (Europe)	Essex (Europe) Ortho Biotech (USA)	Breast and ovarian cancer, Kaposi sarcoma	1995 (conditional)	Sequus Pharmaceuticals, Inc.
Doxorubicin	Myocet	Novartis Pharma AG, Basel, Switzerland	Breast cancer	2000 (EU)	The Liposome Company
Irinotecan	Onivyde	Merrimack Pharmaceutical Inc. of Cambridge, Massachusetts	Advanced pancreatic cancer	2015 (FDA approved; USA)	Merrimack Pharmaceuticals
Verteporfin	Visudyne	Novartis Pharma AG, Basel, Switzerland	Age-related molecular degeneration, pathologic myopia, ocular histoplasmosis	2000	QLT
Vincristine	Marqibo	Spectrum Pharmaceuticals Inc.	Philadelphia chromosome-negative (Ph–) acute lymphoblastic leukemia (ALL)	2012 (FDA approved; USA)	Inex and Enzon

Quality control during liposome production stages and appraisal and method reliability are extremely difficult and very expensive, especially in the production of medicines where the bioactive molecules are enclosed into liposome membranes. Unfortunately for the liposomal products in the market, industries do not release their full methodology or give incomplete data. Despite all these difficulties, there is a great activity and a lot of interest from pharmaceutical industries to develop new liposomal pharmaceutical formulations since liposomal technology offer solutions in drug administration that presents problems (e.g., paclitaxel) and improves their therapeutic index (Table 4.3). Recently, a liposomal formulation of the anticancer bioactive molecule, i.e., irinotecan, has been approved by the FDA against advanced pancreatic cancer (Onivyde®, Merrimack Pharmaceutical Inc. of Cambridge, Massachusetts) (Table 4.2). So, in the near future, scientists expect the approval of more new liposomal anticancer medications.

Liposomes' most important property – apart from their ability to protect the bioactive molecule from the enzyme degradation, the fact that they have low toxicity without immune response and are biodegradable – is that they can accumulate due to the enhanced permeability and retention effect (EPR effect). This phenomenon is based upon the differences of the tumor and the healthy tissue blood vessel network. So tumor vessels have better permeability since they are developed in greater speed to support tumor's fast development. Apart from this fact, cancer cells are not that thickly

Table 4.3 Liposomal anticancer drugs in clinical phases

Encapsulated drug	Trade name	Company	Indication	Clinical phase
Cisplatin	SPI-077	Sequus	Advanced cancer	I/II
Cisplatin	Lipoplatin	Regulon	Lung cancer	III
Oxaliplatin	Aroplatin	Antigenics	Rectal cancer	II
Vincristine	Marqibo	Inex/Enzon	Non-Hodgkin's lymphomas, acute lymphatic leukemia, Hodgkin's lymphomas (phase II/III) metastatic malignant uveal melanoma (phase III)	II/III
Lurtotecan	OSI0211	OSI	Ovarian cancer, microcellular lung cancer	III
Irinotecan metabolite	SN-38	NeoPharm	Rectal and lung cancer	I/II
Topotecan	INX-0076	Inex	Advanced cancer	I/II
Paclitaxel	LEP ETU	NeoPharm	Breast, ovarian, and lung cancer	I/II
Doxorubicin	ThermoDox	Celsion Corporation, Lawrenceville, NJ	Non-resectable hepatocellular carcinoma	III
Binolerbin	NX-0125	Inex	Advanced cancer	I

placed next to each other, like healthy cells, and the tumor's lymph system that removes substances and nanoparticles (like liposomes) from tissues and organs is insufficiently developed. Therefore, nanoparticles like biological macromolecules or synthetic polymers with a molecular weight greater than 30–40 kDa and liposomes with a diameter up to 600 nm can penetrate tumor blood vessels and accumulate in cancer tissue and not in healthy tissues or organs. The phenomenon is called passive targeting. Liposomal technology has a lot to offer in disease therapy. It must be mentioned that a literature review between 1970 and 2007 related to publications and patents through Scopus TM data (Elsevier B.V) presents 95,082 reports on liposomal technology, 30,979 reports on polymer systems connected with bioactive molecules, and 7453 on copolymers that are used as bioactive molecule delivery systems.

4.1.1.2 Solid Lipid Nanoparticles (SLN)

Solid lipid nanoparticles (SLN) that appeared in bibliography in 1991 are alternative bioactive molecule transfer systems to colloidal nanocarriers, i.e., colloidal dispersions like nanoemulsions, liposomes, and polymeric nanoparticles [58]. These nanoparticles having a size range of 50–1000 nm and the newly categories of nanostructured lipid carriers (NCL) combine more advantages in comparison with classical systems. They can be characterized as safe and effective nanosystems due to biodegradability and biocompatibility. Solid nanoparticles of lipid nature are developed using the homogeneity and microemulsion production method. The lipids of choice are tristearins, stearic acids, cholesterol, and cetyl palmitate. When homogeneity method is applied, the bioactive molecule is dissolved in melted lipids in temperature 5–10 °C over the melting point. The method of thermal homogeneity includes the bioactive molecule dissolution and its dispersion in the melted lipids by continuous mixing in a heated surfactant solution, in the same temperature. The preemulsion that is developed is homogenized to get the nanoemulsion and then left to cool down in room temperature. The parameters that can affect the nanoparticle size and the bioactive molecule entrapment percentage are the following:

- The type of homogeneity
- The homogeneity speed
- The cooling rate in case of thermal homogeneity

The method of cold homogeneity is applied for high-sensitivity hydrophilic bioactive molecules. The lipid particles are dispersed in a cold surfactant solution that is homogenized in a temperature less than room temperature. This process minimizes the lipid melting and, therefore, minimizes the hydrophilic bioactive molecule loss in the water medium.

4.1.1.3 Nanoemulsions

Emulsions are considered as liquid-liquid immiscible dispersion systems. The dispersion phase presents as the low-volume percentage. Emulsions with a dispersion phase particle size of nanoscale are called nanoemulsions [21].

According to the characteristics regarding phase transitions, nanoemulsions can be classified into the following:

- Oil/water (oleum/water, O/W) nanoemulsions
- Water/oil (water/oleum, W/O) nanoemulsions

Nanoemulsions differ not only in dispersion phase particle size but in their properties when related to microemulsions whose particle size is in micrometer (μm).

Nanoemulsion development is not a spontaneous process but requires energy like other nanoparticle categories, i.e., liposomes. So they are characterized as thermodynamically unstable dispersion systems and this relates to their stability. The rational nanoemulsion ingredient choice, the production process, the production temperature, and the raw ingredient concentration, especially the emulsifiers, are critical parameters related to their physicochemical stability and effectiveness.

Nanoemulsions differ from microemulsions since they are transparent (light scattering in a non-visible wavelength) due to dispersed particle nanodimensions and depend on the volume fraction of the dispersion phase. In dimensions greater than nanoscale, dispersion systems are cloudy. An important observation to be mentioned is the surfactant concentration, which in microemulsions is greater than 20 % – nanoemulsions can be produced using surfactant concentrations less than 10 %. The use of nonionic polymers as surfactants is useful in order to avoid and neutralize interaction forces between nanoparticles. It should also be mentioned that the oily phase choice of low viscosity presents advantages of smaller dispersed nanoparticle dimension production in nanoemulsions when compared to oils of high viscosity (triglycerides with long fatty acids).

Generally, nanoemulsion stability studies follow the rules governing the dispersion nanosystem stability studies (see Chap. 2).

The nanoemulsion physicochemical property characterization (size, size distribution, surface charge, ζ-potential, osmolarity, conductivity) involves techniques and observations like the ones presented in the nanosystems mentioned previously (see Chap. 2). For commercial use, long-term stability studies should be performed, especially for their use as drug delivery systems.

Nanoemulsions present advantages in relation to classic emulsions. The most important advantages are the following:

- Due to particle nanoscale, they have a greater outside surface and free energy when compared to common emulsions.
- Do not present the dispersion system's common stability problems.
- Depending on their composition, they can be characterized as nonirritants; therefore, they can be used for skin product development.
- The surfactant choice (that has been approved for human use) allows the parenteral route of administration.

According to all of the advantages mentioned above, nanoemulsions can be used as products for skin care and therefore for cosmetic use. They can be used as lipophilic bioactive molecule carriers due to their lipophilic inside, a fact that makes them more favorable in comparison to liposomes. Their applications are related to their act against bacteria, viruses, fungus, and seeds.

The important antimicrobial action relates to concentrations that do not affect the skin functionality. Last but not least, nanoemulsions can be used in applications like detergents, bioactive molecule delivery systems (parenteral, intravenous, per os, etc.), and intraocular systems.

4.1.2 Polymers

Polymers are substances of high molecular weight, consisted of repeated units called monomers that are connected onto a long chain. Polymer molecules can be linear or branched, while the linear or branched chains can be linked with covalent bonds. Polymers that consisted of the same monomers are called homopolymers. Polymers that consisted of more than one type of monomers are called copolymers [3, 70]. The polymer schematic representation is shown in Fig. 4.6**.** Polymers do not form perfect crystals but have semicrystalline and amorphous areas (Fig. 4.7).

Various copolymer types can be consisted of monomers with different orientation. Therefore:

- Various monomers can be oriented in linear chain in random or specific alteration along the chain.
- Linear polymer chains can be in monomer block systems called block copolymers. These can be:
  - Diblock copolymers: AB diblocks
  - Triblock copolymers: ABA or BAB triblocks like poloxamers where the chain A is polyethylene or chain B is polyoxypropylene

$$\mathrm{HO}\left(\mathrm{CH_2CH_2O}\right)_x-\left(\mathrm{CH}\left(\mathrm{CH_3}\right)\mathrm{CH_2O}\right)_y-\left(\mathrm{CHCH_2O}\right)_x-\mathrm{H}$$

– where x and y are the monomer numbers in each block.

Fig. 4.6 Structures of homopolymers and copolymers composed monomers A and/or B. (*1* linear homopolymer, *2* alternating copolymer, *3* random copolymer, *4* block copolymer, *5* graft copolymers) (https://en.wikipedia.org/wiki/Copolymer#/media/File:Copolymers.svg)

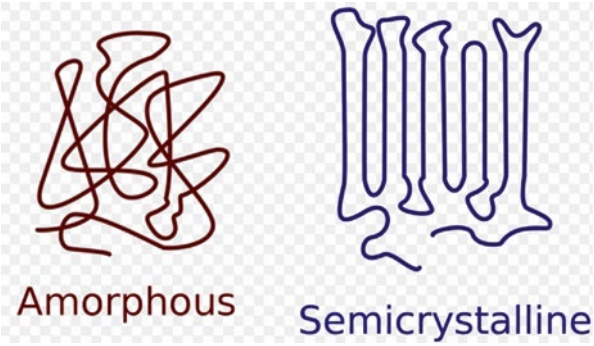

Fig. 4.7 Schematic representation of an amorphous and semicrystalline polymer (https://en.wikipedia.org/wiki/Crystallization_of_polymers#/media/File:Polymerketten_-_amorph_und_kristallinEN.svg)

The chains can be composed of repeated units from a monomer on which there are grafted chains of a second monomer, like a brush. These polymers are called grafted copolymers [78, 86].

The polymer melting point cannot be absolutely defined, like in low molecular weight crystalline solids due to areas not perfectly structured that are melted in a temperature range. Also, polymer melting point presents variation from the glass transition temperature T_g (g: glass). In a temperature lower than the glass transition temperature (T_g), polymer chains are rigid-immobile and the polymer is glassy and fragile.

In temperatures above the T_g, polymer chains present mobility. Polymers are products that are widely used in the pharmaceutical industry in various pharmacotechnological formulations and in bioactive molecule delivery system coating. Polymer behavior is directly related to their chemical structure. Also, their abilities depend on the way that monomers are connected to each other. Polymers can have linear or branched chains that may cross each other. Copolymers are composed of more than one monomer and develop new polymers with completely new properties. An important polymer application is the genetic material DNA transfer. Concluding the polymer section, important facts for their development must be mentioned:

- Possible toxicity due to cationic polymer use that will develop DNA complexes.
- System physicochemical instability during storage and agglomeration development results in polymer nanoparticle changes in size and size distribution.
- Minimization of cell target transfection ability.
- System stability problems while in the organism.
- Possible final product high-cost production in industrial scale (scale-up).

A particular class of polymers is the class of polyelectrolytes. A polyelectrolyte, according to IUPAC, is considered to be a macromolecule that has ionic groups or groups that are able to ionize [35]. In other words, a polyelectrolyte is a polymer that consists of one repeating ionizable group along their backbone. This group can dissociate in a polar solvent such as water and leave counterions resulting in an opposite charge on the backbone. Polyelectrolyte block copolymers constitute an

intriguing class of bio-inspired macromolecules, as they combine the structural properties of amphiphilic block copolymers, polyelectrolytes, and surfactants and provide various possibilities for use as nanostructural delivery platforms of genes and proteins/peptides, through electrostatic complexation of the pharmaceutical agent. The polyelectrolytes' solution shows particular properties and behavior that are different from those exhibited in neutral polymer solutions and solutions of electrolytes [69]. They are water soluble and are considered to be promising drug delivery systems especially therapeutic biomolecules like protein and peptides (see Chap. 5) [10]. They are also used as stabilizing agents in colloidal dispersions and as rheology modifiers as well as in pharmaceutics, as suspending agents [11].

It is important to mention that DNA and RNA are polyanions due to their negatively charged phosphate esters in their backbone [8] and are belonging to the biological polyelectrolytes. Several other biological molecules such as proteins and polysaccharides are charged macromolecules with essential functions for the living organisms. Association of biological polyelectrolytes with synthetic polyelectrolytes or polyelectrolyte complexes is of great importance in drug delivery and in biopharmaceuticals [49]. The application of polyion complex micelles into therapeutic fields is rapidly increasing due to the simple and efficient encapsulation of biopharmaceuticals like insulin, lysozyme, antitumor peptides, and DNA/RNA and outstanding biocompatibility among various polymer-based nanocarrier delivery platforms. Proteins and polyelectrolytes interact, primarily via electrostatic interactions, to form hybrid complexes, which can have widely varied stoichiometries, morphologies, architectures, and shapes. These mixed systems are bio-functional with potential applications in the design and development of delivery systems of biopharmaceuticals.

Polylysine-based block copolymers are suitable nanocarriers to transport and deliver proteins, peptides, and genes. These mixed biomaterials exhibit both pH- and temperature-responsive behaviors and self-assembly properties and are used as nanocarriers with enhanced properties. According to Lee and Kataoka [43], ionic biopharmaceuticals, such as genes and proteins, can interact with ionic block copolymers to form polyion complex micelles with core-shell morphology. Insulin is an attractive biomolecule to encapsulate into polyion complex micelles. It is a polyampholyte with isoelectric point at pH 5.5 and can be either positively or negatively charged due to both basic and acidic groups. The encapsulation of insulin into nanoparticles is a promising strategy that has been developed in order to enhance its absorption and its bioavailability, aiming a successful delivery. Several approaches have been employed in order to realize effective insulin formulations. The structural analysis of insulin in pharmaceutical formulations recently appeared in the literature. An ideal insulin carrier should have reasonably high protein encapsulation efficiency and loading capacity and sustained/controlled release of the loaded protein while retaining bioactivity [7, 30, 79].

4.1.2.1 Polymersomes

The evolutions in the field of controlled polymerization techniques have allowed the design of a new category of amphiphilic membranes composed of block copolymers.

Block copolymers are macromolecules that have one or more polymer types as mentioned earlier. This combination of various polymers resulted in new properties. Polymer amphiphilic bonds have the ability of self-assembly into complex membranes offering stability and improved mechanical properties in comparison to the conventional phospholipid membranes. The simplest structure that these membranes can form is a *pseudo-spherical* shell known as polymersome [84]. Polymersomes are interesting structures that can be self-assembled and can be used for bioactive molecule delivery to the damaged tissues. Their ability to encapsulate hydrophilic molecules and integrate hydrophobic bioactive molecules makes them attractive delivery systems. But they demand specific physicochemical parameter values for their development and a specific ratio of hydrophilic/hydrophobic section for, i.e., a block copolymer to form vesicular structures.

Biophysically speaking, polymersomes – and liposomes as mentioned before – are interesting biological membrane simulation systems (membrane mimics), while intermembrane proteins can be integrated with reconstitution methods leading to proteopolymersomes [59]. On the other side, amphiphilic block copolymers have been widely studied for biomedical and pharmaceutical applications from the controlled, sustained, delayed-release advanced technology to membrane-mimic and bioactive molecule transport.

Polymersomes with dimensions varying from 10 nm to 10 μm have a relatively high control on size distribution around the average value of the dispersion medium. Polymersomes have drowned scientists' attention to study innovative nanosystems for various applications in different scientific fields. More in specific in the field of pharmaceutics, polymersomes have the ability of incorporating a wide range of bioactive molecules and biomolecules and offer control over their release rate. Amphiphilic molecule (like phospholipids) self-assembly with various chemical structures is related to their geometrical characteristics (Thompson 2012). The geometry of these structures is defined from the proportion of hydrophobic-hydrophilic section of the amphiphilic molecule. This self-assembly amphiphilic molecule approach through geometric characterization has facilitated the understanding not only of phospholipids but of low molecular weight surfactants as well. The high molecular weight molecules like amphiphilic block copolymers, polypeptides. etc. can be designed to have the same amphiphilic character as phospholipids and surfactants but also composed of polymer chains covalently linked in series of two or more blocks. It is known that block copolymers can be organized in a great structure range, for example, in lamellar structures. Lamellar structure hydration with water solution results in a steady dispersion of amphiphile block copolymers. Proportional to the packing parameter S_p (see Chap. 4), the factor F corresponds to the hydrophilic fraction and determines the morphology of copolymers formed in aqueous media. In the case of polymersomes (unilamellar polymer vesicles), F is between 25 and 40 %.

Initial copolymer studies with F values that form polymersomes having a molecular weight (MW) between 2000 and ~20,000 Da have shown through cryogenic transmission electron microscopy (cryo-TEM) that the membrane core (d) increases by molecular weight increase from d ~8–21 nm. Simulations that took place revealed that, only in small molecular weight systems, the polymorphic bilayers show a clear

average level of high density that strongly reminds "methyl through" like the one appearing in lipid bilayers. For the high molecular weight copolymers, the two layers of the lipid bilayer are mixed or melted together in a homogenous thick shell. It is possible to produce diblock copolymers, triblock copolymers, multiblock copolymers, and grafted copolymers by controlling their synthesis. This observed chemical architecture variety can be of use when designing various membrane polymersomes with various structures. Polymersomes produced from AB diblock copolymers present interconnected membrane. The powerful entanglement inside the hydrophobic layer can be granted as physical crossed connection enhancing their mechanical strength in comparison to conventional liposomes.

Triblock copolymers BAB, i.e., hydrophobic-hydrophilic-hydrophobic copolymers, are similar with diblock copolymers since there is only one molecular structure that leads to membrane development: hydrophobic parts of the chain are put together to form the membrane and the hydrophilic diblock forms a U-shaped loop. BAB diblock copolymers cannot form a loop where the two hydrophobic diblocks link at the same part of the hydrophilic diblock. The membrane structure can be additionally evolved by using multiblocks that have various polymers and are subjected into phase separation after assemblage. When the proportion of hydrophobic/hydrophilic section favors the membrane development, ABC copolymers are formed into asymmetric membranes that form polymersomes whose inner and outer chemistry is different in order to minimize the surface tension and enhance the vesicle curvature (Fig. 4.8). Polymersomes, when making block copolymers, assemble in lyotropic phases of the liquid crystalline phase, like reverse hexagonal structures, lamellar, hexagonal perforated membranes, *sponge phases*, etc. Figure 4.9 shows a detailed representation where the formed structure is presented as a proportion to the polymersome molecular weight and concentration in water. The polymersome size significantly affects the lyotropic phase development and the dispersed vesicle structures. The evolution from solid to lyotropic liquid crystal shows that small molecular weight copolymers are formed initially in reversible hexagonal structure and then in lamellar, while the high molecular weight copolymers are dissolved directly to lamellar structure. The transition from lamellar to *sponge phase* is not affected by the amphiphilic molecule molecular weight; instead the transition from sponginess structure to vesicles is quantitatively different and depends on the amphiphilic diblock copolymer size. Smaller copolymers form dispersed vesicles, while high molecular weight copolymers initially form vesicle gel bluster diblocks that are finally disrupted in dispersed vesicles. Amphiphilic molecules' molecular weight affects the nature of the developed vesicles. Large amphiphilic molecules form exclusively unilamellar vesicles and smaller copolymers form multilamellar vesicles. Copolymers' molecular weight does not only affect phase transition but the kinetic transition from phase to phase. One of the most important diblock copolymer clusters is their non-ergodic nature, for their formation in organized structures. This practically means that there is no structural unit exchange (polymeric chains) between micelles/vesicles and solution, underlying a critical agglomeration concentration near zero. This is a desired property for polymersomes and causes long lifetime.

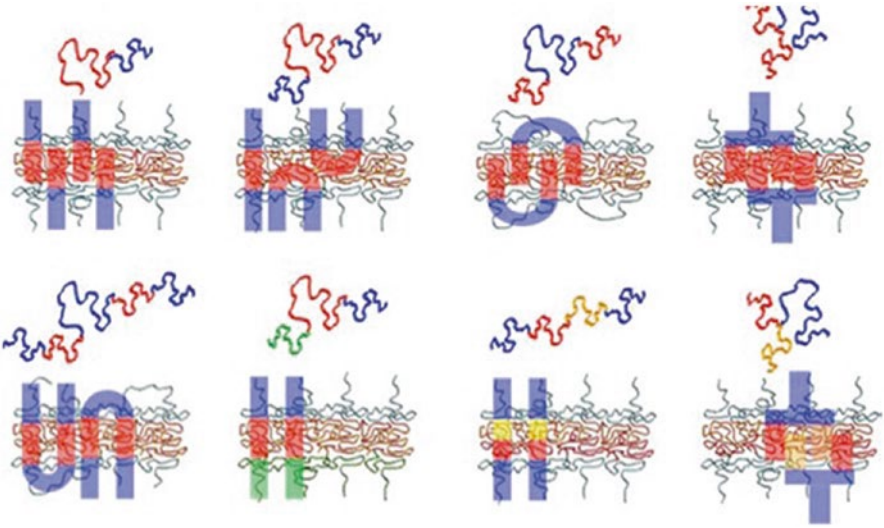

Fig. 4.8 Membrane structure of polymersomes formed by diblock (AB), triblock (ABA, BAB, ABC), multiblock copolymers and micto-arm copolymers (Adapted from [50] with permission from Springer)

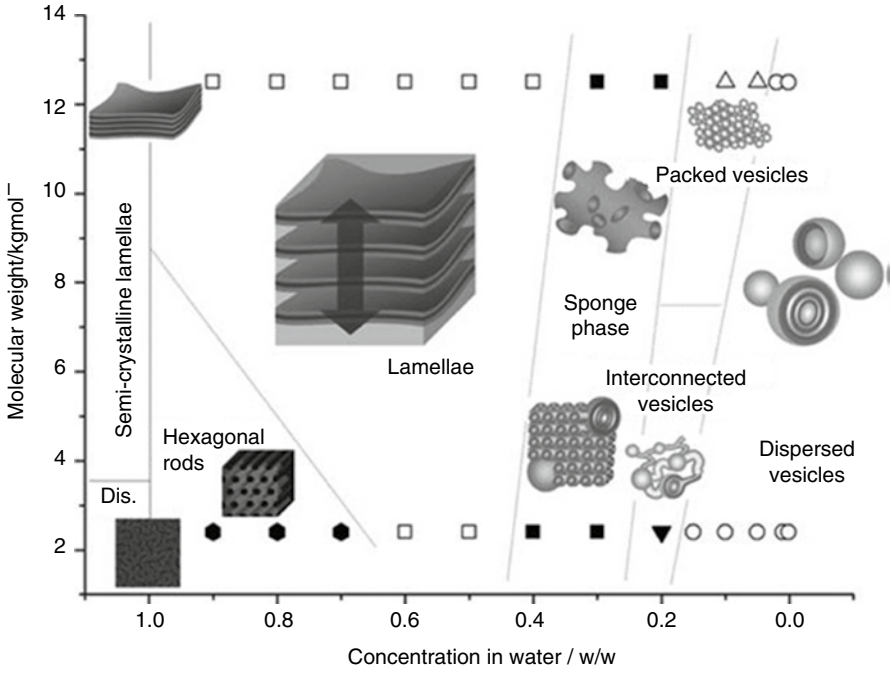

Fig. 4.9 Phase diagram of copolymer in water (Adapted from [50] with permission from Springer)

Polymersomes that form diblock copolymers swell when in contact with water in two different phases. Initially, the water and copolymer are diffused into each another resulting in amphiphilic membrane molecular setup. After a critical time where the polymer's molecular weight is exponentially changed, the amphiphilic membranes will gain balanced structure, and therefore, the formation will follow Fick's diffusion. This kind of complex movement shows why polymersomes were studied later than copolymer micelles. Polymersome production method is presented in the Appendix.

Polymersome Size and Size Distribution Appraisal

Polymersome size and size distribution are critical parameters to study in order to use polymersomes as bioactive molecule carrier and delivery nanosystems. Polymersome size and size distribution appraisal define in vitro and in vivo nanoparticle effectiveness. In vivo, the size defines the nanosystem circulation times and their ability to reach specific targets, the extravasation possibility, their properties, and their final decomposition and clearance. It is not yet completely understood which parameters affect polymersome size and size distribution.

Regarding polymersome formation, it was observed that the proportion of hydrophobic/hydrophilic copolymer section seems like micelle structure (i.e., the copolymer is mostly hydrophilic), the mean vesicle diameter decreases to three times down. This is attributed to the curve formed and it is obvious that the more hydrophilic the copolymer is, the more curved and stable the polymer structures are. On the contrary, crystalline development can show that the polymersome size is reversely proportional to the copolymer concentration. Under mixing conditions, where the concentration is high, polymersomes appear to be in nanometer-size diameter, while in mild mixing conditions, their diameter is in μm size. Experimentally, polymersome size is strictly defined from the method used for their development (Fig. 4.10).

Polymersome Properties and Applications

Polymersomes have the ability to integrate into their membrane an important amount of hydrophobic bioactive molecules. This integration prevents self-aggregation that would normally take place when these molecules were in solution in their free form and protects them from interactions with biological components.

The polymersome amphiphilic nature allows the intake and integration of amphiphilic molecules on their membrane. Polymersome macromolecular nature makes them especially resistant in detergent dissolution, and therefore, the surfactant integration is proposed to be on the polymersome membrane to avoid collapse. Ruysschaert and coworkers [71] observed that this hybrid structure helps implementing phospholipids inside polymersomes, a fact that offers them advantages related to their physicochemical properties and greater effectiveness as bioactive molecule transports. Possibly, the most important example of amphiphilic molecule stabilization is the use of polymersomes for cell membrane protein scaffold. The ability of hydrophilic molecule entrapment into polymersomes is an important

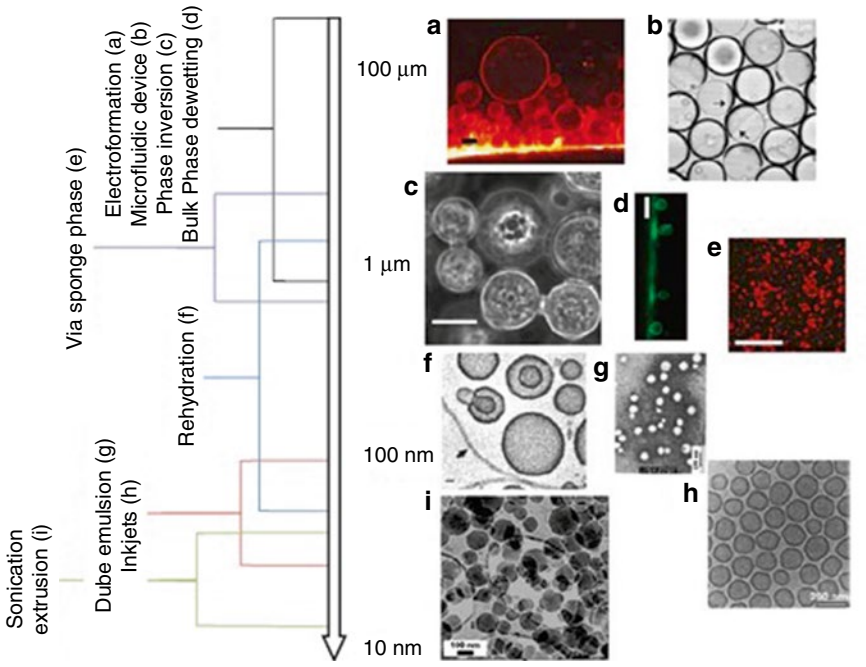

Fig. 4.10 Schematic representation of the size distribution of polymersomes, which depends on their manufacture methodology (Adapted from [50] with permission from Springer)

advantage for their transport and delivery to damaged tissues. Despite all the above, the hydrophobic or amphiphilic molecule entrapment is more or less simple; the complex kinetics when forming polymersomes prevent hydrophilic molecule entrapment. The most important parameter regarding hydrophilic molecule entrapment is the polymer membrane permeability. One of the advantages that polymersomes have is that polymer solution properties are strictly checked according to side groups' chemistry. The same polymer chain has both soluble and insoluble parts and their equilibrium defines total solubility. Therefore, polymer solubility can change from outer stimulants like temperature, pH, ionic strength, light, etc. This polymersome response to the outside environment has been used in polymer mechanics, and many devices have been developed based on polymer solubility. In polymersomes, these properties can be used for structure development that will be dissolved under specific environmental conditions. The simplest approach has been made with hydrophilic polymer connection with hydrolyzed polymers. In contact with water, polymers will be degraded by nonreversible polymersome disassembly and content release. The degradation ability after a period of time or an external stimulant is of critical importance for new carriers and for the modified polymersome diameter development. Reversible disassembly can take place when combining hydrophilic polymers with polymers presenting pH, temperature, and radiation-dependent solubility. For example, polymersomes with structural units of poly(L-glutamic acid)-poly(L-lysine) that respond to pH can be modified reversibly

into weak acidic or basic water solutions. Lastly, similar transition can take place by theuseofsensitivetoradiationgroupslikepoly(ethyleneoxide)-poly(methylphenylsilane) (PEO-PMPS) and azobenzene-containing poly(methacrylate)-poly(acrylic acid) (PAA-PAzoMA). Recently, Mabrouk and his coworkers [48] published structures of asymmetrical polymersomes whose membrane consisted only of one lamellar copolymer sensitive to radiation: poly(ethyleneglycol)-poly(4butyloxy-2-(4-(methacryloyloxy)butyloxy)-4 (4-butyloxybenzoyloxy)azobenzene (PEG-b-PMAazo444). When exposed to radiation, these polymersomes burst. This fact is attributed to thermodynamic changes related to membrane curve.

Polymersomes' Surface Chemistry

Polymersome membrane is the result of amphiphilic diblock copolymer auto-assembly in water, as mentioned earlier. Also chemical structure can be used offering the necessary hydrophilic/hydrophobic conditions to preserve the assembly. As mentioned before, hydrophilic blocks concentrate in brush configurations, which according to polymer nature will modify-control the polymersome surface characteristics and its interaction with the environment. As an example, it can be mentioned that block copolymers have polyethylene oxide (PEO) that assemble in polymersomes with highly hydrated and neutral polymeric brush orientation, having little protein interactions. This orientation allows PEO polymersome structural integrity in biological fluids without immune systems interactions.

Their ability to escape the immune system is known as "stealth" and has been widely used to increase liposomes and other nanoparticles lifetime in the organism. The brush thickness and structure are critical parameters for polymersome stealth ability, as Photos and coworkers [67] have proven since they found different circulation times for different molecular weight PEO polymersomes. Polymers of the polyethylene glycol (PEG) category and other non-fouling polymers are acceptable for biological applications. It has been recently proven that polymersome surface can be additionally modified with molecules characterized as islands and have different chemistry. This is made possible by mixing various copolymers developed to produce polymersomes that separate phases of polymer-polymer. In a second level of complexity, polymersome surface can withhold macromolecular structure biomaterials like proteins, antibodies, vitamins, hydrocarbons, etc. Biotin groups' connection with hydrophilic diblocks is used to group together abidines to predefined polymersomes that will later on connect to biotinyl targeting ligand (since each abidine group has four connection spots for biotin). Using a similar approach, polymersomes with anti-ICAM-1 antibodies have been tested for inflamed endothelial cell treatment.

Polymersome Applications

Some important polymersome applications concern the in vivo tissue imaging by using near infrared (NIR) and protein, DNA, and bioactive anticancer molecule entrapment

on their inside. Apart from an exceptional delivery and a bioactive molecule release system, polymersomes seem to be equally useful in imaging applications.

Additionally, polymersomes offer the ability of hydrophobic and hydrophilic bioactive molecule incorporation, addition of targeting macromolecules on their outer surface, as mentioned and analyzed earlier on, forming the nanocarrier for damaged tissue targeting according to "combined drug delivery" and magnetic resonance imaging (MRI). Another polymersome application is their use as protein incorporation systems, as mentioned earlier. Recent studies on this field concern primary human antibody delivery using fluorine-labeled antibodies. Gene therapy is another important research field mostly due to the polymersome ability to replace or exclude specific gene expression. Intracellular genetic material delivery lacks effectiveness due to their negative charge and size since repulsive interactions exist with the negatively charged plasma cell membrane. Many methods have been explored to avoid interaction with cell membrane by using gene transport carriers inside the cell. Some of these methods include polymersome use [59].

4.1.2.2 Biodegradable Polymeric Nanoparticles

Polymeric nanoparticles are solid, colloidal particles of 10–10,000 nm size. The bioactive molecule can be entrapped inside the nanoparticle, absorbed, or connected on its surface. According to the production method used, polymer nanoparticles, nanospheres, and nanocapsules have different properties and characteristics that affect bioactive molecule release. Nanocapsules are systems where the bioactive molecule is on the inside surrounded by a polymer membrane. Polymeric nanospheres consisted of a matrix where the bioactive molecule is dispersed. The advantages of using polymeric nanoparticles for bioactive molecule administration and transfer are due to their basic properties: small size to achieve small blood vessel penetration, taken over by cells and accumulate in target areas. The use of biodegradable materials allows bioactive molecule steady release rate in the target tissue for a period of time equals days or even weeks.

4.1.2.3 Polymeric Micelles

Amphiphilic copolymers in water solutions can self-assemble, like previously mentioned and form micelles (Fig. 4.11). Polymer micelles have nanometer size, with a hydrophobic core (where lipophilic molecules can be attached) and hydrophilic parts that help in the steady dispersion development in water. Their distribution in the organism depends on its surface properties. Polymer micelles due to their small size (<100 nm) are not recognized from the MPS and can be accumulated in the damaged tissue environment with passive diffusion. Surface macromolecules, like antibodies, can connect on polymer micelles and produce immunomicelles that will present selectivity. Polymeric micelles present advantages over conventional micelles that are

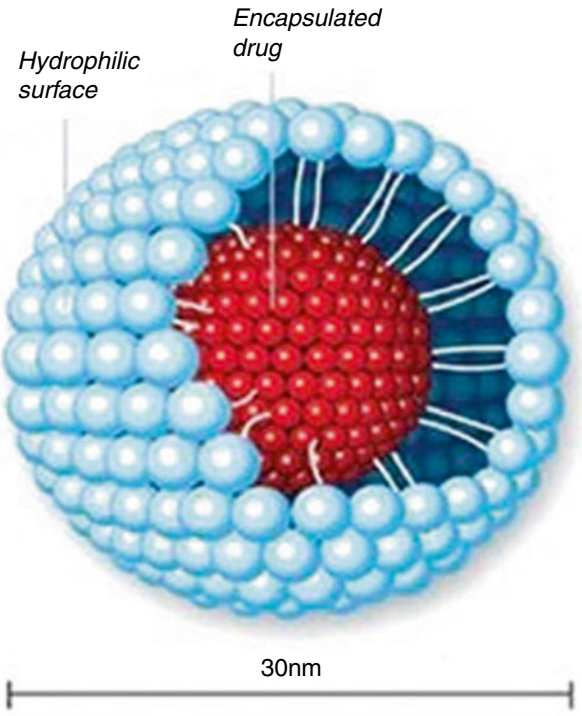

Fig. 4.11 Polymeric micelles (Adapted from www.atrp.gatech.edu/pt18-3/18-3_p3 with modifications)

formed from surfactants because they have better thermodynamic stability in biological solutions, resulting in their slow in vitro disintegration [86].

4.1.2.4 Nanogels

Nanogels are swollen nanosized networks consisted of hydrophilic or amphiphilic polymer chains. Nanogels can protect and transfer bioactive molecules and therapeutic nucleotides and control their release by integrating highly familiar functional groups that correspond in configuration and biodegradable links in the polymer network. Like other nanosystems, nanogels can be easily administered as liquid pharmacotechnological formulations for parenteral administration. Nanoparticle size offers high special surface that is available for bioconjugation with factors targeting special macromolecular targets on damaged tissue surface.

4.1.2.5 Dendrimers

Dendrimers belong to polymers of the fourth generation that were firstly formed in the beginning of the 1980s and are consisted of long chains connected to a central tube.

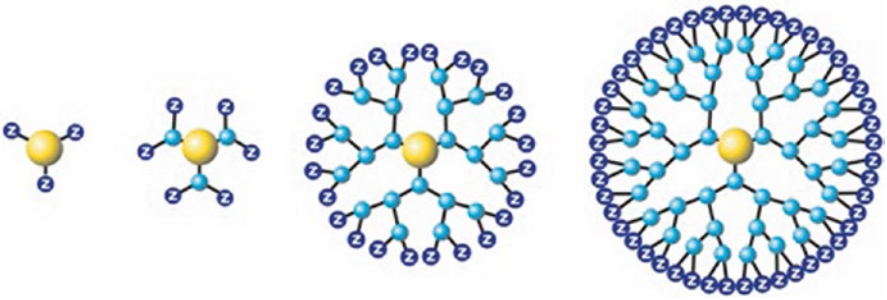

Fig. 4.12 Development of generations of dendrimers (http://www.chemheritage.org/discover/online-resources/chemistry-in-history/themes/microelectronics-and-nanotechnology/tomalia.aspx)

They are structured around a central core and are composed of repeated structural units (Figs. 4.12 and 4.13). They are attractive bioactive molecule transfer nanosystems due to their small size (<10 nm) and the multiple functional groups that can be attached on their surface. Dendrimer interaction with biological environment is defined from their end groups. Dendrimers have special properties due to their shape that looks like a tree and their inner cavities. Bioactive molecules can be enclosed in dendrimer cavities or connect to final surface groups [17, 18, 36]. The term dendrimer comes from the Greek word *δένδρο* (*dendron* (=tree) and *μέρος meros* (=part) and describes a new group of branched macromolecules whose architecture looks like a tree. In 1985 Donald Tomalia and his coworkers [80] published the composition and the full characterization of a new macromolecular group of the poly(amidoamines) [Poly(amidoamine)(PAMAM)] calling them dendrimers. Until this day, the mostly studied dendrimer group is PAMAM generation that is the most commercially used for research applications. Many new dendrimers are composed of various structural units. Dendrimers have an important research interest as these branched polymers of nanometer size offer conventional methodology of bioactive molecule transfer and delivery, i.e., their structural unit chemical control, their molecular weight, their surface characteristics, and biological targeting process control [81–83].

In comparison to conventional linear polymers, dendrimers have specific dimensions, almost spherical shape and molecular weight and not molecular size distribution. In contrast with other polymers, the critical parameters in nanoscale, like size, shape, and final active groups, can be accurately controlled through their architecture, i.e., core, internal branching units, and final surface groups. These properties are not the sum of the monomer properties but are completely different and follow different rules. The term dendrimer phenomenon is used to describe these unexpected dendrimer properties. Dendrimers, as mentioned earlier, have branched units with end chemical groups that have various properties, allowing charge, hydrophilicity, or hydrophobicity alteration. Dendrimer size is extremely small, 2–10 nm, and therefore can be characterized as true nanoparticles. Their dimensions cannot be extremely large because of their branching unit stereochemistry. Branched polymers presented in the 1960s were the precursors of dendrimers since their branched units are simple branched polymers and can be characterized from their molecular

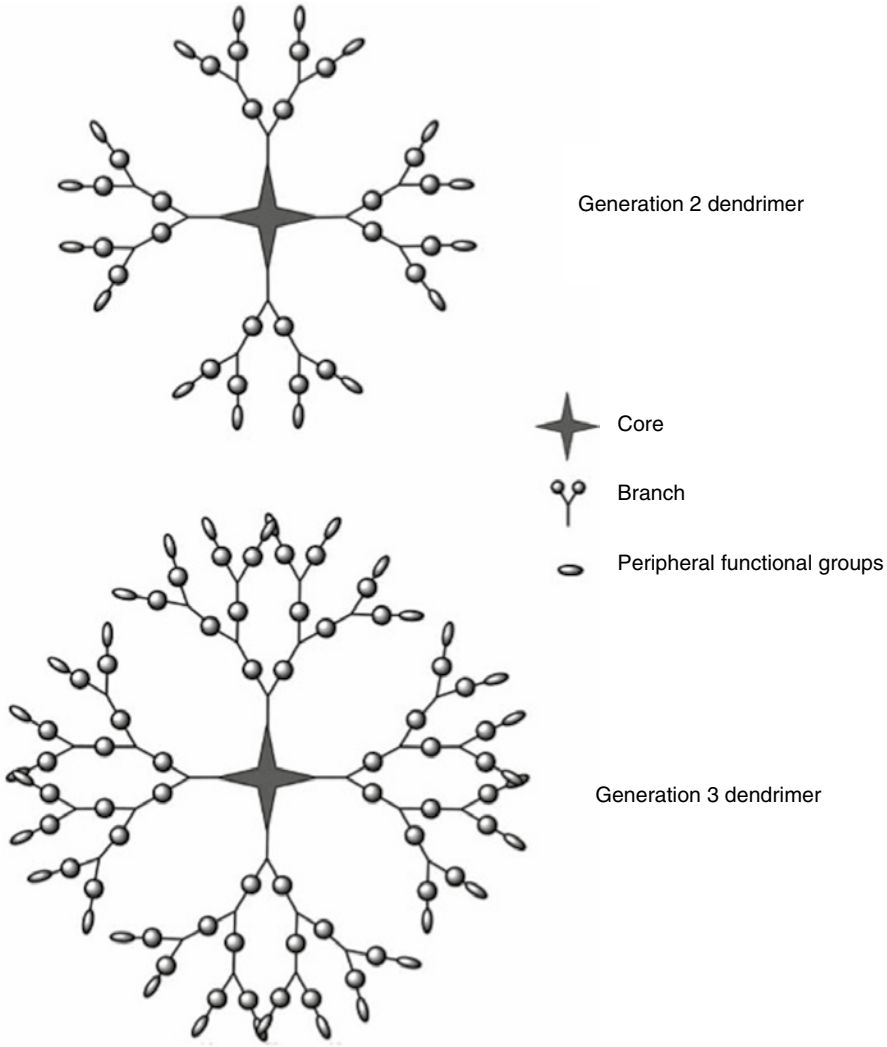

Fig. 4.13 Dendrimer structures (generations 2 and 3) which present the cores, the branches, and the peripheral functional groups (Adapted from [24] with permission from Bentham Science Publishers)

weight, structure, and physicochemical characteristics according to technology evolution and analytical assays developed in the past decade. Biologically speaking, dendrimers seem to present architectural similarities with macromolecules like proteins, polysaccharides, and nucleic acids.

Dendrimers are composed of three distinct areas:

- The core where all the branching units start from
- The branching units that compose the dendrimer
- The outer surface where the active groups are placed

The core and the repeated units define the microenvironment inside the molecules, while the surface groups define the solubility and the physical and chemical interactions with the environment. Therefore, the combination of these three areas defines the physical and chemical properties of the dendrimer molecule. Since the innovative works of Tomalia [80] and Newkome [61] which were published independently, the research in this field created intense interest and concentrated on dendrimer design and development with specific physical and chemical properties, aiming on their application on fields of great scientific and commercial interest such as biomedicine.

Dendrimer Use in Biomedicine

There are a large number of applications of dendrimeric structures in biomedicine with emphasis to drug delivery and imaging. However, dendrimers are used for diagnostic and therapeutic purposes like:

- In diagnostics – Gd^{III} block dendrimers are used in magnetic resonance imaging (MRI).
- In DNA biosensors.
- In therapeutics as controlled release drug delivery.
- In gene therapy (gene transfection).
- In cancer therapy with ^{10}B (boron neutron capture therapy).
- As antimicrobial and antivirus medication.

The advantage of dendrimer use as bioactive molecule carriers is based upon their structure control which is a result of their controlled production. This controlled synthesis allows molecule production with special properties, like hydrophilic groups on the outer surface and internal hydrophobic cavities that offer the possibility of different chemical molecules' entrapment. The dendrimer family that has been studied the most as a bioactive molecule carrier is the PAMAM.

Tomalia [81] and other scientific groups have studied the relation between structure and biocompatibility of PAMAM derivatives. They observed that cationic dendrimers (that have NH_2 groups on their surface) are generally toxic and cause hemolysis even in low concentrations. Also, they observed that toxicity increases in greater generations, i.e., seventh generation is much more toxic than fifth or third generation. In contrary, anionic dendrimers (that have COOH groups on their surface) do not present toxicity. Despite these, negatively charged carboxyl dendrimers are not appropriate for delivery systems as they cannot interact or connect with the negatively charged surface. To overcome this problem, PAMAM derivatives have been developed that had hydroxyl groups on their surface, or polyethylene glycols (PEG) chains were connected to outer groups and used as anticancer bioactive molecule entrapment.

Another dendrimer class that has been studied and developed is poly(ester) dendrimers, firstly produced by Frechet [23, 34]. Poly(ester) dendrimers that are usually asymmetric can enclose anticancer molecules like doxorubicin. Also, the

development of a liposomal nanosystem and dendrimer (PAMAM) has been tested for the entrapment of a large quantity of the anticancer agent methotrexate and doxorubicin. Two different ways of using dendrimers as bioactive molecule carriers have been described: the bioactive molecule entrapment inside the dendrimer (inside the cavities formed from the branches) through hydrophobic interactions and ionic links and the covalent bonding of the bioactive molecule on the dendrimer surface. The development of a multifunctional dendrimer consisted of PAMAM G5 generation connected with the anticancer agent methotrexate or paclitaxel, folic acid, and fluorescein as detection molecules for dendrimer course into the organism is a contribution to nanosystem development that has targeting, imaging, and therapeutic functions against cancer at the same time. It is well established that cancer cells due to their quick multiplication need large quantities of folic acid. They achieve that by increasing the folic acid receptors on their surface. So, by taking on folic acid that is connected to the dendrimer, anticancer cells receive at the same time the bioactive molecule. Lastly, the fluorescent molecule allows dendrimer tracking. The first in vitro experimental results in cancer cells that overexpress folic acid receptors are encouraging. Some of the bioactive molecules enclosed in or connected with dendrimers are indomethacin, methotrexate, doxorubicin, fluorouracil, paclitaxel, and ibuprofen. Dendrimer synthesis is analyzed in the Appendix of this chapter.

Dendrimer Applications

There are more than 50 dendrimer families, each one with unique properties like surface, internal cavities, and core that can be adjusted in various applications. Polymer multiple possible applications are based on their molecular uniformity, multifunctional surface, and internal cavities. All the above properties make dendrimers appropriate for high technology applications in the fields of biomedicine and industry. More in detail:

- Dendrimers have been used in in vitro diagnostics.
- Dendrimers have been used in preclinical studies.
- There are research efforts for dendrimer use in the delivery of bioactive molecule target tissues. Bioactive molecules can be equally incorporated inside dendrimers or connect to outer groups.
- Dendrimers can be used in industrial processes' improvements.
- Dendrimers have been used as carriers known as "vectors" in gene therapy.

Dendrimers belong to a chemical group that presents special characteristics resulting in a great variety of applications. They belong to nanotechnological systems, and their basic characteristic is the lack of polydispersity due to absolute controlled synthetic reaction process that leads to polymer systems of specific molecular weight. Their almost spherical outer surface allows many outside groups with various functions (targeting, detection, delivery of bioactive molecule), while cavities formed inside can enclose bioactive molecules. The application of dendrimers has been studied in several fields of modern technology. It is important to

figure out that the profile of a candidate drug is affected by its solubility in aqueous media, its lipophilicity, degree of ionization, pharmacokinetics and pharmacodynamics, permeability, and its behavior regarding the binding process with plasma proteins. As dendrimers belong to the class of polymers, polymer therapeutics is a term that describes nanosized polymeric carriers that are in clinical phases and practice for diseases such as multiple sclerosis, renal failure, and virucide vaginal formulations [87].

Dendrimers have been used for most hydrophobic anticancer molecule dissolution due to their ability to produce a great amount of hydrogen bonds in water environment. In relation to other nanotechnological systems, liposomes and polymers have been used in order to take advantage of the EPR phenomenon (see Chap. 6). This in vivo anticancer action and the simultaneous toxicity reduction of the known chemotherapeutic agents like doxorubicin and cisplatin when incorporated in dendrimers were attributed to the enhanced permeability and retention (EPR) phenomenon. Poly(glycerol-succinic acid) dendrimers have been used to dissolve 10-hydroxycamptothecin. The system effectiveness was increased in human breast cancer series, but the bioactive molecule quick release (6 h) excluded its systemic administration. Important entrapment percentages in various dendrimer structures have been achieved for anticancer molecule etoposide, paclitaxel, methotrexate, and 6-mercaptopurine. The hydrophobic anticancer molecule entrapment in middle size dendrimers (G4–G6 generation) has been proven to increase their solubility and toxicity. The most basic disadvantage is the lack of kinetic controlled release since these systems release their content in a few hours after systemic administration. Possibly, dendrimer systems with incorporated anticancer agents can be used in the future for infusion directly to the tumor. The method of bioactive molecule covalent bonding on dendrimer surface offers advantages in comparison to their cavities. Different bioactive molecules can be connected to a dendrimer molecule and their release depends in the linkers' nature. With this method, methotrexate, paclitaxel, doxorubicin, and 5-fluorouracil have been covalently bonded with dendrimers of the polyamidoamine group (PAMAM). In all occasions, the results showed effectiveness sustenance, delayed release, selective accumulation on cancer cell, and lower toxicity. Dendrimers can be applied as viruses' inhibitors, and their activities has been shown against HSV, RSV, and HIV, while a dendrimeric formulation as vaginal gel (i.e., VivaGel™, Starpharma) is in clinical trials.

Active Targeting

Taking full advantage of dendrimer architecture allows simultaneous bioactive molecule and target group connection on the same dendrimer molecule. In the field of oncology, targeted transport of chemotherapeutic molecules in cancer cells is translated as side effect reduction since healthy tissues like the liver, spleen, and bone marrow can accumulate bioactive molecule toxic levels. For dendrimer system active targeting, monoclonal antibodies, PEG chains, and imaging agents with the encapsulated drug have been used for the production of multifunctional dendrimers

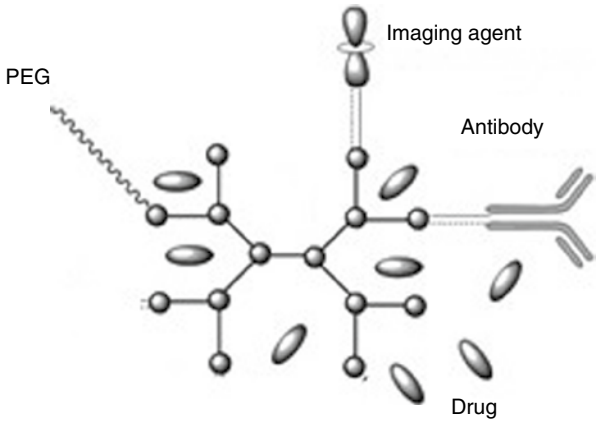

Fig. 4.14 Schematic representation of multifunctional dendrimers carrying on their surface antibodies, PEG, and imaging agents with encapsulated drug

(Fig. 4.14). Dendrimer great surface in relation to their size and high solubility makes them useful as catalysts since they can combine the advantages of homogenous and heterogeneous catalysts.

Dendrimer Application in Imaging with Magnetic Resonance Imaging Technique

An important diagnostic method is the magnetic resonance imaging (MRI) that is the spectroscopy application of the nuclear magnetic resonance (NMR) that offers three-dimensional imaging of the living organs and their blood vessels. The use of paramagnetic metal cations (enhancer parameters) improves the method sensitivity and accuracy. A very common enhancer parameter, the gadolinium salt diethylene triamine pentaacetatic acid (DTPA), is unfortunately diffused outside the blood vessels due to its low molecular mass. To overcome this problem, scientists studied dendrimers with gadolinium salts on their outer region. These studies showed that the modifications of dendrimer characteristics, like size and nuclear or regional composition, can lead to the development of enhancer factors that will be specialized for specific organ imaging or lymph node blood vessel imaging. Additionally, the use of dendrimers makes targeted delivery possible since the dendrimer molecular structure can be transferred to cancer tissues making dendrimers useful tools in cancer imaging. An example of dendrimer application in cancer treatment is in boron neuron capture therapy (BNCT), an experimental method of two steps. Initially, the patient is injected with a boron 10B radioactive isotope which selectively accumulates in cancer cells. Following, the patient receives radiation of a low-energy neutron neutral beam. Neutrons respond to boron inside the tumor and produce α-particles that destroy only tumor cells leaving intact the healthy cells. For this process to be effective, high concentration of 10B inside the cancer cells is needed. This can be achieved by the use of boron incorporated dendrimers and only if they can deliver it to cancer cells.

4.2 Therapeutics and Nanotechnology

Pharmaceutical sciences have a great interest in developing new nanotechnology products, understanding the new material behavior through the development of analytical assays and the possibility of measuring and taking pictures of products and devices in nanoscale. In the field of health, the problems of medicine's safety and efficacy, on time diagnosis and therapeutic confrontation of many diseases, like cancer, lead to the need of maximizing knowledge, aiming to the maximum development in therapeutic potential of the existing bioactive molecules [54].

The National Cancer Institute (NCI) and the US National Institutes of Health (NIH) under the Cancer Nanotechnology Project – a strategic initiative that targets the change in clinical oncology and basic research with immediate application of nanotechnology achievements – have completed the program by entering nanotechnology in biomedical research with encouraging results and started a new program applying nanotechnological achievements in cancer diagnosis, treatment, and prevention, with an ultimate goal of the reduction of deaths caused by cancer. The methodology for decision implementation includes finance securing for exchanging scientific opinions and researchers of all grades between universities and institutes in the fields of clinical oncology, molecular biology, and nanotechnology. The development of nanotechnology, electronics, and robotics is expected to offer important advantages in biomedical applications, like genome therapy, drug administration, imaging, and new drug discovery techniques.

Pharmaceutical nanotechnology orients in developing and evaluating bioactive molecule colloidal system transport, diagnostics, and imaging materials offering solutions in fields like:

- Imaging and diagnosis of diseases like cancer, in early stages.
- Developing systems for evaluating treatment effectiveness in real time.
- Multifunctional systems deliver bioactive molecules to damaged tissues in high concentrations.
- Nanomaterials that can detect molecular changes to prevent cancer metastasis or precancer cell transport. Innovative bioactive molecule delivery that can be used for central nervous system diseases with targeted imaging biomaterials.
- Uncontrolled cell multiplication and gene indicator (promoting cancer cells) detection systems.
- Research tool development that offer possibilities like new target cell identification and possible drug resistance detection.
- Study and development of new nanodimensional herbal medicinal products by improving their solubility in biological fluids.
- Alternative nanotechnological ways for insulin delivery.
- Applications in dental treatment by developing and evaluating new nanodimensional materials of high durability and biocompatibility.

For the pharmaceutical industry, these new technologies that deliver bioactive molecules are a strategic tool for their development. Technology can offer new

solutions for the already existing bioactive molecules, increasing their effectiveness, improving their safety, and offering better patient compliance. Moreover, new drugs being developed under computational chemistry, using the knowledge gained from the human genome decoding program, need administration and delivery to the damaged tissues systems in order to be effective. Nanotechnology offers the possibility of administering bioactive molecules that are practically insoluble to water or unstable to biological environment. Designing and developing delivery nanosystems that target damaged tissues are an emerging direction in therapy. Even though nanotechnology offers the possibility of developing effective delivery systems for all kinds of bioactive molecules, the major directions are the developments in drug administration in diseases of the respiratory, the central nervous, and the cardiovascular system.

The advantages of nanotechnology in pharmaceutical sciences come as a result of controlling the nanomaterials' physicochemical properties and biological action. The cooperation of nanotechnology and biotechnology can lead to the development of nanodevices and nanosystems for studying the physiological processes of the living matter. The most important biomaterial in living organisms is their genetic material and mainly the DNA – also proteins and other biomolecules/biomaterials that participate actively in the existence of the living matter and its functions. The transport of the genetic material that is complexed with nanoparticles can offer solutions in genetic diseases and create appropriate conditions for the development of nanosystems that transfer and deliver genetic material with advantages in the genome therapy.

Nanoparticles consisted from polymers, synthetic or natural, from naturally occurring or synthetic lipids or from inorganic materials. Bioactive molecules that transfer nanoparticles can interact with them (covalent bonds or electrostatic forces) or encapsulate them in order to transfer them to the target tissues. The biomaterials mostly used for biodegradable nanoparticles and nanosystem production are polyacrylates, especially the quickly biodegradable butyl cyanoacrylate, gelatin, polylactic acid, and the polylactic acid copolymers.

Also, molecules of lipid nature that are compatible with human organism and biodegradable are used for nanosystem development, i.e., cholesterol, lipid acids, and phospholipids. The problem arising during bioactive molecule transport is their recognition from the MPS that detects them as foreign biomaterials and destroys them through opsonins' detection mechanism.

Opsonins are proteins functioning as flags, detecting the foreign-unknown biomaterials that are then detected from the macrophages of the MPS and are destroyed. Therefore, their long period of time stay in blood circulation that aims to the bioactive molecule transport to target cells is related to their surface physicochemical properties and their size. Changing the surface characteristics leads to behavior change and possibly reticuloendothelial system (RES) detection while particles of greater size like micro-sized systems or nanosized systems are detected and destroyed. Modern bioactive molecule nanocarriers have the ability of increasing the therapeutic index of the existing drugs by lowering the toxicity in tissues and achieving control release therapeutic levels for a prolonged period of time. Nanocarriers can

increase the solubility of lipophilic bioactive molecules and improve their stability and therefore allowing the use of bioactive molecules that were rejected during preclinical and clinical studies due to short pharmacokinetics and biochemical and physicochemical properties. Lastly, nanotechnology can help in the development of multifunctional nanosystems that transfer bioactive molecules to the targeted area and nanosystems having diagnostic and therapeutic properties at the same time, allowing the simultaneous diagnosis and treatment of the damaged tissue. The multifunctional nanoparticle whose development in the future will offer the possibility of many independent functions. Nanosystems that can be characterized as nanorobotics are very difficult to be developed due to their complexity of their physicochemical properties and their *thermodynamic abnormalities* that lead to their instability. Despite all that, modern technology moves toward that direction. Target detection can occur in organ level or even in a single surface macromolecule target that is specific for some cells like surface antigens. The most common form of identification is the identification in molecular level that is based on the fact that each organ's or tissues' special macromolecules (antigens) can be detected. To achieve that targeting macromolecules can be used, i.e., antibodies capable to interact with a target expressing a special macromolecule (antigen). Nowadays, many protocols for the development and evaluation of innovative medicines are being developed and have various approaches. In some occasions, for damaged tissue targeting, many physiological characteristics of the targeted area can be used. In basic protocols of targeted therapy that have already been used experimentally or in clinical trials, the following approaches are included: the approach of the bioactive molecule to the damaged tissue; the passive accumulation through structural and functional abnormalities of the vessels that appear in areas with tumors, heart attacks, or inflammations; the natural target that is based on the difference of a physical parameter (i.e., pH or temperature) in the target area (such as tumors or inflammation); the magnetic targeting that is connected to paramagnetic carriers, with the effect of outside magnetic fields; and the active target using molecule carrier that are specially related to the damaged tissue or the cancer cell. There are already synthetic nanoparticles (liposomes, nanocapsules, nanospheres, dendrimers, etc.) that can target and enter damaged tissue cells releasing the bioactive molecules. Nowadays nanopharmaceutics and nanomedicine lead to the development of products that offer great advantages and effectiveness. The majority of the candidate bioactive molecules present low solubility in water, preventing their effectiveness. Research has shown that when the bioactive molecules are encapsulated in nanoparticles that have the appropriate physicochemical properties especially on their surface, preventing their aggregation, a stable and effective nanosystem carrier of the bioactive molecule is developed. The nanocrystal technology that big pharmaceutical industries in the United States apply has led to the development of therapeutic products [rapamycin (Rapamune® Pfizer) and aprepitant (Emend® Merck & Co.)] that are based on this technology.

The development of nanotechnology, electronics, and robotics is expected to offer great advantages in biomedical applications like gene therapy, bioactive molecule transport, imaging, and diagnostic techniques. Many bioactive molecules are ineffective due to limited access to target tissue. Nowadays, the major directions of

pharmaceutical and medical research are therapeutics, lab diagnostics, and in vivo imaging diagnostics. There is an important effort in developing innovative medicines according to the interdisciplinary path of nanotechnology for the therapeutic approach for many diseases.

4.2.1 Nanotechnology and Cancer

During the past decades, there is an important progress in understanding and in description of the carcinogenesis mechanisms, while important diagnostic tools have been developed for damage tissue imaging and treatment. Despite this progress, the universal cancer mortality is one of the most important causes of death.

The in-depth knowledge of genetic and industrial changes that are responsible for cancer cell development has changed the therapeutic confrontation strategy. During the past years, new methods in diagnostics are developed that aim to the early malignant diagnosis and its therapeutic confrontation. Scientists have also understood that the microenvironment of cancer cells affects the treatment and provokes drug effectiveness.

Therefore, even if normal cells that neighbor cancer cells do not present changes in their genetic material; can change their physiological activity because they are surrounded and close to the cancer cells. Understanding the microenvironment around cancer cells, and not only their evolution and their behavior, is crucial in order to understand their development and design a strategy that scientists need to follow to develop effective anticancer agents targeting the cancer tumor and the environment where it grows. Moreover, a multifunctional system is developed that includes physical chemistry, biochemistry, and biophysics whose parameters should be evaluated in order to choose the therapeutic strategy. The factors and the procedures of this multifunctional system can be classified as follows:

- Factors related with the development and the multiplication of cancer cells and should be controlled.
- Physiological factors controlling the development and the multiplication according to the genetic information that cancer cells do not respond.
- Cancer cells do not obey the apoptosis procedure (programmed cell death).
- Development of vessel network process (angiogenesis) around tumor to provide oxygen and nutrients.
- Cancer cell metastasis process from the original tumor that results to death of 90 % of the patients.

It is scientifically proven that cancer therapy should be according to its complexity. The recognition of this complexity and the understanding of parameters and processes related to it have created new trends to the development of innovative medicines that are now designed according to the “system” cancer with the parameters and processes that define it. Nowadays, cancer therapeutics is oriented in the interdisciplinary cooperation and new technologies, with which we can detect

sooner the "trace," understand the microenvironment, and design the bioactive molecule transfer, delivery, and target system. Dr. Andrew C. von Eschenbach, director of the National Cancer Institute (NCI), has set a target: to reach cancer therapy until 2015 or, more rationally, to develop effective therapeutic protocols. For this reason, the scientific alliance *NCI Alliance for Nanotechnology in Cancer* was founded in the United States that methodizes the tools and practices related to cancer prevention, diagnosis, and therapeutics with the means that nanotechnology can offer. This aims to bring in collaboration the physical, chemical, biological, and medicinal scientific community in a coordinated effort, so all nanotechnology benefits are directed to cancer patients [54].

NCI in the *NCI Alliance for Nanotechnology in Cancer* and other European-related initiatives emphasize the following sectors:

- Cancer prevention and control. Includes the nanodevice development for delivery of bioactive molecules to target tissues by using liposomes, dendrimers, nanoemulsions, etc. Complex anticancer vaccines will be synthesized using nanodevices for their systemic administration.
- Early diagnostics and proteomics. Implanted stable molecular sensors will be developed in order to find bio-indicators. These biosensors will be evaluated in situ or ex vivo, and the results will be transmitted through wireless technology to medicinal personnel and other databases.
- Imaging diagnostics. Aiming toward sensitive and precise imaging and "smart" injectable nanoparticles that will allow cancer tissue analysis cell by cell to be developed. Nanodevices analyzing the biological diversity of tumor's cancer cell will be also developed.
- Multifunctional therapies. There is a great need of nanosystems that will incorporate a combination of diagnostic and therapeutic functions. For example, nanocrystals can be used for both bioactive molecule transport and performing tissue imaging at the same time. Toward this direction, scientists are designing the development of smart devices in the near future that will control the bioactive molecule release in the particular place and time and evaluate the effectiveness of the treatment at the same time.
- Quality of life improvement during chemotherapy. Nanosystems for the treatment of nausea, pain, appetite loss, and fatigue will be designed.
- Interdisciplinary education. Nanotechnology success is relied upon the scientists' ability from various fields to interact and communicate effectively with each other. Steps like education for chemical engineers, physicists, and chemists on molecular and system biology as well as education of scientists on nanotechnology are very important.

The combination of the above scientific fields has led to the introduction of the term "nano-oncology" in the literature world [28]. Nano-oncology is divided into five basic fields: bio-imaging, gene therapy, thermal ablation, immunotherapy, and drug delivery.

The developments in managing genetic material and the scientific directions concerning its transport are related to the gene expression control during transcription,

translation, and replacement of defective genes with compensatory genes. Despite the many possibilities of gene therapy, its clinical practice has important problems. More than 400 clinical studies have been evaluated during the past 15 years, and most of them have failed to achieve the desired results.

Here we can refer the liposomal vectors that are able to transport genes. It is well established that there are identified huge number of genes that are able to correct diseased phenotypes. Several delivery systems that are defined as nonviral vectors such as micelles, emulsions, and lipidic vectors have been used for gene delivery. A suitable nonviral vector should be stable and biocompatible to efficiently deliver genes to specific tissues, upon administration. The viral vectors despite their high efficiency in transfecting cells present a numerous problems like immunogenicity, toxicity, and difficulties in the scale-up process. However, the exploration of nonviral vectors is a demand. Liposomal vehicles could be systems that offer advantages because of their easy and safe administration by several routes such as i.p., i.v., etc. DNA can covalently attach on the surface of cationic liposome created complexes that are known as *lipoplexes* or can be accommodated into anionic liposomal vehicles. We have to keep in mind that the route of administration plays a key role in the in vivo results. Gene therapy is an emerging scientific field, and liposomes are considered as such nonviral vectors that could play a role in the development and evaluation process in gene therapy [45].

The effective gene therapy demands the combination of two different factors:

- A therapeutic gene that can be expressed in the target cell
- A safe – regarding the gene material safety – and effective delivery system that can transfer the gene in a specific tissue or organ

Even though impressive results have been published and presented when a therapeutic gene is injected on cancer tissue, its systemic administration has been proven to be especially difficult since most systems degrade in biological medium or excreted from the kidneys before reaching the target. Viruses, retroviruses, and adenoviruses that have presented infectious abilities are now abandoned because of the difficulty in incorporating into a large amount of genetic material and the safety matters related to carcinogenesis and immunostimulation. The synthesis of polymer nanosystems that transfer and deliver genetic material seems helpful in the development of innovative nanosystem that will be biocompatible, biodegradable, and effective. Some categories of polymer nanocarriers are the following:

- Poly-L-lysine.
- Synthetic biodegradable polycations.
- Chitosan.
- Cyclodextrins [55].
- Polymeric micelles (see Chap. 4). They have been used in research for gene transport and can be further divided in micelles composed of polyethylene glycol-polyester, polyethylene glycol-poly(amino acid), and polyethyleneglycol-polysaccharide lipid.
- Dendrimers.

Many studies have used dendrimers as gene material vectors through cell membrane to cell core. Liposomes and modified viruses have been extensively used for this reason. During the past years, PAMAM dendrimers have been tested and found to be very stable. They can carry great amount of genetic material in relation to viruses and are more effective than liposomes since they have a strictly defined structure and low pK amine values that help pH stabilization inside the human body. For this reason, PAMAM dendrimers tend to be established in an effective category of polycation synthetic dendrimers for gene transport.

To achieve local hyperthermia in order to destroy cancer cells is a subject that scientists study the past years but presents important problems. A basic problem occurring is the fact that the source of thermal energy might harm the surrounding healthy tissue even in case of targeted radiation. To resolve this problem, materials that selectively heat the tumor using gold nanoparticles absorbing near infrared have been developed. These systems are called nanoshells (see Chap. 3) and consisted of a silicon core surrounded by a thin gold shell. Nanoshells absorb heat (they are heated) during radiation in appropriate wavelength exposure. The characteristics of near infrared radiation have been chosen because the tissue absorbance in this wavelength is in minimum state while light permeation is in maximum state. To achieve hyperthermia, carbon nanotubes (see Chap. 3) have been used (tube structures with one carbon atom wall thickness) after the addition of specific antibodies. The results in breast cancer series were encouraging.

An important chapter in therapeutics is immune response. It has been proven that it can contribute in small tumor termination. But cancer cells have the ability to respond by developing mechanisms that provoke the organism's antigen ability. Research scientists try to develop immune system activation mechanisms against tumors that will possibly give the organism the ability to destroy cancer cells. Tumor antigens are not immune and if administered as vaccines can strengthen the immune system against cancer. The new vaccine toward this direction connects antigens with nanospheres. To increase the effectiveness, nanospheres must have a specific diameter (40–50 nm) and narrow size distribution that will allow them to target dendrimer cells of the lymph node.

The effectiveness of bioactive molecules that are administered to destroy cancer cells can be improved by using nanotechnological approaches. The design of appropriate nanosystems for bioactive molecule transport and delivery is an important research field that aims the development of *Trojan horses* that will not be detected from our immune system and will not affect healthy tissue cells. More in particularly, the following conditions must apply for the cytostatic bioactive molecules:

- Adequate bioactive molecule concentration in biological fluids will allow the effective concentration on the cancer tissue. For greater safety, the concentration of the bioactive molecule free form that is not loaded in the nanosystem must be the smallest possible in the biological fluids.
- The bioactive molecule should have high differential toxicity against cancer cells or at least a favorable therapeutic window.

Nanotechnology research aims to the points mentioned above using the special characteristics of cancer cells. These characteristics allow the passive and active nanosystem targeting cancer tumors.

High heterogeneity in cancer tumor vascularization includes areas of vascular necrosis and areas of high vascularization from where oxygen and nutrients reach cancer tissue. Cancer blood vessels present abnormalities regarding the corresponding normal blood vessels like high proportion of endothelial cells presenting abnormalities in the cell membrane, high blood vessel bending, and defects in the pericytes. Cancer capillary vessels present increased penetration that is mainly regulated by the abnormal secretion of the endothelial vessel growth factor, bradykinin, nitrogen monoxide, prostaglandins, and metal proteins.

Macromolecule transport in tumor microcirculation can take place through interendothelial connections and endothelial channels. The molecular exclusion level of these transport channels is less than 1 μm, and in vivo extravasation studies of liposomes in cancer xenografts have shown molecular exclusion of 400 nm. Generally particle extravasation is inversely proportional to size, and smaller particles (<200 nm) have shown to be more effective for tumor microcirculation extravasation. Lymph cancer network is also defective resulting in fluid retention and increased pressure relative to the fluid flow outside the tissue.

The absence of an intact lymphatic system results in nanocarrier's retention in the intracellular space. The combination of deficient microcirculation and the lack of an intact lymphatic system results into the enhanced permeation and retention (EPR) effect. Nanotechnology takes advantage of this effect for passive cancer targeting through nanosystem concentration into the cancer tissue at significantly higher levels compared with the plasma or healthy tissues. The bioactive molecule release from the nanosystem results in its relatively increased concentration in the tumor as well as the increased toxicity against cancer cells.

The modification of nanosystems' outside surface aiming the cancer tissue active targeting can be achieved by using antibodies, peptides, and small molecules that recognize special cancer antigens in tumor microenvironment. When these nanoparticles are directed at the outside section of the intramembrane cancer antigens, they are possibly taken over from the cancer cell through enhanced receptor endocytosis.

Known anticancer drugs like dimeric indole alkaloids, camptothecin, lignans derivatives, taxanes, and many more are the modern armory against cancer. Questions arising during treatment are the following:

- Why are current treatments not effective and most of the times fail?
- Why cannot we improve or completely eliminate drug toxicity and avoid toxic side effects?
- How can we achieve greater efficacy since the discoveries in the field of cancer molecular approach have noted gene expression in cancer cells leading to specialized protein production?

Current therapies fail to destroy compact tumors for three reasons:

1. At the state of diagnosis, the tumor is already well developed. A tumor of 1 cm^3 size (the smallest clinically traceable tumor) contains one billion cells. To achieve

complete therapy, all cells must be destroyed. Even if we manage to destroy 99.9 % of those cells, one million of living cancer cells will remain in the organism.

2. Fifty percent of patients that will surgically remove the tumor will not be cured due to metastasis. During metastasis, the genetically modified cells will expand from their initial place and through circulation will be installed in new places like the liver and lungs. Usually, metastasis is not traceable due to their small size (<5 mm) and can remain inactive for many years.
3. The third and greatest obstacle in achieving successful treatment is tumor heterogeneity. Tumor contains cells with different genetic materials and biochemical, immune, and biological characteristics. Cells can vary according to surface cell receptors, enzymes, karyotype, morphology, recycle time, sensitivity in various therapeutic factors, and metastasis ability. This heterogeneity sets a limitation in surgery and therapy to achieve total tumor cell destruction. Apart from all the above, especially in the colon, kidneys, and adrenal gland tumors, the gene of P-glycoprotein is overexpressed resulting in tumor drug resistance.

Drug resistance is due to mrd-1 glycoprotein that carries cytotoxic drugs from the inside of the cell through adenosine triphosphate (ATP). It is believed that tumor cell compact structure is another obstacle for drug transportation into the tumor. Compact tumors do not have sufficient lymph system and as a result there is an elevated pressure in tumor's center. It is believed that the inside increased pressure in combination with the fast and abnormal tumor cell increase is responsible for the compaction and the blood vessel exclusion. After entering the organism, the bioactive molecule is not selectively accumulated in the damaged tissue but is distributed in various organs and tissues (depending on the bioactive molecule nature and administration route).

Despite all these, in order to reach its target (organ, tissue), it must come through many biological barriers, like other organs, cell membranes, and intercellular compartments where it can be disabled or present side effect in organs and tissues that are not involved in the pathological process. Therefore, in order to achieve the desired therapeutic concentration in the desired area in the organism, we must administer a great concentration of the bioactive molecule, which most of it will be lost in healthy tissues.

Furthermore, cytotoxic drugs have side effects as they cannot discriminate between healthy and tumorous cells (i.e., the most commonly used anticancer bioactive molecule, doxorubicin, presents cardiotoxicity). During the last decades, efforts are made to resolve the problems above through targeting of the bioactive molecules to the damaged tissues. Generally speaking, bioactive molecule targeting can be defined as the ability to accumulate into the organ target selectively and quantifically despite the route and method of administration. The bioactive molecule local concentration must be greater in the damaged area, while its concentration in the rest of the organs and tissues should not be above a certain level, preventing or limiting side effects.

For target therapy, the following conditions should apply:

- Drug administration protocols should be simple.
- The drug concentration needed for therapeutic result should be as small as possible, to prevent toxicity to healthy tissues.
- Bioactive molecule concentration in target tissue should have the ability to increase enough, without causing side effects in the rest of the tissues.

The theory of target therapy was introduced by Paul Ehrlich 100 years ago, and it involved a hypothetical *magic bullet* with two components: the first should detect the target and attach to it and the second should have the therapeutic effect.

Nowadays the theory of the *magic bullet* to target the damaged tissues involves different ingredients. The most common form of damaged tissue identification is the molecular level identification and is based on the fact that in every organ or tissue special macromolecules (antigens) can be found and only expressed in the particular organ. To achieve targeting, biomolecules/biomaterials can be used that are capable of selectively interacting with the target (e.g., special antibodies for the corresponding antigens).

Nowadays many protocols for targeted therapy are being developed and include a variation of approaches. The basic schemes of a therapeutic approach that have been already used in the lab or in clinical practice include the following:

- The bioactive molecule direct application at the area of the damaged organ (tissue)
- Its passive accumulation through the "incompatibilities-abnormalities" appearing in the vessels next to cancer tumors
- The target process that is based on a physical parameter (i.e., pH or temperature) at the target area (like tumor or inflammation)
- The magnetic targeting of nanosystems that are connected with paramagnetic materials, influenced by external magnetic fields
- The active targeting using molecule carriers that are specially related to the damaged tissue or cancer cell

There are many nanosystems (i.e., liposomes, nanocapsules, nanospheres) that can target cancer cells, enter inside their membrane, and release the bioactive molecules.

4.2.2 Nanobiotechnology

Nanobiotechnology is the interdisciplinary field of nanotechnology with biological systems and includes biophysics data that are described in Chap. 2. In the science of biology, many of the organism's biological structures and biomaterials have the same evolution and development with nanotechnology. The combination of these two fields is the interdisciplinary field of nanobiotechnology aiming at the nanosystem development from materials that possess both properties of nanomaterials and biomaterials, applied in disease therapeutics, the development of "green" energy, and the elimination of environmental pollutants.

The relation between biology and nanotechnology can be found in processes that are common in both scientific fields for the system development and the organization (bio- and nano-, respectively). The basic procedure reported in biological systems and nanosystems is self-assembly that controls various processes kinetically and thermodynamically. The most important biomaterial used by biomedical engineering, the scientific environment of nanobiotechnology in the field of health that combines the biological properties with the nanomaterial properties, is the genetic

material, the DNA. DNA is implicated in numerous biological functions through biochemical paths while, because it is a nanomaterial, we can approach its physical properties through the science of biophysics. Recently, literature suggested that the genetic material plays an important biophysical role in physical phenomena with possible future biological applications. More in particularly, Kunming Xu [90] suggested at his work entitled *Stepwise Oscillatory Circuits of DNA Molecule* published in the *Journal of Biological Physics*, 2009 that the DNA biomolecule functions as a stepwise oscillatory circuit of electromagnetic radiation. One DNA molecule is characterized as a stepwise oscillatory circuit of electromagnetic radiation, where each base pair is a capacitor, each phospho group acts like an electric self-induction, and each desoxyribose acts as an electric switch. The circuit calculates the DNA conductance through charge rebounds between smaller and greater distances according to the experimental results that lead to many stages of mechanical rebounds. Therefore, in a charge rebound that is opposite to the phenomenon, the circuit acts according to the charge transfer mechanisms that reflect the genetic material credibility in electron transfer. The stepwise oscillatory charge transfer through DNA sequence is the one controlling the oscillatory frequency. Another approach of biophysical background concerns the electromagnetic signals that are developed from water nanostructures derived from bacterial DNA nucleotide sequences. Montagnier and his partners published a study presenting a new DNA property that is based in some bacterial sequence property to produce electromagnetic waves in high concentration water solutions. This appears to be a coordination phenomenon due to the low-frequency electromagnetic field wave that is applied. Most pathogenic organisms' DNA contains sequences that produce these signals. This phenomenon gives rise to the development of high-sensitivity detection systems for chronic bacterial infections in human and animal diseases. Most pathogenic organisms' DNA contains sequences that are able to produce these signals. This phenomenon raises new possibilities for the development of high-sensitivity tracking systems in both human and animal chronic bacterial infections. From all the examples mentioned above, the contribution of biophysics in the study of materials that structure the living organisms and are involved in numerous biochemical paths that lead to biological processes is observed. Classic physics, through its principles and laws, offers tools that can be used in understanding the behavior of these materials, not as chemical macromolecules, but as material sections with such a behavior that is based on their physical abilities like electrical conductance and electromagnetic radiation emission. Modern physics, especially quantum mechanics, has set a framework for the DNA behavior interpretation, one single nanobiomaterial that codes the genetic information.

4.2.3 Nanogenomics and Nanoproteomics

The term nanogenomics can be defined as the nanobiotechnology application in an organism's genetic material study. Some of the technologies used when studying the genetic material are presented in Table 4.4**.** Also, important technologies for the

Table 4.4 Examples of application of nanoparticles in gene therapy

Nanoparticle	Application
Poly(D,L-lactide-co-glycolide) nanoparticles with entrapped stem p53 DNA	Inhibition of cellular proliferation in cancers due to sustained expression gene with consequent release of intracellular p53
Intravenous administration of liposomal complexed form with composition of DOTAP: Chol-FUSI for repression of FUSI gene	Inhibition of tumor growth in mouse models with metastatic lung cancer
Cationic gelatin nanoparticles	Nonviral and nontoxic vectors for gene therapy
Calcium phosphate nanoparticles	Nonviral carriers for targeted therapy
Nonionic polymeric micelles composed of poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide)	Gene transfer (gene) in the gastrointestinal tract using oral administration in laboratory animals (mice)
Nanocomposite materials: titanium oxide nanoparticles in combination with DNA oligonucleotides which are activated by light or radiation	Genes encoding antibodies may be transferred to a particular intracellular target and in combination with radiation therapy aimed at killing cancer cells in patients
Combination of gene nanoparticles and surfactants	Gene transfer to the brain through the blood-brain barrier permeation
Integrin-targeted nanoparticles	Targeted delivery of anticancer drugs
DNA nanoparticles (20–25 nm): each DNA molecule covered with positively charged peptides	Crossing nanoparticles by nuclear passages (pores) with millions folds to facilitate gene expression compared to the non-genomic DNA. Used for trans-nasal treating cystic fibrosis
Nanoparticles' complexes with composition: EGF-PEG-biotin-streptavidin-PE-DNA	It presents great post-vaccine (transfection) effectiveness absence of aggregation of nanoparticles
Dendrimers with composition polyamino(amine) which can hold within DNA	Non-immunological carriers for in vivo gene delivery

study of the genetic material and its applications in therapeutics and diagnosis are mentioned in Table 4.4 and in the following section:

- Genetic polymorphism nanotechnological determination
- Nanoparticles for cancer therapy through p53 (immunolipoplex for p53 gene transfer)
- Silicon and gelatin nanoparticles for gene transport

With respect to nanogenomics, nanoproteomics is the application of nanotechnology in proteomics. The term proteomics expresses the protein effect study on absorption, distribution, metabolism, and excretion (ADME) from a bioactive molecule organism. The determination of protein effect with nanotechnology techniques is described as follows:

- Multiphoton detection of proteins
- Nanoflow liquid chromatography
- High-field asymmetric waveform ion mobility mass spectrometry

- Nanoproteomics for study of misfolded proteins
- Use of nanotube electronic biosensor in proteomics
- Nanofilter array chip detection

Some of the most common nanoparticles used to study the mitochondria are the following:

- Biosomes. Self-assembled structures composed of amphiphilic molecules (bola-amphiphile) that develop nanosystems for mitochondrial DNA transport in gene therapy.
- Liposomes are designed for bioactive molecule transport in the mitochondria using ligands with hydrophobic areas.
- Nanoparticles with special ligands that target mitochondria aiming to take control of mitochondrial functions.
- Quantum nanodots with special ligands that target the mitochondria aiming to take control of mitochondrial functions and morphology.

4.2.4 Nanotechnology and Biological Treatments

Biological therapies are those therapies where molecular biology can be applied. Biological therapies include vaccines, gene therapy, antisense, and RNA interference. Some of the biological therapies include the use of nucleic acids and proteins, while others include the genetic material management. The application of nanotechnology in therapeutics is definitive as materials and systems can be managed in nanoscale. Also, biological therapies use therapeutic products and not classical pharmaceutical products (bioactive molecules). The European Medicines Agency (EMA) (see Chap. 7) committee for therapeutic products' approval is called Committee for Advanced Therapies (CAT) (see Chap. 7).

The increased availability of therapeutic biological products in the market, like proteins, peptides, and antibodies, offers an important asset in therapeutics. These therapeutic macromolecules mentioned above have important advantages against conventional bioactive macromolecules, while the route of administration is an important research area for academic institutes and pharmaceutical industries.

Per os administration is an important drawback since these therapeutic products will permeate the surface of the buccal cavity or other biological membranes with difficulty. The sensitivity of their structure and conformation leads to peptide bond cleavage, proteolysis, oxidation, etc., and the disruption of noncovalent interactions resulting to aggregation, sedimentation, and finally immune response is developed. Problems mentioned above flag the macromolecule sensitivity in relation to the conventional bioactive molecules. They require special handling techniques, and the administration route should be chosen taking under consideration the rapid liver xxx that requires controlled doses.

It is obvious that their administration demands proper delivery systems that will enhance the above problems and will try to eliminate side effects. The alternative routes of administration are oral, nasal, and pulmonary. Today, the most common route of administration is the parenteral associated with compliance problems due to the repeated therapeutic dose, especially for chronic diseases. For example, diabetes is a disease that requires repeated doses and, therefore, long-term compliance that is a problem for patients. Biological therapeutic products transferred through mucosal and other administration routes, like oral, nasal, rectal, buccal, transdermal, and ocular, are under study. Tables 4.5 and 4.8 present biological therapeutic products that are in clinical trials or already in the market.

Systems mentioned in Tables 4.5, 4.6, 4.7, and 4.8 offer advantages in the field of therapeutics based on biological products. The development of micro- or nanosystems for drug delivery and administration through the routes mentioned above is

Table 4.5 Development of orally administered therapeutic biological products which are in clinical phases

Company	Product	Clinical phase
Emisphere Technologies	Salmon calcitonin	Clinical phase III
Emisphere Technologies/Novo Nordisk	GLP-1 (glucagon-like peptide)	Clinical phase I
Emisphere Technologies/Novo Nordisk	Insulin	Clinical phase I
Biocon	IN-105 (insulin conjugate)	Clinical phase III (India)
		Clinical phase I (USA)
Fosse Bio-Engineering Development Ltd.	Insulin	Clinical phase III
Generex	Oral-lyn™ (buccal insulin spray)	Approved product for sale on the market in many countries. Experimental products in the United States

Table 4.6 Developed inhaled technological forms of insulin

Company	Product	Clinical phase
Pfizer	EXUBERA®	Withdrawal due to low sales
Novo Nordisk	AERx®	Interruption in clinical phase III
Eli Lilly and Company and Alkermes Inc.	AIR® Insulin	Completion of the clinical phase III
MannKind Corporation	Technosphere® Insulin System	Clinical phase III (USA, Europe, Latin America)
Baxter	Recombinant human insulin inhalation powder (RHIIP) based on Baxter's proprietary PROMAXX formulation technology	Clinical phase I
Ventura and MicroDose Technologies Inc.	QDose insulin	Encouraging results of inhaled insulin will be announced. Prospective clinical studies

Table 4.7 Therapeutic biological products administered by the nasal cavity (nasal administration)

Company	Product	Description
Sanofi-Aventis	Kryptocur®	Nasal administration of luteinizing hormone-releasing Hormone (LHRH)
Novartis	Miacalsin®	Nasal administration of salmon calcitonin
Unigene Laboratories/Upsher-Smith Laboratories, Inc.	Fortical®	Nasal administration of salmon calcitonin
Ferring Pharmaceuticals, Inc.	Desmospray®	Nasal administration of desmopressin (analog of 8-arginine vasopressin (ADH)
Sanofi-Aventis	Suprecur	Buserelin (agonist of LHRH)
Sanofi-Aventis	Suprefact	Buserelin (agonist of LHRH)

a scientific and technological challenge oriented in their development from the pharmaceutical industry. Polymer chemistry and the design/evaluation of drug delivery systems (see Chap. 5) in nanoscale in order to enable biological product transfer will contribute in effectiveness and decreased side effects of these biological macromolecules.

4.2.5 Nanotechnology of Vaccines

The development of nanosystem transferring DNA for the vaccines' development and production, nanoemulsions (see Chap. 4), and nanoaerosols is an important direction in the field of vaccines. Table 4.9 presents examples of vaccines that are currently in the market or in different development stages and their administration is either nasal or per os. Vaccination is one of the most valuable and cost-effective health measures to prevent and control the spread of viral/bacterial infectious diseases responsible for high mortality and morbidity as reported in Strategic Research Agenda for Innovative Medicines Initiative 2 (IMI2) "The right prevention and treatment for the right patient at the right time" and in the report by Vaccine Europe 2013 entitled "Advancing health through vaccine innovation." A significant number of infectious diseases and chronic disorders are still not preventable by vaccination such as HIV, tuberculosis, malaria, healthcare-associated infections (HAIs), cytomegalovirus (CMV), and respiratory syncytial virus (RSV) for which new generation vaccines are needed. Novel technologies such as adjuvants (including immune modulators and molecular targets) can enable safe and effective vaccines for difficult target populations such as newborns, elderly, and the immunocompromised. The adjuvants need to be very well designed in order to avoid excessive response, long-term autoinflammatory diseases and allergy, and secondary effects. Therefore, particulates and especially liposomes could represent a perfect vaccine adjuvant, thanks to the possibility of a high level of customization and control. More recently,

Table 4.8 Development of micro- and nanosystems for mucosal administration of therapeutic biological products

Carrier	Size (nm)	ζ-potential (mV)	Loading method	Biomolecule	Loading (%)	Encapsulation efficiency
Oral delivery						
Chitosan	0.215	20.7	Encapsulation	Insulin	–	49.43
Chitosan			Encapsulation	DNA	–	
Chitosan/HPMCP	0.255	30.1	Encapsulation	Insulin	–	60.88
Chitosan/dextran sulfate	0.497–1.612	−21.5–3.2	Encapsulation	Insulin	–	48.6–96.4
Chitosan/dextran sulfate	0.527–1.577	−20.6–11.5	Encapsulation	Insulin	2.3	69.3
Chitosan/lecithin	0.121–0.347	7.5–32.7	Encapsulation	Melatonin	Up to 7.1	
DEAPA-PVA-g-PLLA	0.200–0.400	7.5–32.7	Self-assembly	Insulin	–	85
Polyacrylic acid/$MgCl_2$	0.278	−23.4	Encapsulation	Calcitonin	–	53.8
Lipid nanoparticles	0.200	−50.3	Encapsulation	Calcitonin	–	>90
Lipid nanoparticles/PEG	0.207–0.226	−36.6–34.8	Encapsulation	Calcitonin	–	>90
Nasal delivery						
Chitosan	0.275	46.7	Encapsulation	Insulin	44.1	46.9
Chitosan	0.040–0.600	18.8–31.1	Encapsulation	siRNA	13.2	9.2
Pulmonary delivery						
Liposomes	0.091–0.104		Encapsulation	Vasoactive intestinal peptide	0.4	
Liposomes/PEG	0.090–0.095		Encapsulation	Vasoactive intestinal peptide	0.4	
Lipid nanoparticles	0.115		Encapsulation	Insulin	–	

Table 4.9 Examples of vaccines on the market or in various development stages which are administered orally (oral administration) or by the nasal route (nasal)

Route of administration	Disease	Type of vaccine	Clinical phase
Oral	Poliomyelitis	Live attenuated	On market
	Typhus	Live attenuated	On market
	Cholera	Live attenuated	On market
	Acute gastroenteritis	Live attenuated	On market
	Diarrhea	Neutral/inactivated	Clinical phase III
	Dysentery	Live attenuated	Clinical phase III
	Ulcers in the digestive system, gastrointestinal cancer	Neutral/inactivated	Clinical phase I
	Anthrax	Vaccine strain	Preclinical phase
Nasal	Influenza	Live attenuated	On market
	Hepatitis B	Vaccine strain	Preclinical phase
	Diseases of the respiratory system	Live attenuated	Preclinical phase
	Anthrax	Neutral/inactivated	Preclinical phase
	Bronchiolitis/pneumonia	Neutral/inactivated	Preclinical phase
	Cervical cancer	Neutral/inactivated	Preclinical phase
	SARS	Vaccine strain	Preclinical phase
		Vaccine strain	

liposomes have found application as vaccine-adjuvants due to their ability to prevent antigen degradation and clearance, coupled with enhancing its uptake by professional antigen-presenting cells (APCs), and have marked liposomes as useful vehicles for the delivery of a diverse vaccine antigen. The majority of vaccines currently in development belong to the category of subunit vaccines, consisting of recombinant or purified pathogen-specific proteins or encoded (DNA) antigens that will be expressed and presented in vivo [66]. Subunit vaccines when administered alone have low efficacy in activating the immune system and require the addition of adjuvants in order to induce a measurable immune response of the antigen, through activation of the innate, and subsequently the adaptive immune system. Ideally, the adjuvant should be able to improve antigen uptake by APCs and induce an antigen-specific immune response while eliciting minimal toxicity [66]. Liposomes (Chap. 4) are a type of adjuvant that can potentially satisfy the above criteria. The adjuvant efficacy of cationic liposomes composed of Dimethyldioctadecylammonium bromide and trihalose dibehenate is well established in the literature. While the mechanism behind its immunostimulatory action is not fully understood, the ability of the

formulation to promote a "depot effect" is under consideration. The depot effect has been suggested to be primarily due to the cationic nature which results in electrostatic adsorption of the antigen and aggregation of the vehicles at the site of injection. Virosomes are liposome-based vaccine formulations that are constructed from viruses without their genetic material. However, they are not able to replicate and to cause infection. Inflexal V (Berna Biotech Ltd.) and Epaxal (Janssen-Cilag Ltd.) are biological medicines that are vaccine-based antigen liposomal delivery nanosystems against influenza and hepatitis A virus (HAV), respectively. They are composed of DOPC (dioleoylphosphatidylcholine) and DOPE (dioleoylphosphatidylethanolamine), and their formulations are classified as suspensions. They mimic the viral infection promoting immune response while both are well tolerated and effective in children [14]. Dendrimers are under investigation as vaccine carriers and/or adjuvants for both infectious diseases and cancer immunotherapy. The adjuvant capacity of mannosylated poly(amidoamine) (PAMAM) dendrimers was documented in the literature as well as glycopeptides dendrimers and phosphorus dendrimers. Additionally, polymeric nanoparticles have been applied in vaccine delivery, showing significant adjuvant effects as they can easily be taken up by antigen-presenting cells.

4.2.5.1 Proteasomes™ as Vaccine Transport Vehicles

Proteasomes (Proteasomes™, GlaxoSmithKline, Brentford, Middlesex, UK) are considered to be vaccine transferring vehicles and create structures and cystic clusters at the size of viruses. The size of nanostructures is between 20 and 800 nm and depends on the type and the amount of antigen that is shaped into proteasome. The proteasome hydrophobic nature can contribute to vaccine transfer by facilitating the interactions between vaccine particles and their uptake from the ESN cells that will result in immune response. This technology is applied for vaccines against viruses, allergens, swines, and viruses of the respiratory syncytium.

Appendix

Liposome Preparation Protocols

Liposomes can be prepared with various techniques that offer energy in order for the phospholipid bilayers to bend and form pseudo-spherical structures.

The methods that are used for liposome preparation include the following basic steps: removal of the organic solvent that the phospholipids or lipids are dissolved, dispersed lipids in aqueous medium, encapsulation of the bioactive molecule into the lipid bilayers or into the aqueous core of liposomes, purification (e.g., using column chromatography) of liposomes encapsulated the bioactive molecule from its free from, and finally analytical methods to determine the encapsulation efficiency

of the liposomal vehicles [60]. The conventional methods that are used for encapsulating the bioactive molecule into liposomes are:

- Reverse-phase evaporation technique [76]
- Ether injection technique [72]
- Freeze-thaw method [68]
- Rapid solvent exchange method [9]

There are two more conventional techniques that are used by the liposomal manufacturers:

- The French press technique [6]
- pH adjustment method [31]

The following liposome preparation protocols depend on the desired characteristics and size.

Thin-Film Hydration Method

This method (Fig. 4.15) is the most commonly used technique for multilamellar vesicles (MLVs). It is primarily based on lipid thin-film production that takes over the greatest possible surface in spherical flask in rotary evaporator under reduced pressure followed by hydration in a temperature greater than the main transition temperature of the phospholipids. The enclosed bioactive molecule to be incorporated or encapsulated is added either in the lipid film during its production if it is lipophilic or in the aqueous medium if hydrophilic [76].

The thin-film hydration method leads to multilamellar vesicles characterized of great size heterogeneity (1–5 μm). These liposomes can undergo size reduction and transform into small unilamellar vesicles (SUV) or large unilamellar vesicles (LUV) with greater size uniformity. This can be achieved by using probe sonication

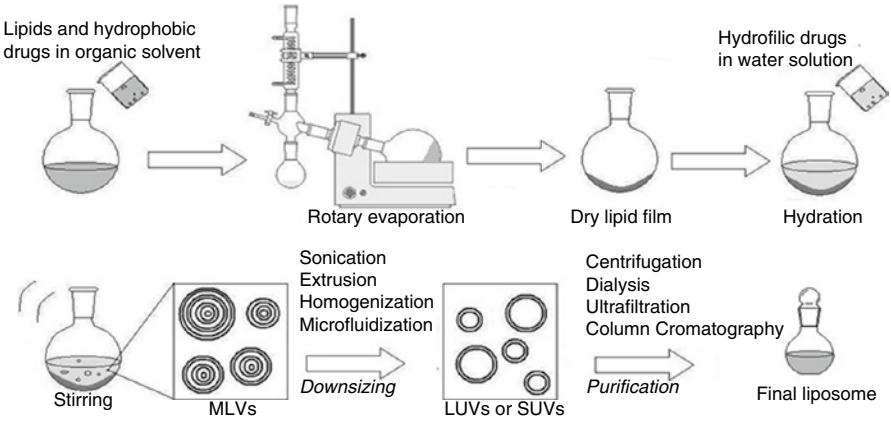

Fig. 4.15 Thin-film hydration method (Adapted from De Arou' jo Lopez et al. [2])

or extrusion through specific porous polycarbonate filters. Both sonication and extrusion provide energy to the system (thermal, mechanical) that is required for breaking the MLVs' liposome bilayers in small lipid vesicles, reducing the hydrophobic part exposure to the aqueous environment. Usually, before the use of polycarbonate filters, the liposomal MLVs' mixture undergoes fusion to reduce their size. The freeze-thaw method includes the rapid change between cold and hot temperatures of dry ice or liquid nitrogen and the basic phospholipid transition temperature used in liposome structural units. These extreme conditions break the bilayers up and produce smaller multilayer lipidic vesicles.

Solvent Injection

Phospholipid solvent injection in ethanol or diethyl ether in water medium is a technique for small or large unilamellar liposome (SUV, LUV) production. Phospholipids are primarily dissolved in a small volume of ethanol or diethyl ether and then quickly or slowly injected in water medium in a temperature greater than phospholipids' transition temperature.

The organic solvent is removed with evaporation, filtration, or dialysis. This method is simple and free from harmful (for the ingredients) chemical or physical processes, which is an important advantage of the method.

Nevertheless, the disadvantages of this method include the extra stage of the organic solvent and solute removal and the production of liposome dispersion systems.

Reverse-Phase Evaporation (REV)

Just like solvent injection method, phospholipids are dispersed in water environment through organic phase. The organic phase is not being removed but forms water in oil (w/o) emulsion. The phospholipid molecules are placed in unilamellars in a reverse micelle formation around the water molecules that during the organic solvent removal stage are agglomerated to form a mixture of unilamellar and multilamellar liposomes. Usually, a part of the water medium used for the liposome production is added into the solution of phospholipids in organic solvent. The rest is added after the organic phase removal. An advantage of this method is the great output in hydrophilic water molecules.

Heating Method

This method for liposome and nanoliposome production has been developed without using toxic chemicals and hazardous processes. This method includes liposome structural component hydration followed by their heating in a temperature of 120 °C in the presence of glycerol [56].

Glycerol is water soluble and physiologically acceptable from the human organism chemical molecule. It has the ability to increase lipid vesicle stability and doesn't need to be removed from the final liposomal product. The heating process is the major stage, and therefore, the method is called *heating method* and the produced liposomes are called heating method vesicles (HMV). By thermal method application, there is no need to sterilize the utensils used; therefore, the time for this method is reduced and the liposome production costs are limited. HMVs can be used as bioactive molecule delivery systems and for biological membrane simulation. In comparison to conventional liposome production, during this method, no volatile organic solutions are used, only glycerol that is a biocompatible and nontoxic parameter already used in pharmaceutical products preserving osmolarity during liposome production. There are cases where structures that are produced with *heating method* resemble the cell membranes of organisms in primordial earth. This is another leading indicator that liposomes are critical parameters in life's origin and determination.

Energy Demand and Approach of Liposome Preparation

To form lipid bilayers and, therefore, liposomal vesicles (i.e., liposomes), no matter the preparation method used, the hydrophilic/hydrophobic interactions between lipids and lipid/water molecules are essential. The energy offered (sonication or heat) in the lipid system results in lipid molecule appropriate orientation through hydrophobic interactions and, therefore, lipid vesicle formation to achieve the necessary thermodynamic balance of the lipid system. Lasic, in his book entitled *Liposomes: from Physics to applications* in 1993 [39], suggested that symmetric membranes favored flat configuration and energy should be offered for lipid bending.

The lipid type used and the presence/absence of cholesterol are parameters defining membrane rigidity. Whether *pseudo-spherical* lipid structures form liposomes in one or two stages (i.e., *pseudo-spherical* lipid vesicles), with specific thermodynamic content, as suggested by Lasic and coworkers, the basic condition for liposome formation is energy offered into the system. To summarize, liposomes are not formed spontaneously during lipid (mostly phospholipid) dispersion in aqueous medium, but extra energy input is required. In 'heating method,' for example, liposomes are formed due to energy offered into the system, and the mixing throughout the heating is performed to facilitate the homogenous ingredient distribution.

Polymersome Preparation Methods

During copolymer hydration, the motivation force for polymersome formation is the concentration gradient between copolymer diffused in water and water diffused into the copolymer. During simple hydration, the concentration degrades exponentially by time. The most common used method for polymersome formation is the thin-film hydration method. This method produces polymer emulsions with great size dispersion but also great amounts of other thermodynamically metastable polymersome structures.

Organic Solvent Method

This method is based on organic solvents and is related to water-in-oil-in-water (W/O/W) double emulsions. This method produces polymersomes with asymmetric membrane, where the inside and outside surfaces are of different nature. This is achieved by stabilizing water in oil (W/O) emulsion using the copolymer for the inside lamellae and producing vesicles permeating the water droplets through a second oil in water interface that consisted of the copolymer in order to form the outer membrane lamellae. Polymersomes are finally formed with controlled organic phase water removal using dialysis through membranes. The disadvantage of this method is that the organic solvent residues can cause biologic toxicity, limiting the produced polymersome application and use.

Dendrimer Synthesis

Many times during dendrimer production, scientists use, as cores bonds with special chemical and/or physicochemical properties, mostly photoactive or electroactive groups like porphyrins, ionic complexes, organ metal bonds, and fluorescent dies. The branches of dendrimers can be composed of repeated structural units. The number of the repeated structural units is controlled and defines the dendrimer generation (G). Each repeated structural unit is connected to the core or to another repeated structural unit through a branching spot. The branching spot between the core and the branch is of high importance since it determines the interaction between them. The branches play an important role in the dendrimer three-dimensional configurations and in the internal microenvironment. The dendrimer outer section ends up in peripheral groups. The part of these groups is also of great importance since they define the interaction between the dendrimer and each surroundings. The number of the peripheral groups increases exponentially as the dendrimer generation increases. Dendrimer formation always includes a series of repeated reaction sequence whose completion provides each time a new generation. Dendrimers are composed of monomers through polymerization and this process includes two methods. The methods can be classified into two basic synthesis methods, the divergent method (Fig. 4.16) and the convergent method (Fig. 4.17), and there are processes that combine these two methods (Fig. 4.18). The divergent method seems to be more effective for large-scale dendrimer synthesis, since the dendrimer weight doubles or triples in each generation. By increasing the generation number, the active group number in the surroundings increases exponentially by the necessary use of large excess of reagents. These reagents have a big difference in their molecular weight with the product and therefore are easily separated from it during reaction treatment. The exponential surrounding groups increase in each generation, increasing the possibility of incomplete reaction or by-product production. In these cases, the by-product separation is usually extremely difficult since they have similar weight

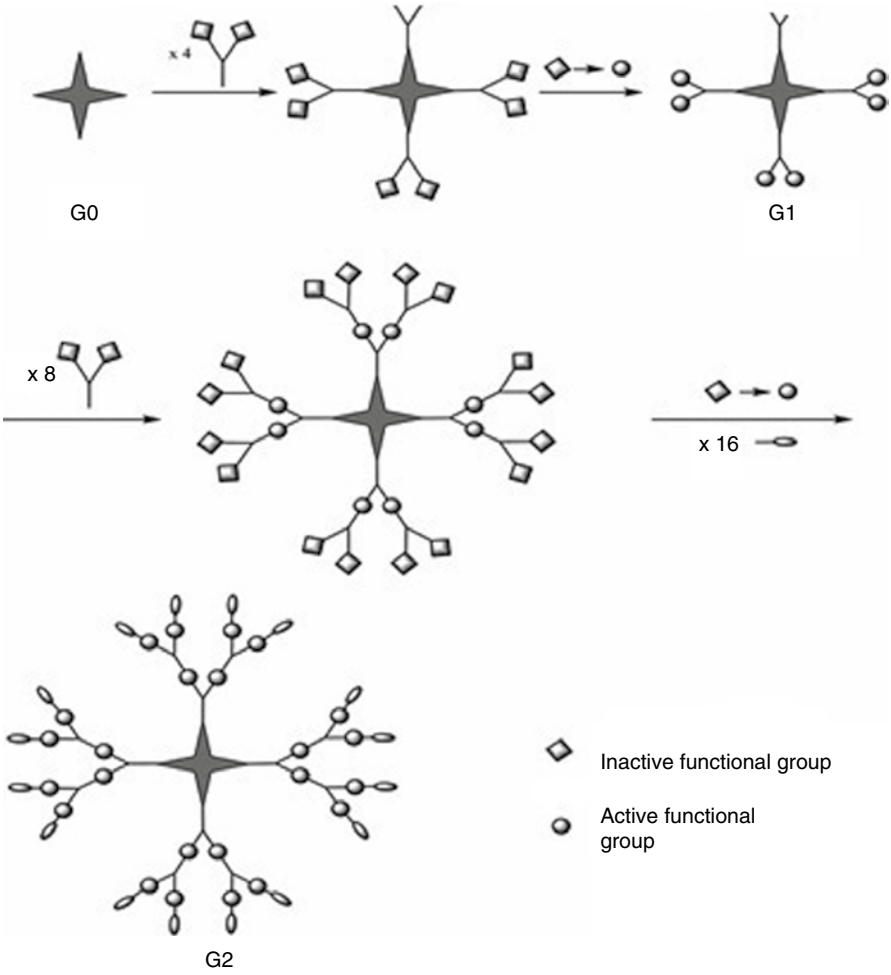

Fig. 4.16 Schematic of divergent synthesis of dendrimers (Adapted from [24] with permission from Bentham Science Publishers)

and structure with the dendrimer. The convergent reagent has more advantages in relation to the divergent method. Fewer reactions per molecule are needed during coupling and activation steps, and therefore, the use of large excess of reagents is not necessary – the cleaning is usually easier and the final product structure flaws are limited. Also, the precise surrounding group setting from the very first step allows the design of more target complex courses. In an effort of developing more efficient and less time-consuming complex courses for dendrimer synthesis, many complex courses combining the convergent and divergent method have been mentioned. This way the disadvantages of both methods will be limited by preserving their advantages. In 1991, Frechet's group [32, 89] mentioned a double-stage convergent

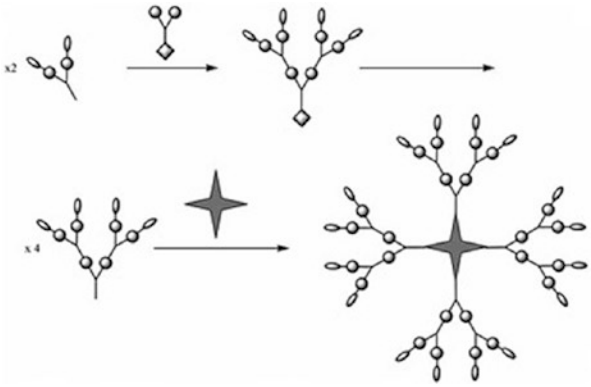

Fig. 4.17 Schematic of convergent synthesis of dendrimers (Adapted from [24] with permission from Bentham Science Publishers)

course (Fig. 4.18) in which the divergent method was used for large core formation (actually it was a low-generation dendrimer) followed by the convergent method for the coupling of the core and the branches that were formed separately.

Another combining approach aiming in complex course development with few steps is the orthogonal coupling method (Fig. 4.19) which targets in activation step removal during branching formation.

Summary

Liposomes are lipidic self-assembled nanostructures that are able to accommodate into their lipid bilayers or into their aqueous core, lipophilic and hydrophilic bioactive and biological molecules, respectively. Liposomes can be used as carriers for imagining and for diagnostic agents.

Liposomes are characterized as nanocolloidal lyotropic liquid crystals, and their *mesophases* that taking place during their thermotropic behavior affect their functions and their effectiveness as drug nanocarriers.

Liposomal membranes are studied based on their thermodynamics and biophysical aspects. Their thermotropic behavior affects their pharmaceutical effectiveness. They have been used to simulate biological membranes and to understand biological phenomena, i.e., the protein fusion in cell membranes.

Thermo- and pH-responsive liposomal vehicles are emerged nanotechnological platforms for drug delivery and targeting.

Polymersomes are composed of bilayers and their structural organization is close to that of liposomal bilayers.

Dendrimers belong to the very last generation of polymeric structures. They are considered as real nanoparticulate systems with very low polydispersity profile.

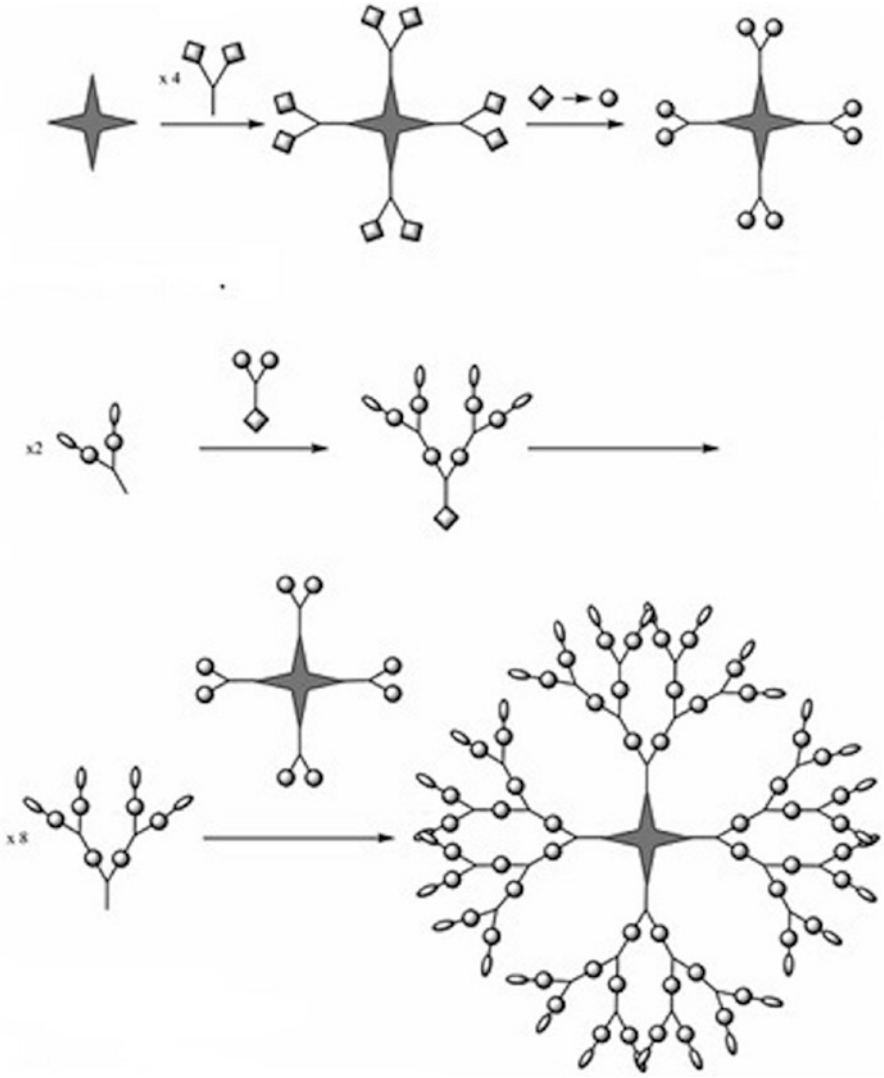

Fig. 4.18 Schematic of double-stage synthesis of dendrimers (Adapted from [24] with permission from Bentham Science Publishers)

They are used in diagnosis, in drug delivery, and as nonviral vectors in gene therapy.

Nano genomics is the term by which we describe as the nanobiotechnological applications.

Vaccines based on nanoparticulate systems are an emerging technology. Liposomes are promoted as a type of adjuvants that can potentially satisfy the criteria of adjuvanicity.

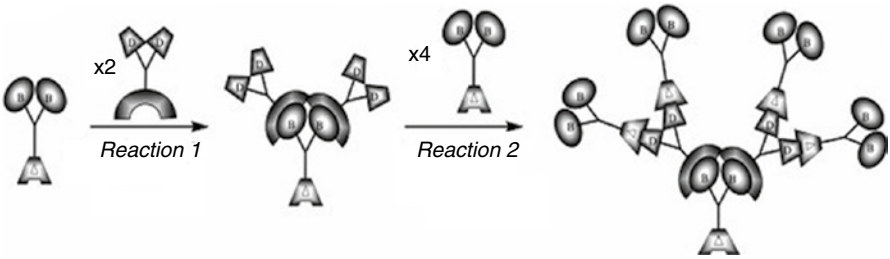

Fig. 4.19 Schematic of orthogonal conjunction synthesis of dendrimers (Adapted from [24] with permission from Bentham Science Publishers)

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