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*Expert Review*

# Nanotechnology: Intelligent Design to Treat Complex Disease

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***Abstract.*** The purpose of this expert review is to discuss the impact of nanotechnology in the treatment of the major health threats including cancer, infections, metabolic diseases, autoimmune diseases, and inflammations. Indeed, during the past 30 years, the explosive growth of nanotechnology has burst into challenging innovations in pharmacology, the main input being the ability to perform temporal and spatial site-specific delivery. This has led to some marketed compounds through the last decade. Although the introduction of nanotechnology obviously permitted to step over numerous milestones toward the development of the "magic bullet" proposed a century ago by the immunologist Paul Ehrlich, there are, however, unresolved delivery problems to be still addressed. These scientific and technological locks are discussed along this review together with an analysis of the current situation concerning the industrial development.

**KEY WORDS:** autoimmune disease; cancer; drug targeting; infections; industrial development; liposome; metabolic disease; nanoparticle; vaccine; nanotechnology.

## INTRODUCTION

In the past 30 years, the explosive growth of nanotechnology has burst into challenging innovations in pharmacology, which is in the process of revolutionizing the delivery of biologically active compounds. The main input of today's nanotechnology in pharmacology is that it allows real progresses to achieve temporal and spatial site-specific delivery. Thus, the concept of the "magic bullet" proposed a century ago by the immunologist Nobel laureate Paul Ehrlich turned out recently to reality with the appearance of several approved forms of drug-targeting systems for the treatment of certain cancer and serious infectious diseases. This breakthrough was made possible by the development of various types of nanosystems resulting from cutting-edge researches based on pluridisciplinary approaches. From the first liposomes proposed in 1974 by Gregoriadis *et al.* (1) and today, there was an explosion in the number of nanodevices suitable for drug delivery, which are either made of lipids or composed of polymers (Fig. 1) (3,4). Recently, new drug delivery systems based on carbon assemblies were also suggested (5–7). These systems are exploited for therapeutic purpose to carry the drug in the body in a controlled manner from the site of administration to the therapeutic target. This implies the passage of the drug molecules and drug delivery system across numerous physiological barriers, which represent the most challenging goal in drug targeting (Fig. 2) (4,8). In general, nanocarriers may (i) protect a drug from degradation, (ii) enhance drug absorption by facilitating diffusion through epithelium, (iii) modify pharmacokinetic and drug tissue distribution profile, and/or (iv) improve intracellular penetration and distribution (see Table I). Nanosystems were also found useful to improve the performance of imaging techniques applied for the *in vivo* diagnosis of tumors. In this case, colloid metals are often incorporated in the nanodevice.

Applications of nanotechnology in pharmacology are now undeniably linked to the potential of drug targeting. Although we are still far from the ideal "magic bullet", today, nanotechnology has already completed several key achievements to reach this goal. The most straightforward application is in cancer therapy with several marketed compounds (Caelyx®, Doxil®), others being currently investigated in clinics (Transdrug®, Abraxane® or ABI-007). Another very demanding field includes infectious diseases [human immunodeficiency virus (HIV), leishmaniasis, malaria, nosocomial infections, all kinds of infections in immunocompromised patients, etc.] with already approved drugs for clinical uses (Ambisome®) (9). Treatments of these severe diseases generally involved highly toxic compounds for healthy tissues, and their uses in therapy are considerably limited by occurrence of dramatic side effects using the traditional pharmaceutical formulations. Nanotechnology also seems to be as a promising alternative to overcome the problems of the administration of peptides and proteins and of the new drug molecules coming out of the discovery pipeline. Many of them are, indeed, poorly soluble in both aqueous and organic media, which results in poor bioavailability with low and/or erratic absorption when using traditional formulations (10). Nucleic acids are other potential candidates for which nanotechnology represents a unique opportunity to be used in therapy. They are rapidly degraded in biological media, and they hardly cross biological barriers. In addition, these molecules, which

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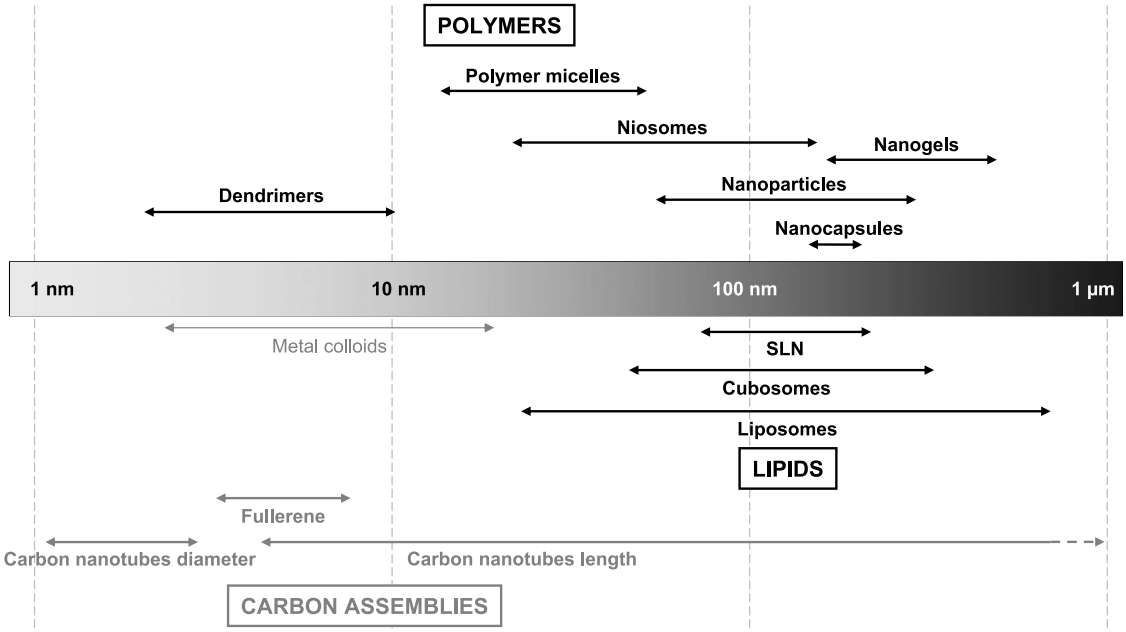


**Fig. 1.** Types of nanotechnology used for drug delivery and targeting. The major components are either lipids or polymers. The incorporation of metal colloids in the other types of systems can be used to confer additional specific properties such as magnetic, superparamagnetic, and thermal properties. The black axis represents the diameter of the nanotechnology.

are even sometimes considered as "undeliverable" compounds, need to reach an intracellular target to achieve their therapeutic effect (11–17). Therefore, the tremendous therapeutic potential of nucleic acids depends on the success to find suitable carriers that will bring them to their target site.

Thus, the core of this review aims to analyze and to discuss the impact of nanotechnology in pharmacology to improve treatments of various diseases considered as the major health threats (cancer, infections, metabolic diseases, etc.). Milestones of achievements will be discussed considering the different challenges that need to be addressed. Present considerations about industrial developments of nanotechnology for pharmacological applications will also be discussed at the end of this review.

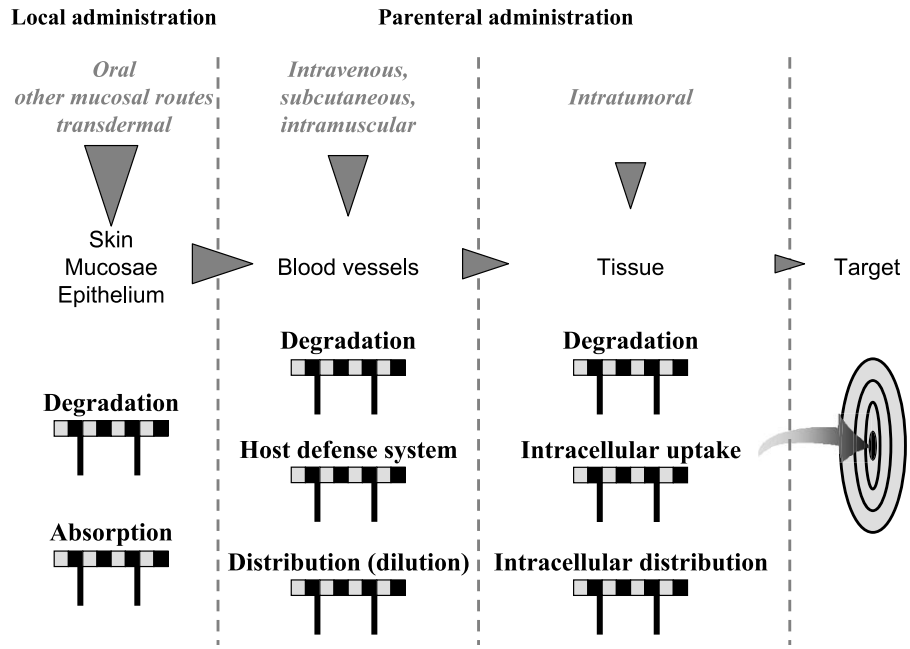


**Fig. 2.** Illustration of the different types of barriers drug molecules and drug delivery systems have to overcome before reaching the biological target into the body. The gray triangle gives a schematic representation of the drug loss while progressing across the different barriers toward the target site.

**Table I.** Therapeutic Challenges Addressed with Nanotechnology to Improve Treatment against Major Human Health Threats

| Disease | Therapeutic challenge | Nanotechnology solution |
|---|---|---|
| Cancer | –Increase efficacy<br>–Reduce toxicity<br>by<br>–Controlling biodistribution,<br>–Improving intracellular penetration | Nanoparticles<br>Liposomes<br>Micelles<br>PEGylated nanoparticles<br>PEGylated liposomes<br>PEGylated micelles<br>Targeted nanoparticles<br>Targeted liposomes |
| Infections | –Increase efficacy<br>–Reduce toxicity<br>by<br>–Controlling biodistribution<br>–Improving intracellular penetration<br>(in macrophages, cell-presenting antigens, dendritic cells...)<br>–Facilitating absorption through mucosa<br>–Improving protection against degradation<br>(antigenic peptides) | Nanoparticles<br>Liposomes<br>PEGylated nanoparticles<br>PEGylated liposomes<br>Antigen-presenting devices |
| Metabolic diseases | –Protection against degradation<br>(therapeutic peptides and proteins)<br>–Improve mucosal absorption<br>–Controlled and sustained release | Nanoparticles<br>Liposomes<br>Nanoparticles<br>Liposomes |
| Autoimmune disease, prevention of graft rejection | –Control biodistribution to target the immune system and/or the inflammatory cells<br>–Controlled and sustained release<br>PEGylated nanoparticles<br>PEGylated liposomes | |
| Pain treatment | –Controlled and sustained release<br>–Improve the bioavailability towards the central nervous system (CNS) | Liposomes<br>CNS targeted liposomes<br>CNS targeted nanoparticles<br>Solid lipid nanoparticles |
| Gene therapy relate diseases | –Protect against degradation<br>–Condense DNA<br>–Improve cellular uptake<br>–Address cytoplasmic/nucleus intracellular compartments<br>Nanocapsules | Cationic nanospheres<br>Cationic polymers<br>Cationic lipids<br>Cationic nanogels |

## APPLICATIONS IN PHARMACOLOGY

### Cancer

#### *Nanotechnologies for the Delivery of Small Anticancer Molecules*

*General Considerations.* Even if new molecules are discovered to treat cancer diseases, the clinical use and efficacy of conventional chemotherapeutics is hampered by the following limitations: (i) drug resistance at the tumor level because of physiological barriers (noncellular-based mechanisms); (ii) drug resistance at the cellular level (cellular mechanisms); and (iii) distribution, biotransformation, and clearance of anticancer drugs in the body. At the tumor level, for example, the high interstitial pressure may lead to an outward convective interstitial fluid flow, which opposes to the diffusion of the drug molecules from the vascular space to the tumoral tissue, the drug transport being governed also by the physicochemical properties of the interstitium (composition, structure, charge) and of the molecule itself (size, configuration, charge, hydrophobicity) (18,19). The fact that some tumor regions are poorly vascularized or that vascularization is often heterogeneous is another concern. At the cellular level, the resistance of tumors to therapeutic intervention may be caused by alterations in the biochemistry of malignant cells including altered activity of specific enzyme systems (e.g., topoisomerase activity), altered apoptosis regulation, or transport-based mechanisms, such as P-glycoprotein efflux system, responsible for the multidrug resistance (MDR) or the multidrug-resistance-associated protein (20,21). Finally, because the body distribution of an anticancer molecule is essentially based on its physicochemical properties, which are not necessarily fitting the characteristics of the diseased area, large amounts of drug have to be given. Toxicity comes then from massive drug penetration into healthy organs and

tissues, which is another important limitation (22). In other words, conventional chemotherapeutics is often limited to inadequate delivery of therapeutic concentrations to the tumor target tissue.

It is therefore of importance to develop new nanotechnologies (liposomes, nanoparticles, polymerized micelles, etc.) for targeted delivery to tumors both at the cellular and tissue levels, thereby improving the therapeutic index of the carried anticancer molecules. Strategies for developing new efficient targeted nanoformulations of anticancer compounds may result from the combined knowledge of cancer physiopathology features and *in vivo* fate and behavior of nanotechnologies.

*Nonsurface-Modified Nanotechnologies for the Treatment of Cancers (Passive Targeting).* At the tissue level, upon intravenous injection, colloids are opsonized and rapidly cleared from the blood stream by the normal reticuloendothelial defense mechanism, irrespective of particle composition (23–25). Thus, the liver acts as a reservoir toward nanoparticles, liposomes, etc., conditioning their rapid first-phase disappearance from the blood and, in case of biodegradable systems, their second-phase release in the body under degraded and excretable form. This biodistribution can be of benefit for the chemotherapeutic treatment of mononuclear phagocyte system (MPS) localized tumors (e.g., hepatocarcinoma or hepatic metastasis arising from digestive tract or gynecological cancers, bronchopulmonary tumors—primitive tumors or metastasis—including "nonsmall cells tumor" and "small cells tumors," myeloma, and leukemia). For instance, the superiority of doxorubicin targeted with the aid of biodegradable poly(alkylcyanoacrylate) nanoparticles has been demonstrated in a murine hepatic metastases model (M5076 reticulosarcoma) (26): irrespective of the dose and the administration schedule, the reduction in the number of metastases was much greater with doxorubicin-loaded nanoparticles than with free doxorubicin, particularly if the treatment was given only when the metastases were well established. Additionally, with this type of nanoparticles loaded with doxorubicin, very impressive results were obtained concerning the reversion of the MDR, likely because the strong adsorption of nanoparticles onto the cell surface induces a microgradient of drug concentration at the membrane, which, in turn, increases the intracellular diffusion of doxorubicin, thus overflowing the PgP detoxification capacity (27,28). This is a very important observation for the treatment of hepatocellular carcinoma, one of the most prevalent cancers worldwide (29), because these tumors are well known as resistant to chemotherapeutic drugs, mostly because hepatocellular carcinoma cells are able to develop resistance mechanisms and to evade the effects of chemotherapy (30). The higher cytotoxicity of doxorubicin when loaded onto poly(isohexylcyanoacrylate) nanoparticles has been shown recently on the X/*myc* transgenic mouse model of hepatocellular carcinoma, which mimics several steps of human hepatocarcinogenesis. In this study, doxorubicin-loaded poly(isohexylcyanoacrylate) nanoparticle-induced apoptosis was specific and restricted to hepatocellular carcinoma tumors because it did not enhance the apoptosis rate of noncancer hepatocytes in peritumor areas (31). Based on these data, a phase II multicentric clinical trial is currently being performed on patients with resistant hepatocarcinoma or liver metastasis. When anthracycline antitumor agents are encapsulated into liposomes, they show reduced cardiac as well as gastrointestinal toxicities because the major part of the injected dose is sequestered into the MPS, which provides lower peak plasma levels while maintaining similar total body exposure [area under the curve (AUC)] than the free drug counterparts (32,33). It is suggested that after the drug-loaded liposomes are captured by the Kupffer cells of the liver, the liposome matrix becomes leaky, and the drug (and its active metabolites) may be released and distributed in free form to the tumor. The therapeutic index is improved because the anthracycline's antitumor efficacy is maintained, whereas acute and chronic toxicities are substantially reduced (33).

*Surface-Modified Nanotechnologies for the Treatment of Cancers (PEGylation).* Despite the promising results obtained with the "first-generation" drug nanocarrier systems, their usefulness is limited by their rapid blood clearance and recognition by the MPS. Recently, a great deal of work has been devoted to developing so-called "Stealth®" particles, which are "invisible" to macrophages ("Stealth®" is a registered trademark of Liposome Technology Inc., Menlo Park, CA, USA). A major breakthrough in the liposome field consisted in the use of phospholipids substituted with poly (ethylene glycol) (PEG) chains of molecular weight from 1000 to 5000 Da (34,35). This provides a "cloud" of hydrophilic chains at the particle surface, which repels plasma proteins, as discussed theoretically by Jeon *et al.* (36). These "sterically stabilized" liposomes have circulating half-lives of up to 45 h, as opposed to a few hours or even minutes for conventional liposomes. They have been shown to function as reservoir systems and can penetrate into sites such as solid tumors (37). A similar strategy has been applied to nanoparticles. PEG can be introduced at the surface in two ways: either by adsorption of surfactants (38,39) or by the use of block or branched copolymers, usually with poly(lactic acid) (PLA) or poly(alkylcyanoacrylate) (40,41). It is noteworthy that not only the surface characteristics of the particles but also their size are the keys for the biological fate of these nanodevices because these parameters can prevent their uptake by MPS macrophages. A high curvature (resulting in a small size, <100 nm) and/or a hydrophilic surface (as opposed to the hydrophobic surface of conventional nanoparticles) are needed to reduce opsonization reactions and subsequent clearance by macrophages (42). Thus, those "second generation" of "Stealth®" nanotechnologies with small size and/or decorated with hydrophilic polymers (PEG, poloxamers, hydrophilic polysaccharides, etc.) are able to selectively extravasate in tumors with a leaky vasculature (43). The mechanisms by which those "Stealth®" nanotechnologies diffuse into the tumors and release their drug content are not completely understood. It is believed that these nanosystems need to be small enough and to circulate for a sufficient period of time to extravasate selectively through the small defects of the fenestrated and leaky vasculature that generally characterize tumor vessels (44). This so-called "enhanced permeability and retention effect" results in intratumoral drug accumulation, which is even higher than that observed in plasma and other tissues (45,46). Particle uptake by the circulating macrophages resulting from the inflammatory process is another possible mechanism involved in colloid translocation through the endothelial barrier (47). A typical illustration of this approach is the clinically approved

Doxil® formulation in which a PEG layer surrounds the doxorubicin-containing liposomes (100 nm). Doxil® has been investigated in various cancer types including breast cancers, ovarian cancer, non-Hodgkin's lymphoma, nonsmall cell lung cancer, etc. In the USA, Doxil® is approved by the Food and Drug Administration for the treatment of metastatic ovarian cancer in patients with diseases refractory to both paclitaxel- and platinum-based chemotherapy regimens, and it may be considered as a drug of choice for patients with advanced ovarian cancer for whom first-line chemotherapy has failed (48). Indeed, pegylated doxorubicin liposomes have demonstrated a significant pharmacological efficacy in the treatment of recurrent or relapsed ovarian cancers in several clinical trials (48–50). Because the long circulating liposomes promote extravasation of the drug, new toxicities may emerge, the most common being the hand–foot syndrome (51). Further investigation has shown that the incidence of hand–foot syndrome is schedule dependent, with shorter dosing intervals leading to increased frequency and severity of occurrence (48,49,51). The combined use of Doxil® with agents with nonoverlapping toxicities should be investigated to increase the efficacy of the treatment, without increasing the toxicity as it was performed recently with carboplatin (52).

*Addressed Nanotechnologies for the Treatment of Cancers (Active Targeting).* A lot of efforts have been devoted in achieving "active targeting" to deliver drugs to the right cells, based on molecular recognition processes. Specific antibodies or ligand targeting proteins expressed on cancer cell membranes or endothelial cells lining the newly generated blood vessels into the tumor are among the possible options to perform the active targeting of nanotechnologies toward tumoral sites. Examples of relevant targets are the folate receptor or the integrin surface receptor. Other examples include galactolipids that bind to the asialoglycoprotein receptor of the human hepatoma HepG2 cells. In some cases, active targeting needs a receptor-mediated cell internalization to occur (53). For example, antibody-coated liposomes have been developed either by direct linkage of the antibody to the liposome phospholipids head group or to the terminus of the PEG polymer. The second approach has been proven to be more efficient because of the better accessibility of the antibody toward its corresponding antigene (54). Coupling the antibody at the terminus of the PEG polymers allows to combine longevity of the liposomes in the blood circulation with its targetability for drug delivery into the tumor (55). Anyway, decorating the surface of the liposomes with antibodies directed against tumor-associated antigens needs to be balanced between a sufficient number of antibody molecules per liposomal surface to achieve efficient binding and recognition on one hand, and not too many antibodies to avoid complement activation and to keep the ability of the immunovesicles to escape from the recognition by the MPS on the other hand. An optimal coating of 10–30 antibody molecules per liposome seems to allow the combination of an efficient delivery with a limited uptake by the MPS (56,57). Here are some examples of the variety of liposomal constructs using antibodies for the treatment of experimental cancers:

- Targeting of immunoliposomes to pulmonary endothelial cells of the lungs was found possible using the IgG monoclonal antibody (34A) directed toward the glycoprotein receptor pp120 (57).
- Targeting of immunoliposomes to circulating B-lymphoma cells was successfully achieved using pH-sensitive liposomes decorated with anti-CD19 antibodies for the specific recognition of the CD19 receptor of the human B cell lymphoma (53).
- Anti-HER-2 immunoliposomes with encapsulated doxorubicin were found to be more efficient against breast cancer xenograft models when compared with single PEGylated liposomes (58).
- Significant tumor accumulation was also observed using the CC52 antibody directed against rat colon adenocarcinoma as targeting moiety of liposomes (59,60).

The use of proteins or peptides for active liposomal targeting to tumors includes the peptide sequence RGD capable of specific recognition of the $\alpha_v\beta_3$-integrin receptor expressed in the neovasculature during angiogenesis of tumor. Thus, the encapsulation of doxorubicin in RGD-addressed liposomes has showed superior anticancer efficacy on the C26 colon cancer xenograft model than RGD-non-addressed liposomes (61). Antagonist G-targeted liposomes increased the targeting of doxorubicin toward the human small-cell lung cancer H69 cell line as revealed by the increased cellular uptake of the targeted liposomes compared with the nontargeted liposomes (62).

Transferrin is also a very useful ligand for liposome targeting to tumors. The main advantage of the transferrin receptor as a target arises from its ability to be cell internalized with its specific ligand (63). When doxorubicin is encapsulated into liposomes coupled with transferrin, an increased antitumor effect is observed on C6 glioma cells (64). The transferrin bearing liposomes also showed the capacity of specific receptor binding and receptor-mediated endocytosis with target colon tumor cells 26 implanted in mice (65).

As stated above, addressing liposomes with the aid of antibodies or proteins includes the risk to confer to the vesicles a certain hydrophobicity, which may account for some opsonization and nonspecific MPS recognition, instead of tumoral targeting. Therefore, small molecules are considered with great attention as targeting moieties for the design of liposomes with specific recognition properties. Thus, by taking advantage of the overexpression of folate receptors on the surface of malignant human cells, folate-conjugated liposomes were developed in the hope that their folate grafting would help them to actively and specifically target cancer cells (66–69). Folated targeted liposomes with encapsulated doxorubicin and daunorubicin have been found to efficiently deliver their cargo into cancer cells, thus increasing cytotoxicity (67,68). Mannose is another small molecule considered to design targeted devices. It was used to target immunomodulators to liver metastasis with mannosylated liposomes (69).

If the concept of active targeting has led to many liposomal-based constructions as detailed above, there are much less data in the literature concerning the active targeting performed with polymer nanospheres for cancer treatment, likely because these nanocarriers were developed later than liposomes. One example of this approach is the design of

PEG-coated biodegradable nanoparticles coupled to folic acid via the PEG terminal amino group of the copolymer, poly[aminopoly(ethylene glycol) cyanoacrylate-*co*-hexadecyl cyanoacrylate] [poly($H_2$NPEGCA-*co*-HDCA)] (71). Interestingly, surface plasmon resonance revealed that folate grafted to pegylated cyanoacrylate nanoparticles had a 10-fold higher apparent affinity for the folate-binding protein than free folate did. Indeed, the particles represent a multivalent form of the ligand folic acid, and folate receptors are often disposed in clusters. As a result, conjugated nanoparticles could display a multivalent and hence stronger interaction with the surface of the malignant cells (71). *In vitro* experiments have shown that PEG-coated nanospheres with folate are internalized by KB cells, which express high amounts of the folate receptor at their surface. In another study, biodegradable nanoparticles based on gelatin and human serum albumin were used as a core, whereas their surface was modified by covalent attachment of the biotin-binding protein NeutrAvidin, enabling the binding of biotinylated drug targeting ligands by avidin–biotin complex formation (72). Using the HER2 receptor-specific antibody trastuzumab (Herceptin) conjugated to the surface of these nanoparticles, a specific targeting to HER2-overexpressing cells could be obtained. Further confocal laser scanning microscopy demonstrated an effective internalization of the nanoparticles by HER2-overexpressing cells via receptor-mediated endocytosis.

### *Nanotechnology for the Treatment of Cancers via the Triggered Release of Anticancer Drugs*

Triggered release by means of nanotechnologies may be performed either extracellularly or intracellularly for the treatment of tumors. Because tumors are often characterized by an acidic microenvironment, liposomes with compositions leading to destabilization at acidic pH have been proposed (73,74) to induce the specific release of the encapsulated anticancer drug, extracellularly in the diseased tissue. However, there are two main limitations to this approach: (i) the highest acidity is in the center of the tumoral tissue, far from the vasculature; and (ii) the pH of the tumor interstitium does not decrease below a value of 6.5, which makes the design of pH-sensitive liposomes in a range of 0.9 unit of pH very difficult (75,76). At the intracellular level, the design of pH-sensitive liposomes to take advantage of the acidic environment of the far endosomes and lysosomes has been more successful because the pH of those intracellular compartments may be below 5.0. A number of pH-sensitive liposomes were developed as reviewed by Fattal *et al.* (77) and Simoes *et al.* (78). A key lipid for the fusion of liposomes with the endosomal membrane is the dioleyl phosphatidyl ethanolamine (DOPE), but other components are generally associated with giving liposomes pH-sensitive properties. This includes carboxylated-PEG (79,80), mildly acidic amphiphiles such as oleic acid and cholesteryl hemisuccinate (CHEM) (77,78), or hydrophobized alkylated *N*-isopropylacrylamide (NIPAM) copolymers (81).

Now, if the pH-sensitive liposomes are efficient for allowing the cytoplasmic delivery of drugs *in vitro*, they are much less efficient *in vivo* because of the opsonization process. Problems about the *in vivo* stability of the pH-sensitive liposomes were addressed by the incorporation of certain types of lipids [dipalmitoyl succinylglycerol, dioleyl succinylglycerol, CHEM] (78) into the formulation or by using polymers such as PEG (82,83) and poly(NIPAM) (81). The introduction of PEG in liposomes prolongs circulation time in the blood avoiding recognition by macrophages of the liver and the spleen (84). Generally, modifications introduced in the formulations increased the stability of pH-sensitive liposomes in plasma, but, on the other hand, they shifted pH of destabilization to lower values (84,85). With certain types of additives the situation is even worse. Although addition of cholesterol increased the stability of liposomes in plasma against leakage, it led to a loss of pH sensitivity (85). A similar effect was reported with ganglioside M1 introduced into the liposomal bilayer to escape recognition by MPS (79). Those modifications in the pH-sensitive liposomes have succeeded in delivering their content in the cell cytoplasm.

The use of local hyperthermia for tumor-specific drug delivery has been proposed since 1978 (86). This approach is based on the design of liposomes with phospholipid compositions characterized by a phase transition temperature just above 37°C. Additionally, the tumoral hyperthermia is cytotoxic *per se*, and it facilitates the tumoral accumulation of liposomes because of an increased tumor blood flow and an enhanced permeability of the tumoral endothelium. Another new approach for the heat-triggered release has been proposed recently by incorporating lysophospholipids in the liposomal membrane in gel phase (87,88). When the liposomes are heated above the phase transition temperature, the lysophospholipids leave the bilayer, inducing a dramatic increase of the liposomal membrane permeability at this temperature. Even if this approach is limited to accessible tumors, it deserves to be further investigated for the treatment of cancers that cannot be removed surgically (56).

Different groups have exploited the feature that many enzymes are up-regulated in tumor tissues for the design of enzyme triggered release. For example, Andresen *et al.* (56,88,90) have conceived site-specific liposomes taking into account that phospholipase A2 is overexpressed in inflammatory and tumor tissue. Cell-associated proteases, elastase (91), as well as alkaline phosphatase (92) have also been considered as targeting enzymes.

Finally, even if the light-triggered activation of liposomes has not yet been proven to be efficient for specific drug release into tumors (56), the use of lipids that either polymerize or fragment upon excitation by light deserves to be mentioned as a laboratory curiosity (93).

### *Iron Oxide Nanospheres for Imaging and Hyperthermia of Cancers*

*Imaging.* Colloidal iron oxides (magnetite $Fe_3O_4$, maghemite $\gamma$-$Fe_2O_3$, or other insoluble ferrites) are superparamagnetic, possessing large magnetic moments when a magnetic field is applied, but retaining no net magnetization when the field is removed. In magnetic resonance imaging (MRI), the presence of superparamagnetic iron oxide (SPIO) nanoparticles leads to a deep shortening of the relaxation time (longitudinal and transversal relaxation times, T1 and T2, respectively) of the surrounding protons as a result of the inhomogeneous magnetic field around the particles. On

the images thus obtained, this effect is evidenced as a negative contrast generated by the reduced MRI signal (94).

The embedding of iron oxide colloids within polymeric matrices (dextran, starch, siloxane, and PEG) for stabilizing purposes prevents their aggregation in physiological media. The first generation of iron oxide colloids, which were generally dextran-coated, possessed large diameters and broad size distributions. These particles had good MRI-enhancing properties and concentrate in macrophages of the MPS (95,96). Thus, such colloidal systems, generally named SPIOs, have become useful tools for the diagnosis of tumors or metastasis in the liver (97,98) and the spleen (99). Imaging of tumors is made possible by the lack of accumulation of the SPIO in the malignant tissue because of the absence of Kupffer cells in these areas, thus providing good contrast with the normal tissue in MRI. Iron-oxide-enhanced MRI of the liver, spleen, and GI tract is now an established clinical application of commercialized SPIO (Endorem®, Lumirem®, Guerbet, Roissy, France) (94). As with polymer nanoparticles (see above), a lot of effort was devoted to developing SPIO able to avoid massive uptake by macrophages of the MPS and hence increased circulation half-life. The observation that a fraction of injected dextran-stabilized SPIO showed a prolonged blood half-life because of their smaller diameter (100) led to the synthesis of ultrasmall superparamagnetic iron oxides (USPIO, sometimes also called monocrystalline iron oxides nanoparticles), with a size below 50 nm and narrow polydispersion (101). These long-circulating USPIO have applications in angiography, including tumor angiogenesis imaging (101), and it was recently demonstrated that USPIO may be used for magnetic resonance lymphograpy to detect cancer metastasis. Products conceived for this application are currently on the market (Sinerem®, Guerbet) (94,101,103,104). Weissleder *et al.* (101) demonstrated, in experiments dealing with glioma cells (as highly malignant tumor model), that iron oxide colloids, after passing through the hyperpermeable vessels of intracranial tumors, are taken up not only by tumor-associated macrophages but also by rapidly proliferating cancer cells (105,106), thus obtaining an enhancement of the tumor/healthy tissue contrast in MRI of the brain. Still more promisingly, the group of E.A. Neuwelt at the Oregon Health and Science University conducted an FDA-approved clinical comparative study on 20 patients with primary tumors or intracranial metastases demonstrating that USPIO provides tumor enhancements comparable to common clinically used contrast enhancers but with a longer persistence of the signal (107).

The third generation of SPIO colloids is represented by ultrasmall nanoparticles in which the surface has been chemically engineered to bear targeting molecules. In this way, enhancement of MRI of tumors in animal models using such third particles generation, in the form of USPIO linked to specific monoclonal antibodies, has been successfully demonstrated (108,109).

*Hyperthermia.* Depending whether administration is local or systemic, two main procedures can be identified to achieve magnetically mediated hyperthermia with magnetic particles.

The first way magnetic particles can mediate hyperthermia is after direct local injection into the tumor tissue. This approach has been adopted by the Japanese group of Kobayashi with the design of magnetic cationic liposomes for hyperthermia (110). Magnetic liposomes, in which colloidal magnetite is encapsulated within the vesicle, have been injected intratumorally in glioma-tumor-bearing rats. Positive charges have been demonstrated to retain liposomes at the site of injection in the tumor, probably because of electrostatic interactions with cell membranes. The complete regression of the tumor was observed in almost 90% of the rats after three hyperthermic periods of 30 min each (111). In a similar study, a direct injection of various magnetic colloids in a subcutaneous breast cancer model in mice has been tested by Hilger *et al.* (112) to assess a tumor ablation therapy for breast cancers. In these experiments, the heating of the tumor mass was much higher than in normal hyperthermia (even >80°C), but maintained only for short periods of time (4 min). Although the results were quite spectacular with a complete regression of the tumor tissue, some improvements are still necessary before any effective clinical application to breast cancer could be proposed. The magnetic fluid hyperthermia is another method that has been proposed for prostate and brain tumors, in particular for the treatment of nonresectable gliomas or residual disease after surgical tumor ablation, and consists in stereotactic deposition of ferrofluids (i.e., suspension of magnetic nanoparticles) in the tumor region for repeated hyperthermic treatment (45°C for 30 min) (113). Nevertheless, the authors have already planned that the hyperthermia should be combined with radiotherapy treatments. A prototype of the device for the application of the alternating magnetic fields that would be used clinically has also been presented (114).

The second approach proposed for the hyperthermic treatment of tumors is intracellular hyperthermia, generally after systemic administration. The idea of an intracellular hyperthermia is fascinating because it implies both the specific targeting of the cancer cells and a more direct damage of the heating on their vital structures. The potentialities of intracellular heating have been shown by Halbreich *et al.* (115) and Bacri *et al.* (116). In some *in vitro* experiments, magnetic nanoparticles (maghemite coated with 2,3,-dimercaptosuccinic acid) have been incubated with human monocytes and mouse macrophages at concentrations that are not able to produce any significant heating of the solution when an alternating magnetic field is applied. Nevertheless, a magnetically induced cytolysis could be observed, likely because of the generation of intracellular hyperthermia following the uptake of magnetic nanoparticles. Using BT20 human mammary carcinoma as a model cell line, Jordan *et al.* (117) demonstrated, in a detailed *in vitro* study, that even when macroscopic heating occurs, an increased cytotoxic effect was obtained in the case of magnetic nanoparticles uptake (aminosilan-coated magnetite particles) in comparison with water bath heating, as control.

Unlike macrophages, tumor cells are not professional phagocytic cells. Thus, to improve the intracellular uptake of colloids, one strategy employed was to bind targeting molecules on their surface. For example, *in vivo* studies have been conducted by Shinkai *et al.* (118) in mice bearing a human renal cell carcinoma, using monoclonal antibodies-targeted magnetic cationic liposomes. A benefit in the survival time of mice treated with these targeted liposomes and hyperthermia in comparison with control groups without

heating or without targeting has been shown. Folate-conjugated superparamagnetic maghemite nanoparticles have been synthesized for the intracellular hyperthermia treatment of solid tumors too (119). The ability of these folate "decorated" maghemite nanoparticles to recognize the folate receptor has been investigated both by surface plasmon resonance and in folate receptor expressing cell lines, using radiolabeled folic acid in competitive binding experiments. Qualitative and quantitative determinations of both folate nanoparticles and nontargeted control nanoparticles demonstrated a specific cell internalization of the folate superparamagnetic nanoparticles.

Recently, an innovative intracellular hyperthermic approach, not based on magnetic properties, has been proposed using metal colloids (120). This method has something in common with photodynamic therapy in the sense that it is based on the use of light-absorbing microparticles (containing iron oxide) or nanoparticles (made of gold) together with short laser pulses (in the range of nanoseconds). The absorption of short laser pulses causes extreme temperature rise of particles ($\Delta T$ >1500 K), sufficient to vaporize thin layers of liquid around them; the microbubbles formed immediately implode, causing cavitational damage to cellular structures and cell killing (121).

*Nanotechnologies for the Delivery of Nucleic Acids*

Cancer is a disease in which alterations occur at the genetic level when the balance between oncogenes (responsible for cell proliferation) and suppressor genes (inducing apoptosis) is perturbed. The development of neoplastic cells is also under the control of the immune system, and it is recognized that cytokines such as interleukin-12 may play a role in boosting the immune response against cancer cells. Thus, gene therapy may be considered as a potential approach for cancer treatment either to transfect a tumor suppressor gene or to inhibit the expression of oncogenes or to induce an immune response. One of the frequently encountered genetic immunotherapy strategies involves the transfer of the genes of the immune-stimulant molecules such as cytokines (for instance, intensive research has focused on the transfection with interleukin-12 gene).

It is noteworthy that, contrary to other pathologies related to gene dysfunction, which needs the prolonged expression of the transgene, short gene expression is sufficient for most anticancer strategies. Although naked DNA may be efficiently transfected by direct intratumoral or intramuscular injection (122), it is inefficient after systemic administration because of the rapid clearance and degradation of this molecule (123), which additionally does not diffuse easily intracellularly. Beside the virus, various nonviral nanotechnologies have been proposed for gene therapy. If their efficiency is lower than that of their viral counterparts, they may overcome the drawback of the virus: oncogene recombination, immunogenicity, toxicity, and difficulties in production. Cationic lipids and liposomes are probably the most popular and ancient nanotechnologies for gene delivery; they interact electrostatically with DNA allowing the condensation of this molecule and cell internalization, likely through endocytosis (11). Cationic lipid dioleoyltrimethylammonium propane (DOTAP) is a typical example of such cationic lipids, which generally consist of two diacyl side chains linked with a propyl ammonium group. The linker between the ammonium and the hydrophobic part of the molecule may be an ester (case of DOTAP) or an ether [in case of *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA)]. Multivalent cationic lipids have been synthesized to further improve the efficacy of DNA condensation (12,124). The hydrophobic part may be also cholesterol-derived molecules (125). Protamine, an arginine-rich peptide, may be used to condense DNA before complexation or encapsulation with the above-mentioned cationic lipids (126). This mixture has been used for the delivery of the tumor suppressor genes *Rb* or *E1A* (127,128). This resulted in tumor cell apoptosis, reduction of tumor growth, increased life span in experimental human xenograft models, and in spontaneous multiple neuroendocrine neoplasia and lung metastasis in Rb +/− mice (128). To escape the endosomal/lysosomal pathway, the helper lipid DOPE may be added. As explained above, it is protonated at acidic pH, allowing a phase transition from a bilayer lamellar structure to a hexagonal nonbilayer fusogenic structure to occur causing the release of the content of the endosomes into the cell cytoplasm. Cationic and fusogenic peptides such as the influenza HA-2 subunit may also be useful for improving the cytoplasmic release of the condensed DNA (129).

Cationic polymers were also used to form nanoparticle complexes with DNA. Poly(lysine) (130,131) and poly(ethyleneimine) (132,133) are the more traditional polymers of this approach. Whereas poly(lysine) was the first cationic polymer employed for intracellular gene delivery (130), poly (ethyleneimine) was claimed to be able to make DNA to escape from the degradation into the lysosomes because of the so-called "proton sponge" effect (131). In fact, when the endosomes become more and more acidic, the buffer capacity of the poly(ethyleneimine) induces a dramatic water diffusion into those vesicles because of the osmotic pressure, which, in turn, enables destabilization of the lysosomal membrane. Chemical modifications allow the linkage of PEG to reduce the interaction with the blood proteins and to prevent the clearance by the liver (134). Specific ligands of tumors, such as transferrin (135), monoclonal antibodies (136), or folic acid (137), may also be attached, which allows better tumor transfection and lower toxicity. For example, Kircheis *et al.* (138) have demonstrated in a nice study that gene transfer after intratumoral administration was much more efficient with transferrin-poly(ethyleneimine) polyplexes in comparison with free DNA. Even after intravenous administration, tumor transfection could occur if PEGylated-transferrin poly(ethyleneimine) was used as delivery system. Transferrin polylysine polyplexes were used as cancer vaccine to induce the production of interleukin-2 *ex vivo* in patient's melanoma cells (139). B4G7 antibody was also used to construct a targeted formulation able to transfect epidermal growth factor receptor overproducing cancer cells (140). It has to be noted that numerous studies have investigated the efficacy/cytotoxicity/biodistribution profile of polyplexes by modifying molecular weight, ionic strength of the solution, zeta potential, degree of branching, and particle size (131,141). Chitosans (142), dendrimers (143), and poly(2-(dimethylamino)ethyl methacrylate) (144) are other cationic

polymers that have been used for gene delivery purposes. For further details on delivery systems in cancer gene therapy, see the excellent review by El-Aneed (145).

Antisense oligonucleotides (AS-ONs) with base sequences complementary (antisense) to a specific RNA and, more recently, small interfering RNA (siRNA) also offer the exciting potential of selectively modulating the expression of an individual oncogene, thus leading to tumor growth inhibition. However, crucial problems such as the stability of AS-ONs and siRNA, in relation to nuclease activity *in vitro* and *in vivo* and the low penetration into cells, have to be solved. Thus, as for DNA, the development of nanotechnologies has been considered as an interesting approach to improve the *in vitro* and *in vivo* efficacy of these short fragments of nucleic acids by protecting them against degradation and by increasing their delivery into the cell interior. For example, *in vitro* growth inhibition was carried out on human tumorigenic cells (HBL100ras1) using an anti-ras AS-ON loaded-poly(isohexylcyanoacrylate) nanospheres (146). In this study, the carrier system consisted of a cationic hydrophobic detergent [cetyltrimethylammonium bromide (CTAB)], which interacted with the AS-ON by ion pairing (147). *In vivo*, tumor growth inhibition was achieved at concentrations ten times lower than those needed with free AS-ON, when the antisense–CTAB complex was adsorbed onto the surface of these nanospheres. More recently, functional nanospheres obtained by free radical emulsion polymerization of methylmethacrylate using quaternary ammonium salt of 2-(dimethylamino) ethyl methacrylate as the reactive emulsifier were used for loading C-myb AS-ON (148). When tested *in vitro* on HL60 leukemia cells, this carrier system induced long and effective inhibition of cells growth through an antisense mechanism. Similar anticancer efficacy was obtained with liposomes. For example, the encapsulation of a 15-mer phosphorothioate oligodeoxynucleotide complementary to the 5′ end of the coding region or to a loop-forming site in the mdr-1 mRNA resulted in a reduced resistance to doxorubicin for multidrug-resistant SKVLB cells (149). PEG–poly(ethyleneimine) nanogels represent another more recent option for the intracellular delivery of AS-ON for mdr gene down-regulation (150).

Particularly interesting is the targeting of junction oncogenes, which are found in cancers such as certain leukemias, Ewing sarcoma, and thyroid papillary carcinomas. These tumors are relevant targets for AS-ON and siRNA because they originate from a chromosomal translocation leading to a junction sequence only found in the tumor cells. For instance, in the Ewing sarcoma, the EWS–Fli1 chimeric protein results in the fusion of the carboxyl-terminal region of Fli1 with the amino terminal region of a putative RNA binding protein EWS. This protein is believed to function as a transcriptional activator (151). However, successful results have never been obtained with AS-ON directed against junction genes on solid tumors *in vivo* (152), probably because of their short biological life and limited cellular uptake. It has been found that the design of *in vivo* efficient AS-ON against these junction oncogenes is only possible if these molecules are delivered to the solid tumor with the aid of nanocapsules containing an aqueous core or with nanospheres (153,154); this result can be explained by the ability of this carrier to escape from the intracellular lysosomes (155).

Even if only very few publications have to do with the use of siRNA associated with nanotechnologies for cancer treatment (156,157), it is expected that investigation on gene silencing obtained by special siRNA-targeted nanosystems will multiply in the next few years.

### Application to Infectious Diseases

Although progress was made during the last century in the treatment of infectious disease because of the major discovery of antibiotics and to the application of large program of vaccination, infections still remain a major public health problem with urgent need of new treatments (9). Causes of infections are multiple, and finding new treatments is complicated by many factors including the constant modification of pathogen patterns because of the emergence of resistant strengths of bacteria, parasites, fungus, and viruses and of the appearance of new pathogens (HIV, Ebola virus, avian influenza, etc.). Another factor is the increasing number of immunocompromised individuals among patients requiring a treatment against pathogens. This can be explained by the aging of the population, an increased number of patients infected by HIV virus, and an increased number of patients treated by immunosuppressive therapy for cancer or after organ transplantation. For instance, infections by cytomegalovirus has become the leading cause of mortality in transplant patients, whereas the virus can remain silent in healthy individuals. Tuberculosis bacteria and the malaria parasites are still responsible for millions of deaths each year. In such a context, several strategies are investigated to find new efficient treatments. However new active molecules against pathogens are also toxic for healthy tissues, and the use of colloidal carriers is more than required for diverting drugs from sites of toxicity.

Beside anti-infectious treatments, vaccination against pathogens was found in the past as an efficient way to control infection disease. For example, after the introduction of public health measures, smallpox was eliminated, and polio, with 500 cases reported on the earth for a 9-month watch period in 2005, is on the verge of extinction (158,159). Vaccination can also be used to control the spreading of the pathogens among the population and across the continents because of the increased number of worldwide travelers. It is now considered as the major preventive treatment against viral infections like influenza.

In this part of the review, the first section will consider the use of nanotechnology for the treatment of infectious disease, whereas the second section will focus on the strategies in vaccination. In both cases, targeting cells of the MPS is the major goal to achieve the control of the infection. Indeed, many of the pathogens that need to be eliminated from the body are located in phagocytes. For vaccination, antigens must be delivered to immunocompetent cells, which are also part of the MPS system. This is an ideal situation because conventional delivery systems from nanotechnology can be used straightforward without spending much effort to design sophisticated targeted systems as discussed earlier. However, it has to be remembered that, in severe infections such as tuberculosis, non-MPS cells or the extracellular compartment may also serve as reservoirs, which sustain the infection. Now, nanotechnologies based on long circulating systems may offer

different tools to reach these territories and to improve efficacy of existing therapeutic approaches (160).

*Treatments Against Infections*

The discovery of novel molecules is probably the leading strategy in finding new treatments for infectious diseases. However, these active molecules need to reach, at the cellular/subcellular stage as well as at the tissular level, the exact site in the body where pathogens are hidden. This is sometimes a challenge because of the physicochemical properties of the molecule itself as explained in Fig. 3. The toxicity of the active component is also a real problem because the more active agents against devastating fungus, parasites, bacteria, and viruses are also very toxic.

*Fungus and Parasite Infections.* Systemic delivery of antibiotics with liposomes was extensively investigated against parasites responsible for severe infections including visceral leishmaniasis, candidose, and malaria (161–166). In general, liposomal formulations seemed superior for the treatment of all kinds of infections (i.e., fungal, parasite, bacterial, viral) compared to treatment with the free drug. In many examples, the toxicity of the antibiotic was dramatically reduced by targeting larger amounts of drug to the infected tissue. The efficacy of the treatment was also improved by increasing the dose that can be administered to patients because of the safety profile of the liposomal formulation. For instance, after formulation in liposomes, toxicity of amphotericin B, which is the leading compound against leishmaniasis and fungus, was reduced by a factor of 50- to 70-fold (167). This allowed the administration of more than 5-fold of the drug compared with conventional treatments. Thus, the liposome formulation, which was marketed in 1996 under the name Ambisome® (NeXstar now Gilead, Foster City, CA, USA), is today the more efficient treatment for leishmaniasis and other fungal infections (163). Besides the considerable reduction of the toxicity, the success of this formulation is also because of the small size of the liposomes making the Ambisome® particles (<100 nm). This allows a large portion of the injected dose to escape immediate clearance by the macrophages of the liver and the spleen. Thus, these liposomes carrying amphotericin B remain in the blood circulation and can distribute a high enough concentration of the drug in the infected tissues: lungs, liver, kidney, and brain. The formulation also presents the advantage that it can kill both phagocytized and nonphagocytized microbes.

After several years of use in clinics, Ambisome® is now considered as an excellent treatment for visceral leishmaniasis, and it has been proposed for many other therapeutic indications as far as fungal infections are concerned (163). The main problem with Ambisome® remains its cost (estimated at 800 euros per 1 injection per day), and this drug is still not affordable for many of the infected people who need such a treatment in the developing countries (168,169). Among the strategies proposed to reduce the cost of such a treatment, the use of cheaper liposomal formulations and the development of alternative nanoparticles are in progress (170). A new lipid formulation of amphotericin B was proposed in which the

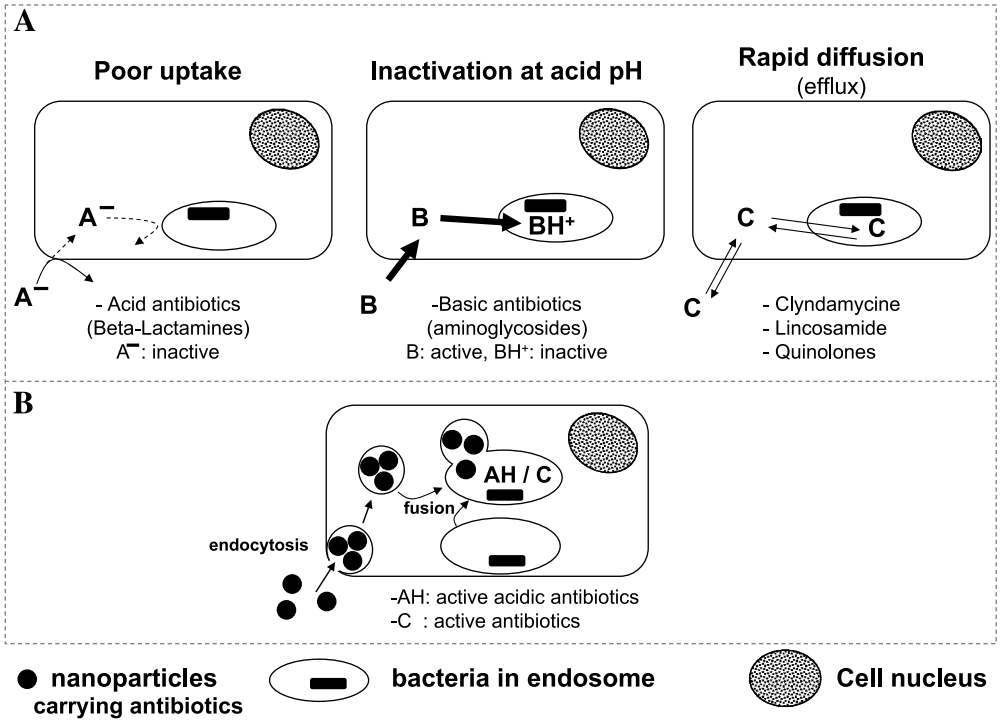


**Fig. 3.** Schematic representation of how nanodevices can improve the intracellular delivery of antibiotics. Three main hurdles are identified in (A) the poor intracellular diffusion (incl. diffusion into lysosomes), the drug inactivation by the lysosomal acidic pH, and, depending on their physicochemical character, their segregation into these intracellular vesicles. Nanodevices may allow to resolve these problems by (B) increasing the intracellular uptake (i.e., through endocytosis) and/or by allowing the drug to diffuse freely into the cell (i.e., by lysosomal fusion mechanisms).

total amount of lipids was reduced by a factor of 3 compared to the concentration used in the Ambisome® formulation. A commercial form, Abelcet®, is available, but it does not compete with Ambisome® in terms of the activity. Recently, Larabi *et al.* (170) showed that nanodisks (250 nm in diameter, 3 nm in thickness) containing amphotericin B may be obtained using a lipid composition very similar to that used in the Abelcet® but changing the method of preparation of the amphotericin B/lipid complex. By testing this new system *in vivo*, the authors obtained very promising data compared to other lipid formulations. Although it was still slightly less active than Ambisome®, it seemed more efficient than Abelcet® to reduce the infection by *Leishmania donovani* in a model of visceral leishmaniasis developed in mice. Compared to Ambisome®, the new formulation requires far less lipids and can be prepared in only two steps: mixing of phases and organic solvent elimination. The combination of both the reduction of expensive phospholipids used and the easy preparation method should contribute to decrease considerably the cost of such a formulation, which may appear as a competitor for Abelcet® in the future. Formulations based on niosomes are also considered as suitable alternatives to liposomes. They are made of polymer surfactants, which are much cheaper than lipids, less toxic, biodegradable, and nonimmunogenic. *In vivo*, the activity of antileishmanial compounds associated with niosomes was found to be higher than that of the liposomal form. From an economic point of view, the biggest challenge is believed to come from polymeric carriers made of cost-effective biodegradable polymers or of natural polymers (171). Although progresses to find suitable polymer nanoparticle formulations of antiparasitic or antifungal agents are less advanced, several systems were reported to show very interesting activity as evaluated in different models of infected animals (172). Poly(isohexylcyanoacrylate) nanospheres loaded with primaquine increased by 21-fold the activity of the drug against intracellular *L. donovani* (173). The toxicity of dehydroemetine, another drug candidate for the treatment of visceral leishmaniasis, was reduced after linkage with these nanospheres (174). More recently, nanospheres made of poly(epsilon caprolactone) containing amphotericin B were evaluated for their therapeutic efficacy against systemic candidiasis in neutropenic mice (175). Similar to what was observed with Ambisome®, the polymeric formulation decreased the *in vivo* antifungal activity of the free drug, and higher concentrations of amphotericin B were necessary to obtain the same therapeutic effect. However, effective doses could be administered because the formulation reduced the toxicity of the drug molecule. Such formulation could be marketed at a reasonable price. Another study compared the efficacy of the indigenous drug arjunglucoside incorporated either in a hydrophilic nanogel (made of cross-linked random copolymer of NIPAM and *N*-vinylpyrrolidone) or in hydrophobic nanospheres (made of PLA) against experimental leishmaniasis. The nanoparticle formulation both reduced the hepatotoxicity and the nephrotoxicity of the free drug, whereas they promoted its activity. Almost no difference was measured between the two nanoformulations, which showed rather similar activity. They reduced the parasite payload of the spleen by 79% for the nanogels and by 75% for the PLA nanospheres, whereas the free drug could only reduce it by 38% (176).

In the case of malaria, the situation is quite different. Parasites are located in red blood cells. Thus, the nanotechnology used to reduce the toxicity of the drug molecules needs to either remain in the blood stream or to interact with infected red blood cells. A couple of studies have considered the administration of antimalarial drug with liposomes targeted to infected red blood cells. The binding of liposomes to the red blood cells via a grafted F(ab′) fragment was very effective in reducing parasitemia. The chloroquine-loaded liposomes could even cure chloroquine-resistant infections (166). Long circulating formulations including PEG-coated liposomes and nanocapsules also seemed superior to the free drug to treat malaria (177,178). For instance, various nanocapsule formulations of halofantrine were evaluated in *Plasmodium berghei*-infected mice, which are a relevant animal model to investigate antimalarial drugs (177). Halofantrine is one of the new antimalarial molecules developed because of the emergence of chloroquine resistance *P. falciparum*. The nanocapsule formulation seemed superior to the drug solution in severely infected mice because it could mask the toxic side effects of the drug. The results from the pharmacokinetic study showed that the nanocapsule formulation, especially those coated with PEG, provided a more favorable halofantrine profile in plasma. Indeed, the AUC for halofantrine in plasma was 6-fold higher with the nanocapsule formulation compared to the solution.

Having drug delivery devices with prolonged blood residence time may be important to increase concentration of antifungal drugs at sites of fungal infections outside the MPS, such as the kidneys and the lungs. Several formulations of PEG-coated amphotericin B-loaded liposomes were tested on severely infected mice with neutropenia. In some of the infection models, the long circulating liposomes showed equivalent activity than Ambisome®, whereas in other models, a single dose of the long circulating liposomes gave similar therapeutic efficacy than repeated administration of Ambisome® (178). This demonstrated that the long circulating liposomes may further improve the performance of the Ambisome® and, at the same time, may reduce the cost of the treatment by reducing the number of administration.

*Bacterial Infections.* Nanotechnologies are also attractive candidates for the delivery of antibiotics in infections caused by bacteria. Generally, the encapsulation of antibiotics in liposomes or in nanoparticles increased the maximal tolerated dose and the therapeutic index of the antibiotics compared with the free drug (179–181). This can be explained by a modification of the pharmacokinetic profile of the antibiotic when encapsulated as well as by a modification of its biodistribution. For instance, in a liposome formulation of amikacin (Mikasome®, Gilead), which is in clinic evaluation, the antibiotic was found 2- to 6-fold more active than the free drug and the free streptomycin in an acute experimental model of murine tuberculosis in which bacteria were located into macrophages. In a model of mice infected by *Mycobacterium avium*, amikacin in liposomes could reduce viable bacterial count in liver, spleen, and, to a lesser extent, lungs by approximately 3-$\log_{10}$ compared with the untreated control (182–184). In another example, the entrapment of ampicillin in poly(isobutylcyanoacrylate) nanoparticles increased by 120-fold the efficacy of the antibiotic in an experimental acute infection of mice by *Salmonella typhimu-*

*rium*. In this model, 100% of the infected mice treated with a single dose of the nanoparticles survived, whereas all the untreated animals died after 10 days. Such high activity was explained by a complete sterilization of the organs where the intracellular bacteria were located. Treatment with liposomes was less efficient. The survival of mice did not exceed 60%, and the infected organs were never completely sterilized in mice that survived (185–187). Ampicillin-loaded nanoparticles were also found more efficient than liposomes for the treatment of listeriosis in a model of chronic infection of mice by *Listeria monocytogenes* (188). In this case, it was shown that the spleen was not totally sterilized, and a reinfection occurred after several days whatever the treatment was. In this model, reinfection was believed to occur from nondividing bacteria, but even nanoparticles loaded with ciprofloxacine, a fluoroquinolone with antibacterial activity against both dividing and nondividing bacteria, could not totally eradicate the infectious reservoir (189). This shows the extreme difficulty to eradicate all bacteria from the body even when they are *a priori* located in the MPS. Another difficulty is to reach infections, which develop outside the MPS and outside macrophages. Indeed, it was suggested that part of the clinical trials, which aimed to treat patients infected by tuberculosis with Mikasome®, failed because the antibiotic was released in macrophages that were too far from the extracellular bacilli clustered in cavity caseum in the human infection (184). In case of tuberculosis, it is now known that targeting the nonreplicated persistent bacilli still remains a challenge to be addressed (190). Further improvements of drug delivery systems are still needed to enhance the targeting of the extracellular infectious sites.

So far, most of the very promising data were obtained by treating experimental animal infections with antibiotics associated with nanodevices in comparison with the free drug. However, in front of the somewhat disappointing results obtained with Mikasome® during clinical trials, questions about the relevance of the animal experimental models (with intact host defense and with highly susceptible bacteria to the antibiotics) were raised. Indeed, in clinical practice, treatments are often given to patients with impaired host defenses and who may be infected with bacteria of low antibiotic susceptibility. Only a few studies considered experimental models on animals with impaired host defenses (188). *In vitro* models must also be handled cautiously because they were not always predictive of the *in vivo* activity. Indeed, the activity measured *in vitro* may be found dramatically reduced or significantly promoted because of synergies with lymphocytes when tested *in vivo* in animal models (179). Nevertheless, for a systemic treatment of bacterial infection in which the target cells are the MPS macrophages of the liver and the spleen, conventional liposomes and nanoparticles can be suggested as the most relevant delivery systems for antibiotics. The efficacy of liposomes was found very dependent on their physicochemical characteristics. For instance, specific composition may affect the bactericidal activity by interaction with the infected organism (191). In contrast, such formulations seemed of limited value to treat infections in which bacteria are located outside the main MPS organs (i.e., liver, spleen, and bone marrow), and more efforts are still required to address this goal. Indeed, targeted systems to extracellular bacteria and to other reservoir organs may contribute to make progress in the battle against bacterial infections. It is also needed to develop appropriate strategies to eliminate persistent bacteria, which are either in inaccessible sites or in a state of dormancy within macrophages. Some attempts were made using targeted liposomes with mannose to promote recognition by human phagocytic cells (192). However, the design of a targeted device seemed very delicate to find the right length of spacer between the targeting moiety and the surface of the device and to balance between the number of mannose residues on the lipid surface.

Finally, only a few investigations have considered comparative experiments performed with liposomes and other nanosystems. The nanoparticles seemed more efficient than niosomes, which were, in turn, more efficient than liposomes (172,187). This superiority of nanoparticles may be explained by a higher stability in biological media. In the future, the problem of stability of delivery systems in biological fluid may become even more important in view of the systemic delivery of targeted antibiotics by the oral route. This is another challenge that emerged and is still poorly documented at the moment (193).

Liposome formulations of antibiotics were also evaluated for the local delivery of antibiotics to be used as controlled release system at the site of the infection. They have proven to be of interest for readily accessible infected tissues such as the eye, wound, and lungs (180). This strategy was suggested in surgical wound prophylaxis (194,195), in the treatment of keratitis using liposomal formulation of tobramycin in eye drops (196), in the treatment of endophthalmitis by intravitreal injection of amikacin-loaded liposomes, and in lung infections by aerosol delivery of the liposomal formulation of antibiotics (197,198). Recently, a bioresorbable composite pellet of calcium sulfate and hydroxyapatite nanoparticles was studied as a material for local and sustained delivery of antibiotics in bone infections (199). In this system, the nanoparticles of hydroxyapatite changed the properties of the material by increasing the specific surface of the device and by allowing a higher loading of antibiotics. The nanoparticles incorporated in the material could also advantageously modify the released profile of antibiotics permitting the release of the total dose of the antibiotic incorporated in the material at the end of the process. This was actually not the case with the material devoid of nanoparticles, which retained up to 25% of the dose of the antibiotic after 10 days. Finally, the tolerance of the material modified by the nanoparticles was improved because the quantity of acid produced by the dissolution of calcium sulfate and responsible for an inflammatory response was reduced. This example illustrates advantages brought when nanotechnology is associated with other technologies to improve the pharmacological properties of a material used as an implant.

Beside what could be considered as "artificial" nanotechnologies including liposomes and nanoparticles, some authors considered the use of "natural" nanotechnologies to fight against resistant bacteria by using bacteriophages. This approach was used once in human with an unexpected success in combination with ciprofloxacin for local treatment of patients with wounds infected by multidrug-resistant *Staphylococcus aureus* (200). Very recent data obtained on infected animal models suggested that bacterial infections

can be circumvented only with functional phage specific to the bacterial strain (201). The formidable activity observed was suggested to result from the functional capability of the phage only and not due to a nonspecific immune effect of the host defense (202). At the moment, no side effect was reported about the use of phages, but the number of studies remained very limited. The level of antibodies against phages found in the rescued animals was not substantially elevated.

*Viral Infections.* Viral infections are caused by noncellular agents. They are probably the most difficult infections to treat, and finding suitable efficient therapies requires new resources. So far, the most promising innovative strategies are based on the use of oligonucleotides either for the development of therapeutic vaccines (16) or for the development of treatments based on molecular biology approaches (16,203–209). Antisense therapy constitutes a general alternative to all other antiviral treatments for the major viral infections in human: HIV, hepatitis B and C viruses, herpes simplex virus, papillomavirus, respiratory syncytial virus, and cytomegalovirus. This is because the identification of viral gene sequences able to hybridize with AS-ON, ribozymes, or siRNA seems to be a more straightforward approach to develop new drugs (210). Moreover, it is believed that the emergence of multidrug-resistant strains because of a single mutation on the viral genome may not be sufficient for escaping the antisense inhibition unless it will occur in the target sequence of the antisense agent. Even if this might occur, the problem of the emergence of resistant viral strains may be addressed by the introduction of the corresponding change in the sequence of the antisense agent. The rational behind the development of therapeutic vaccines is to stimulate the immune response of the infected host. This can be achieved by the intracellular delivery of oligonucleotides containing unmethylated CpG sequences mimicking immunostimulating properties of bacterial DNA found in traditional vaccines. These synthetic molecules are considered as the most potent immunostimulating agent known to date. They were found to promote immune response against antigens, thanks to the stimulation of dendritic cells (13,14,16,211,212). The strategy based on therapeutic vaccine does not interfere with the viral cycle and should be insensitive to the emergence of multidrug-resistant strains as well. Moreover, it should be very effective against the high immunogenic variability observed with certain viruses (211,213). So far, antisense strategies are the treatment of choice in the battle against HIV, cytomegalovirus, hepatitis C, and respiratory syncytial virus, however immunostimulating approaches have recently been receiving much attention as treatment for HIV, herpes virus, and influenza virus.

The first AS-ON was marketed in 1998 for the treatment of cytomegalovirus infection in HIV-infected patients. This medicine, Vitraven®, is delivered locally in the eye by intravitreal administration as a solution of a free AS-ON. However, the low stability and poor intracellular penetration of oligonucleotides impose to repeat the number of injections, which may be harmful and damaging for the eye. Thus, liposomes and nanoparticles were proposed as sustained released formulations to improve intravitreal treatment with AS-ON (208,212,215). In cell cultures, albumin nanoparticles were shown to deliver AS-ON into the nucleus of cells, while free AS-ON accumulated within vesicular compartments (208). It was suggested that a fusion-promoting peptide was produced during the degradation of the albumin nanoparticles. This fusion-promoting peptide produced during degradation of the nanoparticles in the lysosomal compartment of the cells may then destabilize the lysosomal membrane at the acidic pH, inducing the release of the AS-ON out of the lysosomes. Although this was a benefit for the delivery of AS-ON, the mechanism by which it is then translocated into the cell nucleus is not yet elucidated. *In vivo*, these nanoparticles were well tolerated for intravitreal administration (208). After intravitreous administration, PEG-coated liposomes also protected and prolonged the residence time of oligonucleotides in the vitreous (216,217). Up to 37% of the injected dose of the intact oligonucleotide can still be detected in the vitreous 14 days postinjection.

Apart from local delivery, tools from nanotechnology were also developed to improve access and intracellular release of oligonucleotides to target sites in view of systemic administration for the treatment of HIV or hepatitis infections. In general, liposomes and nanoparticles offer good protection of oligonucleotides against nuclease attack (77,218). They can also help the oligonucleotide to bypass the plasma membrane of cells but generally deliver their cargo into the endosomal compartment by penetrating into cells by the endocytic pathway. For their activity, oligonucleotides need to escape from the endosomal compartment before fusion with lysosomes. Efforts were made to design nanodevices able to destabilize the endosomal compartment or to fuse with the endosomal membrane thanks to the decrease of pH, which occurs before fusion with liposomes. As in chemotheraphy, pH-sensitive liposomes were suggested to deliver AS-ON directed against viruses into the cell cytoplasm (77–81). After endocytosis by cells, the pH-sensitive liposomes destabilize in the late endosomes where the pH decreases. This event induces a fusion of the liposome membrane with the endosomal membrane, and the content of the liposomes is then directly unloaded into the cell cytoplasm. A pH-sensitive liposome formulation of an AS-ON directed against the env mRNA of the Friend retrovirus was able to control the viral infection in cells more effectively than the non-pH-sensitive liposome formulation, whereas the free control and AS-ON lack antiviral activity (219). It was shown that the cell uptake of the pH-sensitive liposomes depended greatly on the virus exit process leading to the conclusion that it may result from the activation of cell pinocytosis because of the virus budding (220,221). The pH-sensitive liposomes were also found superior to inhibit the virus replication with an AS-ON in monocyte-derived macrophages infected by the virus HIV-1 5′LTR compared to the non-pH-sensitive liposome formulation and to the free oligonucleotide (210). So far, there is no demonstration that the antisense strategy is able to control viral infections *in vivo*. This is partly because of the fact that the pH sensitivity of the liposomes was modified when the liposomes are in contact with blood. As explained earlier, several strategies were suggested to improve the *in vivo* stability of these liposomes (78,79,81–85).

Generally, nanoparticles are valuable alternatives to address stability problems encountered with liposomes. So far, only a few works considered nanoparticles as a mean of AS-ON delivery against viral infections. For example, poly

(lactic-*co*-glycolic acid) (PLGA) nanoparticles were found to enhance the intracellular uptake of phosphorothioate AS-ON in cells infected by HIV-1 reducing the viral activity (222). However, the main drawback of these nanoparticles was that they released the AS-ON very fast in the cell culture medium. Thus, the mechanism by which AS-ON entered into the cells remained unclear. The controlled released properties of this system need to be improved before the nanoparticles can be used for long-term treatments with AS-ON. Other nanoparticles were designed for the delivery of antiviral AS-ON or of immunostimulating oligomers with CpG sequence, but they were not tested at present to control viral infection (223–225). Fusogenic liposomes are other nanotechnology that may be suitable for the delivery of AS-ON to control viral infections (224,226). Recently, an even more sophisticated system has been proposed combining fusogenic liposomes with AS-ON-loaded nanoparticles (227). In this system, the nanoparticles, which will be released in the cell cytoplasm thanks to the fusogenic liposomes, would serve as intracellular reservoir of AS-ON controlling their release and prolonging their action in the cells. The system was found particularly interesting to achieve a prolonged regulation of the expression of the target gene in the cell (228). Now, all these inventive systems need to be evaluated for their efficacy to reduce viral infections *in vivo*.

Waiting for more results coming from these new treatments, nanotechnologies were also widely proposed to improve the existing treatments against viral infections that are still far to be satisfactory. Both liposomes and nanoparticles were suggested to achieve a better control of the targeting of toxic antiviral drugs to enhance their bioavailability to infected cells. For instance, liposomes targeted to HIV-infected cells with the ligand CD4 grafted on their surface increased by almost 10-fold the efficiency of a protease inhibitor, whereas, at the same time, it decreased the toxic side effects (204). Another aim is to improve the stability or the solubility of the antiviral drug in the gastrointestinal tract. Indeed, HIV was reported to be active in the associated lymphoid tissue of the gut in which immunocompetent cells are an important target for the virus during the period of clinical latency. The stability of a major anti-HIV agent, 3′-azido 3′ deoxythymidine (AZT), was enhanced in digestive media by association with poly(alkylcyanoacrylate) nanoparticles (228). For saquinavir, a potent HIV-1 and HIV-2 protease inhibitor, the association with nanoparticles was suggested to resolve the many problems at the origin of its low bioavailability. Cyclodextrin was found to promote saquinavir incorporation into poly(alkylcyanoacrylate) nanoparticles by improving by 400-fold its solubility in water (229). Hidden in nanoparticles, the drug might now be able to bypass the efflux mechanism of the MDR transporter P-glycoprotein and the hepatic first-pass metabolism initially responsible for the low bioavailability reported when the free drug was administered by the oral route. Actually, in Caco-2 cell monolayers, this system improved significantly the amount and kinetic of saquinavir transported from the apical to the basolateral site (229). The encapsulation of saquinavir in poly(alkylcyanoacrylate) was also beneficial to promote the intracellular uptake of the drug in macrophages infected by HIV allowing a clear reduction of the viral activity, whereas it remained unchanged when the infected macrophages were treated by the free saquinavir (230). *In vivo*, pH-sensitive nanoparticles were found to increase the bioavailability of a new very poorly water-soluble HIV-1 protease inhibitor, CPG 70726, given by the oral route to beagle dogs. The best absorption of the nanoencapsulated drug depended on the fed and unfed state of the animal (231); the best effect was reported for the fed state. The pH-sensitive nanoparticles designed in this study were made of poly(methacrylic acid-*co*-ethylacrylate) (commercial name, Eudragit® L100-55), which is a pH-dependent dissolving polymer. The release of a drug incorporated in such nanoparticles occurred almost instantaneously at the pH of dissolution of the polymer. Thus, the selective release of the drug at the pH of the gastrointestinal tract allowed the creation of high concentration gradients close to absorption sites, which could explain the observed improvement of the bioavailability of the drug.

For local treatment of cytomegalovirus infections and delivery in the eye, several antiviral molecules, such as ganciclovir and acyclovir, were associated with nanoparticles. The albumin nanoparticles were well tolerated after intravitreal administration (232), whereas the tolerance of poly (alkylcyanoacrylate) nanoparticles toward the cornea could be improved by coating with PEG (233). In both cases, the nanoparticles slowed down the clearance of the drug and increased its availability in the healthy ocular tissue, but evaluation in infected tissue remains to be carried out.

The principal limitation of using low molecular weight antiviral compounds is the rapid emergence of viral resistance. Antiviral strategies are currently developed based on the stimulation of the natural host defenses by using interferon or PEG-interferon combined with low molecular weight compounds. However, many patients do not respond to this therapy (16,234–236). Further improvements were obtained by encapsulation of interferon in liposomes (16).

Application of nanotechnology for antiviral therapy is still in its infant age. The few data already obtained on infected cells predict that new antiviral strategies applied in combination with nanotechnologies should provide promising efficient treatments. Further evaluations both *in vitro* and *in vivo* are still required to confirm the first very encouraging data. However, the high level of safety procedures required for handling highly infectious viruses is an obstacle for the rapid progress because only limited numbers of groups have access to safe laboratory facilities. Another limitation is the lack of convenient animal models of viral infections that need to be developed to make these evaluations possible.

### *Vaccines*

Progresses in immunology, biology, virology, and microbiology from the last 30 years allowed a complete reconsideration of vaccine design (237–240). From a safety perspective, new generations of vaccines will be prepared from recombinant protein antigens, which will replace whole pathogens or inactivated subunits found in the traditional vaccines. However, purified peptide and protein antigens are far less immunogenic than the corresponding crude inactivated microorganism. Their association with performant adjuvants and immunostimulating agents is necessary to improve their immunogenicity. Another key problem in

vaccinology is the way antigens are presented to the immune system. Thus, better comprehension of the immune system led to revisiting vaccine formulation introducing new types of adjuvant and new antigen-presenting methods. Although the majority of vaccines are administered by injections, innovations also concern the development of vaccine formulation for other routes of administration. Mucosal immunization, which is accessible by oral or nasal administration, offers several advantages including easier and safer administration, reduced side effects, and potential for frequent boosting. Mucosal immunization may induce a local immunity at the initial sites of pathogen infections as well as an immune response at the systemic level (239). Stability of antigens at the level of mucosa constitutes the main limitation for the success of mucosal vaccine as well as for their delivery to the mucosal associated lymphatic tissues. It seems from a recent paper that optimal formulations of new generation of vaccines should include a delivery system able to target both immunostimulating agents and antigens to antigen-presenting cells to promote a long-lived B- and T-cell memory response (239).

In this move, the use of nanotechnology was suggested to resolve some of the problems that emerged with novel antigens produced by biotechnology or following innovative vaccine approaches (241–247). Adjuvants based on nanotechnology were developed as early as in the 1970 using liposomes and nanoparticles. They were first proposed as encapsulation method to simply insure protection of protein antigens against degradation by peptidases prior to their uptake by macrophages (248,249). Today, the immunoadjuvant role of liposomes and nanoparticles is much wider. These systems can be designed in such a way that the most sophisticated ones resemble closely to empty virus envelopes or capsides. They can be obtained thanks to progresses in fundamental research resulting, for instance, in a better comprehension of self-assembling mechanisms of lipids to produce vesicles and of proteins to reconstitute empty viral capsides (237,239,250). For instance, the new marketed hepatitis, Epaxal®, and influenza, Inflexal®, vaccines (Berna Biotech, Zurich, Switzerland) are formulated with virosomes, which correspond to a new technology platform consisting of a liposomal carrier for antigens (243,246,247,251–253) (Fig. 4A). They are formidable antigen-presenting devices made of unilamellar vesicles with a mean diameter of approximately the size of a real virus (150 nm). Two functional glycoproteins are intercalated in the phospholipid bilayer membrane: the influenza hemagglutinin and neuraminidase. They play a key role for the unique immunoadjuvant properties of virosomes providing fusion capabilities that enable virosomes to fuse with cell membranes and to deliver antigens in the cytoplasm of antigen-presenting cells, i.e., macrophages and dendritic cells (243). The influenza glycoproteins also enhance the immune response of the carried antigen by promoting opsonization of virosomes and uptake by immune cells after administration (243,247). The method of preparation is based on phospholipid vesicle reconstitution by elimination of surfactant (247) from a blend of natural lipids, synthetic lipids, influenza-derived hemagglutinin and neuraminidase, and the purified antigen to be incorporated, which will give the specificity of the vaccine. Because of this method of preparation, virosome is a versatile technology; antigens can be incorporated into the virosome vesicle, adsorbed onto its surface, or inserted into the lipid bilayer membrane. For instance, influenza vaccine is yearly formulated using hemagglutinin and neuraminidase taken from the viral strains recommended by the World Health Organization as antigens (243). So far, virosomes are only one of the three adjuvant systems approved for human vaccine by the authorities that has carrier capability and in which additional compounds may be incorporated (247).

Virus-like particles (VLPs) also look very similar to empty viruses (254,255). They fundamentally differ from virosomes in the sense that they result from the reconstitution of a protein viral capside obtained by spontaneous self-

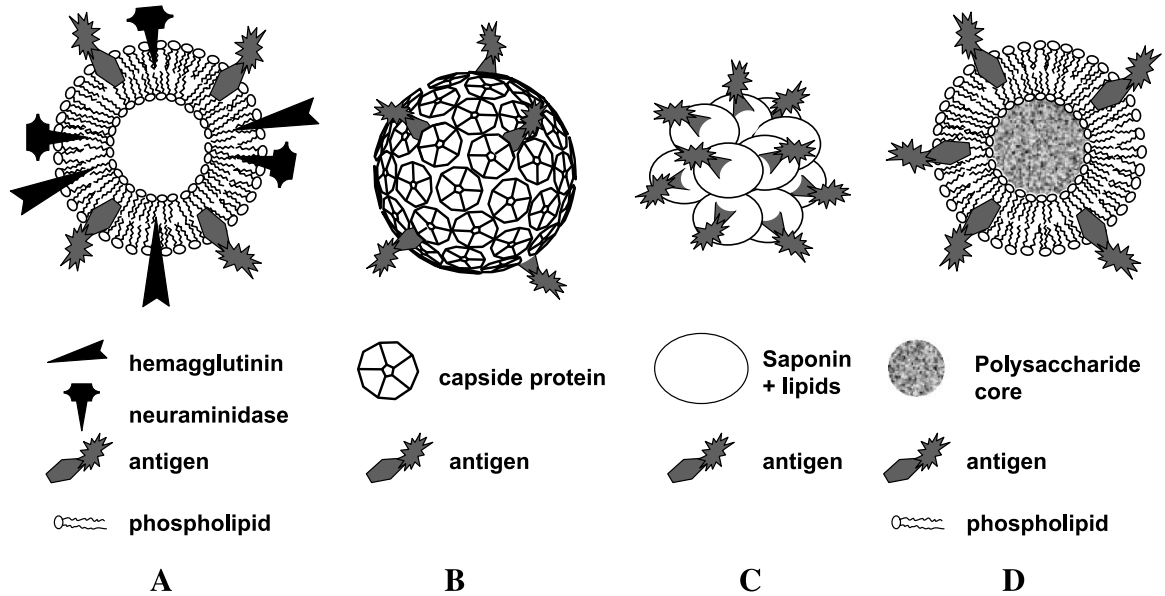


**Fig. 4.** Antigen-presenting systems derived from nanotechnology. (A) Virosomes [adapted from (254)]; (B) virus-like particles [adapted from (255)]; (C) immune-stimulating complex [ISCOM®; adapted from (257)]; D: SupraMolecular Biovector™ (SMBV).

assembly of recombinant viral coat proteins (Fig. 4B). Such VLPs were prepared from recombinant proteins arising from various viral strains (254) and using immunogenic epitopes of several pathogens such as the human papilloma virus, Ebola virus, HIV, or hepatitis B (255). They are currently evaluated in clinic for human vaccination against human papilloma virus. It is believed that the immunogenicity of VLPs is caused by their interactions with dendritic cells allowing both humoral and cellular immunologic response (255). As in virosomes, VLPs can be used to deliver small molecules. For instance, immunostimulating agents such as synthetic bacterial CpG oligonucleotides were incorporated into VLPs in which the epitope p33 of the lymphatic choriomeningitis virus glycoprotein was incorporated as antigens. The whole particle protected mice from viral challenges enhancing the immunostimulatory capacity of the antigen and reducing side effects of the CpG oligonucleotides (256).

Other adjuvants derived from nanotechnology are nanoparticles made either of lipids or of polymers. The immunestimulating complex (ISCOM®, a registered trademark of CSL Limited, Victoria, Australia) corresponds to a nanosized aggregate (40 nm in diameter) of saponin, lipids, and antigen held together by hydrophobic interactions occurring between the three components (257) (Fig. 4C). This system is marketed for horses and cattle vaccination and is currently under development for human vaccine against HIV. After parenteral administration, the ISCOM® induced an efficient response of the T helper cells including both Th1 and Th2 cells and of the cytotoxic T lymphocytes. The SupraMolecular BioVector® (SMBV), developed in the late 1990s by the French company BioVector Therapeutics S.A. (Labege, France) is another example of antigen-presenting system. It was formed by a polysaccharide core surrounded by a phospholipid bilayer in which antigens may be inserted. The whole particle showed a diameter ranging from 50 to 100 nm and presented similarities with empty viruses (Fig. 4D). These systems were developed up to a phase I clinical trials for the nasal vaccination against the influenza viruses. It was demonstrated that the SMBV constituted a platform for the delivery of antigens into cells, whereas it did not act as an immunomodulator or as a simple adjuvant. The nasal vaccination with SMBV generated serosal immunity, local mucosal immunity, and a systemic cytotoxic T lymphocyte response (258,259). Using an antigen from the group C meningococci, this immunization strategy induced an Ig-A-mediated bactericidal activity at the mucosal level, which can be very effective in fighting the infection at its portal of entry (260). All data demonstrated the high potential of SMBV for use as nasal delivery for various antigens, and further developments are still expected.

All these systems and many others were also developed for mucosal vaccines, which requires the protection of antigens from protease degradation at the mucosa level (240,257–266). It is now accepted that the optimal size range of artificial particles, which are taken up by the associated mucosal lymphoid system, is lower than a few hundreds of nanometers (266–271). It even seems to be in the viral size range (40–50 nm) (272). Size dependence was clearly observed for transcellular uptake of nanosized systems by the M cells of the mucosa and for transfer of antigen carriers to the immunocompetent cells of the associated lymphoid tissue. However, the role of the surface characteristics, the composition, and the architecture of the carrier still need to be clarified for further optimization of delivery systems (266,267,271). Nevertheless, the superiority of nanoparticular antigen delivery systems has already been demonstrated resulting in a significant enhancement of the response following oral or nasal administration (261,266,273). Safety is a key issue for prophylactic immunization of healthy individuals (237,264). This explains that among the many experimental adjuvants that have reached the advanced clinical trials, only a few were approved by the authorities. On the other hand, adjuvant approved for parenteral immunization may cause serious concerns for mucosal immunization. Indeed, a nasal formulation of virosomes, Nasalflu®, was quickly withdrawn from the market after several months of clinical use because it induced unexplained facialis parese, whereas virosome vaccines for parenteral administration could be applied without side effects in vaccination programs against influenza each year (246,274,275). This illustrated the difficulty and the complexity to find new suitable adjuvants for mucosal vaccine delivery despite the fact that mucosal immunization represents promising approaches to protect an individual against mucosal infectious agents. Numerous other polymer- and lipid-based nanoparticular systems (264,265) hold promise for the development of mucosal vaccines, but a lot of work is still needed to prove their safety and to completely elucidate their mechanism of action.

Another issue of nanotechnology to prophylactic vaccination is the control of the immune response by using a synergistic combination of cytokines or costimulatory molecules together with antigens (276). At the moment, as already mentioned for the therapeutic vaccines, the most promising candidate of immunostimulating molecules is the unmethylated CpG containing oligonucleotides (13,14). Their packaging into liposomes, VLPs, or polymer nanoparticles improved their pharmacodynamics and prevented the occurrence of systemic side effects described for the free form. It also interestingly enhanced their immunostimulatory capacity when they are combined with a viral antigen improving the efficacy of the corresponding vaccine (5,256,277–279).

DNA plasmids encoding for antigens are also considered as exciting alternatives for the development of innovative vaccines (17,270,271). Of course, the type of the immune response induced by DNA vaccines is of prime importance for protection from intracellular viral infection. However, the expression of the antigen is dependent on the entry of the DNA plasmid into the cells. Although formulation of DNA vaccines using nanotechnology is now recognized as a valuable strategy to enhance the immune response against the encoding antigen, only few systems have yet been evaluated including virosomes, VLPs, and different polymer particles (252,280–282). Moreover, promises expected from immunization studies performed with small animals turned out very disappointing after the recent clinical trials. Thus, investigations are now shifted to a better understanding concerning the differences of performance of the DNA vaccine in mice and in bigger animals. It is hoped that this should help transfer, in the future, the success obtained with small animals to humans (17). Another aspect that deserves further research efforts concerns the route of administration of the formulated DNA vaccines. Indeed, no optimal route of

administration can be proposed from the only few studies that have considered nanoparticular DNA vaccine formulations (281–285).

In conclusion, nanotechnology offers interesting tools for fighting the many types of infectious agents and presents considerable interest for the development of both drug delivery systems and prophylactic vaccines. For the delivery of anti-infectious agents, further efforts are needed to find systems able to carry the drug down to the deeper infectious centers, especially in the case of chronic infections. Nanotechnology also provides systems for prophylactic immunization, which can be designed to mimic pathogen structure. However, further investigations are still needed to better understand the interaction of these nanodevices with the immune systems. Indeed, the more recent data clearly suggest that nanotechnology can be used to control the type of the immune response produced by antigen-associated liposomes or nanoparticles. Controlling the type of immune response by using appropriate carriers is also part of a new challenge, which consists in finding new treatments of chronic infections based on therapeutic vaccination (238,239). The rational behind this emerging concept is to stimulate the patient's own immune system to fight against chronic viral infections by reducing the level of viral replication and eliminating infected cells (286,287). For instance, a recent therapeutic vaccination approach developed against the chronic hepatitis B virus suggested to target the induction or the stimulation of $CD4^+$ and $CD8^+$ T-cell responses and the induction of proinflammatory cytokines capable of controlling viral replication (287).

### Application to Metabolic Diseases

Metabolic diseases include several major public health threats. For example, diabetes concerns approximately 150 million patients worldwide (288), and osteoporosis is the most prevalent metabolic bone disease. Osteoporosis has a high incidence in postmenopausal women and can also affect patients receiving corticosteroid therapy. It leads to bone fragility and an increased susceptibility to fractures. Treatments of metabolic diseases require the administration of proteins or peptides to palliate the metabolic disorder. In the case of diabetes, multiple injections of insulin are based on self-monitoring of blood glucose levels, in combination with a specialized diet and exercise regimen (289,290). The administration of the hormone by other routes than injection makes it difficult to obtain normal levels at the precise moment and at the right place during feeding, rest, and exercise (290). For the osteoporosis, calcitonin, a naturally occurring peptide, is used since many years, being administered by the parenteral route too (291).

As a general problem, these treatments based on multiple daily injections of peptides and proteins are source of patient noncompliance, which considerably limits the success of the therapies (289). To improve treatments, methods that allow a more sustained delivery of the peptides have been the subject of intensive investigations (289,291–293). In parallel, improved delivery systems for mucosal administration have been searched to provide convenience and to enhance patient compliance. Indeed, new formulations are currently being investigated to allow oral, nasal, or pulmonary administration of insulin and calcitonin. For insulin, oral formulations would provide the hormone directly to the liver by the hepatic portal circulation. This will be a major advantage because this pathway mimics the physiological traffic of the hormone when it is secreted by the pancreas of healthy individuals (294). However, mucosal routes are extremely challenging for the administration of peptides and proteins because these generally hydrophilic macromolecules are unable to overcome mucosal barriers by themselves and are degraded before they can reach the blood stream.

Liposome technology was introduced as early as 1976 with the primary aim to protect insulin against proteolysis degradation in the gastrointestinal tract (295,296). Results obtained with liposomes the following years were, however, disappointing because of a lack of stability of these formulations in the conditions prevailing in the gastrointestinal tract (297). Nanoparticle technology was, then, proposed for the first time in 1988 for successful oral delivery of insulin (297). Although preliminary, these related pioneer's works opened the door to the use of various tools offered by the nanotechnology including more stable formulations of liposomes, nanospheres, nanocapsules, and many other systems in the nanosize range (292,298–310).

Polymer nanoparticles were the subject of considerable experiments especially for the delivery of proteins by the oral and nasal routes. It was demonstrated that particles of size below 1 μm in diameter can be absorbed across the intestinal epithelium after oral administration and can be used to transport peptides and proteins across the barrier (300,311–313). However, results were sometimes contradictory because of the various types of insulin-loaded nanoparticles tested (314–316). The highest activity was found with insulin nanocapsules made of poly(alkylcyanoacrylate). In diabetic rats, it induced a spectacular reduction of the glycemia remaining over 20 days (298). In dogs, results depended on the animal: several dogs did respond very well, whereas others did not show a clear reduction in the glycemia (317). The peak absorption of insulin appeared between 30 min and 1 h in agreement with the rapid transit of the nanocapsules in the intestinal tract, but the intensity of the absorption greatly depended on rat individuals (318–320).

Many parameters may influence the success of therapies requiring the administration of peptides or proteins after administration by a mucosal route. Thus, protection of the drug against degradation may differ according to the type of nanoparticles and depending on the release profile of the peptide from the nanodevice (304,308,309,319,321,322). For example, poly(alkylcyanoacrylate) nanocapsules resisted well in the gastric fluid retaining the encapsulated insulin inside the nanocapsules. In the intestinal medium, the nanocapsule envelope can be degraded by intestinal esterases, and most of the encapsulated insulin can be released in less than 30 min (319,323). Other types of nanoparticles made of PLGA and of poly(fumaric anhydride-*co*-sebacic anhydride) seemed less effective to protect orally administered insulin from the harsh conditions of the gastrointestinal tract. They released the peptide even in simple saltine buffer in less than 2 h. The coencapsulation of $Fe_3O_4$ in PLA nanosphere (321) or the coating of the nanodevice with hydrophilic polymers like chitosan or poly(ethylenoxide) (309,324) can improve the protection of the encapsulated peptide against degradation.

Protection against degradation is necessary but not enough. Another very important factor for the delivery of therapeutic concentration of peptides is the amount of intact peptide absorbed by the mucosa whatever free or still associated with the carrier. Major questions remaining unaddressed concern the fate of the nanoparticles after they arrived at the level of the mucosa, in close contact with the epithelium and the amount of peptide actually delivered to the blood. Indeed, the efficacy of the transport remains a controversial issue, whereas peptides and proteins need to be delivered at a reproducible therapeutic concentration. This requirement is far different from that for the delivery of a small amount of antigen to the lymphoid-associated tissue for inducing an immune response. If a lot of work has focused on the mechanisms of particle uptake by the M cells found in the Peyer's patches, this lymphoid tissue represents only a small fraction of the total surface area available on the intestinal mucosa for absorption. Thus, more efforts should concentrate on other portions of the intestine better designed for the absorption of exogenous molecules (313). Moreover, polymer nanoparticles were shown to be able to cross the epithelial barrier of the intestine at the level of epithelial cells; but the extent of particle absorption remains unknown and is still difficult to be quantified *in vivo* (312,313,325–327). Mechanisms of particle absorption are under evaluation using cell culture models including the enterocyte-like Caco-2 (292,308,322,328,329) and the mucus-secreting MTX-E12 cells (328). Although the *in vitro* models may not always be predictive, they are helpful to understand passage of nanosystems across a monolayer of epithelial cells (292,330). According to a recent study by Prego *et al.* (292), the mucoadhesive properties of the carrier and its interaction with the mucus are key factors to provide an efficient *in vivo* transport of the nanodevice.

Apart from the mucoadhesion, the mechanism of absorption of nanoparticles across the mucosa may also depend on the nature of the nanoparticles. For instance, poly(alkylcyanoacrylate) nanoparticles were absorbed as intact entities at the level of the intestinal epithelium devoid of Peyer's patches, whereas they were found greatly degraded in the vicinity of M cells of the Peyer's patches (312). The other types of biodegradable nanoparticles were mainly described to be taken up at the level of the Peyer's patches, whereas less attention was really paid on their absorption by other portion of the intestine. Knowing the route and the mechanism of absorption of the nanoparticle is important to direct correctly therapeutic peptides in the organism. Indeed, we certainly would like to prevent the delivery of therapeutic peptide to antigen-presenting cells, which may induce adverse unwanted hypersensitivity reactions. It can be expected that the absorption may be influenced by the physiopathology of the animal and of the patient. Thus, it is still questionable whether or not a relevant concentration of peptide can be administered in a reproducible manner using nanotechnology, by a route of administration that involves the mucosa. The fate of the nanoparticles after absorption remains also unknown. This is another important issue to elucidate for optimal delivery of therapeutic peptides and proteins. For

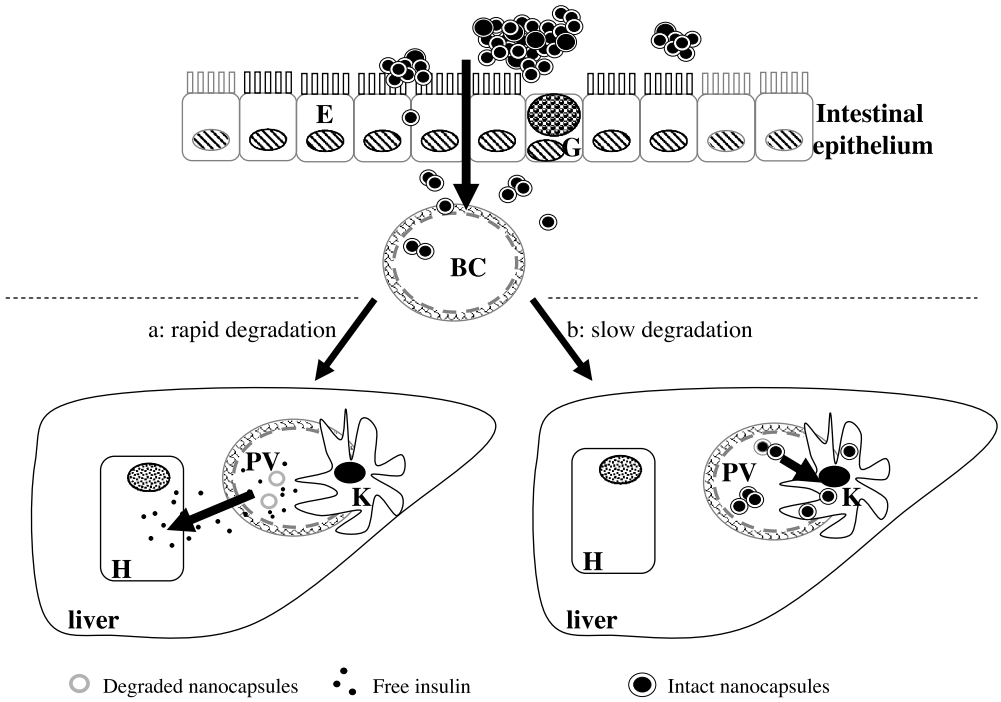


**Fig. 5.** Absorption of insulin-loaded poly(alkylcyanoacrylate) nanocapsules by the epithelium of the intestinal tract (E, enterocytes; G, Goblet cells) was observed by transmission electron microscopy (upper part) (312). The nanocapsules are delivered to the blood capillary through paracellular or transcellular pathways. Hypothesis about the fate of the nanocapsules arriving in the liver by the portal vein (PV) depending whether they are rapidly degraded (a) or slowly degraded (b) in the blood (lower part). In case of rapid degradation (a), free insulin will be released in the blood and will be delivered to the hepatocytes (H). On the contrary (b), still intact nanocapsules will be taken up by the Kupffer cells (K). BC: blood capillary.

instance, considering the poly(alkylcyanoacrylate) nanocapsules loaded with insulin, which were found in the blood vessels, the fate of the carried peptide may depend on a competition between the rate of degradation of the nanocapsules in the blood inducing the release of insulin and the velocity of transport of the nanocapsules by the blood to the liver. As illustrated in Fig. 5, it can be assumed that if the nanocapsules are degraded before reaching the liver by the portal vein, insulin will be released from the nanocapsules and will reach the liver under the free form. If so, the free released insulin may be taken up by hepatocytes, which will serve as a reservoir to control the insulin concentration in the peripheral blood. This will be the ideal situation because the fate of insulin will follow the physiological pathway. On the contrary, if the nanocapsules arrive in the liver by the portal vein almost intact, they will probably be recognized as foreign particles by the macrophages of the MPS including the Kupffer cells and be removed from the blood stream. In this figure, it is assumed that only a small part of the absorbed insulin will actually be efficient. Thus, knowing the fate of the peptide may help to improve the strategy of targeting the gut portion to obtain the right postabsorption pathway and the maximum activity of the peptide. An approach using lectine-modified insulin liposomes has been recently suggested in this sense (310).

Therapies provided with calcitonin and insulin by the nasal or pulmonary routes were also a subject of interest because of the high permeability and low enzymatic activity of these mucosa compared to the intestinal one. Although a couple of clinical trials have been performed on nasal/pulmonary administration of free peptides, the major problems were related to the poor amount of drug absorbed and/or the low reproducibility of the absorption (331,332). Thus, the use of liposomes or nanoparticles loaded with insulin was considered to enhance absorption and reduce early clearance at the level of the mucosa. For example, insulin-loaded nanoparticles made of chitosan were able to reduce glycemia by 50% of the initial value in rabbits, 30 min after administration (333). The amplitude of the effect was far less pronounced with solutions of insulin containing dissolved chitosan. Recently, transport of PEG-coated PLA nanoparticles across the nasal mucosa was clearly demonstrated (334). Factors promoting the intramucosal transport of such nanoparticles were a small size and a high density of the PEG coating. Nanocomplexes of insulin obtained with the amine-modified poly(vinylalcohol)-graft-PLA also increased significantly the bioavailability of the peptide resulting in a reduction of glycemia both in healthy and diabetic rats (335). The pharmacodynamic profiles found with these systems were very similar to those obtained with the chitosan nanoparticles (333). In general, the more hydrophilic the nanocomplexes were, the more important was the absorption. Intranasal administration of insulin nanoparticles seems interesting in that it provides a rapid supply of the peptide, which is absorbed in about 30 min and for a short duration of the effect (from 1.5 to 2 h). For a more sustained delivery of insulin, it was suggested by Tanaka *et al.* (289) to deliver the gene of insulin by the nasal route using liposomes. A sustained production of insulin was obtained in diabetic mice following daily treatment thanks to the successful transfection of the alveolar epithelial cells of the lungs. In this study, the lungs seemed to be the major tissue in which gene expression occurred after liposome-mediated gene transfer via nasal administration. The level of insulin produced was sufficient to correct the hyperglycemia of the diabetic mice without producing hypoglycemias or any kind of adverse side effects. Liposomes were also considered for the pulmonary administration of insulin (336) as promoters of absorption (337). Although the pulmonary epithelium represents an attractive site for the administration of drugs because of its exchange surface of approximately 80 $m^2$, its primary physiological function is not absorption. Thus, in the current move, all nanosystems employed to enhance absorption of drugs through the pulmonary (and nasal) mucosa may be suspected to be hazardous for health (338). The situation is not yet clarified, but the design of liposomes with lung surfactant can be considered as a clever approach to improve the biocompatibility of these systems (336).

The evaluation of the new nanoformulations of insulin and calcitonin given by mucosal routes also requires investigations on reliable animal models. Calcitonin activity is often evaluated by measuring the serum level of calcium in the rat, which reflects the activity of this peptide, whereas the insulin activity is usually determined through the measure of the glycemia in diabetic rats. Only few studies considered the bioavailability of insulin after mucosal administration (320,335). It seems that the widely used streptozotocin-induced diabetic rat is far from ideal and is quite difficult to handle in practice. On one hand, insulin resistance may occur, and, on the other hand, reduced level of the glycemia could not always correlate with insulin concentrations found in the blood of the treated animals because glycemia is highly susceptible to stress or fed status (302,320,339,340). A more systematic measure of the insulin blood concentration is therefore recommended, including the difference between free insulin and the insulin hidden in drug carriers or adsorbed onto blood cells. To evaluate the total amount of insulin absorbed after oral administration, it would also be better to determine the insulin concentration in the portal hepatic vein before regulation by hepatocytes occurs. Whatever its defaults, this model remains the more accessible diabetic animal model for research laboratories. It will continue to be used for the evaluation of new oral formulations of insulin, but we need to be aware that correlations between data obtained with this rat model and with bigger animal models may be hazardous. Alternative models are still actively searched (341).

From the work carried out so far, a few general rules to enhance the mucosal absorption of peptide-based nanoformulations can be pointed out:

(i) Transport of peptides across the nasal and the intestinal barriers is improved using polymer particles of small size (less than 200 nm), preferably coated with hydrophilic bioadhesive polymer (PEG or chitosan).

(ii) Poly(alkylcyanoacrylate) nanoparticles and chitosan-based nanosystems seemed to provide the best protection of the peptide in contact with mucosa especially with the intestine.

(iii) At present, parenteral substitution of peptide therapy is still imperfect, and, before the dream of a nonparenteral formulation of insulin or calcitonin comes to reality, this ambitious challenge requires additional research efforts.

## Other Applications

### *Autoimmune Disease and Prevention of Graft Rejection*

For both treatment of autoimmune disease and management of graft rejection, the current therapeutic strategy is based on the modulation of the immune response by using immunosuppressive compounds. The chief of file of the immunosuppressive drug is cyclosporin A. Its activity is to inhibit selectively the release of interleukin-2 resulting from the T-cell activation process to suppress cell-mediated immune response. It is used orally to prevent graft rejection and, by topical administration, to control the progression of the ocular autoimmune diseases. This molecule has a poor bioavailability because of a low absorption pattern through epithelia. For oral administration, liposomes and nanoparticles cannot compete with the present dosage form based on a microemulsion (342) because they display a relative bioavailability of only 20–40% as compared to the microemulsion. However, their interest for the delivery of cyclosporin A to the eye is supported by the fact that the local bioavailability of this compound is improved when it is associated with colloidal drug carriers (343–345). These formulations, indeed, act as drug reservoirs allowing a local sustained release of cyclosporin A and promoting either the local intraocular penetration of the drug or its ocular surface residence, thus limiting systemic side effects. For instance, liposomes improved the intraocular bioavailability of cyclosporin A without showing toxicity on the retina (343). Using chitosan nanoparticles, therapeutic concentration of cyclosporin A could be maintained for at least 48 h at the ocular surface, mainly in the cornea and the conjunctiva. Negligible or undetectable levels of cyclosporin A could be measured in the inner ocular structures (iris, ciliary body, and aqueous humor) and in the blood (344). Among the different types of nanoparticles that were evaluated, those made of chitosan presented the highest performance for the delivery of cyclosporin A in the periocular tissue, together with a good ocular tolerance. It is interesting to point out that it is now possible to design nanotechnology able to target different regions of the eye allowing, for instance, the precise delivery of cyclosporin A to the intraocular compartment for the treatment of the ocular uveitis or to the external tissues for the treatment of extraocular diseases, such as the keratoconjunctivitis sicca. Retention of the nanoparticles by the extraocular tissue is assumed to occur by an absorptive-mediated endocytosis, but uptake by transcellular route may only occur in the first two cell layers (346).

Several other compounds were combined with nanoparticles to control the progression of autoimmune diseases. PEG-coated liposomes were used to deliver high concentrations of glucocorticoids to the brain to circumvent progression of neurodegenerative disorder, which are increasingly attributed to the occurrence of autoimmune diseases (347). Increase in the efficacy of the drug was obvious because the effective concentration of prednisolone carried by the liposome was 5-fold lower than the one required with the free methylprednisolone (348). Similarly, poly(PEGcyanoacrylate-*co*-hexadecylcyanoacrylate) nanoparticles were found to be able to translocate into the brain tissue of rats with autoimmune experimental encephalomyelitis (47). For the treatment of ocular uveitis, the use of tamoxifen, a nonsteroidal estrogen receptor modulator, associated with the same nanoparticles was found to dramatically reduce the inflammatory process. Administered by the intraocular route, the intraocular concentration of tamoxifen could be maintained for a long period of time. The beneficial activity of tamoxifen released from the nanoparticles was explained by a direct activity on the local immune system, thanks to a manifestation of the immune privilege of the eye that protects the ocular tissue from the deleterious effects of ocular inflammation (349). Results obtained with the tamoxifen-loaded nanoparticles are important for the evolution of treatments of autoimmune diseases. Indeed, the more recent knowledge on mechanisms by which the progression of the disease might be stopped or attenuated suggests the development of new therapeutical strategies based on vaccination aiming to boost the protective autoimmune response. This will constitute a revolution in the treatment of all autoimmune diseases including the eye glaucomatous, multiple sclerosis, and diabetes. As suggested by the hypothesis of De Kozac *et al.* (349) and by some of their experimental data, the delivery of compounds that may improve the protection of the degenerative tissue from destruction by promoting a protective immune response may constitute a new therapeutical approach. Several compounds showing such an activity have already been identified like Cop-1 (a synthetic polymer that weakly cross-reacts with a wide range of self-reacting T cells and which is FDA-approved for the treatment of multiple sclerosis), whose main activity is to boost a T-cell-based response, inducing a protective autoimmunity against the destruction of the optic nerve in glaucoma. The delivery of this compound was shown to even promote the recovery of the damaged optic nerve (350). As discussed in a previous part of this paper, the input of the nanotechnology to stimulate an immune response is now well demonstrated. There is no doubt that nanotechnology will constitute the keystone of the development of these very new therapeutic approaches to improve treatments of autoimmune diseases. However, because all these findings are very recent, more fundamental research is still needed to further improve the overall knowledge about the whole mechanisms involved in the induction of the protective immunity. Today, nanotechnology is also already used to help in these investigations when magnetic resonance imaging techniques are needed to monitor cell homing in living organism over a period of up to 20 days (351).

Waiting for these prospective and innovative therapies, the current treatments of glaucoma with pilocarpine or betaxolol may be improved using nanotechnologies too [see (352) for review]. Several types of systems were tested on animal models to improve the local delivery of these drugs to the eye. The different nanoparticles were all performant to reduce systemic dissemination of the drug and to increase the concentration in the ocular tissue, but they differed regarding their toxicity. The best tolerated nanoparticles were those made of chitosan. However, toxicity of the nanoparticles against the cornea could be reduced by incorporating the nanoparticles in a polyacrylic gel.

### *Inflammation*

One of the main drawbacks of the major anti-inflammatory agents, diclofenac and indomethacin, is their local

toxicity in contact with tissues. They induce ulcers on the gastrointestinal mucosa when they are administered by the oral route and cause damage on the corneal epithelium after instillation or on the muscular tissue after intramuscular injection. The local toxicity can be considerably reduced by encapsulation into nanocapsules or in liposomes while the pharmaceutical activity of the nanoencapsulated drug was not influenced by the encapsulation (353–356). After oral administration, the nanocapsule formulations exhibited drug pharmacokinetic profiles similar to those obtained with the free drug. This was independent of the nature of the nanocapsule polymer nature (353). This major improvement can be explained by a slow release of the drug from the nanodevice and by a better spreading of the dose on the mucosa so that local concentrations are very low, hence reducing the ulcerative effect (355). After intramuscular administration, the protection from toxicity was shown to depend on the nature of the oil entrapped in the nanocapsules. Thus, diclofenac-loaded benzylbenzoate nanocapsules were as toxic as the free drug, whereas diclofenac-loaded mygliol® nanocapsules were able to reduce considerably the muscular damage caused by diclofenac (357). In the case of the ocular treatments, an increase of the bioavailability of indomethacin by 300% was reported using poly(epsilon-caprolactone) nanocapsules (358). By confocal microscopy, it was shown that the nanocapsule formulation enhanced the penetration of the drug in the corneal epithelium by an endocytosis mechanism. It was suggested that the components forming the nanocapsules act as penetration enhancers or as an endocytotic stimulator. The investigation of the ocular pharmacodynamic profile of ibuprofen-loaded Eudragit nanoparticles also leads to the conclusion that the nanosystem increased the bioavailability of the anti-inflammatory drug and ultimately its pharmacological activity (359).

Anti-inflammatory treatment is also hampered by the poor solubility of efficient drugs including ibuprofen or naxopren. By using nanotechnology, the exchange surface between the drug and the surrounding medium can be considerably increased (360–362). This favors the dissolution of the drug and its deposition at different places on the absorption sites especially when it is given orally (361,363,364). The combination of these two effects leads to an improvement of the bioavailability of the active compound (361,363,364).

*Pain*

Quite recently, liposomes have attracted much attention for pain management, mainly to develop sustained released delivery systems for anesthetic compounds. They are then used either by topical administration (365,366) or by single injection at the time of surgery (367,368). For local anesthesia achieved by topical administration, the liposomal formulation (lidocaine, tetracaine,...) is incorporated in a cream and applied onto the skin surface at the place where the anesthesia is needed (369). The efficacy of such delivery systems was evaluated in dermal-related procedures in clinical trials consisting of puncture of the intact dermal surface with a needle including intravenous cannulation, venipuncture, arterial cannulation, and insertion of spinal needle (365). Results concluded that both tetracaine- and lidocaine-loaded liposomes were at least as efficacious as eutectic mixtures of local anesthetics. They allow an easy needle-free administration and display a fast onset of action compared to the other formulations in use. Today, the lidocaine-loaded liposomes are marketed in the USA. Its routine use seems to be of lower cost than the eutectic mixtures of local anesthetics. This is an important economic factor contributing to the development of a new drug delivery formulation. Following the more recent clinical trials, the conclusions are suggesting that the liposomal formulation of lidocaine should be recommended as the new standard for cutaneous anesthesia in children (366).

Liposomes are also part of the emerging techniques for postoperative analgesia in orthopedic surgery (367,368). They are proposed for the delivery of morphine as alternative to the current other pain treatments using morphine (367). The increasing knowledge about pain management makes clear that adequate pain control is associated with key clinical benefits and contributes to reducing the cost of treatments, thanks to shorter hospital stays and improved rehabilitation after surgery (367,368). The liposomal formulation of morphine proposed today for postoperative pain management consists of an extended release of morphine for epidural administration. The liposome technology used consists of lipid-based particles with closely packed internal chambers separated by lipid membranes (Depofoam®, SkyePharma, Inc., San Diego, CA, USA) containing the drug (368,370). The results from a clinical trial (phase III) concluded that when these liposomes were injected once in the epidural space, up to 48 h of pain relief was provided (368). This is a considerable progress for hip arthroplasty. It should now be investigated whether the easy administration of this formulation compared to the current marketed products may favorably impact the anesthesia preparation time, physical therapy, and activity of daily living, which are all factors affecting the cost of treatments.

Apart from liposomes, a very recent study suggested the use of solid lipid nanocapsules containing ibuprofen for pain management (371). Given to rats either by the oral or by the intravenous route, these nanocapsules induced a pain relief over a prolonged period of time of at least 2 h. One advantage of this formulation is that it offers an injectable carrier of ibuprofen, which is an interesting alternative to other formulations containing cosolvents and surfactants, which may be questionable from a toxicological point of view.

Along the same line, Betbeder *et al.* (372) showed that it was possible to deliver morphine to the brain by intranasal administration of the SupraMolecular BioVector™, thanks to the existence of a direct pathway between the olfactory mucosa and the central nervous system. This study deserves to be revisited now in the light of the more recent progresses in the understanding of pain management at the level of the brain (373). Additionally, although nanodevices can be optimized in terms of size and bioadhesiveness for the intranasal administration of drugs (292), the design of blood–brain barrier targeting technology is less advanced (374). It is noteworthy that systems using receptor-mediated transport hold promise. However, the discussion remains open concerning the best strategy either to target the transferrin or insulin receptor (375,376) or to design nanoparticles coated with different surfactants (poloxamer and polysorbate), which are believed to be endocytosed by the low-density lipoprotein receptor of the endothelial cells lining the blood–brain barrier capillaries (377).

*Oxygen Carriers*

Nanosystems were thought to be useful to develop artificial erythrocytes for blood substitute. The main indication would be the rapid supply of hemoglobin when blood is not available after excessive blood loss because of an accident or surgery. The main advantage of these nanosystems is their immediate availability after wounding without need for special storage conditions. Therefore, they would be available anywhere where emergency situation would need it. The market of blood substitute is very important because of looming shortage of blood donors and safety problems because of possible transmission of viral infections during transfusion. Hemoglobin-based oxygen carriers were developed by encapsulating the hemoprotein in liposomes (378–380), nanocapsules (381), or by loading into nanoparticles (382,383). All these systems are designed to be long circulating carriers. However, the main challenge is to preserve oxygen affinity and functionality of the hemoglobin when it is associated with the carrier. Another problem to circumvent is the irreversible oxidation in met-hemoglobin, which generally occurs rapidly when hemoglobin is extracted and purified from red blood cells. To prevent oxidation, the coencapsulation of hemoglobin with a multiple enzyme complex including superoxide dismutase and catalase was proposed in nanocapsules (381). The nanocapsules were designed in such a way that they allow exchanges of small molecules between the outside and the inside compartment containing the proteins. This property of the nanocapsule envelope seems important to insure the regeneration of functional hemoglobin when it is oxidized in met-hemoglobin, providing nanocapsules that function almost like red blood cells. Suspensions of nanocapsules containing up to 150 g hemoglobin per liter could be obtained, and degradation produces lactic acid corresponding to a physiological metabolite. The amount produced by the degradation of 500 mL of nanocapsule suspension will represent less than 1% of the normal production of lactic acid of the resting body (381). With the optimized system, the maximal non-red blood cell systemic hemoglobin reached in rats was 3.66 g/dL. The concentration fell to 1.67 g/dL after 24 h (384). Liposomes' ability to transport oxygen comparable with blood was also demonstrated, and the safety of the carrier was tested in preclinical trials, which concluded with the nonantigenicity of the carrier and with the absence of toxicity. *In vivo*, the degradation of hemoglobin carried by liposomes occurred in the MPS, which is the physiological compartment for degradation of senescent red blood cells and heme detoxification pathway. It was controlled that the degradation of high doses, which are required for clinical uses, can be processed without causing toxicological problems. Coencapsulation of a flavin mononucleotide in the hemoglobin-containing liposomes was a good method to restore the oxygen-binding capacity of hemoglobin when it is transformed in met-hemoglobin through a photoreduction process using visible light (385). This carrier for hemoglobin transport is comparable to native red blood cells in the sense that they are formed by phospholipid membrane. *In vivo*, the hemoglobin half-life was around 22 h with this carrier as determined in rats (379).

So far, nanocapsules and liposomes are the most advanced hemoglobin carriers issued from the nanotechnology as blood substitute, and they are now close to reach the clinical trial stages. However, motivations to find other suitable carriers are still active. In the more recent studies, hemoglobin was entrapped in a layer of carbohydrates at the surface of nanoparticles, thanks to the observation that sugars provide structural stability to the protein against conformational changes, which is another important factor for keeping intact the cooperative functionality of hemoglobin. One system emerging from this observation was built from a hydroxyapatite core (diameter 100–200 nm) coated with poly(amidoamine) dendrimers in which trehalose and hemoglobin were entrapped together (382). In another study, nanoparticles were designed with a core (diameter 80 nm) of poly(alkylcyanoacrylate) and overcoated with a heparin layer in which the hemoglobin could be entrapped (383). In these systems, oxygen affinity and cooperativity of hemoglobin were well preserved, and they were stable, in terms of hemoglobin desorption, despite the fact that the protein was adsorbed at the surface of the nanoparticles. The nanoparticles having the hydroxyapatite core preserved hemoglobin stability over a period of 30 days (382). The stability of hemoglobin associated with the poly(alkylcyanoacrylate) nanoparticles coated with heparin has not yet been evaluated, but the polymer used in their constitution has been widely employed for drug delivery purposes, which make these nanoparticles very attractive as hemoglobin carrier. They are also the nanoparticles that showed the highest hemoglobin-loading capacity because they were capable of transporting up to 40 mg hemoglobin/g nanoparticle, whereas the loading capacity of the hydroxyapatite core nanoparticles was only one third of this value (13.7 mg hemoglobin/g nanoparticle). Because these nanoparticles are coated with heparin, it is highly expected that they will also show long circulating properties in the blood as heparin-coated nanoparticles containing a poly(methyl methacrylate) core made according to the same polymerization method (386).

## INDUSTRIAL DEVELOPMENT

### Development Perspectives

It is expected from different sources that the global market of nanotechnology will expend very fast. This year, it should reach $1 trillion (387). Applications in pharmacology are included in this action, and the National Institutes of Health estimated that by the year 2010, more than 50% of all biomedical advances will be in the nanotechnological sector (387). Several factors will contribute to the achievement of these previsions.

First, in the move of the present industrial pharmacy's view, methods to achieve an efficient drug delivery are considered as an essential product characteristic. This is new from the past. The new drugs coming out from the discovery processes often present special delivery challenges; this has to be taken into account in the drug development strategy. Indeed, for proteins, peptides, genes, AS-ON, or si-RNA, which all represent an important potential market, the most prominent barrier is recognized to be their delivery, which highly complicates their clinical success (388). Therefore, these compounds will undoubtedly be part of the key growth drivers for the development of new nanotechnologies in

pharmacology. For the existing compounds, pharmaceutical industries are also looking for new strategies to extend product life cycle. The development of new formulations enabling better performance of an existing drug or finding new therapeutic indications is one of the research strategy that may be considered as low risk/high reward. In this case, risks inherent to the drug molecules are known, whereas the only unknown part of the development process is focused on the delivery problems (389).

Second, a pharmacoeconomic analysis performed on clinical oncology trials has recently pointed out that, in practice, the use of nanotechnologically engineered drugs may be high competitors to more traditional chemotherapies. Indeed, in certain cases, the overall cost of a chemotherapy performed with existing liposome drugs (Caelyx®, Doxil®) was lower than the classical chemotherapy given similar activity because it required less frequent administration and less intervention for toxicity (390). Thus, it can be expected that the use of nanotechnology in clinics will be encouraged when targeting a drug is crucial to benefit both the efficacy of the treatment and the reduction of side effects. This will also contribute to boost the market of the nanotechnology in pharmacology especially as far as treatments of cancer and of severe infectious diseases are concerned. Other pharmacoeconomic analysis indicates that a considerable reduction of cost in favor of the development of nanotechnology may be expected to come from the reduction of the time of hospitalization in countries in which hospitalization and medical care costs are substantial (168,169,365,368).

Third, the pioneering successful experience of three liposomal companies in the 1980s (Liposome Company Inc., now Sequus Pharmaceutical, NeXstar in San Dimas, CA, USA, now Gilead, and The Liposome Company Inc. in Princeton, NJ, USA) will importantly impact clinical practices in the near future. Solid experiences are now available with several approved formulations of liposomes. Routine productions of conventional and Stealth® liposomes are well established, and the *in vivo* fate of these systems is well described as well. These first experiences raised confident climate for new nanosystems to get into the development pipelines. As a consequence, it already opens doors to several new approved liposomal formulations, and others are under development (55,365,368,391). At least 29 clinical trials are currently recruiting patients for phase II and phase III investigations of liposomal formulations of anticancer drugs, anti-infectious drugs, and analgesic compounds (392). Several new companies emerged with the goal of developing liposomes. Polymer nanoparticles are also now taking part of this challenge. Two types were approved for clinical trials for the treatment of cancer: Transdrug® (Bio-Alliance Pharma, Paris, France) and Abraxan® or ABI-007 (American Bioscience Inc., Santa Monica, CA, USA). The main advantages of using polymer nanoparticles instead of liposomes are that polymers are considered as cheaper materials than lipids, and that polymers offer wider chemical engineering solutions. In addition, the stability of polymer particles can be much better controlled than the stability of liposomes upon slight modifications of the formulation. Therefore, nanoparticles may appear as alternative carriers considering the design of more sophisticated systems including multifunctional types of device.

Today, the market of nanotechnology developed in pharmacology is mainly focused on the development of solutions to transport the drug in the body from the site of administration to the site of action. In the future, novel nanotechnology may be introduced. New concepts will emerge from new types of interdisciplinary collaborations. The development of strategies based on nanomachines or nanorobots and the introduction of computer science may lead to a new revolution in the improvement of treatments and diagnostic methods (393–395).

## Obstacles to Developments

Obstacles to the growth of nanotechnology for medical applications greatly depend on three major concerns: cost, registration procedures, and some kinds of fears.

The problem of *cost* results in funding research. Today, most developments are carried on by small entrepreneurial firms including many spin-ups that cannot support themselves as yet on current revenues, whereas big pharmaceutical companies seem still awaiting for more successes. Fortunately, governments are strongly convinced by the potential economic impacts that nanotechnology can raise in the medical field by reducing hospitalization and medical care cost (168,169,365,368). Thus, all big countries open large funding programs to support the cost of research. They encourage the building of strong partnerships at the national and international level among academic and industrial partners with multidisciplinary expertise (387). This allows the small entrepreneurial firms to find valuable financial complement to their venture capital from government grants. The problem of cost is also somehow linked to the management of the intellectual property rights. For the survival of a company, it is economically essential to build a relevant intellectual property strategy taking patents that will protect the technology and the commercial interests on both an offensive and a defensive standpoint (396). The nanosystems designed for the delivery of drugs are part of the nanotechnology that is subjected to the intellectual property rights (397). The issue is believed to have a huge impact in the future of the drug delivery sciences that companies are generally very cautious about these aspects. Universities and government institutions also hold several patents and promote transfer technology to company.

To come to market, all new drugs must receive *approval from authorities* [Food and Drug Administration (FDA) in the USA, European Agency for the Evaluation of Medicinal products (EMEA) in Europe, Pharmaceutical and Medical Device Agency, KIKO (PMDA, KIKO) in Japan]. It is only recently that the FDA adopted a clear position about regulations that may apply to product coming out from nanotechnology. FDA has identified a couple of regulated products that are expected to be impacted by nanotechnology including drugs (new molecular entities or novel delivery systems), medical devices, biotechnology products, tissue engineering products, vaccines, cosmetics, and combination products (398). Among these, applications in pharmacology include drugs, vaccines, and combination products. The general concerns of FDA on nanotechnology products are about safety, quality, and characterization of material and environmental impact. Many questions are addressed. How-

ever, it seems that there are currently no testing requirements that are specific to nanotechnology products. If research identifies toxicological risks that are unique to nanomaterials, additional testing requirements may become necessary. At the moment, the FDA is not anticipating any new guidance documents regarding nanomaterials in the near future. It indicates that the process of approval for nanomaterials will be the same as that used for other products making the same claims. It would not be surprising if the present regulation will change in the near future because a large debate was recently opened to evaluate the real benefits to risk of the enlargement of nanotechnology for applications and to identify possible hazards (399).

The third obstacle to development is related to the *general fears* of skeptical public related to the outcome of any new kinds of technologies that can become virtually ubiquitous and may impact almost all domains of applications (399,400). Presently, this is the case with nanotechnology in general, and it can slow down processes to turn new potential applications to real ones. As a general skepticism, people are aware that chemical properties may become toxic at the "nano" level. In the case of pharmacology and especially as far as treatment of cancer is concerned, it has to be taken into account that toxicity is useful owing it is targeted.

Focusing on pharmacological applications, fears include the feeling that engineering delivery systems will increase time and cost. It is true that research programs on these systems cost a huge amount of money mainly because a lot remains to be discovered to improve and to extend their uses. However, it can be pointed out that the cost getting into formulation strategies may be easier and cheaper than staying in chemistry for a long period of time to find better physicochemical properties for a potential new molecule. As it was discussed earlier, formulation can be part of a strategy to extend product life of an existing molecule. It can be expected that the previous success of several liposome formulations will boost reluctant people to look at nanotechnology more closely. It can also help them to get rid of the idea that liposome and nanoparticle formulations are over-engineered delivery systems.

Finally, application of nanotechnology often requires development of partnerships with experts outside the company. For an optimal success, it is even recommended that this partnership will start at the earlier stage of the drug development process. Many drug discovery companies are afraid to share information that they consider as highly risky with a third party. For a successful collaboration, the management of the intellectual properties must be considered very carefully at the beginning of the business relationship, defining clear intellectual properties ownerships in a contract that could help overcome this reluctance (389,396). In many cases, new challenges arise during the execution of pluridisciplinary collaborations that really merit overcoming difficulties to settle alliance agreements.

## CONCLUSIONS

The introduction of nanotechnology in pharmacology has revolutionized the delivery of drugs, allowing the emergence of new treatments with an improved specificity. Nanotechnology is now widely implanted in the move of revisiting drug delivery methods. These new nanosystems can be tailor-made according to the desired functions and duty thanks to parallel progresses in the synthesis of colloidal systems with perfectly controlled characteristics. They can be administered by all routes of administration for systemic or local treatments. Their values are the control of the drug release and distribution, the enhancement of drug absorption (by mucosa or cells), and the protection of drugs from degradation. They offer so many advantages to improve the precision of the treatment that some were marketed during the last decade. So far, there is still no universal platform that is suitable for the delivery of all kinds of drugs. It is expected that in the future, several platforms will emerge, each being specific for either a type of drug (i.e., peptides or nucleic acids) or for a specific biodistribution. Additionally, some nanosystems may have special physical properties, such as colloid metal-based systems, which may be exploited to kill cells or to improve imaging techniques for diagnostic purposes.

The various indications of nanotechnology proposed so far already impacted new thinking on the way of delivering drugs today. Comprehension of biological disorders causing disease will definitively help in making further progress to identify very precise new targets and to enter the age of gene therapy. It may be expected that the major immediate scientific and technological lock to overcome includes the understanding of the functioning of the immune system as a whole. Because it seems to be involved in many of the physiopathological disorders, its resolution will boost the appearance of innovating treatments for numbers of diseases and for controlling the biodistribution of drug nanocarriers. Another important lock is the identification of specific cell targets to allow more selective performance.

Although the introduction of nanotechnology has obviously permitted to step over numerous milestones toward the development of the "magic bullet," a lot of work remains to be performed. Next improvements will certainly come from the introduction of new materials including stimuli-responsive polymers to elicit the challenge of targeting the drug to its specific site of action, to retain it for the desired duration, and to release it according to the correct time schedule. It may also be expected that more sophisticated and multifunctional systems will be conceived allowing with a single system to perform *in vivo* diagnostics and to release the targeted drug on demand. Finally, the development of strategies aiming to develop entities existing in Mother Nature and based on biomimetism should also participate to major progresses in the next few years.

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