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Review

Colloidal drug carriers: achievements and perspectives

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Abstract. Colloidal drug carriers such as liposomes and nanoparticles are able to modify the distribution of an associated substance. They can therefore be used to improve the therapeutic index of drugs by increasing their efficacy and/or reducing their toxicity. If these delivery systems are carefully designed with respect to the target and route of administration, they may provide one solution to some of the delivery problems posed by new classes of active molecules such as peptides, proteins, genes, and oligonucleotides. They may also extend the therapeutic potential of established drugs such as doxorubicin and amphotericin B. This article discusses the use of colloidal, particulate carrier systems (25 nm to 1 μm in diameter) in such applications. In particular, systems which show diminished uptake by mononuclear phagocytes are described. Specific targeting of carriers to particular tissues or cells is also considered.

Key words. Drug delivery; liposome; nanocapsule; nanoparticle; nanosphere; poly(ethylene glycol); polymer; targeting.

Introduction

The fate of a drug after administration in vivo is determined by a combination of several processes: distribution, metabolism, and elimination when given intravenously; absorption, distribution, metabolism, and elimination when an extravascular route is used. Regardless of the mechanisms involved, the result depends mainly on the physicochemical properties of the drug and therefore on its chemical structure. During the last few decades, much work has been directed toward the development of delivery systems which will allow the fate of drugs within the patient to be controlled by modifying these processes. Although drug carriers usually act on the second process – drug distribution within the organism – they may also affect absorption, metabolism, and elimination.

Drug delivery systems can be classified according to either their physical form or to their functional properties. In the latter case, a division into first-, second-, and third-generation has been proposed [1].

Classification of drug delivery systems

The so-called first-generation systems are capable of delivering the active substance specifically to the intended target but cannot be considered as 'carriers' because they have to be implanted as closely as possible to the site of action. Microcapsules and microspheres for chemoembolization belong to this group, as do similar systems used for the controlled release of proteins and peptides or for drug delivery within the brain.

In contrast, 'second-generation' systems are true carriers and are usually soluble or particulates less than 1 μm in diameter. They are capable not only of releasing an active product at the intended target but also of carrying it there after administration by a general route. This group includes so-called passive colloidal carriers such as liposomes, nanocapsules, and nanospheres, and certain 'active' carriers such as temperature-sensitive liposomes and magnetic nanospheres, which release their contents after a specific signal. However, after intravenous administration, most colloidal carriers are rapidly removed from the

circulation by phagocytic cells in the liver and spleen. This limits their potential to deliver their contents to specific sites. Over the last 10 years, systems whose surface properties have been modified to reduce the deposition of plasma proteins and which show diminished recognition by phagocytes have been developed. These are known as sterically stabilized carriers (or 'Stealth™' carriers[1]) and may remain in the blood compartment for a considerable time. Although such colloidal particles cannot cross normal continuous capillary endothelium, they have been shown to extravasate into sites where the endothelium is more permeable, such as solid tumors or regions of inflammation and infection.

The systems referred to as 'third-generation' are also true carriers and, furthermore, are capable of specific recognition of the target. For example, monoclonal antibodies belong to this group, as do certain second-generation particulate systems (liposomes, nanocapsules, nanospheres) piloted by monoclonal antibodies or other ligands. Of course, targeted colloidal carriers will be much more effective if they are also sterically stabilized.

As far as the physical form of drug delivery systems is concerned, these can be molecular or particulate. Molecular carriers include soluble polymers to which drug molecules have been covalently attached, sometimes with targeting moieties coupled to the same molecule, drug-antibody conjugates such as immunotoxins as well as conjugates with other naturally occurring macromolecules, and lipophilic prodrugs. Drugs trapped within the central cavity of water-soluble cyclodextrins can also be considered as belonging to this category. Although molecular carriers allow a wide distribution of the associated drug, one limitation to their use is the payload of drug which can be carried by each molecule. In contrast, particulate delivery systems can carry a large number of drug molecules in one entity. The most 'natural' particulate carriers are cells from the patient which have been loaded ex vivo; these can be resealed erythrocytes or lymphocytes which have been loaded by electroporation. The disadvantage of this approach is the complexity of the procedures involved, not least the regulatory aspect, since each preparation can be considered as a separate 'batch'.

The potential of synthetic particulate drug delivery systems depends on their size. As explained above, polymeric microspheres with a diameter of more than 1 µm cannot be given by a general route and have to be implanted close to the intended site of action. On the other hand, submicronic particles, often referred to as 'colloidal drug carriers' can be given by parenteral routes, including intravenously, and may be able to deliver the drug to a site distant from the site of administration, subject to some limitations which will be discussed below. The best-known member of this group is liposomes.

[1] Stealth™ is a registered trade mark of Liposome Technology Inc.

Liposomes

Liposomes consist of one or more phospholipid bilayers enclosing an aqueous phase. They were first proposed as carriers of biologically active substances in 1971 [2], and have been comprehensively studied since. They can be classified as large multilamellar liposomes (MLVs), small unilamellar vesicles (SUVs), or large unilamellar vesicles (LUVs) depending on their size and the number of lipid bilayers. Water-soluble drugs can be included within the aqueous compartments, while lipophilic or amphiphilic compounds can be associated with the lipid bilayers. In some cases, the resulting objects are better described as lipid complexes than liposomes, since they do not contain an internal aqueous phase. Methods for the preparation of liposomes are reviewed in Gregoriadis [3]. Non-ionic surfactant vesicles or niosomes are similar systems obtained from synthetic surfactants and cholesterol [4].

Nanoparticles

Nanoparticles, based on biodegradable polymers and of similar size to liposomes, show some advantages over the latter in terms of stability both during storage and in vivo [1]. They may consist of either a polymeric matrix (nanospheres) or of a reservoir system in which an oily or aqueous core is surrounded by a thin polymeric wall (nanocapsules) [5]. Polymers suitable for preparing nanoparticles include poly(alkylcyanoacrylates), poly(methylidene malonate 2.1.2), and polyesters such as poly(lactic acid), poly(glycolic acid), poly(ε-caprolactone), and their copolymers. Lipophilic drugs, which have some solubility in the polymer matrix or in the oily core of nanocapsules are more readily incorporated than hydrophilic compounds, although the latter may be adsorbed onto the particle surface. Methods for the preparation of nanoparticles can start from either a monomer or from a preformed polymer. They are reviewed in Barratt et al. [1]. Nanospheres can also be formed from natural macromolecules such as proteins and polysaccharides, from nonpolar lipids, and from inorganic materials such as metal oxides and silica.

Other systems

Another type of submicronic drug delivery system attempts to mimic lipoproteins. Both modified natural lipoproteins [6] and synthetic systems [7] have been used. Microemulsions and self-emulsifying systems destined for use by the oral route can also be considered as drug delivery systems, in this case influencing the absorption of the associated drug rather than its distribution [8].

Scope of the review

This review will be limited to liposomes and related lipid complexes and nanoparticles prepared from biodegrad-

able polymers, although some reference will be made to microparticulate preparations from the same polymers. The fate of these 'second-generation' systems after administration by various routes will be discussed and the resulting therapeutic potential described. This will be followed by an account of different strategies which have been employed to modify the distribution of colloidal drug delivery systems to enlarge their domain of action and achieve true 'targeting'. There is now an enormous amount of literature in this area and no single review could give comprehensive coverage. Therefore, I have tried to indicate the main areas of research with some selected examples. I apologize in advance to colleagues whose work has been omitted through lack of space.

Distribution and fate of colloidal drug carriers in vivo

Intravenous administration

The fate of liposomes after intravenous administration has been discussed by Poste et al. [9]. The authors describe three different types of capillary endothelium and point out that colloidal particles cannot extravasate except in tissues with a discontinuous capillary endothelium; i.e., the liver, spleen, and bone marrow. In these particular capillaries, called sinusoids in the liver, the basement membrane is discontinuous or absent and the endothelial cells are perforated by pores (fenestrations) of about 100 nm in diameter. This means that only the smallest nanoparticles can penetrate into the tissue. On the other hand, liposomes are more flexible and, depending on the composition, vesicles as large as 400 nm can penetrate the capillary endothelium of the liver and reach the hepatocytes [10, 11]. Colloidal carriers can extravasate into solid tumours and into inflamed or infected sites, where the capillary endothelium is defective. However, for 'conventional' colloidal carriers, the usual fate is opsonization by plasma proteins followed by uptake by phagocytic cells: either polymorphonuclear leukocytes in the blood or fixed macrophages, particularly the Kupffer cells in the liver [12–14]. Liposomes which penetrate the endothelial barrier can also be taken up by hepatocytes, and since these cells are more numerous than Kupffer cells, this can account for a considerable proportion of the dose [10]. Complement activation by the alternative pathway is an important component of carrier recognition and uptake [15], but opsonization by other plasma proteins, for example non-specific adsorption of IgG, also intervenes. Liposomes are also destabilized by interactions with circulating lipoproteins, especially high-density lipoproteins [15]. The stability of liposomes in plasma is determined by the phase transition temperature of the phospholipids and is increased when the bilayer is stabilized by cholesterol [12].

After uptake by phagocytic cells, the drug carrier systems will be localized in the acidic environment of endosomes and lysosomes and will be degraded by lysosomal enzymes. If the associated drug is not able to escape from this compartment, it may also be degraded and will not reach its site of action unless this is within the lysosomes and the drug is stable in this environment.

Oral administration

The most convenient route of drug administration is the oral one. However, this route presents a number of barriers to the use of colloidal carriers, since conditions within the gastrointestinal tract can disrupt many of them. The concerted action of duodenal enzymes and bile salts destroys the lipid bilayers of most types of liposome, thus releasing the drug [16]. MLVs prepared from phospholipids with phase transition temperatures above 37 °C and which contain cholesterol in their bilayers are the most resistant to degradation. Polymeric nanoparticles are more stable, although there is some evidence that polyesters can be degraded by pancreatic lipases [17].

Even if the carrier is stable, anatomical considerations mean that only a small proportion of the administered drug carrier systems can be absorbed intact across the intestinal mucosa into the circulation or the lymphatics. Passage across enterocytes by diffusion is restricted to small, lipophilic molecules, and transcytosis, which is rare, to particles less than 200 nm in diameter. Passage by the paracellular pathway is impossible if the tight junctions are intact. Nevertheless, a number of studies have reported the appearance of particles in the circulation after oral dosing [reviewed in ref. 18]. The current consensus is that uptake occurs via Peyer's patches, which are specialized areas of the gut-associated immune system. Transcytosis of particles occurs across M cells, delivering them to the underlying lymphoid follicule. This antigen-sampling mechanism is important in developing a mucosal, and sometimes also a systemic immune response [19], but is probably not an important therapeutic route. Most particles are phagocytosed by antigen-presenting cells, although some nanoparticles may find their way into the portal circulation and hence to the liver [20].

Polymeric nanoparticles also show bioadhesive properties, that is, they may be immobilized within the mucus or in contact with the epithelial cells and hence undergo a slower clearance from the gastrointestinal tract than material remaining in the lumen [21]. This bioadhesion can be increased by specific targeting [22, 23].

Other routes of administration

When colloidal drug carriers are administered by other routes, e.g., subcutaneously or by intramuscular injection or topical application, they are generally retained at the

site of administration longer than free drug. When a liposome-associated drug is applied to the skin, the amount penetrating into the superficial layers may be increased compared with free drug, while its passage to the systemic circulation may be reduced [24]. After subcutaneous or intraperitoneal administration, small liposomes and nanoparticles are taken up by regional lymph nodes [25–27]. Retention of carriers instilled into the eye also occurs, leading to important therapeutic potential in this area. As well as a bioadhesive effect, some evidence has been presented to show that nanoparticles can penetrate through the corneal epithelium [28].

Therapeutic potential of colloidal drug carriers

The therapeutic potential of drug carrier systems is influenced by their distribution, as described above. This section describes the possible spheres of application of 'conventional' liposomes and nanoparticles by different routes of administration. Structural modifications designed to modify their distribution will be discussed in the following section.

Administration by the intravenous route

The potential applications of colloidal drug carriers by the intravenous route can be summarized as concentrating drugs in accessible sites, rerouting drugs away from sites of toxicity and increasing the circulation time of labile or rapidly eliminated drugs (e.g., peptides and proteins).

Delivery to macrophages

Since colloidal drug carriers are naturally concentrated within macrophages, using them to deliver drugs to these cells is logical. A good example is the delivery of muramyldipeptide (MDP) and chemically related compounds to stimulate the antimicrobial and antitumoral activity of macrophages. MDP is a low-molecular-weight, soluble, synthetic compound based on the structure of peptidoglycan from mycobacteria, and, although it acts on intracellular receptors, it penetrates poorly into macrophages. Furthermore, it is eliminated rapidly after intravenous administration. These problems can be overcome by encapsulation within liposomes or nanocapsules. Early work using soluble MDP within liposomes showed activity against pulmonary [29] and liver [30] metastases in mice. More recently, lipophilic derivatives such as muramyltripeptide-cholesterol [31] or muramyltripeptide-phosphatidylethanolamine [32] have been developed to increase the efficiency of encapsulation. In vitro studies have shown increased intracellular penetration of muramyl peptides into macrophages when these compounds are associated with colloidal particles, leading to an increase in macrophage effector functions (e.g., nitric oxide, cytokine, and prostaglandin production) and increased cytostatic activity against tumor cells. Activity against hepatic metastases has been observed in a number of mouse models; however, this treatment is only curative when the tumor burden is low [31, 32]. Liposomes containing muramyltripeptide-phosphatidylethanolamine have also been shown to be effective against bacterial infections, for example, by *Klebsiella pneumoniae* [33].

Liposomes or nanoparticles can be used to concentrate antibiotics at the site of infection for direct treatment of bacterial and parasitic infections, particularly when the microorganism is within the lysosomes [reviewed in ref. 34]. For example, nanoparticles containing ampicillin were more effective than the free drug against both *Salmonella tymphimurium* and *Listeria monocytogenes*. Co-localization of the particles and bacteria was seen in *Salmonella*-infected macrophages in vitro [34]. A liposomal formulation of amikacin (MiKasome) is currently in clinical trials against complicated bacterial infections and is reputed to be better tolerated than the free antibiotic.

The potential of liposomes as immunological adjuvants was recognized as early as 1974. In the case of protein antigens, encapsulation increases capture by antigen-presenting cells such as macrophages [35]. In an alternative strategy, immunogenic peptides have been coupled to the surface in order to activate B and T cell clones directly [36]. Liposomes have also been used as carriers in DNA vaccines [37].

Reduction of toxicity

Colloidal carriers can also be used to divert drugs from sites of toxicity after intravenous administration. For example, the anticancer drug doxorubicin (adriamycin) is active against a wide spectrum of tumors, but provokes dose-limiting cardiotoxicity. Encapsulation within liposomes or nanoparticles reduces this toxicity, by reducing the amount of drug which reaches the myocardium [38, 39].

A corollary is that concentrations of doxorubicin in the liver increase considerably. In one study in mice, this was not associated with any overall toxicity [39]. However, another group reported a temporary depletion of Kupffer cells, and hence the ability to clear bacteria, in rats, which was less marked when long-circulating liposomes (see below) were used [40]. A systematic study using unloaded nanoparticles confirmed a reversible decline in the phagocytic capacity of the liver after prolonging dosing, as well as a slight inflammatory response [41]. Thus, altered distribution may generate new types of toxicity and this must be borne in mind when developing carrier systems.

Accumulation of carrier-associated drug in the liver may also influence its elimination, since this organ is the site of metabolism and biliary excretion. The biliary clearance of indomethacin was increased threefold by inclusion in

nanocapsules [42]. Nanoparticle-associated doxorubicin also accumulates in bone marrow, and led to a myelosuppressive effect in one study [43]. However, this tropism of carriers can also be used to deliver myelostimulating compounds such as granulocyte-colony-stimulating factor [44].

'Indirect' targeting of doxorubicin

The efficac of doxorubicin in mice was considerably greater following treatment with doxorubicin-loaded poly(alkylcyanoacrylate) nanoparticles compared with free doxorubicin [45]. Tissue pharmacokinetic studies showed that the particles were initially concentrated within Kupffer cells, from which the drug was progressively released, reaching the tumor cells in the hepatic parenchyma [46]. This can be considered as 'indirect targeting' to an accessible site which acts as a reservoir [46].

Interestingly, the association of doxorubicin with poly(alkylcyanoacrylate) nanoparticles also reversed the resistance to doxorubicin in a large number of multidrug-resistant cell lines [47, 48]. The nanoparticle-associated drug accumulated within the cells and appeared to avoid P-glycoprotein-dependent efflux. This reversal was only observed with poly(alkylcyanoacrylate) nanoparticles and was not due to particle endocytosis. Rather, the formation of a complex between positively charged doxorubicin and negatively charged polymer degradation products seemed to favor diffusion across the plasma membrane.

Liposome-based commercial formulations of doxorubicin and daunorubicin are already available.

Lipid formulations of amphotericin B

Amphotericin B (AmB), an antifungal drug used in deep-seated systemic disease, is another drug with specific dose-limiting side effects, in this case to the kidneys. In 1984, association with colloidal lipid systems was noted to increase the maximum tolerated dose of AmB and thereby enhance activity [reviewed in ref. 49]. Many different colloidal delivery systems for AmB have been developed since then: emulsions, nanoparticles, liposomes, and lipid complexes, some of which are now on the market. Reduced toxicity of these systems is probably the result of both an altered distribution and the physicochemical state of the AmB in the carrier systems. Lipid or polymer association maintains AmB in its monomeric form which is less disruptive to mammalian cell membranes than the self-associated form, while maintaining its antifungal activity toward fungal cell walls [49]. Association of AmB with lipid carriers also reduces its transfer to low-density lipoprotein, thereby lowering its renal toxicity [50]. Colloidal preparations of AmB also have an increased therapeutic index against *Leishmania* infections [51]; this is an intracellular infection and the use of carriers increases the concentration of drug within macrophages. On the other hand, lipid association also reduces some immunostimulating effects of AmB (such as nitric oxide and tumor necrosis factor-α production) compared with free AmB at the same dose, which may contribute to the reduced toxicity [52]. However, the different lipid carrier systems for AmB are not equivalent: their behavior depends on the size and composition of the particles and on the AmB:lipid ratio.

Prolongation of circulating half-life

Although 'conventional' colloidal carriers are rapidly cleared from the circulation, they are still capable of increasing the plasma half-lives of some labile or rapidly eliminated molecules. These could be proteins, peptides, or small molecules. For example, the circulating half-lives of cytokines such as gamma-interferon [53] and interleukin-2 (IL-2) [54] are prolonged by encapsulation within liposomes. As an example of a small molecule, the encapsulation of ATP in liposomes increases its circulating half-life, allowing it to protect rats against repeated episodes of cerebral ischemia [55].

Administration by the oral route

Colloidal drug carriers can be used to protect a labile drug from degradation in the gastrointestinal tract or to protect it from toxicity due to the drug. They may be able to improve bioavailability, particularly for highly insoluble drugs, by increasing the surface area for dissolution, and as a result of bioadhesion. Finally, particulate carriers can be used to deliver antigens to Peyer's patches for oral immunization.

Protection of active compounds

Colloidal systems have been shown to protect insulin from enzymatic degradation in the gastrointestinal tract. In the 1970s and '80s, many studies with insulin encapsulated in liposomes gave controversial results, which may have been due to differences in liposome composition, affecting their stability in digestive fluids [reviewed in ref. 18]. Later studies involved insulin encapsulated in poly(alkylcyanoacrylate) nanocapsules. Although these formulations were ineffective in reducing glycemia in normal rats, they were effective in diabetic rats and in normal rats loaded with glucose [56]. A 2-day lag was observed between administration and the fall in glucose levels, and the duration of the hypoglycemia (up to 20 days), but not its intensity, was dose dependent. These nanocapsules were shown to protect insulin from degradation by digestive enzymes in vitro [57]. Furthermore, microscopic evidence was obtained for the passage of intact nanocapsules across the intestinal mucosa [58]. The sustained effect was possibly due to the sustained release of the encapsulated insulin from an as yet unidentified site. Another explanation might be a local effect of insulin on intestinal cells. En-

capsulation within nanocapsules also improved and prolonged the therapeutic effect of a somatostatin analogue given by the oral route [59].
The bioadhesive properties of nanoparticles could potentially be used to improve the absorption of poorly water soluble drugs. Incorporation within submicronic carriers can increase the surface area and thereby facilitate dissolution in gastrointestinal fluids, while bioadhesion can increase the residence time in the gastrointestinal tract [60, 61]. Targeting of particles to specific regions of the mucosa has also been envisaged [21].

Reduction of gastrointestinal toxicity
Nanocapsules have been shown to be effective in protecting the gastrointestinal mucosa of rats from the ulcerating effects of non-steroidal antiinflammatory drugs after oral administration. Two major non-steroidal antiinflammatory agents, diclofenac [62] and indomethacin [63], have been encapsulated in poly(lactic acid) nanocapsules with the aim of reducing their side-effects on the gastric mucosa. Their pharmaceutical activity after oral administration does not seem to be influenced by the encapsulation of the drug in nanocapsules. In fact, nanocapsules containing these two drugs exhibited drug concentration-time profiles in the plasma of rats similar to those obtained with the corresponding aqueous solutions. These observations seemed to be independent of the nature of the polymer shell, whether poly(isobutylcyanoacrylate) produced by interfacial polymerization or preformed poly(lactic acid) [64]. In contrast, the gastrointestinal side-effects of both drugs were dramatically reduced by encapsulation in nanocapsules [62, 63], compared with the ulcerative effect on the mucosa observed with the drug solutions. Fawaz et al. [65] also observed reduced rectal irritability of indomethacin after administration of nanocapsules by the rectal route. These results suggested that mucosal side-effects after oral administration were of local rather than systemic origin, since intravenous administration of nanocapsules did not reduce the gastrointestinal side-effects. This protection could be attributed to a slow release of the drugs in the acidic gastric environment or to the reduced toxicity of the acidic form of diclofenac or indomethacin encapsulated in nanocapsules compared with the sodium salt in the aqueous solution [62].

Oral immunization
A number of groups are using particulate systems to elicit mucosal immune responses, for example, to increase resistance to microbial infection by this route [66]. This subject area is vast and merits a separate review. In general, the particles which are most readily captured by M cells are 5–10 μm in diameter (and are therefore not really colloidal systems) and hydrophobic in nature [19]. Chitosan nanoparticles have been used for DNA vaccination by the oral route [67].

Administration by other routes
Colloidal drug carrier systems have been used to concentrate interferon-γ in the skin for the treatment of cutaneous herpes. The cytokine accumulated in the stratum corneum, rather than remaining on the surface as occurred after administration of a simple solution [68].
Application of carrier formulations to the eye retards elimination of drug from the corneal surface. This has been demonstrated for β-blockers [69] and cyclosporin A [70] within nanospheres and nanocapsules. Nasal absorption of insulin has been improved by its encapsulation in chitosan nanoparticles [71].
Subcutaneous [72] or intraperitoneal [73] administration of anticancer agents in liposomes has been shown to deliver the drug to lymphatic metastases. The biotin-avidin system has been used recently to aggregate liposomes within lymph nodes and thus increase targeting [74]. The accumulation of nanospheres and nanocapsules in lymph nodes has also been described [26, 27]. The use of nanocapsules also has been shown to reduce drug-related irritation, for example, after administration by the intramuscular route [75].
Recently, nanocapsules with an aqueous core have been developed for the encapsulation of antisense oligonucleotides. These carriers effectively protect the oligonucleotide from degradation in biological fluids and when loaded with phosphorothioate oligonucleotides directed against EWS Fli-1 chimeric RNA have allowed a true antisense effect against the experimental Ewing sarcoma in mice after intratumoral administration [76].

Active carriers
As far as so-called 'active' carriers are concerned, a comprehensive account is outside the scope of this review. Recently, thermosensitive liposomes have been described which are able to accumulate in tumors because of their long-circulating properties (see below) and to release doxorubicin in response to local heating [77].

Drug carriers with modified distribution

Despite encouraging results with these 'conventional' carrier systems, much research has been devoted to designing carriers with modified distribution and new therapeutic applications. One major axis is the development of sterically stabilized, or 'Stealth™', carriers which undergo greatly reduced opsonization and uptake by the mononuclear phagocyte system and therefore open up new perspectives for applications by the intravenous route. Second, since internalization of colloidal carriers usually leads to the lysosomal compartment, modifying the intracellular distribution of the carrier may be necessary. This is particularly true when the encapsulated drug is a nucleic

acid. Delivery systems are necessary for this type of molecule because they are susceptible to nuclease-mediated degradation in the circulation and penetrate poorly through membranes. However, they are also susceptible to nuclease attack within the lysosomes and their site of action is either in the cytoplasm in the case of an antisense strategy or in the nucleus in the case of gene replacement or antigene therapy. Thus, systems have been developed which either fuse with the plasma membrane or have a pH-sensitive configuration which changes conformation in the lysosomes and allows the encapsulated material to excape into the cytoplasm. Finally, the ultimate goal would be to be able to direct the drug carrier system to a specific cell type; that is, to develop a third-generation carrier.

Drug carriers with reduced uptake by phagocytic cells

These carriers have been termed 'Stealth™' particles because they are 'invisible' to macrophages.

Liposomes

Early work with liposomes defined some factors which led to increased persistence in the circulation: small size, inclusion of cholesterol and/or phospholipids with a high phase transition temperature, and use of some negatively charged lipids such as the ganglioside GM_1 [78]. However, a major breakthrough in the liposome field consisted in the use of phospholipids grafted with poly(ethylene glycol) (PEG) chains of molecular weight from 1 to 5 kDa [79]. This provides a 'cloud' of hydrophilic chains at the particle surface which repels plasma proteins, as discussed theoretically by Jeon et al. [80]. These 'sterically stabilised' liposomes have circulating half-lives of about 20 h in rodents and up to 45 h in humans, as opposed to a few hours or even minutes observed for conventional liposomes in rodent models. Their ultimate fate is, however, the same as that of conventional liposomes and the majority will eventually be taken up by the liver and spleen. They have been shown to function as reservoir systems and can penetrate into sites such as solid tumors [81–83].

Nanospheres

A similar strategy has been applied to matrix-structured nanospheres. PEG has been introduced at the surface in two ways, either by adsorption of surfactants [84] or by using block or branched co-polymers, usually with poly(lactide) (PLA) [85, 86]. The latter strategy is preferable because it avoids the possibility of PEG desorption on dilution or after contact with blood components.

As far as the adsorption of hydrophilic surfactants onto the particle surface is concerned, Illum et al. [84] studied the use of surfactants with polyoxyethylene blocks, such as the poloxamer and poloxamine series, onto polystyrene latex surfaces. In particular, they found that coating with poloxamer 407 reduced uptake by Küppfer cells but promoted uptake by the bone marrow [87]. Moghimi and coworkers [88] found that poloxamine 908 reduced liver uptake of polystyrene particles, which they interpreted as being due to reduced adsorption of opsonins and increased adsorption of dysopsonins. This surfactant, even when not associated with particles, also activated phagocytic cells so that a second dose some days later was cleared rapidly by the liver. However, with the more hydrophilic biodegradable polymeric surfaces such as poly(lactide-co-glycolide) (PLGA), reversible adsorption was observed in vivo [89]. On the other hand, biodegradable nanospheres prepared from PLGA coated with PLA-PEG diblock co-polymers showed a significant increase in blood circulation time and reduced liver uptake in a rat model, compared with naked PLGA nanospheres [90]. Although interesting results have been obtained with adsorbed surfactant, e.g., concentration of phthalocyanines in tumors using nanocapsules coated with poloxamer 407 [91], covalently linked co-polymers would seem to be a better choice since they are less easily desorbed from the surface.

The surface characteristics (length and density of PEG chains) of nanospheres prepared from PLA-PEG co-polymers have been optimized to reduce their interactions with plasma proteins and to increase their circulating half-life [85, 86]. The average distance between two terminally attached chains had to be 2.2 nm or less for protein repulsion [85] and although PEG of 5 kDa was more effective than PEG of 2 kDa, increasing the chain length further did not confer any additional advantage at optimal surface density. Lipophilic drugs, such as lidocaine and cyclosporin A, and proteins, have been encapsulated and are released in a controlled manner [92]. PEG chains have also been attached covalently to poly(alkylcyanoacrylate) polymers by two different chemical strategies, and both types of particle have shown long-circulating properties in vivo [93]. This type of particle has been loaded with tamoxifen, with a view to its use in the treatment of hormone-dependent tumors [94].

Nanocapsules

Recently, we have applied the same approaches to the reservoir-type polymer-based drug carrier nanocapsules with the aim of creating long-circulating systems with a high loading capacity for lipophilic drugs. By analogy with the work on nanospheres described above, we used PLA-PEG diblock co-polymers to form the wall around the oily core, We compared these particles with 'conventional' nanocapsules stabilized by Poloxamer 188, which could be considered surfactant-coated particles. We used co-polymers containing two lengths of PEG chain: 5 and 20 kDa. By blending co-polymers and PLA homopolymer we obtained different PEG contents, but the most physically stable nanocapsules were those prepared with PLA-

PEG co-polymer alone. In contrast to observations with nanospheres, the negative zeta potential of the nanocapsules was not completely masked by the presence of PEG on their surface. This was due to the presence of lecithin, which remained necessary for nanocapsule stability. Nevertheless, studies of nanocapsule interactions with the complement system show that their complement-rejecting properties are superior to those of nanospheres at equivalent surface area [95]. Furthermore, nanocapsules containing PLA-PEG showed reduced association with a macrophage-like cell line (J774 A1) whatever the dilution and irrespective of incubation time up to 24 h. Both PEG chain length and density were found to be important in reducing interactions with these phagocytic cells [96]. On the other hand, nanocapsules stabilized with Poloxamer 188 showed some phagocytosis-retarding properties at low dilution, but these were lost at higher dilution.

The pharmacokinetics and distribution of these nanocapsules were evaluated after intravenous administration to mice, using [^{3}H]-PLA as a tracer. The formulation which showed the lowest capture by macrophages in vitro also gave good results in vivo, yielding a plasma area under the curve 15 times higher than that obtained with nanocapsules stabilized by adsorbed Poloxamer F68. Persistence in the blood compartment was mirrored by delayed liver and spleen uptake [97].

Biomimetic systems

Another strategy for preparing long-circulating colloidal systems can be considered as biomimetic in that it seeks to imitate cells or pathogens which avoid phagocytosis by reducing or inhibiting complement activation. One example is the development of liposomes with a membrane composition similar to that of erythrocytes, e.g., liposomes containing GM_1 [78] and those coated with polysialic acids [98]. These systems may show circulating half-lives as long as liposomes bearing PEG. Another polysaccharide which can be used to modify the surface of nanoparticles and provide a biomimetic effect is heparin, the anionic polysaccharidic anticoagulant, because it can inhibit several steps of the complement cascade. Heparin was introduced as the hydrophilic part of amphiphilic diblock co-polymers capable of forming nanospheres. In the first studies, the hydrophobic segment was poly(methylmethacrylate) (PMMA), a non-biodegradable polymer which was, however, suitable for validating the concept [99]. These systems indeed showed reduced complement activation, as shown by two-dimensional immunoelectrophoresis of C3 [100]. Using a fluorescent marker randomly co-polymerized in the PMMA segment, reduced uptake by macrophages in vitro [101] and an increased half-life in vivo (5 h or more depending on the injected dose, compared with a few minutes for PMMA nanospheres) for the heparin-bearing systems could be demonstrated [102]. Of note is that nanospheres prepared from a diblock co-polymer with dextran, intended as controls, also showed long-circulating properties; thus a steric hindrance effect caused by a brush-like arrangement of end-attached polysaccharide chains may also contribute to the long-circulating properties of these nanospheres. Recently, similar nanoparticles have been developed using a biodegradable poly(alkylcyanoacrylate) as the hydrophobic block [103]. Depending on whether the polymerization is anionic or radical mediated, branched or linear co-polymers are formed. The encapsulation of drugs within these nanoparticles has not yet been studied.

Applications of long-circulating drug carriers

Such long-circulating drug carrier systems can be used as circulating reservoirs of drug and to deliver drugs to intravascular targets. They can also pass through the vascular endothelium in some circumstances, one of the most important being the case of solid tumors. The rapidly expanding tumor vasculature often has a discontinuous endothelium with gaps between the cells which may be as large as several hundred nanometers [104]. This, combined with the fact that tumors often lack effective lymphatic drainage, means that particulate matter can be trapped within this zone by the so-called 'enhanced permeability and retention' effect. Other pathological situations in which the vascular endothelium is more permeable are infection and inflammation, when the endothelial cells have been activated by chemokines and cytokines to allow extravasation of leukocytes, and hypoxic areas after myocardial infarction.

Prolongation of circulating half-life

Prolongation of the circulating half-life by encapsulation in liposomes has been shown for both a small peptide (vasopressin [105]) and for a cytokine (IL-2 [54]). In the first case, the antidiuretic activity of the peptide administered in PEG-containing liposomes appeared after a 2-day lag period and was prolonged for 4 days. This was interpreted as sustained release from liposomes taken up by a compartment other than phagocytic cells [105]. In the study of IL-2-loaded liposomes, the biological activity of the cytokine was evaluated at the same time as its circulation time. Although long-circulating liposomes increased the plasma half-life of the protein more than conventional liposomes, the latter were more effective in increasing leukocyte levels in the blood and in providing adjuvant activity for sub-unit vaccines [54]. This illustrates that the circulation time of the drug carrier is not the only important factor; its ability to interact with the target cells and to deliver the encapsulated material is also important.

Nanoparticles prepared from PLGA PEG co-polymers have been shown to increase the circulating half-life of cisplatin [106].

Treatment of intravascular disease

To test their efficacy in treating an intravascular disease, nanocapsules prepared from PLA-PEG co-polymers have been used to encapsulate an antimalarial drug, halofantrine, with the aim of obtaining a well-tolerated injectable form for the treatment of severe disease. In mice at an advanced stage of infection with *Plasmodium berghei*, the area under the curve for plasma halofantrine was increased sixfold when the drug was presented in nanocapsules, whether they contained PLA-PEG or were simple PLA stabilized by Poloxamer 188, compared with a solubilized form of the drug. The toxicity of halofantrine was reduced by incorporation into nanocapsules; up to 100 mg/kg could be administered intravenously without problem, whereas all mice given this dose of free halofantrine died instantly. Both nanocapsule formulations increased the therapeutic efficacy of halofantrine: the 'conventional' nanocapsules showed a more rapid onset of effect, whereas that of the PLA-PEG nanocapsules was more sustained [107].

Another intravascular application of long-circulating drug carriers would be the treatment of leukemia. This has been demonstrated by Lopes de Menezes et al. with a human B cell lymphoma in nude mice [108].

Treatment of solid tumors

The main application to date for long-circulating liposomes has undoubtedly been the treatment of solid tumors, because of the enhanced permeation and retention effect mentioned above. Cytosine arabinoside, vincristine, epirubicin and doxorubicin are among the drugs which have been formulated in this way [81, 82]. A doxorubicin-containing formulation based on 'Stealth™' liposomes, Doxil™, is commercially available for use in AIDS-related Kaposi's sarcoma.

Long-circulating nanoparticles and nanocapsules can also be used to deliver lipophilic drugs to solid tumors. This has been demonstrated using photosensitizers: phthalocyanines in PEG-coated nanoparticles [91] and meta-tetra(hydroxyphenyl)chlorin in PLA-PEG nanocapsules [109].

Applications in inflammation and infection. As well as accumulating in solid tumors, long-circulating liposomes can extravasate into sites of inflammation and infection [110]. This provides possibilities for delivering antibiotic and antiinflammatory agents to these sites [111], as well as the possibility of imaging using liposomes labeled with gamma emitters [112].

Extravascular applications. Long-circulating liposomes may be useful for prolonging the residence time of drugs administered by the subcutaneous [113] and intraocular routes [114]. Nanoparticles prepared from PLA-PEG are stable in digestive fluids and can protect an encapsulated antigen [115]. They therefore have potential as protein carriers by the oral route. Antigen delivery by the nasal route has also been demonstrated with this same carrier system [116].

Carriers avoiding the lysosomal compartment

It may be sufficient for a carrier system to concentrate the drug in the tissue of interest. However, in the case of hydrophilic molecules which cross the plasma membrane with difficulty (e.g., nucleic acids [117]), intracellular delivery is required. If the carrier is taken up by endocytosis, its ultimate destination will be the lysosomes, in which hydrolytic enzymes will degrade both the carrier and its contents. Therefore, a number of liposome-based systems have been developed to avoid the lysosomal compartment either by fusing directly with the plasma membrane with the help of fusogenic proteins or peptides [118, 119] or by destabilizing the endosome. Endosome disruption can be achieved using pH-sensitive liposomes, in which the lipid undergo a phase change at acid pH [120], or by cationic liposomes [121]. In this way, the encapsulated material can be delivered to the cytoplasm. These systems are particularly appropriate for the delivery of genes and antisense oligonucleotides, as a non-immunogenic alternative to viral vectors. For example, an antisense oligonucleotide against the Friend leukemia virus was found to have a better antiviral effect in vitro when encapsulated in pH-sensitive rather than conventional liposomes [120]. The use of lipid-based systems has been reviewed recently by Liu and Huang [122].

A somewhat analogous system involving cationic polymers is the so-called 'proton sponge' approach. Polyethylenimine (PEI) is a polymer with a high amino group content. About 20% of these are protonated at pH 7, allowing DNA complexation; while about 45% are protonated at pH 5, the lysosomal pH. The flow of protons into the lysosome causes swelling and rupture, allowing the PEI/DNA particles to escape, making this polymer a versatile reagent for gene transfer [123, 124]. Increased specificity can be achieved by incorporating targeting agents such as mannose [125] or folate [126]. PEG has been used to shield positive charges and thus improve the pharmacokinetic properties of the complexes [127].

Systems targeted to specific cell populations

As stated above, an ideal drug carrier system would contain a specific 'homing group' capable of being recognized by the target cells. Much work has been devoted to coupling specific ligands to the surface of liposomes. Monoclonal antibodies or fragments thereof have often been used because of their specificity [reviewed in ref. 128]. Other targeting systems which have been investi-

gated are sugar-lectin interactions, e.g., the mannose/fucose receptor of macrophages and the galactose receptor of hepatocytes, hormone and growth factor receptors, and receptors for cell nutrients such as transferrin and folic acid, which are over-expressed in some tumors. Impressive results have been obtained in vitro [128] but, with the exception of targeting to the liver, these have often not been confirmed in vivo, since the use of specific ligands cannot overcome physiological constraints. First, even targeted systems will be recognized by macrophages if their surfaces are not modified to reduce opsonization. On the other hand, PEG chains can mask a ligand attached directly to the liposome surface. This has been overcome by attaching the targeting group to the end of the PEG chain and, ideally, using a longer PEG chain to carry the ligand than the PEG chains used to confer 'Stealth™' properties [129]. The degree of substitution has to be limited to avoid recreating a surface on which opsonization can occur easily [130]. Second, the permeability of the vascular endothelium still has to be taken into account. Third, if intracellular delivery is required, the targeting ligand must not simply be bound to the surface, but also internalized after binding, and the carrier system must be small enough to be taken up by receptor-mediated endocytosis in non-phagocytic cells, that is, 200 nm or less [128]. Furthermore, if this internalization occurs, it will lead to the lysosomal compartment unless an endosome-disrupting element is present.

In the light of such constraints and considerations, the most suitable targets for drug carriers would seem to be cells in accessible sites such as liver metastases, circulating cells (e.g., leukemia) and cells in sites where the endothelium is leaky (tumors, inflammation, infection, including within the blood-brain barrier). Another strategy is to target the carrier to a particular region of capillary endothelium, to concentrate the drug within a particular organ and allow it to diffuse from the carrier to the target tissue. A refinement of this approach is to choose a receptor which mediates transcytosis across the endothelium.

Targeting to macrophages and liver cells

Although 'conventional' carriers are naturally captured by phagocytic cells, the extent and rate of capture can be increased by the presence of a ligand for a receptor expressed by these cells. The receptor most often exploited for this purpose is the mannose/fucose receptor of macrophages [for a review see ref. 131]. For example, an immunomodulator can be delivered efficiently in mannose-grafted liposomes to stimulate the antitumoral properties of macrophages [132]. More recently, this strategy has been applied to the delivery of AmB to macrophages [133]. Another targeting ligand which has been used in a similar application is the tetrapeptide tuftsin (Thr-Lys-Pro-Arg) [134]. This peptide has the advantage of being both a targeting element and a macrophage activator. The antileishmanial activity of the drug is thus reinforced by macrophage-mediated effects.

Another type of carbohydrate receptor which has been employed for targeting to the liver is one recognizing galactose. Proteins able to bind this sugar are expressed on both macrophages and hepatocytes. Although targeting to hepatocytes can be achieved in vitro [135], capture by Kuppfer cells seems to predominate over that of hepatocytes [136]. Nevertheless, liposomes coated with 1-amino-lactose have been proposed for targeting to hepatoma cells [137]. On the other hand, apolipoprotein E, which mediates lipoprotein uptake by hepatocytes, has been shown to give some specificity [138].

Targeting to tumor cells

For the treatment of leukemia, effective targeting of sterically stabilised liposomes containing doxorubicin and bearing anti-CD19 to malignant B cells has been observed in vitro and in vivo in mice [108]. The same liposome system has also been used to target circulating myeloma cells, in order to prevent relapse after bone marrow transplant [139]. Another cell-surface receptor which has been targeted by such 'immunoliposomes' is the her2 (ErbB2) antigen [140]. In this case, a phospholipid bearing a PEG chain terminated by an anti-her2 antibody fragment was inserted into preformed commercially available doxorubicin-loaded liposomes (Doxil™). Binding to this receptor is followed by internalization and in this way the drug concentrations can be dramatically increased in her2-over-expressing tumor cells.

However, antibody-targeted systems do not always show an advantage over simple sterically stabilized liposomes in solid tumors, as observed by Allen and Moase [141] in a human ovarian tumor growing in nude mice, probably because the larger antibody-decorated particles diffuse less easily within the tumors, where the hydrostatic pressure is increased due to the lack of lymphatic drainage [104]. Furthermore, such immunoliposomes can in some cases be opsonized and cleared rapidly from the circulation [128] and may even give rise to immunological and pseudoallergic reactions [143, 144]. An approach which avoids coupling a large protein to the carrier surface is a two- or three-step procedure using bispecific antibodies, which combine two immunological recognition functions in the same molecule. The antibody can therefore act as a bridge between the target cell and a carrier bearing a low-molecular-weight ligand. For example, in the protocol described by Cao and Suresh [145], a bispecific antibody was engineered to recognize a tumor-specific antigen and biotin. This was given intravenously and after allowing time for distribution, conventional, multilamellar liposomes bearing biotin were given. These bound excess antibody in the circulation and carried it to the liver, but could not reach antibody already bound to tumor cells. A few hours later, small liposomes bearing PEG chains and

biotin were administered; these could circulate, extravasate and bind to the antibody present on the surface of the tumor cells. This approach can concentrate the carrier within the tumor, but will not necessarily promote internalization, since for the procedure to function, the bispecific antibody must remain at the cell surface. However, this may be sufficient in many cases, for example, if the carrier is loaded with a radioisotope to provide local irradiation.

Targeting to the transferrin receptor

Many proteins and peptides other than antibodies have been used to target carriers to specific cell types. The transferrin receptor system has been widely studied because it is over-expressed on many tumors. Thus, long-circulating liposomes bearing transferrin have been shown to be taken up by receptor-mediated endocytosis in Colon 26 cells [146] and to deliver doxorubicin to C6 glioma cells in vitro [147]. However, although transferrin-bearing PEG-liposomes accumulated more in HeLa cells than in non-targeted ones, they were less efficient at delivering a photosensitiser, hypericin, because the drug leaked from the liposomes [148].

Targeting with low-molecular-weight ligands

Another receptor which is over-expressed on many tumor cells is the folate receptor. Folic acid has some advantages over transferrin or antibodies as a ligand for long-circulating carriers because it is a much smaller molecule which is unlikely to interact with opsonins and can be coupled easily to a PEG chain without loss of receptor-binding activity. PEG-liposomes bearing folic acid are effective carriers for intracellular delivery of nucleic acids and anticancer drugs to tumor cells in vitro [149]. Folate has also been coupled to cationic liposomes to deliver a plasmid coding for the tumor suppressor p53, in order to sensitize the cells to chemotherapy and radiotherapy [150]. This targeting strategy has also been applied to long-circulating nanoparticles prepared from a cyanoacrylate-based polymer [151]. Conjugation of folic acid to the distal end of the PEG chain does not affect its ability to bind to its receptor, as determined by surface plasmon resonance.

Another relatively small molecule which has been used to target long-circulating liposomes is antagonist G, a hexapeptide analogue of the neurotransmitter substance P, which blocks the action of several neuropeptides by binding to their receptors. This ligand promotes liposome binding to and internalization by a human small-cell lung cancer cell line, H69, and improves the nuclear delivery of encapsulated doxorubicin [152].

Targeting to endothelium

An example of targeting to endothelium is the use of sterically stabilized liposomes containing AmB bearing an antibody specific for pulmonary endothelium at the end of the PEG chains [153]. Accumulation of antibiotic in the lungs was observed, as opposed to its remaining in the blood in the case of non-targeted PEG-bearing liposomes or accumulating in the liver in the case of conventional liposomes. This was accompanied by increased efficacy against experimental aspergillosis in mice.

The adhesion molecules selectively expressed on endothelial cells during infection or inflammation, which promote leukocyte arrest and extravasation, can be considered as useful targets for drug carriers. Immunoliposomes directed against ICAM-1 are bound and internalized by interferon-γ activated bronchial epithelial cells [154]. Both liposomes [155] and nanospheres [156] have been targeted to selectins on activated endothelial by means of monoclonal antibodies.

Kamps et al. [157] have developed liposomes with a high specificity for hepatic endothelial cells by modifying their surface with anionized human serum albumin. The majority of the available free amino groups of the protein were derivatized with cis-aconitic anhydride before coupling to conventional liposomes of about 100 nm in diameter. The high uptake by endothelial cells was attributed to their high level of expression of scavenger receptors.

Carbohydrate-coated systems such as those described above under the heading of biomimetic systems might also be useful for targeting to endothelial cells. Heparin-binding proteins have been identified on endothelial cells [158] and liposomes coated with modified dextrans interact with human endothelial cells [159] and with vascular smooth muscle cells [160]. However, Lestini et al. [161] saw no targeting advantage with oligodextrans expressed on liposomes. The same authors described liposomes conjugated with a short peptide (RGD, Arg-Gly-Asp) directed toward the GPIIb-IIIa integrin expressed on activated platelets. This should target the liposomes to regions of damaged endothelium [161]. A similar concept has been developed by the group of Torchilin, in which liposomes bearing antibodies to the cytoskeleton protein myosin accumulate in damaged regions of the myocardium, where this protein is exposed. The presence of the liposomes is sufficient to 'plug' the lesions, and protection of cardiocytes from hypoxic injury has been demonstrated in vitro [162].

The vascular endothelium of tumors would be another valid target for drug carrier systems. Benzinger et al. [163] have designed immunoliposomes directed towards an accessible domain (KDR) of the vascular endothelial growth factor receptor with the aim of delivering antiangiogenic drugs.

Targeting to the brain

The group of Pardridge have pioneered the concept of using receptor-mediated transcytosis to carry drug across the

blood-brain barrier [164]. They recently used sterically stabilized liposomes with 1% of the PEG chains modified with a monoclonal antibody against the transferrin receptor to introduce a plasmid encoding the β-galactosidase gene into the brain. Widespread expression of the transgene in the brain, but also in peripheral tissues such as liver and spleen, was observed after intravenous injection [165].

Conclusion

Colloidal drug delivery vehicles have been studied in the laboratory for almost 30 years, but the few liposome-based formulations already on the market are mainly concerned with reducing the side-effects of the encapsulated drugs. Now that the interactions between particles and biological milieu are better understood, 'Stealth™' liposomes and nanoparticles which show diminished phagocytosis have been developed and the range of sites which can be reached has been extended. Even without specific targeting technologies, sites of inflammation and infection and solid tumors can be reached, as can intravascular sites. If specificity for a particular cell type is required, ligands such as monoclonal antibodies, sugars, lectins, or growth factors can be coupled to these long-circulating systems.
Colloidal drug carriers are particularly useful for formulating new drugs derived from biotechnology (peptides, proteins, genes, oligonucleotides) because they can provide protection from degradation in biological fluids and promote their penetration into cells. Carriers also provide an ultradispersed form of small hydrophobic molecules without the use of irritating solvents and allow rapid drug dissolution. The ability of carrier systems to retain a drug at the site of administration and their bioadhesive properties means that their use is not restricted to the intravenous route. Commercial products able to improve the efficacy of both established drugs and new molecules should, therefore, soon be available. As well as therapeutic systems, carriers loaded with contrast agents, ferrofluids, or radioisotopes could be useful diagnostic agents. Finally, specifically targeted systems could be appropriate tools for ex vivo cellular therapy.

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