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REVIEW ARTICLE

Formulation, Quality Control and Safety Issues of Nanocarriers Used for Cancer Treatment

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Abstract: Cancer is becoming a leading cause of death in the last years. Although we have seen great advances, most human cancers remain incurable because many patients either do not respond or relapse to treatment. Several lines of research are disclosing new therapeutic targets which lead to new active drugs. However, there are still unsolved problems related to stabilization of the pharmaceutical ingredient in aqueous and biological media, pharmacokinetic and pharmacodynamic profiles and cellular uptake to name just a few. In this context, nanotechnology with the emerging tools of nanoengineering offers many possibilities to guide the design of new products with improved safety and efficacy. The presence of several reacting groups and the sensitivity of their properties to small changes in composition make nanocarriers tunable not only to modify their stability in a particular environment but also to respond to changes in biological situations in the right place and time frame.

This review summarizes the main preparation methods and formulation strategies of nano and microcarriers designed for drug delivery applications for cancer treatment and will attempt to give a glimpse on how their structure, shape, physico-chemical properties and chemical composition may affect their overall stability and interactions with biological systems. We will also cover aspects of nanoengineering that are opening new opportunities for the development of more effective nanomedicines, emphasizing on the challenges that have to be kept in mind when dealing with biological activities of nanocarriers that depend not only on their chemical composition but also on those of the structures formed by them and by their interactions with biological systems. From this, a very important issue that emerges is that nanocarriers frequently display an intrinsic bioactivity (i.e.: immunomodulatory). Therefore, it should be stressed that nanocarriers cannot be considered as inert, biocompatible excipients. Furthermore, their biological activity will mostly depend on the physical and chemical properties of the structures of the nanoparticles that are presented to living systems. As an approach to the rational design of new pharmaceutical products, nanoengineering is providing new tools for the precise control of the properties of nanocarriers for cancer treatment.

Keywords: Drug delivery systems, liposomes, micelles, nanoparticles, self-assembly.

1. INTRODUCTION

In December 1959, physicist and soon-to-be Nobel laureate Richard Feynman gave a now famous lecture at Caltech entitled "There is plenty of room at the bottom" [1]. In that lecture he suggested the manipulation of matter at the atomic scale, introducing the possibility of build more compact computers, microscopes with atomic resolution and nanoscale machines [1]. Moreover, he discussed in the lecture an idea of his graduate student Albert Hibbs, that of "swallowing the doctor", implying a nanoscale surgical robot [1]. Several of the challenges posited in that and subsequent lectures have been tackled throughout the intervening decades. Electronics was the first discipline to incorporate the scientific advances in miniaturization, first at the microscale and now approaching nanoscale. For a variety of reasons, mainly related to the complexity of the system to be treated, it took far longer for the first nanomedicines to reach the market. It was not until 1995 that the first nanocarrier was approved for the delivery of an anticancer drug (Doxil® - doxorubicin containing liposomes) [2].

Although there is no single scientifically agreed definition for the size-range of a nanocarrier, it has been usually accepted that their size diameter is between 1-1000 nm and in nanomedicines for cancer treatment (mostly for the permeation of leaky tumor vasculature and for sterilization purposes) it is generally $\leq$ 100 nm. Notwithstanding these arbitrary ranges, a nanocarrier can be defined as such when it's scale, in and of itself, confers special properties to the intended product. Size-, shape- and structural-dependent properties that are vital in the development of nanocarriers and nanoengineering for pharmaceutical purposes include:

a) those that depend on the surface/volume ratio like solubility rate (bioavailability), phase-transition temperature (i.e.: melting point), etc. These are by far the most intuitive properties that are considered when speaking of nanoscale derived properties;

b) those that depend on the shape. Curiously, one of the properties that depend on the shape is the proportionality between surface area and volume. The simplest example of this is to consider two cuboids of 1000 cm^3, one of 10x10x10 cm, which presents a total surface area of 600 cm^2, and another of 100x10x1 cm, that has a total surface area of 2200 cm^2. Therefore, a simple conclusion is that a change in shape can radically alter the surface/volume ratio which in turn can greatly affect the properties of the final product.

c) From the pharmaceutical point of view, pharmacokinetics and pharmacodynamics are highly dependent on the size, shape and surface properties of nanocarriers, and by complex mechanisms that will be discussed in more detail below.

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The basic concept of a pharmaceutical product is that it should be designed for curing, more than simply treating or alleviating the symptoms of a disease. With this in mind, researchers all over the world look every day for new compounds and new strategies. Knowing the basic fundamental biochemical processes that are altered in a particular disease is the natural and logical source for the design of new active pharmaceutical ingredients (APIs). In many circumstances this logical process works well in the laboratory. However, it is anything but straightforward when the same processes and approach are extrapolated to a real environment with human beings, who can present different degrees and stages of a disease like cancer. With the final purpose of curing a disease, and after selecting an API, there are a series of situations that have to be kept in mind. First, how to keep the API chemically and physically stable. Second, how to deliver it to the right target in the body and in the proper amount. This last issue raises the question of how to keep the API stable within biological fluids and cells and how to avoid its interaction with non-target tissues (which are the main causes of side effects). These are just a few of the issues that should be kept in mind when designing a new formulation of a therapeutic agent to cure a disease. In order to aid in the formulation process, several excipients have been progressively studied over the years. Excipients have been used to aid in the solubilization of the API, to mask flavors or odors, to impair chemical degradation of the API, etc. When excipients display an intrinsic biological activity or modulate the activity of the API as in the case of nanocarriers, some new considerations have to be taken in count as will be discussed in the following sections.

2. NANOENGINEERING, FABRICATION TECHNIQUES AND FORMULATION OF NANOCARRIERS USED FOR CANCER TREATMENT

2.1. Nanoengineering as a New Concept in the Formulation of Nanocarriers

There are several types of nanocarriers that have been used with different purposes for medical and pharmaceutical applications. To name just a few these are: protein-derived and polymeric nanoparticles, lipid-derived nanoparticles (liposomes, micelles and lipid nanoparticles), dendrimers, metal nanoparticles, etc. From the historical perspective, the first nanocarriers were mostly designed to solve solubility problems associated to hydrophobic APIs. Thus, liposomes and lipid derived structures were the first to be exhaustively characterized and to reach the market [2-5].

The basics of nanoengineering require considering the energetics, dynamics and chemical structure of the components to be used, because altogether they will determine the free energy changes involved in the main chemical and physical processes (therefore the thermodynamic stability) and their reaction kinetics, and how they are affected by the nanoscale, which will determine their overall stability. The possibility to have molecules with different chemical groups, all within a single supramolecular assembly leads inevitably to several conformations that minimize the energy. This leads to the concept that *nanocarriers are always fluctuating structures whose properties are represented by average values* [6]. In this context, it has been demonstrated that when an API or a component of a biological system interacts spontaneously with a nanocarrier this is generally followed by a reduction in the free energy of the system leading to structures that are thus thermodynamically more stable [7-11]. Therefore, *the structures formed can have different biological activity than those of the original nanocarrier.*

From the production point of view, it is generally accepted that self-assembled structures (bottom-up) are thermodynamically stable while those prepared by top-down strategies are thermodynamically unstable and have to be kinetically stabilized [6, 8-11]. It has been also shown that, depending on how the changes are performed and how they are repeated through cyclic operations different metastable states can also be formed. Therefore, kinetic stabilization can be attained through the use of additives that avoid fusion or clumping of the nanocarriers and also by the adoption of metastable states that have kinetic stability *per se* [12].

From the thermodynamic point of view, considering the many interactions that can be formed in a multimolecular nanocarrier between its components and also with the solvent, the general scenario is that all the processes that take place with bond formation are led by a decrease in enthalpy that will also decrease the number of conformations and distribution of energy leading thus to a reduction in entropy and *vice versa* [6, 7]. When designing a nanocarrier to be used as a nanomedicine for cancer treatment, it is important to keep in mind that they should be able to release the API in the right target cells [6]. Therefore, nanocarriers with great thermodynamic stability can be very stable in solution but usually lack the potential to release the API in the right place and time frame when studied *in vivo* [6].

2.2. Engineering Nanocarriers

From the historical perspective, the first nanocarriers consisted of materials that were basically biocompatible and without reported toxicity. After those, a second generation of nanocarriers were produced with materials with surface chemistries optimized to confer them improved stability and targeting in biological systems. The last generation that is appearing involved the design of what are called "intelligent" materials which are responsive to environmental changes therefore improving efficacy (Scheme **1**) [13].

In this context, the most exciting property that nanocarriers show is the possibility to target a specific tissue or cell type with a response that can be even tuned to allow for personalized medicine [14].

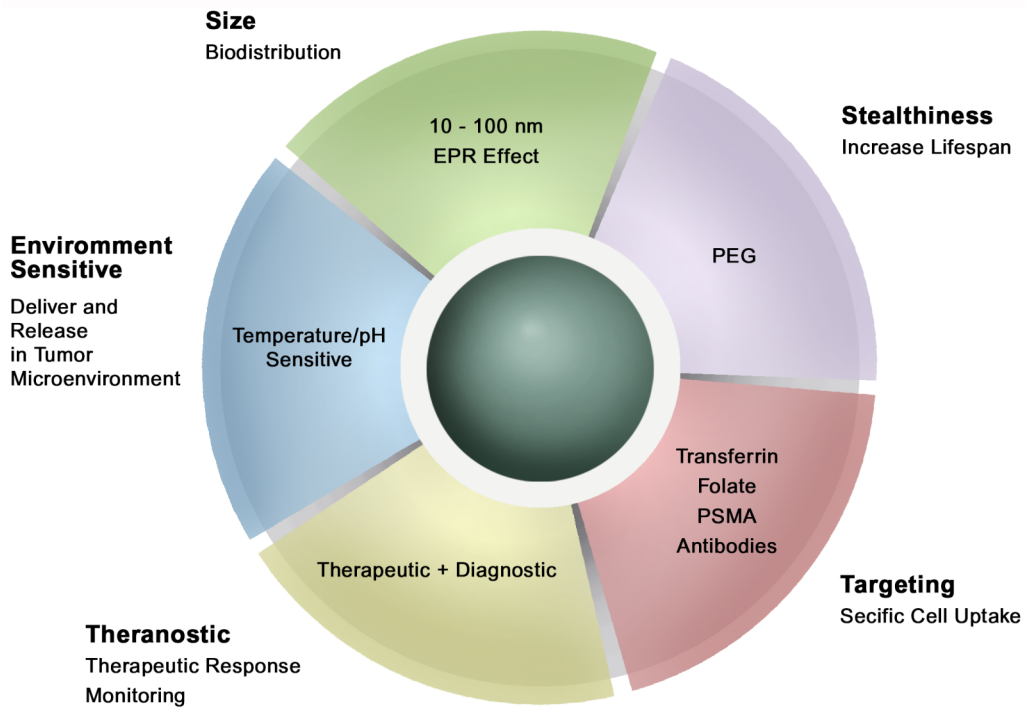
Regarding targeting, a key issue to be considered is the selection of an optimal targeting ligand density on the surface of the nanocarrier in order to optimize binding affinity to the tumor. It is well known that increasing ligand density does not necessarily increase binding and cellular uptake.

From the engineering perspective, there are two basic approaches to prepare modified nanocarriers: a) precoupling and b) postcoupling. Precoupling involves the formation of the nanocarrier using molecules that already have the targeting ligand covalently attached. On the other side, postcoupling involves a first step in which the nanocarrier is prepared and a second step in which the targeting ligand is coupled to the surface of the nanocarrier.

The employment of synthetic polymers to coat nanocarriers increases their stability and solubility with advantageous effects of decreasing cytotoxicity or inflammatory responses following administration. Active molecules can be encapsulated (adsorbed or bound) into the coated carriers to protect them from metabolizing enzymes. Specific properties of their surface can confer protection to non-target tissues from a possible toxic action of the drug payloads leading to side effects of the pharmaceutics. On the other hand, they prevent the drug from premature release or degradation in the biological environment of the body, preserving their efficacy.

Tailoring the size of nanocarriers, allows to modify the interaction with cell surface and intracellular organelles, which would be relevant for delivering a payload to the target. For example, they must be small enough to avoid filtration by the spleen as phagocytotic cells do and also to be able to pass through the liver avoiding Kupffer cell sieve. Liposomes have an increased lifespan, in part due to their ability to extravasate through splenic and liver fenestrae. However, liposomes having 50-70 nm in diameter are taken up rapidly by macrophages [15].

Modification with PEG is the most frequently used alternative to modify nanocarriers. The main purpose of PEG coupling to the surface of nanocarriers is to reduce protein adsorption that is involved in clearance from blood via macrophage phagocytosis.



Scheme 1. Engineering nanocarriers.

There are several examples of nanocarriers modified with PEG: liposomes (Doxil®/Caelyx®), polymeric micelles (Genexol-PM®) and PEGylated lipid nanoparticles (ALN-TTR2®) [2-5].

Nanoparticles can be also functionalized with targeting ligands to allow specific cellular uptake or guide their distribution to cancer cells or to angiogenic microcapillaries growing around the tumor [13]. For instance, various nanocarrier systems have been targeted to the folate receptor because of its overexpression in tumor cells for enhanced delivery as well as diagnosing and imaging malignant masses [15, 16]. Nanocarriers containing transferrin on their surface have been designed in order to improve targeting to melanoma [17]. By this approach, the nanocarriers bind to transferrin receptors that are typically upregulated on cancer cells and trigger cellular uptake via clathrin [17].

Furthermore, targeting nanoparticles to molecules that are differentially expressed in certain tissues, has been exploited for improving the specificity of treatment, such as the polymeric nanoparticles containing docetaxel that have been engineered to bind to prostate-specific membrane antigen (PSMA), a tumor antigen expressed on prostate cancer cells and on the neovasculature of most nonprostate solid tumors [18].

Another ligand for functionalizing nanocarriers is specific antibody. The increasing availability of monoclonal antibodies that have been approved for cancer treatment is promoting the development of antibody-coupled nanocarriers [13]. As an example of this approach, nanocarriers have been prepared coupling a monoclonal antibody against CD33, an antigen expressed on cells from acute myeloid leukemia (AML) [19].

Nanoparticles can also be constructed with ability to react to external stimuli such as the pH or the temperature in the system (i.e. smart), allowing release the drug into the target cell even into particular intracellular compartment [19]. For example, the nanoparticle coupling with anti-CD33 monoclonal antibody, mentioned above, consisting of polymeric pH-sensitive liposome, designed to release its content inside the endosomes [19].

An evolution in the concept of nanoengineering is the design of theranostic particles that combine the possibility of imaging and therapeutic functions into a single nanoparticle, thus allowing to follow the carriers throughout the body or the patient [13].

3. NANOPARTICLE FABRICATION TECHNIQUES

3.1. Self-Assembly

This is by far the ideal fabrication method because it does not require the use of sophisticated equipment and leads to products with great stability. Detergents and lipids with a big polar head group like glycolipids tend to self-aggregate as micelles. These structures have been widely used to deliver hydrophobic APIs [8-11]. One of the main drawbacks of micellar structures is the possibility of disassembly upon dilution leading to precipitation of the API (Table 1). Interestingly, as the API is incorporated as part of the self-assembled structure it has been shown that this can improve the stability of the micelles upon dilution (Table **1**) [8-11]. As an example, the incorporation of paclitaxel to ganglioside micelles leads to the formation of mixed micelles which are very stable in solution and do not disassemble when diluted in dextrose or human plasma [8].

An alternative strategy is to induce structural changes in a natural product (i.e.: a protein) exposing hydrophobic residues, then adding the hydrophobic API and afterwards re-adjusting the conditions as to regain the native structure [12].

3.2. Homogenization and High-Pressure Extrusion

It is well known that when bilayer forming lipids are hydrated in water they spontaneously assemble as multilamellar vesicles (MLV) [20]. These structures are usually big, up to 10 μm, and very heterogeneous in size and shape. Therefore, their use is very limited and to be suitable for drug delivery their size has to be reduced. The most widely used size-reduction techniques are ultrasound and high-pressure homogenization [20]. Sonication has been used mainly to prepare liposomes for research purposes but as it is not easily scalable its use for industrial purposes has been very limited.

Table 1. Pros and Cons of Nanocarrier fabrication techniques.

Method	Advantages	Disadvantages
Bottom-up Self-assembly	- Does not require use of special equipment - Easy to scale-up - Cost and energy efficient - High stability in solution and upon freeze drying	- Size and size distribution are not easy to modify - Trace of solvent impurities in final product - May have low stability upon dilution
Top-down High-pressure homogenization	- Easy to scale up with small variation between batches - Size and size distribution can be precisely controlled	- Time and energy consuming (High number of cycles are frequently required). - Tends to rise temperature of product - Final product unstable requires the incorporation of stabilizing excipients - Not always stable upon freeze drying

On the other side, high-pressure homogenization/extrusion has been the technique of choice by all the pharmaceutical companies that produce liposomes for industrial purposes (Table **1**). The general procedure to prepare small unilamellar liposomes with a controlled size and narrow distribution involves the following steps:

a) dissolution of the lipids in a suitable organic solvent,

b) drying the lipid mixture under vacuum (usually in a rotary-evaporator),

c) hydrating the lipid mixture in a selected aqueous solvent,

d) passing this mixture through a high-pressure homogenizer during a number of cycles that is standardized for each liposome preparation,

e) extruding the liposomes through polycarbonate filters with defined and selected pore sizes, also during a number of cycles that have to be standardized for each product.

Some researchers include freeze-thaw cycles prior to homogenization in order to increase the proportion of unilamellar vesicles in the population [21].

3.2.1. Formulation Strategies for the Stabilization of Liposomes During Freeze-Drying

Given their intrinsic thermodynamic instability, the search for stabilization of liposomes began almost at the same time than the research that led to the formulation of them as carriers for APIs [21-27]. The origin of the first stabilizers was based on research performed on the stabilization mechanisms of biological membranes of organisms that survive extreme dehydration [28]. Thus, it was found that large quantities of disaccharides like sucrose and trehalose were particularly effective at stabilizing liposomes [28]. Afterwards, it has been found that other sugars and polymers can also be used and work as well as disaccharides.

There are two basic properties that should be attained in order to stabilize a membrane for freeze-drying: a) fusion between vesicles should be inhibited and b) the melting point (*Tm*) of the lipids should be depressed in order to keep the membranes in the same lipid-phase upon dehydration. It has been frequently observed that upon dehydration the *Tm* of the lipids rises. Therefore, upon rehydration there are phase transitions that turn the membrane transiently leaky which is critical for products in which the API is intended to be encapsulated in the aqueous compartment of liposomes [2]. Remarkably, one of the first nanomedicines that was approved was AmBisome® which is a lyophilized liposomal formulation of amphotericin B that is stabilized by sucrose [29].

3.3. Spray Drying

Due to its scalability and low cost the use of spray drying has been extensively applied in the food industries to stabilize proteins by dehydration and for the production of microparticles for different applications [30]. The basic idea of the spray-drying method is to produce a spray of a solution that is injected into a current of hot air which evaporates water with the final result of a fine powder of the product. Therefore, contrarily to what could be thought, as the exposure to heat is very short in time, even thermo-sensitive compounds like proteins have been formulated using this approach. However, this technique has been only used so far for the preparation of relatively large microparticles and, as far as we are aware, it has not been adapted for the production of nanocarriers.

3.4. Emulsion Based Strategies - Coacervation

The different alternatives of this method involve the formation of an emulsion, which will be named according to which is the continuous phase [6, 31]. Thus, the most common production strategies are:

a) Oil in water (O/W) emulsions: in this method an organic phase containing the polymer (or carrier forming molecules) and the selected API is emulsified in an aqueous phase containing a surfactant. Afterwards, the droplets containing the polymer and API are hardened when the organic solvent is removed. The intended size of the product is mostly controlled by changing the size of the droplets and by the mean molecular weight of the polymers forming the carrier. The drawback of this alternative is that it has a very low encapsulation efficiency for hydrophilic APIs;

b) Water in oil in water (W/O/W) emulsion: this method is a modification of the O/W method that is used when the selected API is hydrophilic. Therefore, in order to improve encapsulation efficiency the API is dissolved in water and the polymer in an organic solvent. Both phases are mixed forming a W/O emulsion which is then slowly added over an aqueous medium containing an emulsifier like polyvinyl alcohol. After the organic solvent is removed the particles are finally formed. Specially selected conditions of this strategy have been adapted for the production of microspheres of polylactide-glycolide (PLGA) for the sustained delivery of peptides like leuprolide (Lupron Depot®) or octreotide (Sandostatin®) and for the production of microemulsions for the delivery of propofol (Diprivan®) [32-34].

4. GENERAL PROPERTIES OF LIPIDIC NANOCARRIERS AND POLYMERIC MICROSPHERES

4.1 Liposomes

Because of their excellent properties as model membranes, the easiness of their preparation and the possibility to be loaded with APIs with different water solubility, liposome based drug carriers

have been developed for parenteral, pulmonary, ocular and subcutaneous administration [4, 35]. There are various methods for preparing liposomes. The choice of either method depends on the type of particle that is required, in terms of size and shape (Table **1**) [4, 13]. Among them, we can mention:

High-pressure homogenization and extrusion. This is by far the most widely used method for the preparation of liposomes to be used as nanocarriers for drug delivery applications (see above) [36-39]

Self-assembly: Amphiphilic lipidic compounds dispersed in water can form liposomes or micelles by self-assembly. The aggregation state or supramolecular structure formed by amphiphilic molecules is a complex function of their chemical structure and charge. It has been demonstrated that the shape and size of the structure that will be spontaneously formed by a particular amphiphilic molecule or molecules in a mixture can be predicted on the basis of geometric considerations [40, 41]

By these methods it is possible to prepare structures with a hydrophilic coating, a hydrophobic membrane and a water core that may be useful to carry both polar and nonpolar drugs [4, 13].

The small size and large surface area to volume ratio of extruded liposomes gives them unique properties, so that they are being used in many products and the development of new applications in pharmaceutical products, mainly aimed at cancer treatment [4, 13].

Two methods have been standardized for loading drugs into liposomes: incorporating the drug as the particle is being prepared, or after the particle is already formed. In the latter case, the drugs can be actively loaded into the interior of the liposome as in doxorubicin containing liposomes [2]. Loading reaches generally a greater amount of drug when it is hydrophobic and is therefore incorporated during the preparation process, but this may entail certain drawbacks related to the final shape and size of the structure [29].

Table 2. Approved Nanocarriers and microcarriers for cancer treatment.

Nanocarrier	API	Trade Name	Indication	Approval date - Status
Liposomes	Doxorubicin	Doxil/Caelyx	Metastatic ovarian Breast cancer Kaposi's sarcoma	1995 FDA
	Doxorubicin	Myocet	Metastatic breast cancer	2000 Europe & Canada
	Vincristine	Marqibo	Philadelphia chromosome-negative (Ph-) Acute lymphoblastic leukemia (ALL)	2012 FDA
	Cytarabine	DepoCyt	Lymphomatous meningitis	2007 FDA
	Daunorubicin	Daunoxome	Kaposi's sarcoma	1997 FDA
	Irinotecan	Onivyde	Metastatic adenocarcinoma of pancreas	2015 FDA
	Paclitaxel	LEP-ETU	Ovarian cancer	2006 Orphan drug designation Europe 2015 Orphan drug designation FDA
	Mifamurtide	Mepact	Osteosarcoma	2009 Europe
Polymeric Micelle	Paclitaxel	Genexol-PM	Breast cancer Non-small cell lung cancer Ovarian cancer	2007 Korea & Europe
Albumin-stabilized Nanoparticle	Paclitaxel	Abraxane	Breast cancer Non-small cell lung cancer Pancreatic cancer	2005 FDA 2012 FDA 2013 FDA
Microspheres	Leuprolide	Lupron Depot	Prostate cancer	1985 FDA
	Octreotide	Sandostatin	Carcinoid syndrome VIPomas	1998 FDA

One of the advantages of using liposomes to carry drugs is that by varying the chemical composition and preparation procedure it is possible to prepare structures with different release rates of the drug, thus achieving a controlled/sustained release. Furthermore, these nanocarriers can be modified to make them pH sensitive, thermo-sensitive or may be supplemented with various additives conjugated on the surface to achieve greater accuracy in targeting [4, 13].

Among some of the most important pharmaceutical applications of liposomes can be highlighted: their use in cancer therapy (i.e., doxorubicin, vincristine, paclitaxel), ocular delivery (i.e.: prostaglandins), mucosal delivery (i.e., insulin) and subcutaneous administration (i.e.: clodronate) (Table **2**) [4, 13].

4.2 Micelles

As stated above, depending on the geometric properties and balance between the polar (hydrophilic) and the hydrophobic groups some amphiphiles (mostly lipidic or polymeric) self-aggregate in the form of micelles. The main features of these structures as compared to liposomes is that they have a gradient of hydrophobic (inside) to hydrophilic (outside) without an interior aqueous compartment. Another important property of micellar structures is that their dynamic equilibrium with the monomers is relatively fast (as compared with that of a liposome or a natural membrane). This property has benefits and drawbacks (Table **1**). The benefits are related to the detergent like effect, making them very good candidates to solubilize in aqueous media those molecules that are highly hydrophobic. On the other side, as the critical micellar concentration increases, the high equilibrium rate between monomers and micelles turn them highly unstable upon dilution. Therefore, they tend to disassemble when they undergo the natural dilution to which any pharmaceutical product is exposed upon administration. Although this has been frequently observed with polymeric micelles, it has been recently observed that the incorporation of a hydrophobic API can turn them very stable upon dilution [8-12].

From the nanocarrier perspective, it should be emphasized that detergent like molecules have been used as excipients to dissolve highly hydrophobic APIs since long ago. For instance, Taxol® and Taxotere® are two commercial formulations of taxanes (paclitaxel and docetaxel, respectively) whose preparation involve the use of additives that form micelles in water. However, they have not been initially considered as nanomedicines since their structure was not originally designed as to confer them any special pharmacokinetic or pharmacodynamic property. Although still an open question, it should be emphasized that whenever a new physical structure is formed upon formulation of an API it is not licit to consider them as inert components and their properties should be thoroughly characterized.

4.3. Microspheres - Microcapsules

When the treatment option is to deliver the API at a relatively constant rate during a long period of time (in the range of months) depot formulations consisting of microspheres are the first option of choice. Thus, throughout the last decades several commercial products have been developed mostly based in biodegradable microspheres of some derivative of polylactide and/or polyglycolide. Well-known examples are Lupron Depot®, Trelstar Consta®, Risperdal Consta® and Sandostatin® (Table **2**) [32, 33, 42, 43]. Given a desired release profile, a microsphere size is frequently selected. As mentioned above, PLGA has been the polymer of choice because of its excellent biocompatibility and the possibility to modify its properties by changing the ratio of lactide to glycolide and the mean molecular weight. By playing with these two variables it has been possible to modify the diffusion rate of the active ingredient and the degradation of the polymer. Therefore, when the release rate is reduced over time it is compensated by the degradation of the polymer, which usually increases over time. From the design point of view, the size of the particles will affect their remotion from the injection site by macrophages. It has been shown that the lower limit is around 5 μm. Thus, particles with mean diameters above this size do not migrate to other tissues [44]. From the production point of view, encapsulation efficiency usually decreases as the average size of the particles is reduced, but considering syringeability the smaller the particles the better. A very important factor that has to be considered when designing formulation conditions is the possibility to have API loosely bound to the particles. This will lead to an important initial burst of release of API that may or may not be wanted [33].

5. INTERACTIONS OF NANOCARRIERS WITH BIOLOGICAL SYSTEMS

5.1. Nanocarrier, Becoming a Drug

The introduction of nanocarriers as vehicles for pharmaceutical drugs, resulting in improved solubility and bioavaibility, and decreased toxicity, has led to an unexpected road revealing how active can be these delivery systems. We have learned that nanocarrier composition can modify the fate of the vehiculized API into the body, opening the possibility to engineer nanocarriers to improve drug performance/efficacy. Nevertheless, the benefits of nanocarriers for drug delivery are obviously limited by safety concerns on their acute and chronic toxicity and their interactions with components of the immune system (Scheme **2**).

Since the immune system evolved to protect the body from dangerous entities, through specialized mechanisms that end in their removal, the impact of nanocarriers on its normal function is an important factor to be considered in biocompatibility and biodistribution studies.

Understanding how nanocarrier physcochemical properties are related with their *in vivo* behavior and how they can be manipulated to acquire desirable characteristics will certainly allow exploiting the potential of these unique drug delivery systems.

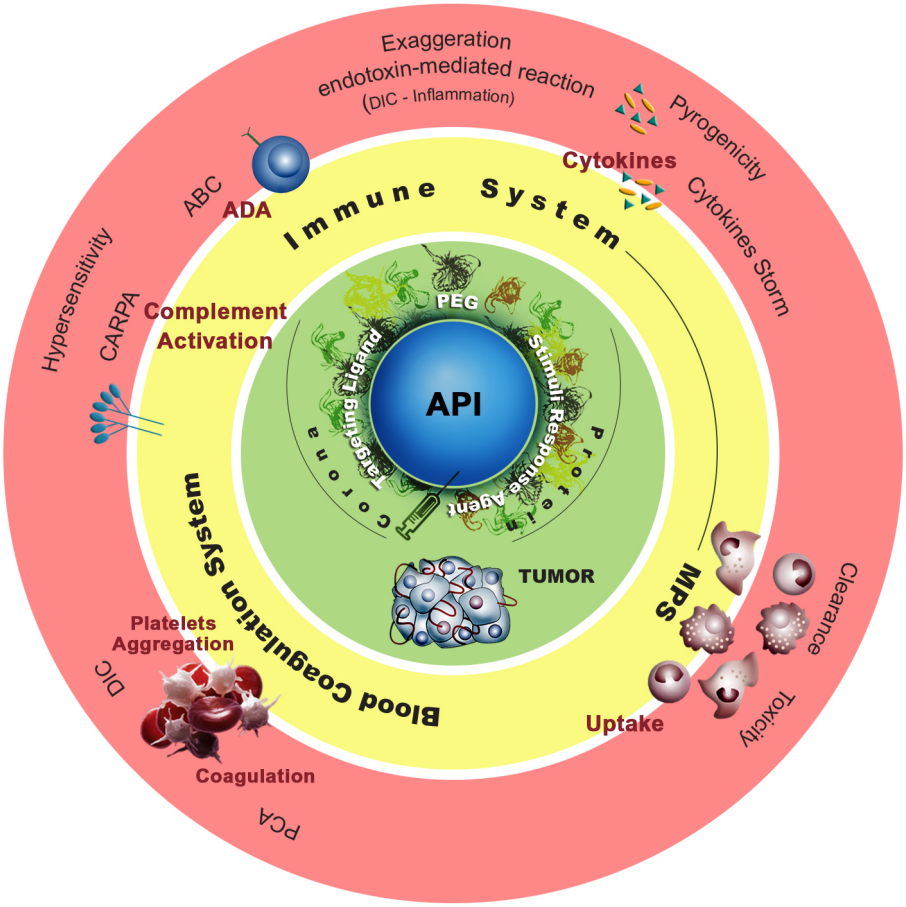
Immediately upon nanocarriers enter systemic circulation they encounter proteins, blood cells, and endothelial cells, as well as the coagulation and immune system components (Scheme **2**). These interactions can be deleterious, but also can be helpful for their therapeutic proposal as will be shown below.

5.2. Interactions with Proteins

Of particular interest are the interactions of nanocarriers with proteins, because their large surface-to-volume ratio implies that more proteins will bind to them than to larger particles. Bound proteins form a corona that modify its surface and increases its diameter influencing the overall effect of the nanocarrier [45].

Protein coronas are complex and variable, depending not only on nanocarrier characteristics (surface, including curvature, charge, and hydrophobicity) but also on the bound protein and the environment [45, 46]. They can involve irreversible or quick reversible binding of proteins forming a hard or soft corona, respectively [45].

Each of these environments represents unique proteomes that go shaping nanocarriers, besides other properties such as pH, ionic strength, and enzymatic activities, as they come in contact with it while traveling throughout the body. As most of the nanocarriers are administered parentally, one important environment to be considered is the plasma proteome consisting of over 3700 proteins. The major identified plasma proteins bound to nanocarriers are serum albumin, immunoglobulins G, fibrinogen and apolipoproteins [47]. These abundant proteins may initially dominate the nanoparticle surface. However, the existence of other minor proteins that have slower binding kinetics but higher affinity will later displace them leading to the presence of other proteins on nanoparticle surface [47]. Furthermore, it has to be considered that in the



Scheme 2. Nanocarrier interactions with biological systems.

tissues, the environment will be determined by proteins secreted by the different cells and the composition of extracellular matrix [46].

Current knowledge about the influence of nanocarrier physicochemical characteristics on the protein binding comes from studies on different nanoparticles with a determined surface. All together seems to point out that the charge and hydrophobicity of particles are the most important parameters influencing protein adsorption. For example, it has been demonstrated that neutrally charged particles have a diminished phagocytic uptake in comparison with charged particles, especially nanoparticles with a positive charge [48]. Also, it has been shown that as the charge density increased, more proteins were adsorbed [49]; but surface chemistry seems to have no influence on the species of bound protein [49].

How the protein corona profile of defined nanoparticles is and how this particular corona profile influences the biological system interaction, is not yet well understood.

However, it is now well established that total protein binding can serve as an indicator of particle "stealthiness." Particles with protein bound will be faster cleared by the mononuclear phagocytic system (MPS) than the stealthy particles. On the other hand, although the identity of the proteins bound to the nanocarriers may not be critical for understanding its clearance, it may be important for understanding their interaction with particular cells as well as their toxicity [50].

The interaction of proteins with nanocarriers can also generate aggregates that lead to an increased phagocytosis and also a decrease in the amount that reaches tissues due to size-restricted passage through the vasculature [46]. Indeed, whenever nanocarriers aggregate, changes in the way in which they interact with cells and tissues should be expected [46].

On the other hand, nanocarriers may induce conformational changes in the structure of adsorbed proteins altering its function. For example, negatively charged poly(acrylic acid)-conjugated gold nanoparticles bind to and induce unfolding of fibrinogen. This promotes the Mac-1 receptor activation and consequently the release of inflammatory cytokines [51].

5.3. Interactions with Cells

The hallmark of nanotechnology is the delivery of cancer therapeutic increasing targeting efficiency into tumor while reducing detrimental side effects to normal tissues.

It has been successfully demonstrated that nanoparticles can passively target tumor through the phenomenon widely known as EPR effect (enhanced permeation and retention) that is mostly due to the presence of a leaky vasculature surrounding a tumor region and to a dysfunctional drainage [52].

However, whenever a nanoparticle encounters a cell, it will be quickly internalized through endocytosis, an energy-dependent process, competing with the targeting process [53].

The endocytic pathways can be classified into clathrin and caveolae mediated endocytosis, phagocytosis, macropinocytosis, and pinocytosis. Clathrin and caveolae mediated endocytosis pathways involve the binding to cell surface receptors, thus also called RME (receptor mediated endocytosis) [53]. As nanoparticles are

coated with proteins upon contact with biological fluids, these represent the main pathways for nanoparticle internalization [54]. All cell types can use the mentioned pathways, excepting phagocytosis that is only performed by specialized cells, such as macrophages, monocytes, and neutrophils, belonging to MPS (macrophage phagocytic system). MPS is part of the immune system function, involving organs such as the liver, spleen, and bone marrow.

Once nanocarriers are endocytosed will be transported, entrapped in vesicles along the endolysosomal network [55]. During the intracellular trafficking, the nanocarrier has to be degraded or disassembled to release the drug within the cytosol or nuclei [54]. Furthermore, it may interact with intracellular components triggering a particular response. For example, if immune cells engulf a nanocarrier, it can activate cytoplasmic multiprotein complexes called inflammasomes, which are involved in the initiation of inflammatory responses [56].

Intracellular trafficking and therefore the fate of nanomaterials into the cell are linked to endocytic pathways [55]. For example, nanoparticles taken up by clathrin-mediated endocytosis are typically destined for lysosomal degradation; whereas, clathrin-independent internalization leads to endosomal accumulation and sorting to a nondegradative path [55].

Therefore, understanding the intracellular interactions of nanoparticles could allow engineering nanoparticles for highly specialized drug delivery to particular intracellular compartments.

One delivery system exploiting the selecting fate of nanoparticle through the endocytic pathway is the PGA-paclitaxel conjugate using the Gly-Phe-Leu-Gly linker [57]. Paclitaxel is mainly released through lysosomal cathepsin B from the polymer backbone.

Furthermore, the design of nanomaterials that can be internalized by receptor-mediated endocytosis and thus release their active drugs inside subcellular organelles might provide a useful means to circumvent efflux pump-mediated drug resistance [58].

Of particular importance for nanoparticle biodistribution is the interaction with MPS. The interaction with MPS along with renal clearance represent the major routes for the removal of nanoparticles from the body [53].

However, if nanoparticles that are phagocyted by MPS in related organs, eventually remain in them for a long time after being taken up, may increase the likelihood of acute or chronic toxicity [56].

In addition, positively charged nanoparticles once inside the cell will slow down the acidification of endosomes, thereby delaying the endosome-lysosome transition. Moreover, they can cause more pronounced disruption of plasma-membrane integrity, stronger mitochondrial, lysosomal damage, and increase number of autophagosomes. Positively charged nanoparticles may also form complexes with the negatively charged nucleic acids raising genotoxicity concerns [56].

5.4. Interactions with Blood Components

Beyond the advantages of using nanocarriers for therapeutic purposes of coagulation disorders they can also cause adverse effects through interaction with the blood coagulation system [59, 60].

Nanocarrier toxicities on coagulation can be mediated through interaction with coagulant factors, platelets and endothelial cells, each of which can be altered in a fashion dependent on their size, charge and chemical composition. These interactions can result in activation or inhibition of the coagulation system leading to hemorrhage or thrombosis, respectively.

This issue is particularly important in cancer since it has been reported that tumor cells can induce coagulation through different mechanisms: activating platelets, inducing proinflammatory cytokines or even more directly expressing procoagulant molecules in their surfaces [61].

There are an increasing number of studies reporting that engineered nanomaterials may cause severe toxicity by perturbation of the coagulation system [60]. In this context, an important disorder that should be ruled out is disseminated intravascular coagulation (DIC) or consumptive coagulopathy. DIC is characterized by the systemic activation of coagulation, mediated by several proinflammatory cytokines (mainly IL-6, TNF-alpha), leading to widespread intravascular deposition of fibrin and subsequent depletion of platelets and coagulation factors. As a result, thrombosis and severe bleeding may occur [62].

Some nanomaterials have been shown to induce DIC after their parenteral administration in animal models. A related disorder that has been reported with some nanocarriers is deep vein thrombosis (DVT), which is produced when clots are formed in deep veins, dislodge and end in the lungs causing pulmonary embolism [63].

In vitro studies have shown that cationic PAMAM dendrimers induce leukocyte procoagulant activity (PCA) that is accepted as an important component in the onset of DIC, while anionic and neutral dendrimers did not [64].

Liposomes are considered to have less effect on coagulation compared to those described for other nanomaterials [60]. It has been observed that negatively charged liposomes can interact with coagulation factors accelerating whole blood clotting time and can also induce reversible platelet aggregation *in vivo* and *in vitro* that is not considered to be of clinical relevance. But, positive and neutral liposomes had no effect on coagulation system [65]. Likewise, negatively charged liposomes induced transient thrombocytopenia, which can occur because of the interaction between liposomes and platelets; whereas neutral and positively charged liposomes did it in minor extension [66].

5.5. Interactions with Immune System

While nanocarriers have been shown to reduce the immunotoxicity of some APIs by masking them, they can also interact with immune system components triggering immunostimulatory or immunosuppressive responses. These effects have been exploited for medical applications, such as vaccines or inflammatory and antiallergy therapeutic treatments, respectively [67].

Particularly, the research on nanocarriers as adjuvants in vaccines to stimulate specific immune responses has become a field of fast development in the pharmaceutical industry and has contributed to the knowledge about the relationship between nanocarrier characteristics and immunostimulatory reactions [68]. For example, several studies reported that small particles (20-200 nm) elicit stronger immune responses than their larger counterparts [69]. It has been also found that small (< 100 nm) nanoparticles were taken up preferentially by dendritic cells (DCs), while large (1 μm) particles were more frequently internalized by macrophages [69]. Furthermore, some lipid nanocarriers were shown to contribute to the drug's immunostimulation activity when used to deliver nucleic acids [70].

However, when a nanocarrier is not designed to interact with the immune system, as frequently occurs in cancer treatment, this possibility turns to be an important concern because many of the components of nanocarriers (e.g. shell, core, surface-decorating moieties, and cargoes) may be recognized as dangerous, and initiate an immune response through a complex process involving different organs, cells and molecules that eventually lead to toxicity in the host and/or lack of therapeutic efficacy [71].

One of these effectors is the complement system, a network of over 30 soluble and membrane-bound proteins, which connects innate and adaptative immunity [72]. It is well known that the activation of complement protein cascade can be harmful because it may lead to hypersensitivity reactions [67].

In this context, a transient acute hypersensitivity reaction named complement activation-related pseudoallergy (CARPA) has been reported in some patients after the injection of liposomal drugs, such as Doxil® and products containing the lipidic vehicle, Cremophor EL®, i.e. Taxol® [73]. CARPA improves upon subsequent exposures and reducing the infusion rate, different from anaphylactic reaction involving IgE that can be a serious life threatening condition [74].

Indeed, contrary to original assumptions, it has been shown that the PEG chains on nanocarriers (i.e.: Doxil®) cannot avoid complement activation [75].

The negative charge present on the phosphate of the lipid-PEG conjugates will be the culprit of such effect. It was found that the removal of the negative charge by methylation of the phosphate oxygen of lipid-PEG conjugates prevented complement activation [76].

A long thought regarding complement activity is that it contributes to eliminate tumor cells directly by the membrane attack complex (MAC), a process usually known as complement-dependent cytotoxicity (CDC), or enhancing the antibody-dependent cell-mediated cytotoxicity (ADCC) [77]. However, recent observations point out that complement activation can promote tumor growth and progression. It was demonstrated that complement anaphylatoxin and chemoattractant C5a production, promoted recruitment of myeloid-derived suppressor cells, deregulating or suppressing cytotoxic CD8+ T cells [78].

This unexpected observation arise new questions about nanocarrier safety in cancer therapy [79].

The design of nanocarriers with C3/C5 convertase inhibitors in conjugation with surface PEGylation has been suggested to prevent complement activation [75]. Furthermore, this strategy will allow the prevention of CARPA, which is an issue to be considered in cardiac patients since one of the main manifestations of complement activation is cardiopulmonary distress. In addition, in some patients it can be severe and even lethal.

As mentioned before, complement activation can also lead to a faster clearance by the MPS through the opsonization of the nanocarriers.

A related phenomenon named accelerated blood clearance (ABC) has been reported to occur in animal models upon repeated injection of PEGylated liposomes [80]. This is caused by the binding of specific IgM to nanocarriers that act as opsonin thereby activating complement [81]. Several properties of nanocarriers (chemical nature of API, physicochemical properties and dose) have been shown to determine this phenomenon [81, 82]. For example, cytotoxic payload as is the case of cancer treatment, seems to abrogate the ABC effect because their deleterious effect on B cell affects the IgM production [82].

It is worth mentioning that anti-PEG antibodies have been recently found in the blood of healthy donors in a proportion as high as 25 % of them. This could be due to better detection methods or to the increase in the exposure to PEG and derivatives that are present in cosmetic and pharmaceutical products [83].

Therefore, the generation of specific antibodies against any of the chemical structures present in nanocarriers is an important issue to be considered in drug formulations requiring repeated administration.

Another effectors that mediate and regulate the immune response are cytokines. These proteins are generally recognized as biomarkers of immunotoxicity [71].

An uncontrolled production of cytokines may cause severe acute reactions that under some circumstances can be life-threatening such as what is known as “cytokine storm” and DIC [84].

The measurement of cytokine levels is emerging as a useful tool for evaluation of nanoparticle safety [77]. For example, they are used as biomarkers for acute inflammation and also as markers of pyrogenic response, e.g. IL-1β and IL-6 [85].

Understanding the underlying mechanisms that induce the secretion of cytokines and the associated adverse reactions is essential in the design of safe nanocarriers [85]. However, there are different reports indicating that the induction of cytokine response by nanocarriers may not be due to their chemical components or structure but rather to the presence of low concentrations of endotoxins [67, 71].

In fact, some nanocarriers can potentiate endotoxin-mediated inflammation, although they cannot induce inflammation *per se*. For example, cationic polyamidoamine (PAMAM) dendrimers exaggerate endotoxin-induced leukocyte procoagulant activity, which is an essential prerequisite to DIC [85].

From the quality control perspective, it should be stressed that due to the hydrophobic active moiety of endotoxins they can be incorporated into the nanocarriers becoming invisible from the detection of routine assays designed to determine their presence. Moreover, nanoparticles interfere with traditional analytical endotoxin-specific assays [86].

Another related safety concern is the presence of minute amounts of chemical impurities like by-products of synthesis reactions and metal catalysts which could start an inflammatory reaction upon contact of the nanocarriers with biological systems [67, 71]. Therefore, before establishing that a nanocarrier is toxic or inflammatory it should be ruled out that these effects are not due to the presence of minute amounts of chemical or biological contaminants [67].

5.6. Oxidative Stress

It has been reported that some nanocarriers can cause the production of reactive oxygen species (ROS), which in high levels could be harmful [87]. The higher surface/volume ratio of nanocarriers as compared to microparticles make them more prone to induce the production of ROS [71].

Many types of NPs have been shown to produce ROS which activate inflammatory response. For example, Cremophor-EL® has been described to induce oxidative stress that elicit the production of IL-8 by monocytes [87].

Other nanoparticles formed by metals and carbon have been also shown to release cytokines mediated by ROS [89]. It was found that the inflammatory response and the production of cytokines could be inhibited by catalase, an enzyme that breaks down hydrogen peroxide preventing oxidative stress [87].

6. CHEMICAL AND PHYSICOCHEMICAL ISSUES TO BE CONSIDERED FOR THE QUALITY CONTROL OF NANOCARRIERS

6.1. Chemical Composition

From the regulatory perspective this is by far the most widely studied and characterized property of any pharmaceutical product (Table **3**). In the special case of nanocarriers, it is also known that their properties will be in part dependent on the chemical composition. Due to regulatory issues, mainly from their proved biocompatibility most of the approved nanomedicines involve the use of lipids, proteins, polysaccharides and a few approved synthetic polymers [6]. These include the natural or derivative forms of: phospholipids, cholesterol, albumin, gellatin, alginates, chitosan, starch, cellulose derivatives, acrylate derivatives, polylactide (PLA), PLGA, among others.

From the formulation perspective there are other ingredients that should be considered because of their possible influence not only in the final properties of the nanocarrier but also in the proper-

Table 3. General aspects to be considered for the quality control of nanocarriers.

MATERIALS	**API** **Excipients**	Specifiy Impurities Endotoxin NC Components Characterization
NANOPARTICLE	**Composition** **Physicochemical** ***In vitro* release**	API and NC Quantities Free and Encapsulated Drug API Distribution Size/Size Distibution Shape Morphology Phase State / Transition T Sruface Zeta Potential Ligand PEG
MANUFACTURING PROCESS	**Nanoparticle Formation** **Size Control** **API Loading** **Purification** **Sterilization Process**	
FINAL PRODUCT	**pH/Osmolarity/Viscosity** **Residual Solvent** **Sterility/Endotoxin** **Stability (API, excipients, structure)**	

ties of the intermediate products that are formed during the preparation procedure. These are: salt, buffering agent, sugars and surfactants. Last but not least, it should be kept in mind that the chemical nature and proportion of API can affect the structure and properties of the product [6-11].

From the production point of view, it has been demonstrated that the order and physical state of the chemical components that have been selected to prepare the nanocarrier will condition their spatial distribution and final structural properties of the product [6]. From the perspective of controlling or modulating the release of API its distribution (homogeneous, encapsulated inside a compartment, distributed differentially between nanocarriers, etc.) will have to be considered when designing the characteristics of the reservoir of API. From the chemical point of view, the interactions that the selected API can establish with the components of the nanocarriers will certainly determine the kinetics of its release in different biological scenarios. If the API is a protein or a big flexible molecule, its physical entanglements with the structure of the nanocarrier are also elements to be kept in mind because they can be affected by the chemical composition and also by the fabrication procedure. Altogether, the modulation of these properties can be used to tune the final biological activity of the nanomedicine, mainly in terms of pharmacokinetics, pharmacodynamics and biocompatibility.

From the nanoengineering point of view, the selection of chemical ingredients of a nanocarrier will be determined by: a) the size and structure, b) the charge and functional groups, c) manufacturing procedure (scalability, sterilizability, potential interaction with production equipment and instruments), d) regulatory issues and e) accessibility and cost.

From the quality control aspect the chemical integrity of API and excipients is by far the most studied and will not be considered further in this review.

6.2. Physicochemical Properties of Structured Nanocarriers

As mentioned above, enthalpic and entropic components have been shown to contribute to the overall free energy change involved in the stability and general properties of nanocarriers [6, 7]. It is always important to stress the fact that the final biological activity of a nanomedicine will be determined not only by its chemical properties but also by its physicochemical and structural properties that will be also dependent on the composition but also on the fabrication process [6, 7].

In this context, the Food and Drug Administration (FDA) of USA has recently released documents intended to establish the criteria to determine the bioequivalence of liposomal doxorubicin products. In these documents it is required to perform a thermodynamic characterization of liposomal products. By far the most widely used technique to characterize the thermodynamic parameters of nanocarriers is high sensitivity differential scanning calorimetry (DSC). The relatively low lipid concentration (around 10 $mg.mL^{-1}$) and small size were probably the main reasons that impaired the obtention of thermodynamic parameters of these liposomal nanocarriers by DSC until this year when, by using a high sensitivity DSC it was possible to demonstrate not only the thermotropic phase transition of the lipids present in pegylated liposomal doxorubicin but also the melting of doxorubicin-sulfate crystals that are present in the aqueous compartment of the commercial products [88]. It was also possible to demonstrate that the nanovolume of the

liposome interior improves the formation of doxorubicin-sulfate crystals and that the presence of cholesterol is critical for the formation of a non-leaky membrane [88].

6.2.1. Structure of the Particle - Size, Dimensions and Distribution of Components

It has been clearly demonstrated that the fate and biological activity of nanocarriers are very sensitive to their size and size distribution [6]. From the biological perspective, it has been shown that not only their targeting and uptake mechanism by target cells but also the interaction with the immune system are affected by their average size and shape of the particles [6]. As for cancer treatment, approved carriers range in size from a few nanometers (Doxil®, mean particle size 90 nm) to several micrometers as in the case of PLGA microspheres in Lupron Depot® (mean particle size 7 μm) and Sandostatin® (mean particle diameter 50 μm). From the quality control perspective, it is should be stressed that it is important to determine not only the average size and dimensions of the nanocarriers but also their size distribution. In this regard, the most widely used technique for the characterization of spherical liposomes, micelles and other nanosized particles is dynamic light scattering (DLS). However, this technique is to be used with caution when dealing with non-spherical particles like those present in some products (i.e.: ribbon-like structure of Abelcet® for the delivery of Amphotericin B).

When dealing with non-spherical nanocarriers there are several microscopic techniques that can be used in order to evaluate not only the average size and distribution but also the shape, external appearance and internal structure of the particles [6].

It is important to mention that in those nanocarriers that incorporate the API as part of the structure, not only their size and shape but also their dynamics (i.e.: stability upon dilution) can be affected by how the API is incorporated and where it ends located [6].

6.2.2. Electrical Properties

Considering that most biological surfaces (biopolymers, extracellular matrix and cell membranes) are negatively charged the presence/absence of net electric charges in a nanocarrier is an important issue that has to be considered in their design and quality control. First of all, it should be kept in mind that the dissociation constant of ionizable groups is going to be dramatically affected by their incorporation within the same supramolecular structure. Thus, it is reasonable to expect that even strong acidic groups that in solution have a dissociation constant close to 1 end with a dissociation constant around 0.2 when incorporated in a micelle or liposome. This aspect has been thoroughly studied with negatively charged detergents when their concentration is raised above their critical micellar concentration [6]. The pH and salt dependency of the ionization state of the chemical components that end in the surface of the nanocarriers are aspects to be considered for the formulation of the product (because their presence or absence will affect the stability of the nanocarriers in solution) and also for their effect on the interactions that can be established within living systems. The presence of electric charges is also important to confer stability to the particles in solution as the repulsion between them prevents aggregation and clumping.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The evolution of nanotechnology provides new tools that allow for a more precise and controlled design of products for specially intended applications. Altogether, these led to the new concept of nanoengineering. In the case of nanocarriers, there are several aspects that are to be considered: a) nanocarriers are always fluctuating structures whose properties are represented by average values; b) nanocarriers can display an intrinsic bioactivity; c) the structures formed by their interaction with biological systems lead to the formation of new entities that can have different biological activities than those of the original nanocarrier; d) their biocompatibility and efficacy not only depend on their chemical nature but also on the physical properties of the structures that end presented to the target tissue and to the rest of the living system.

Improvement in the knowledge of the thermodynamic and kinetic aspects involved in the structuring process of nanocarriers is leading a field that is finding many new preparation procedures and applications. Studies conducted so far indicate that subtle differences in structural parameters of nanoparticles can affect their interaction with biological systems in a still unknown way. The advent of new and more precise techniques to assess the possible outcomes when a nanocarrier enters in contact with a living organism will surely improve the number of successful products that arrive to clinical trials. It is also reasonable to speculate that in the near future the accessibility to molecular diagnostic techniques will improve the precision of cancer diagnostic and therefore in the selection of the appropriate pharmaceutical treatment. Research that is conducted on the molecular mechanisms of cancer progression and metastasis is disclosing new alternatives that will surely open the possibility to specific treatments.

Although we are still far from the idea of "swallowing the doctor" the emerging techniques of nanoengineering that allow for a precise control of the structural and biological properties of nanomedicines gives us a reasonable hope that we will see the appearance of true cures for cancers in the near future. In this direction, the 2016 Nobel Prize in chemistry honored the design of molecular machines. In the words of Bernard Feringa, one of the laureates, controlling movement at the molecular level will allow us to build nanomachines, tiny robots that the doctor of the future will inject and go to search (and kill) a cancer cell.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

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