Expert Opinion

Protein delivery: from conventional drug delivery carriers to polymeric nanoreactors

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Due to their low bioavailability, many naturally occurring proteins can not be used in their native form in diseases caused by insufficient amounts or inactive variants of those proteins. The strategy of delivering proteins to biological compartments using carriers represents the most promising approach to improve protein bioavailability. A large variety of systems have been developed to protect and deliver proteins, based on lipids, polymers or conjugates. Here we present the current progress of the carriers design criteria with the help of recent specific examples in the literature ranging from conventional liposomes to polymeric nanoreactors, with sizes from micrometer to nanometer scale, and having various morphologies. The design and optimisation of carriers in the dual way of addressing questions of a particular application and of keeping them very flexible and reliable for general applications represent an important step in protein delivery approaches, which influence considerably the therapeutic efficacy. We examine several options currently under exploration for creating suitable protein carriers, discuss their advantages and limitations that induced the need to develop alternative ways to deliver proteins to biological compartments. We consider that only tailored systems can serve to improve proteins bioavailability, and thus solve specific pathological situations. This can be accomplished by developing nanocarriers and nanoreactors based on biocompatible, biodegradable and non-toxic polymer systems, adapted sizes and surface properties, and multifunctionality to cope with the complexity of the *in-vivo* biological conditions.

Keywords: liposomes, nanoreactors, polymeric carriers, protein delivery

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1. Introduction

Most therapeutic proteins that are administered in the native form and without any protection are susceptible to biodegradation, have low membrane permeability, or can be rapidly eliminated from the blood circulation. As a result, these proteins need to be protected, typically by using drug delivery carriers, in order to improve their uptake at the desired biological site [1-3]. There are several known issues associated with protein-based drug therapeutic applications that should be strongly considered: short protein half-life *in vivo*; side effects caused by the multiple or high doses administered in order to reach the desirable concentration in the cell; and possible denaturation of the protein during manipulation. To address these problems, the carrier acts as a depot for high concentrations of therapeutics and provides a solubilising and protective environment, either for local delivery, where it is retained at the site of delivery, or for systemic delivery, where it circulates through the vasculature and should increase the circulation lifetime [4].

Although the criteria by which new delivery carriers are designed depend on the protein and intended route of administration, there are some general requirements

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Article highlights.

- Design and optimisation of carriers represent an important step in protein delivery approaches, influencing the therapeutic efficacy.
- Almost all carriers have been characterised in vitro, but only a few have been further tested in animals.
- Polymeric carriers are generally better suited for delivery than liposomes due to their higher stability, possibility to tune their properties, or to allow for multiple functionalization.
- Nanoreactors avoid the problems of release profile optimisation, or the release of therapeutics in inappropriate compartments compared with the conventional carriers.
- Efficient carriers and nanoreactors can be developed with biocompatible, biodegradable and non-toxic polymer systems, adapted sizes and surface properties, and multifunctionality properties.

This box summarises key points contained in the article.

to be fulfilled: i) a non-cytotoxic, biocompatible and biodegradable carrier material; ii) a preparation method that does not affect the protein activity; iii) sufficient robustness of the carrier–protein assembly to protect the protein from physical and chemical degradation; iv) a high loading efficiency and a controlled release profile; and v) ability of the carrier to be targeted to and/or retained at the desired site in the body [5]. There are various systems that have been proposed to protect and deliver proteins, based on lipids, polymers or conjugates. Ranging from micrometre to nanometre sizes, the delivery systems have various morphologies, such as micelles, vesicles, particles, tubes and gels, and represent different approaches to solving the complex situations of therapeutic protein applications [3,6-8].

To the authors' knowledge, delivery systems to accommodate proteins are not commercially available or widely used, but are actively under development. In this respect, this review focuses on the current progress of the design criteria and mechanistic considerations that make possible effective protein encapsulation and protection with the help of recent specific examples in the literature, even though these systems are in the early stages of development. The design and optimisation of carriers in the dual way of addressing questions of a particular application and of keeping them very flexible and reliable for general applications represent an important step in protein delivery approaches, which influence considerably the therapeutic efficacy. Therefore, in this review various carrier types are presented with the characteristics that make them suitable for protein delivery, and examples are given of model proteins, enzymes and vaccines that have been reported recently. In addition, the concept of nanoreactors for therapeutic applications as well as the advantages of multifunctional nanocarriers are discussed. The authors are interested in distinguishing the advantages and limitations of various systems that induced the need to develop alternative ways to deliver proteins for therapeutic applications.

2. Liposomes as protein carriers

Liposomes are polymolecular aggregates (vesicles) formed in aqueous solution on the dispersion of certain bilayer-forming amphiphilic lipidic systems. Under osmotically balanced conditions, the vesicles are spherical, self-closed structures composed of curved lipid bilayers, which entrap part of the aqueous phase in their interior. These molecular bilayers have the hydrophobic part of the amphiphiles forming the hydrophobic interior of the bilayer while the hydrophilic part (the polar head group) is in contact with the aqueous phases, both exterior and interior.

By trapping therapeutic entities, either in the lumen or in the membrane of the vesicles, liposomes can act as carriers for those entities in support of their use in therapeutic applications. A large variety of liposome-based carriers have been developed, ranging from conventional ones based only on lipids to polymer–lipid conjugates. This use is very well documented (see, e.g., [9-11]); only a few new examples of liposome applications in relation to protein encapsulation are presented here.

2.1 Conventional liposomes as carriers

The lipids used to make conventional liposomes are generally safe, biodegradable and FDA approved [12]. As a result, investigators seeking to overcome the problems encountered in protein delivery have often turned to conventional liposomes as delivery vehicles. Depending on the preparation procedure, various types of liposome have been proposed to serve as carriers for enzymes: unilamellar vesicular systems (ULV), multilamellar vesicular systems (MLV) and recently a few cases of multivesicular systems, so-called DepoFoam™ (Pacira Pharmaceuticals, USA) particles [9]. Various enzymes have been encapsulated and tested in liposomes with the aim of developing efficient therapeutic applications. The stability and reactivity of the enzymes were preserved inside the liposomes [13-16]. For example, amyloglucosidase inside dipalmitoylphosphatidylcholine (DPPC) MLVs or large ULVs retained 60% of its enzymatic activity even after many activity cycles [13]. In the case of yeast alcohol dehydrogenase, its encapsulation together with the cofactor nicotinamide adenine dinucleotide in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) MLVs increased its stability even at high temperatures (45 and 50°C) [15].

A higher stability was obtained for catalase when encapsulated in POPC MLVs because this prevents the dissociation of its tetrameric structure, which typically occurs for the free enzyme [14]. Even better stability and reactivity have been achieved by the formation of protein–lipid conjugates via the covalent conjugation of catalase at the outer surface of the lipid bilayer of ULVs composed of POPC, cholesterol and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-glutaryl (NGPE). The covalent conjugation prevents the dissociation of catalase at low concentration, which causes its

Table 1. Recent protein delivery systems based on liposome–polymer conjugates.

Liposome formulation	Polymer conjugate	Payload	Proposed application	Mode of administration	Trial status	Ref.
POPC, cholesterol and NGPE	NGPE	Catalase	Enzyme stabilisation	-	*In vitro*	[16]
DSPC, DCP, cholesterol	Chitosan-aprotinin	Calcitonin	Peptide delivery	Oral	*In vivo* (rats)	[23]
PC, TO, cholesterol	Alginate	BSA	Controlled release	-	*In vitro*	[24]
DSPC, DCP, cholesterol	Chitosan	Calcitonin	Protein delivery	Oral	*In vivo* (rats)	[25]
SPC, cholesterol	TMC	Coenzyme Q_{10}	Protein delivery	Ophthalmic/topical	*In vivo* (rats)	[27]
DMPC, DPPC	PLLc and PMAc	β-lactamase	Drug delivery, micro reactors	-	*In vitro*	[30]
DOPE, CHEMS	NIPAM-*co*-AA	GO	Trigger release	-	*In vitro*	[31]

CHEMS: Cholesteryl hemisuccinate; DCP: Dicetyl phosphate; DOPE: Dioleoylphosphatidylethanolamine; DPPC: Dipalmitoylphosphatidylcholine; DSPC: Distearoylphosphatidylcholine; NGPE: 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-glutaryl; NIPAM-co-AA: Poly(isopropylacrylamide-*co*-acrylic acid); PC: Egg phosphatidylcholine; POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SPC: Soy phosphatidylcholine; TO: Triolein.

inactivation [17]. However, anchoring enzymes to the surface of liposomes can alter the enzyme thermal stabilities, as shown for α-amylase and guanylasekinase [18].

The enhancement of activity by encapsulation in MLV liposomes can be obtained both by protein protection, which increases the half-life of the system, and by facilitation of the intracellular delivery into cytoplasm, as shown for the co-encapsulated CpG oligodeoxynucleotides with the recombinant Leishmania major stress-inducible Protein 1 antigen [19]. However, the encapsulation efficiency in conventional liposomes is < 30%. To increase the encapsulation efficiency, the electrostatic interaction between the protein and the lipid bilayer has to be increased by controlling the liposome overall charge. In the case of negatively charged acetyl cholinesterase, its encapsulation in positively charged POPC liposomes showed higher encapsulation efficiency (~ 35%) than in the negatively charged palmitoyl-oleoyl phosphatidylserine liposomes (~ 15%). A further increase of the encapsulation efficiency can be obtained by specific interactions, such as metal–hystidine interactions, as reported in the case of hystidine tagged acetyl cholinesterase and liposomes containing 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)imidodiacetic acid)succinyl (nickel salt) (DOGS-NTA-Ni) lipids [20].

Even if the enzyme preserves its activity *in situ*, its overall reactivity depends on the substrate permeability across the lipid membrane as a limiting factor. To increase the substrate permeability, either the membrane is destabilised with detergents [21] or membrane channel proteins are reconstituted in the membrane [22]. However, both methods have to be carefully controlled in order to avoid the complete destabilisation of the lipidic membrane and the loss of the encapsulated enzymes [22]. In addition, the easy disruption of the lipid bilayer caused by the presence of defects and subsequent severe leakage of the entrapped protein represent a drastic limitation for the use of conventional liposomes [23].

2.2 Polymer–liposome conjugates

Liposomes face complex challenges *in vivo*: i) the bilayer can be disrupted by interaction with high-density lipoproteins in the blood or by pulmonary surfactants in alveoli [24]; and ii) the liposomes are taken up by the reticuloendothelial system, which limits their circulation time and leads to the release of the protein in the blood. To increase the circulation time, liposomes are coated with a layer of polyethylene glycol (PEG) [10]. In addition, the approach to increase the circulation time of liposomes is to encapsulate them in microcapsules based on a polymer matrix, such as chitosan [25], or alginate [26], usually applied for oral administration. Several examples are presented in **Table 1**. However, most of the examples have been characterised *in vitro*, and only a few have associated tests in animals [27].

Chitosan was used to modify the surface of liposomes because of its bioadhesive and permeation-enhancing properties, which prolong the residence time at the site of adsorption and thus improve the bioavailability [28]. Anionic MLVs coated with chitosan possess an increased mucoadhesiveness and a higher encapsulation efficiency owing to the electrostatic interactions with the superoxide dismutase (up to 65%), compared with cationic or neutral MLVs (60 and 39%, respectively). Anionic liposomes were also more efficient at delivering superoxide dismutase (SOD) to A2182 cells compared with the others [29]. In addition, anionic liposomes coated with chitosan-aprotinin (protease inhibitor) were able to inhibit proteolytic enzymes in the intestine without decreasing the mucoadhesive properties [25]. However, the insolubility of chitosan at physiological pH remains problematic and was only partially solved by using partially quaternised derivative N-trimethyl chitosan chloride (TMC) to coat the liposomes in the case of coenzyme Q_{10} encapsulation [30].

In general, liposome formulations have relatively low encapsulation efficiency (< 30%) and the untrapped protein

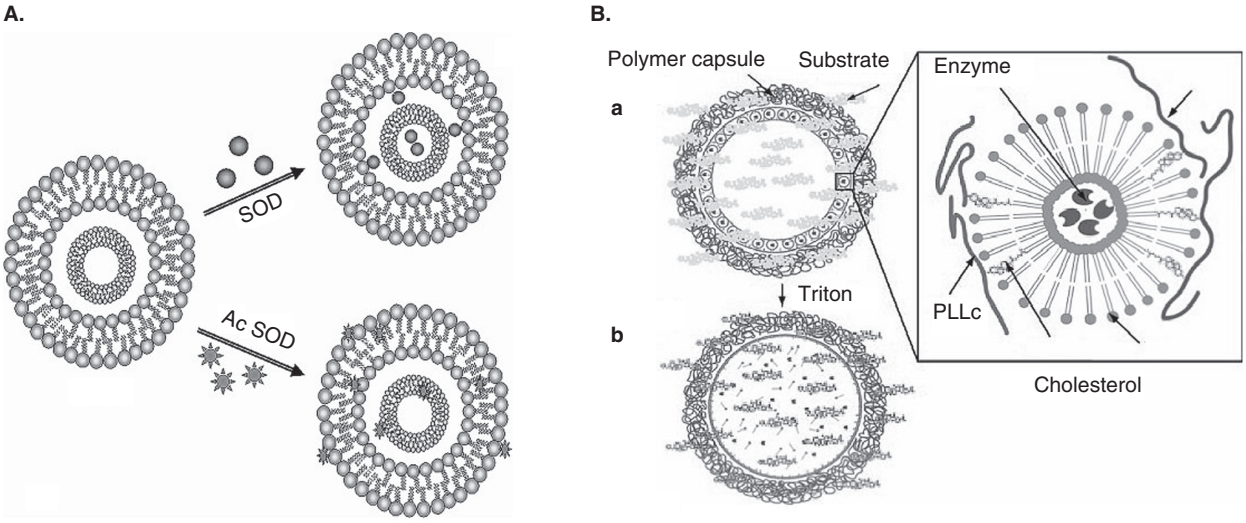

Figure 1. A. Schematic representation of superoxide dismutase and acylated superoxide dismutase (II) localisation in liposomes. **B.** Representation of a capsosome [32]. **a.** The enzyme β-lactamase is preloaded into liposomes and is sandwiched between two cholesterol-modified polymers, which are then embedded inside a polymer capsule. **b.** On addition of Triton X, the liposomes are destroyed and the enzyme is released, thus causing the hydrolysis of nitrocefin.

must be removed by gel permeation chromatography or centrifuge, resulting in loss of protein. To overcome this, Dai *et al.* developed a method to encapsulate the protein-loaded MLVs together with untrapped protein in alginate, forming the so-called liposome in alginate system (LIA). In this way a significant increase of encapsulation efficiency was achieved (up to 95%), and the release kinetics have been tuned to avoid any initial burst and to promote a sustained release, as shown for the delivery of bovine serum albumin (BSA) [26].

The ultimate reactivity of the encapsulated enzyme depends on its release on liposome disruption at the desired biological site. Several methods have been used to deliver liposomes to specific locations and ensure a complete release. For example, cationic liposomes are more adhesive to the intestinal epithelium than neutral ones, and their use improved the delivery of encapsulated SOD [31]. In this way the enzyme is able to exert its therapeutic action while circulating in the organism, regardless of the integrity of liposomes. The acylated SOD (Ac-SOD) exposed at the surface in MLVs (**Figure 1A**) showed an increased bioavailability (43 – 47%) compared with SOD or PEGylated SOD encapsulated inside conventional liposomes (< 9%), when it was intradermally injected to mice [27].

Capsosomes represent a new approach to improving the stability of liposomes. Capsosomes are created by first preparing liposomes containing the desired enzyme and then encapsulating the liposomes into polymer capsules (**Figure 1B**). As an example, cholesterol-modified lipids, poly(L-lysine) (PLLc) and poly(methacrylic acid)-*co*-(cholesteryl methacrylate) (PMAc) were used to improve the loading of β-lactamase in the polymer multilayers, up to 70% [32].

2.3 Trigger liposomes

The triggered release of enzymes from liposomes has been recognised as an attractive therapeutic approach to improve targeted delivery. In this approach, the liposomes do not release their contents until the membranes are destabilised by an external trigger, such as temperature or pH. For example, glucose-sensitive liposomes were prepared by incorporating hydrophobically modified glucose oxidase (GO) into the bilayer of dioleoylphosphatidylethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS)-based liposomes. When the concentration of glucose increases, the pH of the liposomal suspension decreases because of the increased amount of gluconic acid produced by the enzymatic reaction, thus resulting in a release of the encapsulated protein [33]. The response to the triggers is intended to increase the intracellular bioavailability by rapid release of the payload in the endosome and/or facilitating the protein transit to the cytoplasm. The major disadvantage of this strategy *in vivo* is that even under pathological conditions, the pH of tissue interstitium rarely declines below 6.5. Hence, it then becomes difficult to design drug delivery systems that release the encapsulated protein in such a narrow pH range.

2.4 Liposome reactors

The simultaneous reconstitution of a membrane channel protein, such as OmpF, in the lipid bilayer together with the encapsulation of a hydrophilic protein, such as with β-lactamase, inside the cavity represents the basis of a liposome microreactor design (**Figure 2A**) [22,34]. Substrate molecules (ampicillin) effectively pass through OmpF channels into the cavity, where they are subjected to β-lactamase-catalysed hydrolysis [22].

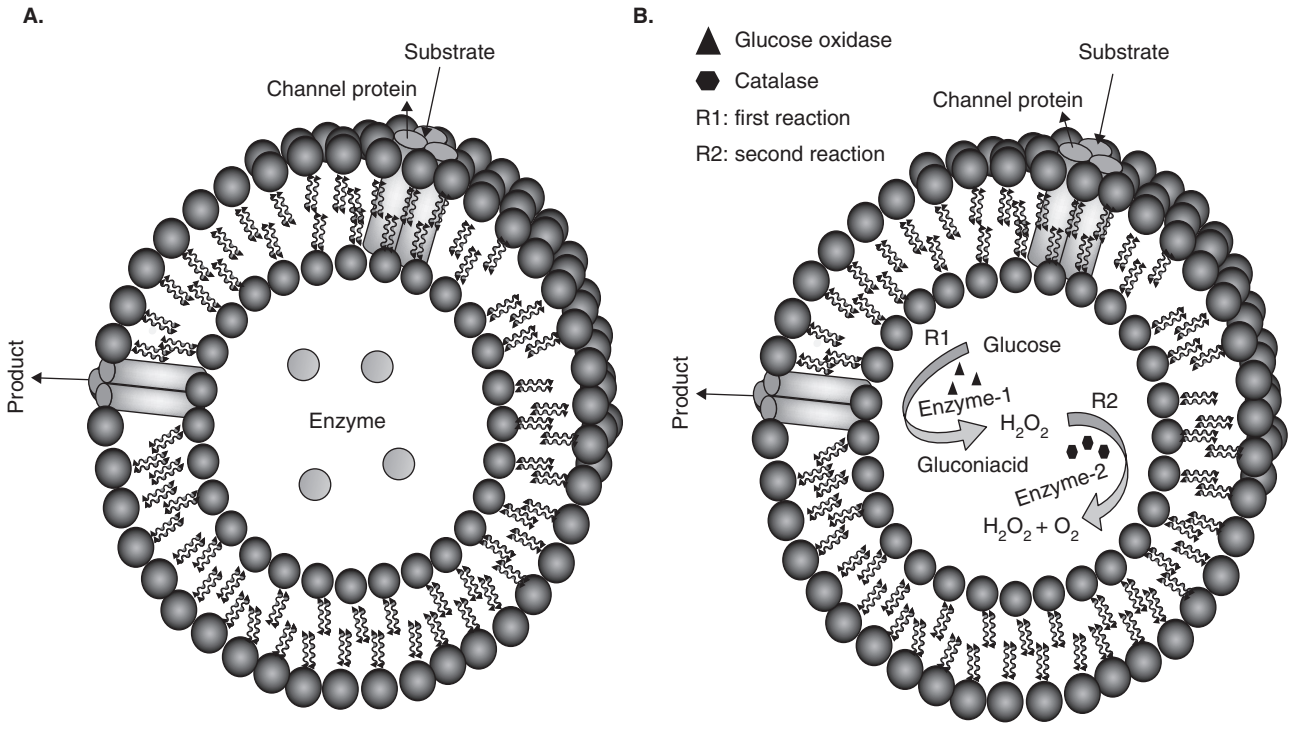

Figure 2. A. Liposome reactor based on one-enzyme encapsulation. **B.** Liposome reactor based on cascade reactions.

Liposome reactors have been proposed as a new strategy for prodrug therapy. Liposomes incorporating OmpF channels were prepared by encapsulation of nucleoside hydrolase of *Trypanosoma vivax*. The prodrug 6-methyl-purine riboside was able to pass through the OmpF channels into the cavity, where it was converted into cytotoxic 6-methylpurine. This subsequently diffused out of the reactor, and thus the system could serve to fight against tumours [34].

A further step was realised when the microreactor was based on co-encapsulation of enzymes acting in tandem (**Figure 2B**). Such a microreactor, developed to improve the reactivity of GO, contained GO and catalase inside the cavity of liposomes whose membrane contained OmpF channels [35]. Hydrogen peroxide produced by the oxidation of glucose by GO (first step of the reaction) was decomposed by catalase, which converted it into oxygen and water (second step of the reaction). The presence of OmpF increased the transport of glucose from the exterior of liposomes to their interior by a factor of 17, compared with liposomes without protein channels, and thus significantly improved the efficiency of the microreactor. However, the well-known instability of lipid membranes together with sizes in the micrometre scale prevent a larger therapeutic application of microreactors based on liposomes [36].

3. Polymeric carriers for proteins

Polymeric formulations have emerged as versatile drug carriers, owing to their improved properties compared with conventional liposomes, such as increased stability, modulated site specificity [37,38] and improved blood circulation stability [39,40]. Their chemical diversity enables the modulation of physicochemical properties for appropriate drug release (delayed, prolonged, triggered) [41] and biological behaviour, including targeting, bioadhesion and cellular uptake [3]. Several methods for the preparation of polymeric carriers have been reported, depending on the polymer structure and the physicochemical properties of the encapsulated protein, as well as the chemical stability of the system and the requirement to preserve the biological activity of the protein [42-45].

3.1 Microcarriers

Microcarriers are polymeric vehicles that serve as drug delivery systems, with sizes on the order of micrometres and homogeneous morphologies, and made of biocompatible and biodegradable (co)polymer(s). Different methods to entrap drugs inside microcarriers are commonly reported: the single or double (W1/O/W2) emulsion method [43,46]; solvent evaporation, a combination of the emulsion method and spray-drying [44]; the extrusion/external gelation method [47]; and coacervation [48]. Their selection is determined primarily by the necessity to prevent denaturation of the protein as well as by solubility and stability of the protein in various solvent mixtures.

A new way to encapsulate efficiently bioactive substances is the isoelectric precipitation method, which stabilises the protein conformational mobility [45]. Various enzymes, such as α-chymotrypsin, peroxidase, lyzozyme and β-galactosidase, were encapsulated in poly(lactic-*co*-glycolic acid) microparticles

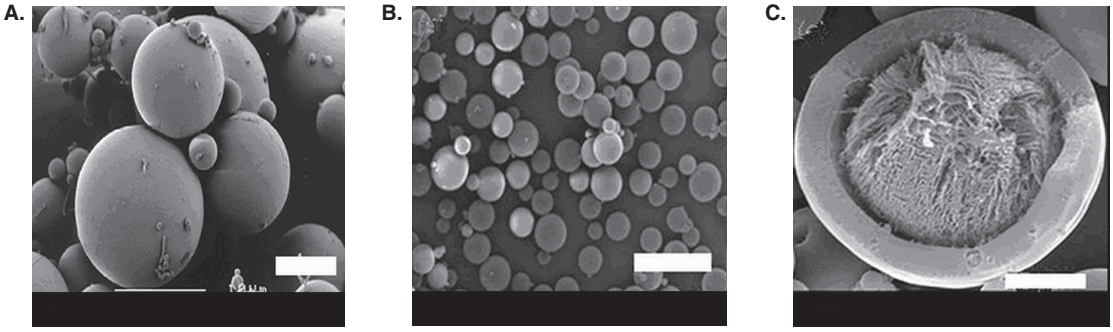

Figure 3. Scanning electron microscopy images. A. Lysozyme-loaded microspheres (scale bar 10 μm). **B.** Freeze-dried microcapsules (50 μm size) loaded with insulin (scale bar 200 μm). **C.** Cross-section of an insulin loaded microcapsule (scale bar 50 μm).
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(**Figure 3A**), with a high encapsulation efficiency and preserving ~ 95% of the activity, without using a stabiliser (glycofurol).

Another new method to form a protein/polymer delivery system is based on the formation of poly(lactic-*co*-glycolic acid) microcarriers using a concentric nozzle ultrasonic atomiser. This was used to form insulin microdroplets in aqueous solution, as a new insulin delivery system (**Figure 3B, C**) [49]. The microcarriers had an encapsulation efficiency of 49% and were injected into type 1 diabetic rat, where they maintained plasma insulin concentrations for 30 days. Enhanced circulation kinetics and controllable release of insulin *in vivo* are desirable to increase patient compliance and to reduce the need for intravenous injections.

Various types of biocompatible polymer are used for microcarriers in order to deliver the encapsulated proteins. Ghassemi *et al.* investigated the suitability of hydroxylated aliphatic polyester, poly(lactic-*co*-hydroxymethyl glycolic acid) as lyzozyme-loaded microparticles for controlled release [50]. By varying the polymer concentration it was possible to control and provide a sustained release of lyzozyme [51]. As poly(ε-caprolactone) (PCL) is known for long-lasting delivery because of its slow degradation rate, PCL microcarriers were loaded with BSA to obtain a slow release of the protein [52]. However, the inner structure of BSA-loaded microspheres is significantly affected by the surfactant concentration used during preparation procedure, which can compromise the delivery.

Polyanhydrides are widely used in controlled drug release systems because they are non-mutagenic, non-cytotoxic, non-inflammatory and are able to undergo surface erosion, enabling control of the release [53]. For example, a slow release profile was obtained for human serum albumin (HSA) entrapped in poly(1,3-bis-(*p*-carboxyphenoxy propane)-*co*-sebacic anhydride) (P(CPP-SA)) microspheres [54].

Hydrophilic chitosan microspheres were proposed to protect sensitive enzymes such as superoxide dismutase [55]. However, the SOD release was fast, reaching ~ 80% within the first day, which suggests that the proposed chitosan microspheres are not yet adapted for therapeutic applications.

Microspheres are usually administrated by subcutaneous or intramuscular injection. By tuning their composition in order to optimise the physicochemical properties, microcarriers could be a choice for local drug delivery. However, microspheres sometimes interact nonspecifically with the encapsulated protein, which limits free diffusion of the protein and thus affects the release profile. Moreover, their large size prevents them from being used for delivery over long periods, or for the delivery in a specific internal biological compartment [56].

3.2 Core-shell particles

As the use of complex coacervate core micelles in biomedical applications has been reviewed extensively [57-59], only the most recent examples are presented here in relation to proteins. Core-shell particles have various advantages that make them interesting as carriers: they can be rapidly produced under mild conditions, have a surface charge that can be modulated, and provide a sustained release of the entrapped protein.

Haidar *et al.* reported a new protein delivery system that consists of a suspension of stable, monodisperse cationic particles obtained by layer-by-layer deposition of alternating polyelectrolytes on cationic liposomes [60]. The system served to entrap BSA both in the aqueous core and within the polyelectrolyte shell, with high encapsulation efficiency (~ 80%).

Stimuli-responsive carriers designed for an efficient release in particular conditions, such as pH, or temperature, or in specific locations, such as, for example tumours, have been reported recently. pH-responsive core-shell microcapsules were prepared using membrane emulsification and interfacial polymerisation methods, and were used to encapsulate GO in order to protect it, and thus to prevent the loss of enzymatic activity [61]. Microcapsules are composed of a core-shell porous membrane and linear grafted poly(isopropylacrylamide-*co*-acrylic acid) chains located in the membrane pores. As the polymers are pH sensitive, they control the permeability of glucose, depending on the pH of the medium.

Table 2. Polymeric nanoparticles recently used as carriers for proteins.

Polymer	Morphology	Payload	Proposed applications	Mode of administration	Trial status	Ref.
PEG-PLA	Filaments, spherical nanocarriers	Catalase	Proteolysis	Intravenous	*In vivo* (mice)	[62]
Chitosan	Nanoparticles	FITC-BSA	Vaccine	Oral	*In vitro/in vivo* (mice)	[115]
Chitosan	Nanoparticles	Prodylase	Disorder of comm. tissue	Topical	*Ex vivo* (skin fibroblasts)	[116]
Chitosan	Nanoparticles	BSA	Model system	-	*In vitro*	[117]
Gelatin	Nanoparticles	FITC-BSA	Model system	-	*In vitro*	[118]
Polyurethane	Nanoparticles	Ova	Vaccine	-	*In vitro*	[119]
PEG-PLGA	Nanoparticles	Proteolyses	Against proteolysis	-	*In vitro*	[120]
PBCA-PLGA	Nanoparticles	SOD b-galactosidase	Protein delivery	-	*In vitro*	[69]
PLGA	Nanoparticles	SOD	Oxidative stress	-	*In vitro*	[70]
PLGA	Nanoparticles	SOD	Oxidative stress	Intavenous/intrajugular/ internal carotid,arterial	*In vivo* (rats)	[71]
PLGF, PLAF	Nanoparticles	BSA	Model system	-	*In vitro*	[123]
Poly(maleic anhydride)	Nanoparticles	Hb	Oxygen carriers	-	*In vitro*	[124]
Poly(acrylamide) and derivatives	Nanoparticles	Ova	Vaccines	Intravenous	*In vivo* (mice)	[125]

3.3 Nanocarriers

Polymer structures at the nanometre scale, such as micelles, vesicles or particles, are mostly used for drug transport and release owing to their mechanical stability, low permeability, the possibility to tune their properties through the polymer chemistry, size and geometry, making them suitable for both local and systemic administration routes [3,62,63]. Nanoparticles are mainly prepared using emulsion/solvent evaporation techniques or emulsion polymerisation techniques, whereas vesicles and micelles are obtained by self-assembly processes. The self-assembling systems are based on amphiphilic molecules that associate under aqueous conditions through weak non-covalent interactions, electrostatic and hydrogen bonding; these form various architectures such as micelles, vesicles and tubes. Amphiphilic molecules with low hydrophobic content tend to form micelles above the critical micelle concentration (CMC), whereas those with intermediate levels of hydrophobicity self-assemble into vesicles [64]. Hydrogels, representing one of the largest structures that is formed through self-assembling, are not discussed here, but they have been well reviewed [65,66].

3.3.1 *Nanoparticles*

Nanoparticles, also called nanospheres, are solid, colloidal matrices consisting of macromolecular substances that vary in size from 10 to 1000 nm, but only those with a diameter < 200 nm are suitable for use as carriers [67]. Depending on the method of preparation, they can be designed to possess various properties and release characteristics for high encapsulation efficiencies and adapted release. The therapeutic agent is typically dissolved, entrapped, adsorbed, attached and/or encapsulated into or onto a nanomatrix [3,68]. In addition to the advantages of size and higher entrapping efficiency, nanoparticles reside longer in circulation and have relatively higher cell uptake when compared with microparticles. These advantages make them suitable for systemic delivery. Protein release occurs by erosion or degradation of the polymer matrix, which follows after initial release of absorbed molecules on the nanoparticles surface. Various proteins have been entrapped in nanoparticles, either as model systems, or as possible therapeutic applications (**Table 2**).

Enzymes, such as β-galactosidase entrapped in nanoparticles of polybutylcyanoacrylate (PBCA) [69], or superoxide dismutase loaded in poly(DL-lactide-*co*-glycolide) nanoparticles [70,71], were delivered to neurons by means of polymeric nanoparticles administered to the brain. SOD-loaded nanoparticles were prepared to neutralise the deleterious effects of Reactive Oxygen Species (ROS) formed following ischemia-reperfusion. They were administered at the time of reperfusion through the intracarotid route, which allowed an increase of their localisation in the brain [70]. The neuroprotective effect of SOD-loaded nanoparticles was compared with PEGylated SOD, known for its increased stability over the free enzyme. The results indicated a significant neuroprotective effect of the SOD-loaded nanoparticles, whereas the PEGylated SOD showed no effect. Another model for SOD delivery, based on polyketal microparticles made of acid-degradable polymers, was reported recently [72]. Although the synthesis procedure is simple and the encapsulation efficiency of SOD is ~ 36%, the polyketal microparticles have a high

Table 3. Various proteins encapsulated in polymersomes as a function of the block copolymer type.

Polymer	Morphology	Payload	Proposed application	Ref.
Mode of action – protein delivery				
PEG-PBD	Polymersomes	Insulin	Model system	[63]
PEO-PCL-PAA	Polymersomes	BSA	Model system	[84]
PEO-PBD	Polymersomes	Hb	Oxygen carriers	[85]
PEO-PBD	Polymersomes	Porphyrin	Imaging applications	[121]
PEG-PBD	Polymersomes	HSF, HSAF	Iron reserves	[87]
Mode of action – in situ product formation and release				
PEG-PPS-PEG	Polymersomes	GO	Sensing devices	[90]
PS-*b*-PAA	Polymersomes	trypsin	Model system	[89]
PS-PIAT	Polymersomes	GO and HRP	Model system	[102]
PMOXA-PDMS-PMOXA	Polymersomes	Serine protease	Therapy	[122]
PMOXA-PDMS-PMOXA	Polymersomes	SOD	Antioxidant therapy	[91]
PMOXA-PDMS-PMOXA	Polymersomes with reconstituted OmpF	β-lactamase	Antibiotic production	[94]
PMOXA-PDMS-PMOXA	Polymersomes with reconstituted OmpF	Acid phosphatase	Model system	[99]
PMOXA-PDMS-PMOXA	Polymersomes with reconstituted OmpF and Tsx	Nucleoside hydrolase	Prodrug therapy	[95]
PMOXA-PDMS-PMOXA	Polymersomes with reconstituted FhuA	HRP	Model system	[96]
CCMV	Virus cage	HRP	Model system	[104]

polydispersity, which imposes extra studies to obtain a monodisperse size distribution, and thus to serve as a therapeutic carrier.

Garnacho and co-workers designed a new delivery carrier for enzyme replacement therapy, based on acid sphingomyelinase encapsulation in polystyrene and poly(lactic-*co*-glycolic) acid polymer nanocarriers [73]. The system was designed to target an endothelial surface protein, ICAM-1, which is upregulated in many pathologies and provides vascular accumulation. The system showed an enhanced enzyme delivery to target organs (primarily the lung) in mice.

The medical utility of proteins, for example therapeutic enzymes, is greatly restricted by their labile nature and inadequate delivery. Designing multifunctional nanocarriers, which combine long-circulating systems with targeting approaches (passive or, better, active), might overcome these limitations. In this respect, Dziubla and co-workers designed endothelial targeted polylactic/polyglycolic acid (PEG–PLGA) nanocarriers loaded with catalase, and tested their activity against vascular oxidative stress [74]. Encapsulated catalase was protected from external proteolysis and exerted the enzymatic activity *in situ* because its substrates, H_2O_2 and *ortho*-phenylendiamine, were able to diffuse through the polymer system.

3.3.2 *Micelles*

Copolymers with amphiphilic character are known to assemble in aqueous environments into polymeric assemblies, such as micelles, vesicles or tubular structures. Micelles have a fairly narrow size distribution and are characterised by their unique architecture, with hydrophobic segments segregated from the aqueous exterior to form an inner core, and the hydrophilic segments surrounding it. Polymeric micelles are generally more stable than those formed from lipids, with a remarkably lower CMC and a slower rate of dissociation.

This enables longer retention of loaded molecule, and eventually higher accumulation at the target site. Size is a crucial factor in determining their distribution in the body, especially when an enhanced permeation retention effect (EPR effect) is involved. Polymeric micelles have received intense attention as colloidal carrier systems for delivery of poorly absorbable drugs and proteins. Their core-shell morphology accounts for a high loading capacity and high water solubility, while their small size allows long circulation times [75]. Proteins can be released from micelles by either micelle disassembly or degradation of the micelles, but not by passive diffusion as in the case of small entrapped molecules [76].

PEG is most often used as the hydrophilic block because it is FDA approved, biocompatible and demonstrates notable ability to resist protein adsorption and cellular adhesion [6]. Various polymers have been proposed as hydrophobic blocks, such as polycaprolactone (PCL), polylactic acid (PLA), polyethylene oxide (PEO), polyethylene and oxide – polybutyleneoxide. The modification of their chemical properties, such as the block length, or hydrophilic/hydrophobic ratio induced a large variety of loading systems as well as release profiles.

The chemical composition of polymeric micelles can be modified to obtain the appropriate physicochemical properties for protein incorporation [77]. For example, heparin-conjugated Tetronic® (BASF, Germany)-PCL block copolymer micelles were used to design an injectable carrier for delivery of basic fibroblast growth factor (bFGF), known for its essential role in the regulation of various biological signalling pathways [78]. It was found that bFGF bound specifically to heparin on the surface of tetronic-PCL-heparin micelles, and thus modulated its release. To achieve a sustained release, Miller and co-workers explored the ability of poly (ε-caprolactone)-block-poly(2-vinyl pyridine) (PCL-*b*-P2VP) block copolymer to self-assemble in organic solvents [79]. The autoassembly in an oily phase represents a unique example of this approach to generate micelles. It has been used to entrap protein in the P2VP corona, with an efficiency of 7.8%. However, the method cannot be applied for sensitive enzymes, which could be inactivated during the preparation procedure.

Reverse polymeric micelles based on alkylated star-shaped poly(glycerol methacrylate) polymers have been proposed as carriers because they are able to increase significantly the solubility of various proteins, as reported for vasopressin, myoglobin and albumin [80]. Note that the nature of the hydrophobic segment and the guest molecule has a significant influence on the ability of the reverse micelles to solubilise the protein in order to trap it inside their structure. Other micelles have been designed from diblock copolymers that are stimuli responsive, for example to pH or temperature changes, and proposed as anticancer drug delivery systems [81], but only a very few examples are related to protein loading [82].

3.3.3 *Polymersomes*

The ultimate achievement in enzyme protection and delivery is represented by polymeric vesicles, so-called polymersomes [41]. Encapsulation of proteins within their aqueous cavity can take advantage of the extended circulation kinetics and controlled release properties of polymersomes [64]. In addition, they are able to co-encapsulate proteins acting in tandem, to serve for complex catalytic reactions or proteins and hydrophilic drugs to be delivered simultaneously, because of their inner water cavity. Together with the higher stability compared with liposomes, this makes them more suited as carriers, both for local and for systemic delivery. The release from the cavity can be achieved when the vesicles degrade and expel their contents. However, during polymersome formation proteins are exposed to hydrophobic interfaces (i.e., organic solvent, air, etc.), which can induce inactive protein conformations, as reported previously for other polymeric delivery systems [83]. To avoid this, a control of the self-assembly process of formation of protein-loaded polymersomes in various environments, such as buffer solutions or anaerobic conditions, has to be performed.

The ability of neutral triblock or charged triblock polymersomes to encapsulate larger, globular proteins has been tested with various proteins, such as Fluorescein isothiocyanate (FITC)-labelled BSA, myoglobin and haemoglobin (**Table 3**) [84,85]. Encapsulation of functional proteins was possible without affecting their activity, as in the case of encapsulated haemoglobin, which preserved its oxygen affinity similar to the haemoglobin of human red blood cells [85].

Insulin served as a challenging first test of therapeutic protein encapsulation in PEG-based polymersomes [63]. The encapsulation of FITC-labelled insulin into polyethyleneglycol-polybutadiene (PEG-PBD) polymersomes has been proved by fluorescence microscopy, which showed that the polymersomes were stable for months at 4°C.

Another way to deliver the encapsulated protein to the biological compartment is to use polymersome formation stimuli-responsive polymers. The presence of the stimulus will serve as a delivery trigger for the encapsulated protein, which will be released from the carrier [86]. Surprisingly, there are very few examples of responsive vesicles that have been used for protein delivery. In this respect, Robbins *et al.* prepared photoactive polymersomes based on PEG-PBD, which contained *meso*-to-*meso* ethyne-bridged bis[(porphinato)zinc] (PZn_2) located in the membrane, as a light-responsive chromophore. These were used to entrap horse spleen ferritin (HSF) or iron-free apoferritin (HSAF) [87]. Experiments demonstrated that 25 – 50% of encapsulated proteins were released from vesicles after a short time of light exposure. However, the simultaneous presence of both ferritin and PZn_2 caused irreversible morphological changes of the carriers, ranging from the formation of new bends or 'arms' to total polymersome destruction (**Figure 4**).

Another approach to develop protein carriers is based on filamentous or tubular polymer nanocarriers, inspired by their morphological similarity to filamentous viruses [62,88]. However, the tubular structures made of PCL as the shell and PEO as the core could not retain enzymes as small as alkaline phosphatase (80 kDa), which limits their use to high-molecular-mass enzymes [88]. Catalase and PEG-catalase were successfully loaded into filamentous and spherical polymer nanocarriers and their loading efficiency and activity were compared: a higher activity was found for filamentous nanocarriers containing PEG-catalase, even if they offered less protection against proteases [62].

3.4 Nanoreactors

Polymeric nanoreactors are polymersomes designed to bear enzymes inside their cavity, which constitutes the enzymatic reaction site, but without the necessity to release the encapsulated enzyme. Nanoreactors serve both to protect the protein inside their water cavity and simultaneously to allow them to act *in situ* (examples are included in **Table 3**). In the case of nanoreactors, various scenarios for the substrate are possible: i) the substrate is encapsulated together with the enzyme [89]; ii) the polymer membrane is permeable to the substrate molecules [62,90-93]; and iii) the transport across the membrane is facilitated by a channel protein [94-96], as shown in **Figure 5**.

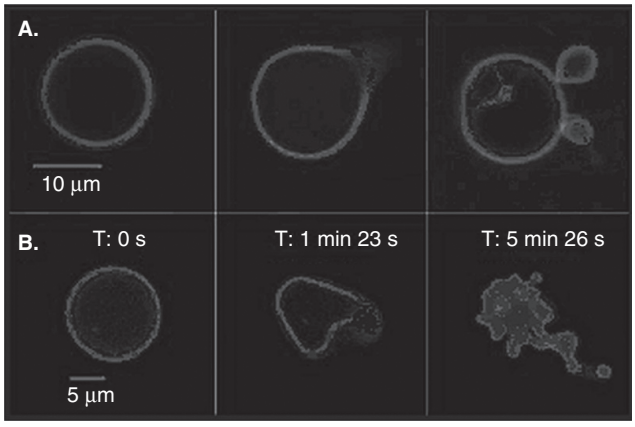

Figure 4. Photo-destruction of loaded HSAF polymersomes, as indicated by confocal microscopy.
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Only nanoreactors with permeable membranes or with channel proteins that make the membrane permeable to the substrate have potential for therapeutic applications. In this respect, nanoreactors represent a new strategy to cope with the necessity to increase the concentration of the protein at biological sites: the protein acts inside the nanoreactor with the membrane acting as a shield, and no release is required in order to obtain the biological effect. The viability of simultaneously shielding the enzymes while maintaining their activity has been tested on model systems. Compared with the liposome reactors, polymeric nanoreactors offer all the advantages of polymersomes, such as stability, functionalisation, or versatility, and thus are expected to be more efficient in *in vivo* tests.

In the case of highly impermeable polymer membrane such as poly (methyloxazoline)-poly (dimethylsiloxane)-poly (methyloxazoline) (PMOXA-PDMS-PMOXA) [97], different membrane proteins (OmpF [94], Tsx [95], FhuA [96], AqpZ [97], LamB [98]) were reconstituted to overcome the diffusion barrier presented by the polymer membrane. Different molecules (β-lactams, DNA, nucleosides) and their products were passively transported across the polymer wall through the channel proteins. In all the above-mentioned situations the enzymes were retained inside the nanoreactors; therefore, one expects an extended activity compared with delivery systems that release the enzyme and thus expose it to proteolytic attack. Using this approach, a therapeutic nanoreactor (PMOXA-PDMS-PMOXA) was designed containing a prodrug-activating enzyme TvNH (purine-specific nucleoside hydrolase) that produced cytotoxic molecules [95]. Another example of such a versatile nanoreactor is obtained by encapsulation of acid phosphatase. Acid phosphatase is inactivated by high pH, therefore by tuning the pH its activity can be controlled. By combining a pH stable polymer (PMOXA-PDMS-PMOXA) with reconstituted OmpF as a diffusion gate, a pH switchable nanoreactor was created [99]. As the nanoreactor concept was introduced recently, these studies are at the *in vitro* stage, and are under development.

GO [90], lipase [92], urease [93], catalase [62] and SOD [91] are enzymes that have been entrapped in polymersomes that enable a controlled transport of molecules owing to the permeability of the polymer membrane. The encapsulation of SOD in polymersomes with oxygen-permeable membranes represents the first example of an antioxidant nanoreactor able to detoxify superoxide anions present in the environment of the vesicles, and that penetrate inside the cavity (**Figure 5A**) [91]. Highly active, even at a level of a few encapsulated enzyme molecules, the nanoreactor does not affect the activity of SOD compared with the free enzyme, as obtained by pulse radiolysis assay. Another example of nanoreactor involved trypsin encapsulation in polyguanine acid (polyG) functionalised PMOXA-PDMS-PMOXA vesicles, and its *in situ* activity indicated using a fluorescent derivative substrate. Owing to its hydrophobicity, the substrate penetrates the vesicle wall in the aqueous cavity where it is converted by the encapsulated enzyme. The product, however, is accumulated inside the vesicle and will inhibit the enzyme or oxidise the polymer, resulting in vesicle rupture and enzyme release [90].

Coupled reactions were achieved in nanoreactors by different positioning of enzymes inside the cavity, and in the polymer/polyelectrolyte wall [100,101]. Tandems of GO–horseradish peroxidase, or lipase B–GO–horseradish peroxidases were studied in such two-compartment nanoreactors. The enzymes encapsulated or positioned in the wall are indeed protected, but the drawback is represented by the harsh conditions (organic solvents) that the enzyme embedded in the polymer wall is subjected to during the preparation procedure. To overcome this, van Hest and collaborators encapsulated GO and horseradish peroxidase in separate vesicles. Their results confirm that communication between different vesicles can be achieved, but again this communication is rather limited to the permeability of the species though the polymer membrane, which restricts the choice of enzymes used for the coupled reactions [102]. In addition, it is hard to see how they can be used for therapeutic applications because of the difficulty in controlling their simultaneous presence and communication in biological compartments.

The accumulation of the product inside the cavity of the nanoreactors can be avoided by reconstitution of channel proteins in the polymeric membrane, such as OmpF and FhuA [94,96,99]. Two important parameters are to be considered in nanoreactor optimisation: the number of membrane proteins reconstituted and the encapsulation efficiency of the enzyme. These parameters will ultimately determine the efficiency of the nanoreactor. Moreover, channel proteins can be blocked with molecules of different size for a controlled release [103]. The tunable properties of each component of the nanoreactors (polymer: length and chemical structure; membrane protein transport properties; enzyme) make them highly versatile for use in medicine, and it is expected that they will

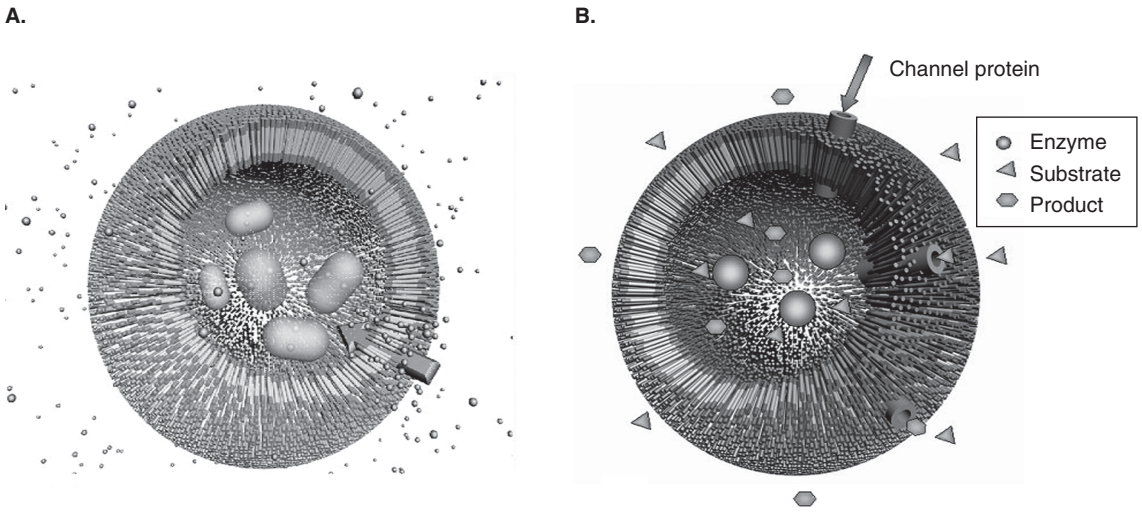

Figure 5. Nanoreactors with a membrane (A) permeable to the substrate, (B) incorporating channel proteins, to allow the substrate passage.

represent a valuable strategy for pathological situations. The major requirements for polymeric nanoreactors to be useful for enzyme delivery remain their biocompatibility and biodegradability in the body, as well as high stability and high encapsulation efficiency.

A different system proposed as carriers are viral capsids, which represent biological counterparts of liposomes and polymersomes. Viral capsids as nanoreactors containing horseradish peroxidase have been studied [104] as a model system. The protein cages are available in nature and their surface can be functionalised, but their thermal and chemical stability still restrict the applications [105].

4. Targeting approaches

The best way to improve the therapeutic effect is to deliver the proteins at the appropriate biological compartment, in a so-called active targeting strategy. In this respect, various specific ligands have been used to target different receptors: growth factors and vitamins, peptides such as transferrins and integrins, sugar derivatives such as heparin sulphate or chondroitin sulphate, and antibodies. They have been reviewed recently, and therefore are not detailed here [38,106,107]. Owing to various targeting ligands attached to the surface of the nanocarriers, they could prolong circulation time in particular cases, increase drug bioavailability, reduce undesirable side effects and minimise nonspecific uptake, and thus allow for specific targeting to certain cells within the cancer sites or even intracellularly to specific organelles Depending on the properties of the targeting ligands attached to the surface of the nanocarriers, they improve the delivery process by increasing the protein bioavailability, or by minimising the nonspecific uptake, and in the special case of antibodies, they can prolong circulation time. These properties allow a specific localisation to certain cells or even to particular organelles [39]. In special cases, such as antibodies, they improve the delivery by increasing the circulation time. Whereas in the case of liposomes only a few studies focused on active targeting [108], owing to the difficulty of functionalising them, in the case of polymeric carriers this is the normal way to address a therapeutic application.

In the case of liposomes the passive targeting approach based on specific properties of the lipidic surface, such as change character, represents the conventional way to improve the delivery at a specific site (for details, see anionic liposomes described in Section 2.2). Most liposomes are known to deliver their content to the cytosol of mammalian cells, but recently it has been found that fusogenic liposomes deliver lactoferrin in the endoplasmic reticulum by means of a microtubule-driven route [109]. Jayanna and co-workers proposed an active targeting approach based on the fusion of liposomes with phage coat proteins to obtain nanocarriers for specific targets in tumours [37].

In the case of polymeric carriers, various functional moieties were used, such as folic acid, heparin sulphate, chondroitin sulphate, peptides or different antibodies [110,111]. Conjugation of carriers with antibodies allows a specific binding to determinants exposed at the cellular surface. By selecting the antibodies it is possible to optimally deliver the proteins to certain cellular destinations (e.g., the cell surface versus intracellular compartments) [112,113].

Owing to the nature of proteins (size, hydrophilicity and physical and chemical fragility), a crucial hurdle for all kinds of systemic delivery is the permeation of the protein across cell barriers. The difficulty of achieving this depends on the nature of the cell layer. Whether the delivery takes place through the

nasal or buccal epithelium, the different types of epithelium in the gastrointestinal system, in the skin, or in the alveola in the lung, the therapeutic protein must permeate through the cell lining. Therefore, it is often necessary to add enhancers, which support the cell membrane penetration, such as protein transduction domains, or cell-penetrating peptides.

In order to enhance their uptake at cellular level, the carriers' surface must be modified, either by a passive strategy of coating with hydrophilic stabilising bioadhesive polymers (chitosan, polyacrilic acid), or by an active one, based on surface functionalisation with ligands for target receptors expressed at cell surfaces, such as WGA lecitin (for erythrocytes), or bacterial adhesions, antibodies (for M cells).

Successful targeting strategies must be determined experimentally on a case-by-case basis, which is laborious. In addition, systemic therapies using nanocarriers require methods that can overcome nonspecific uptake by mononuclear phagocytic cells and by non-targeted cells. Therefore, therapeutic efficiency of targeted nanocarriers is still far from an established approach that is widely used in medical applications. Although using genomics and proteomics technology to choose appropriate targets is an active area of research, so far no clinically effective targets have been identified.

5. Expert opinion and conclusion

The strategy of delivering active proteins to biological compartments using carriers represents an efficient way to improve a patient's quality of life by improving protein bioavailability, reducing the frequency of administration and expanding the effective routes of administration from local to systemic. A large variety of systems has been developed, ranging from conventional drug delivery systems based on liposomes or liposome/polymer conjugates to polymer carriers and nanoreactors; here, recent relevant examples are presented in order to see the state of the art, and which challenging problems remain to be solved in this field. Note that almost all carriers have been characterised *in vitro*, but only a few have been tested further in animals [27,62]. In this respect antibody neutralisation has to be addressed, thus the carriers should be both inert for antibodies' action and not allow their penetration to the encapsulated enzyme. Therefore, although several new approaches, such as nanoreactors, have emerged, further studies are needed to explore the *in vivo* behaviour and develop efficient protein delivery systems.

Even though liposomes are well established as conventional drug carrier systems, we find their inherent characteristics limit possible applications. Limitations of liposomes, such as interaction with high-density lipoproteins, significant leakage resulting from structural defects in lipid bilayer, or mechanical instability, result in rapid release of the encapsulated entity [11]. In comparison with liposomes (of any type), polymeric carriers are generally better suited for delivery in various conditions, even at acidic pH values, owing to their higher stability, the possibility of tuning their properties and the ability to allow for multiple functionalisation. Their chemical diversity enables the modulation of physicochemical properties for appropriate protein release (delayed, prolonged, triggered), and biological behaviour, including targeting, bioadhesion and cellular uptake. An important factor for delivery is the size of carriers, and in this respect nanoparticles demonstrate improved uptake compared with microcarriers, making them suitable for systemic delivery. However, there are some limitations concerning polymeric carriers, such as difficulties in handling, storage and administration owing to a susceptibility of aggregation, or the probability of accessing unintended cellular compartments, with harmful consequences on their integrity [114].

Self-assembling synthetic materials, such as micelles, vesicles and tubes, have emerged as versatile drug carriers because of their increased stability, site specificity, blood circulation resistance and thus overall potential therapeutic capabilities compared with liposomes. These advantages emerge from the ability to tailor their composition at the monomer level and from the ability to control the self-assembly process, in order to achieve the desired properties and release profiles. In addition, their robust nature (a 10-fold increase in critical areal strain before rupture compared with liposomes) makes them more suited for technological development. New combinations based on polymeric materials need to be evaluated further, both *in vitro* and *in vivo*, for real progress in protein delivery. In this respect, the concept of nanoreactors should be developed and tested in biological conditions to determine how they react and to see how to progress from model systems to therapeutically useful protein-based reactors. The unique promise of nanoreactors is that they avoid the problems of release profile optimisation, or the release of therapeutics in inappropriate compartments due to the dissociation or degradation of the carrier.

It is awkward to compare results from different studies in order to draw a general conclusion about the best characteristics a polymeric carrier needs to be optimally endocytosed. A lack of clinical studies makes it even more difficult to assess the relevance of those properties to the use of the carriers in humans. However, there are some general factors clearly relevant to cell uptake: choice and development of biocompatible, biodegradable and non-toxic polymer systems; adapted sizes and surface properties of nanocarriers; and multifunctionality to cope with the complexity of the *in vivo* biological conditions.

In the authors' opinion there remain many interesting challenges for the future of polymeric carriers and nanoreactors in order to optimise performance for specific applications or particular routes of administration. Considering the complexity of interactions at different levels, it was assumed that only tailored systems can serve to solve specific pathological situations. This can be accomplished by understanding the relationships between polymers' physical and chemical properties, and their function or behaviour when combined with therapeutic proteins in biological conditions, indicating that further fundamental study is

needed. In addition, the authors expect that the design of multifunctional carriers or nanoreactors by combining stimuli-responsive polymers and targeting orientated receptors will improve the ability of these systems to cope with complex biological conditions.

Declaration of interest

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