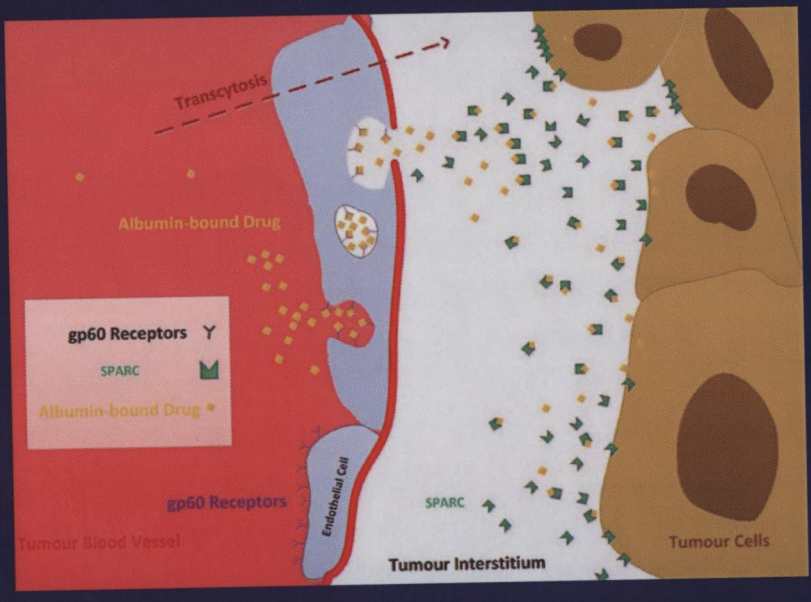
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U.S. Patent No. 9,943,096

Advances in PROTEIN CHEMISTRY and STRUCTURAL BIOLOGY

Protein and Peptide Nanoparticles for Drug Delivery



VOLUME 98

Edited by
Rossen Donev

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Advances in Protein Chemistry and Structural Biology

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VOLUME NINETY EIGHT

ADVANCES IN PROTEIN CHEMISTRY AND STRUCTURAL BIOLOGY

Protein and Peptide Nanoparticles for Drug Delivery

Edited by

ROSSEN DONEV
Biomed Consult Ltd
Swansea, United Kingdom



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First edition 2015

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ISBN: 978-0-12-802828-5
ISSN: 1876-1623

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CHAPTER SEVEN

Polysaccharide Nanoparticles for Protein and Peptide Delivery: Exploring Less-Known Materials

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Advances in Protein Chemistry and Structural Biology, Volume 98
ISSN 1876-1623
http://dx.doi.org/10.1016/bs.apcsb.2014.11.003

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Abstract

Finding adequate carriers for protein and peptide delivery has become an urgent need, owing to the growing number of macromolecules identified as having therapeutic potential. Nanoparticles have emerged in the field as very promising vehicles and much work has been directed to testing the capacity of different materials to compose the matrix of these carriers. Natural materials and, specifically, polysaccharides have been taking the forefront of the challenge, because of several favoring properties that include the higher propensity to exhibit biodegradability and biocompatibility, and also the high structural flexibility. The majority of works found in the literature regarding polysaccharide nanoparticles uses very popular materials like chitosan or hyaluronic acid. This review is aimed at describing and exploring the potential of polysaccharides that are not so well known or that are less explored. For those, the main properties will be described, together with an overview of the reported applications as nanoparticle matrix materials.

1. INTRODUCTION

The strong evolution of the biotechnology industry has resulted in a great number of macromolecules identified to have therapeutic potential, many being protein based. Alongside with the new therapeutic possibilities, questions arose on the feasibility of their administration, because of the inherent physicochemical and biopharmaceutical properties that make their delivery a challenging issue. The therapeutic effect of proteins is not only limited by the potential degradation in biological environments, but is also compromised by their low ability to reach the therapeutic site of action (Antosova, Mackova, Kral, & Macek, 2009; Casettari & Illum, 2014; Janes, Calvo, & Alonso, 2001). These limitations are related with the presence of a high number of functional groups susceptible of chemical degradation and, also, with the high hydrophilic character of the proteins (de la Fuente, Csaba, Garcia-Fuentes, & Alonso, 2008; Li et al., 2013). Soon it was, thus, assumed that specific carriers were needed to provide an efficient delivery. The efforts of many scientists have turned to the task, resulting in the development of a wide variety of drug delivery systems. Nanoparticles, which are solid submicron-sized carriers (Pinto Reis, Neufeld, Ribeiro, & Veiga, 2006), are one of the most promising and popular systems. This is due

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to several interesting features that include the high surface-to-volume ratio and the surface functionality, thus offering potential for the association of macromolecules (Csaba, Garcia-Fuentes, & Alonso, 2006). Additionally, nanosized carriers provide increased contact with epithelial surfaces (Desai, Labhasetwar, Amidon, & Levy, 1996) and, regarding protein delivery strategies, an improvement of proteins' stability, bioavailability, targeting, uptake, and biological activity has been shown (Dombu & Betbeder, 2013). Finally, biological transport processes, anatomically and down to the cellular and subcellular levels, are considered to be affected by the physical attributes of the nanocarriers (Elsabahy & Wooley, 2012).

On selecting the appropriate material for drug delivery approaches, several requirements must be met: biodegradability, biocompatibility, versatility, and low overall costs of production (Chiellini, Piras, Errico, & Chiellini, 2008; Karewicz, Bielska, & Nowakowska, 2012). By definition, nanoparticles can be produced with either synthetic or natural materials, but the latter are known to comply more easily with the mentioned requisites (Beneke, Viljoen, & Hamman, 2009; Malafaya, Silva, & Reis, 2007). Actually, it has been claimed that one of the ways of avoiding the potential toxicity of nanocarriers, an issue believed to be one of the most relevant on preventing diverse clinical applications so far, may be using natural materials (Anwunobi & Emeje, 2011). Moreover, these have some remarkable merits over synthetic ones, namely improved capacity for cell adhesion and mechanical properties similar to natural tissues (Pollard et al., 2008). Additionally, they are among the most versatile building units of drug delivery systems, as their properties can be easily tailored to meet specific requirements.

Polysaccharides are a class of natural materials, along with proteins and lipids. Many are obtained from plants or marine organisms, therefore being less probable to induce adverse immunological reactions, as compared with proteins (Karewicz et al., 2012). Importantly, they are economical, readily available, usually biodegradable and with few exceptions, also biocompatible (Beneke et al., 2009; Copetti, Grassi, Lapasin, & Pricl, 1997). These are the reasons justifying that polysaccharides assume a relevant role as matrix materials for drug delivery systems and, namely, nanoparticles.

In this review, polysaccharide nanoparticles developed for protein and peptide delivery will be described. The emphasis will be placed on materials that were less explored or that are not so well-known regarding the development of nanoparticulate systems, thus excluding polymers like chitosan, alginate, cyclodextrins, and hyaluronic acid. For those polysaccharides selected to be described, a report on their main properties will be presented.

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Additionally, an overview of their applications on the production of nanoparticles aimed at protein delivery will be provided.

2. POLYSACCHARIDES AS NANOPARTICLE MATRIX MATERIALS

Nature provides an impressive array of polymeric materials that have been finding very interesting applications in the biomedical field and, namely, in drug delivery. Polysaccharides are complex carbohydrates, composed of monosaccharides joined together by glycosidic bonds (Karewicz et al., 2012; Mizrahy & Peer, 2012). As mentioned above, they are one of the material classes applied more frequently in the production of nanoparticles. The most common basic unit of polysaccharides is the monosaccharide D-glucose, although D-fructose, D-galactose, L-galactose, D-mannose, L-arabinose, and D-xylose are also frequently present. Some polysaccharides comprise simple sugar acids (glucuronic and iduronic acids) and also monosaccharide derivatives in their structure, like the amino sugars D-glucosamine and D-galactosamine, as well as their derivatives *N*-acetylneuraminic acid and *N*-acetylmuramic acid (d'Ayala, Malinconico, & Laurienzo, 2008). The presence of several of these sugar units on the side chain of carbohydrate polymers makes them good candidates for targeted delivery by carbohydrate recognizing receptors found on the surface of several cells (Zhang, Ma, & Sun, 2010).

As evidenced in Table 1, polysaccharides have several possible origins, namely algal, plant, microbial, and animal.

Notwithstanding the variety of origins, they benefit from the general properties of natural polymers, including the propensity for biocompatibility, the low cost and hydrophilicity (Anwunobi & Emeje, 2011; Liu, Jiao, Wang, Zhou, & Zhang, 2008; Mizrahy & Peer, 2012). Table 2 presents the pros and cons of polysaccharides as nanoparticle matrix materials.

The general propensity for biocompatibility and biodegradability is perhaps the major advantage of these materials. Actually, carriers based on polysaccharides are expected to be easily eliminated from the organism, as these will be processed to result in small sugar units that integrate conventional metabolic processes, thereby permitting elimination or reabsorption (Anwunobi & Emeje, 2011). Polysaccharides present diverse physicochemical properties, deriving from multiple chemical structures that also translate into different molecular weights (MW) and intrinsic characteristics. Ionic nature, for instance, is one of the greatest items of variation, as all cationic,

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Table 1 Main origin and sources of polysaccharides

Polysaccharide	Origin	Examples of sources
Cellulose	Plant	Cotton, wheat, potato tubers, sugar beet pulp, soybean stock
Pectin	Plant	Citric fruits, apples, pears
Starch	Plant	Potato, wheat, rice, maize
Mannan	Plant	Coffee bean, ivory nut
Glucomannan	Plant	Scotch pine, orchid tubers, *Amorphophallus konjac*[a]
Arabic gum	Plant	Species of *Acacia* (*Acacia senegal*)[a]
Tragacanth gum	Plant	Species of *Astragalus* (*Astragalus gummifer*)[a]
Carrageenan	Plant, algae	Red seaweed
Fucoidan	Plant, algae	Brown seaweed (*Fucus vesiculosus*)[a]
Pullulan	Fungus	*Aureobasidium pullulans*
Chondroitin sulfate	Animal	Cartilage
Heparin	Animal	Mucosal tissues

[a]Main source.

Table 2 Pros and cons of polysaccharides as nanoparticle matrix materials

Advantages	Limitations
Structural flexibility, stability	Inter-batch variability
Low cost	Limited availability (if widely used)
Bioadhesion capacity	Complex and varied composition
Hydrophilicity	Difficult to process
Biocompatibility, biodegradability	Immunogenicity

anionic, and neutral polysaccharides can be found (Beneke et al., 2009; Mizrahy & Peer, 2012). The property of hydrophilicity is of particular importance, as it permits the production of carriers by methods avoiding organic solvents. Additionally, polysaccharides also benefit from a great structural flexibility, forming either linear or branched structures and easily

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permitting chemical modifications (Karewicz et al., 2012; Malafaya et al., 2007). This characteristic might be beneficial for several nanoparticle production methods or the development of targeting strategies. The more sophisticated applications, which include controlled or triggered release, or even targeted delivery, demand chemical modifications of polysaccharides. These usually encompass the introduction of ionic or hydrophobic groups, degradable bonds, spacers, or targeting moieties (Karewicz et al., 2012). The ability to adhere to biological surfaces includes a relevant advantage in drug delivery applications (Mizrahy & Peer, 2012), as these frequently imply an interaction with cell surfaces. In this regard, the reactive functional groups of polysaccharides allow the formation of noncovalent bonds with cell surfaces, affording enhanced residence time and, consequently, increased drug absorption (Anwunobi & Emeje, 2011; Liu et al., 2008).

Notwithstanding the relevance of the mentioned advantages, some drawbacks should also be taken into account, such as the possibility of generating immunogenic responses and the polymer variability related to origin and supplier (Pollard et al., 2008). Regarding the latter, plant-derived polysaccharides pose potential challenges, as structural differences might occur according to the location and plant collection season (Beneke et al., 2009).

Traditionally, polysaccharides were included in formulations as inert materials, but in modern pharmaceutical design they increasingly assume relevant roles, endowing the dosage forms with multifunctional abilities, such as controlled release, stabilization, emulsification, or bioadhesiveness, among others (Beneke et al., 2009; Mizrahy & Peer, 2012). Nevertheless, the set of polysaccharide properties confers a relevant versatility that adds to a wide range of biological abilities, thus contributing to an increased application in drug carrier production.

Below, a description is given for each polysaccharide focused in this chapter as matrix material of nanoparticles aimed at protein delivery. The distribution of polysaccharides is presented according to their main origin, as described in Table 1, and the chemical structure of the polysaccharides is described in Fig. 1. The structure of arabic and tragacanth gums is not shown, as these are composed of polysaccharide mixtures.

2.1. Cellulose

Cellulose is the primary structural component of plant cell walls and it can also be extracted from marine invertebrate animals (tunicate), algae, fungi,

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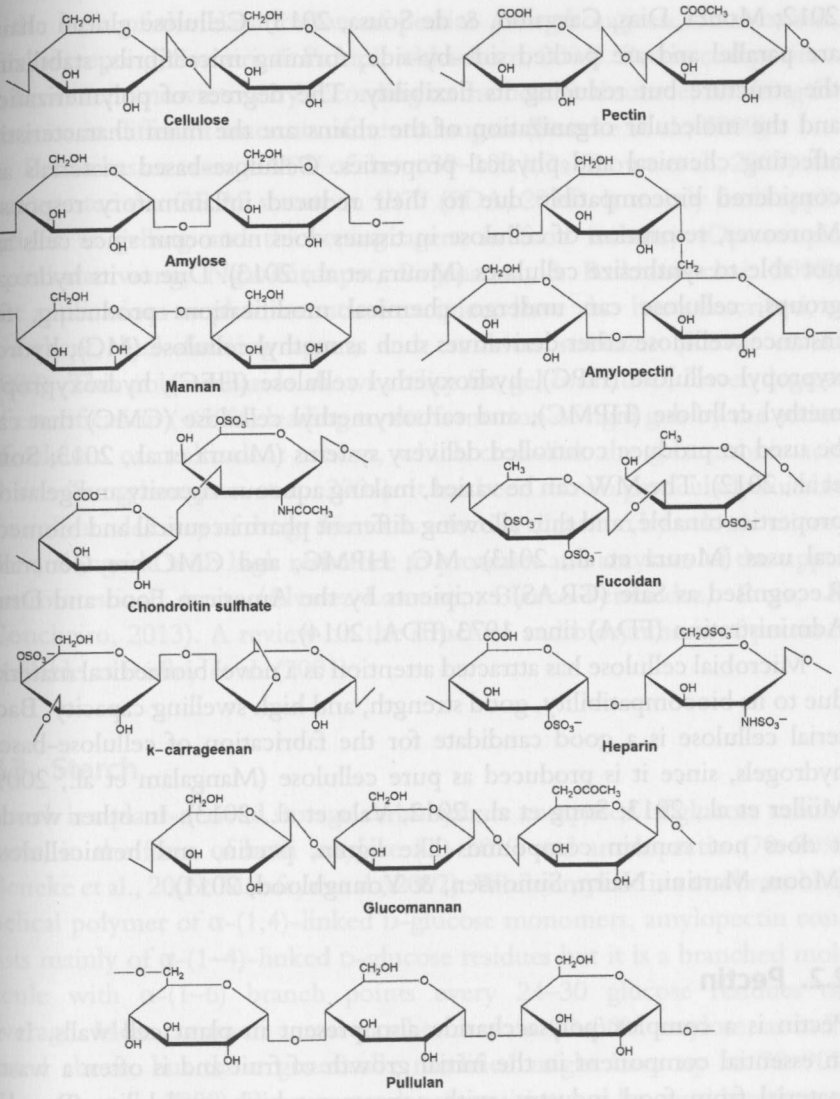


Figure 1 Chemical structure of polysaccharides.

and bacteria (Lavoine, Desloges, Dufresne, & Bras, 2012; Mangalam, Simonsen, & Benight, 2009). It is the most abundant polysaccharide available in the world, being thus readily accessible and relatively cheap (Ding et al., 2012; Padalkar et al., 2010; Song, Zhou, & Chen, 2012). The basic structure of cellulose consists of a linear chain of β-(1,4)-linked D-glucose units which are joined to form cellobiose repeating units (Ding et al.,

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2012; Moura, Dias, Carvalho, & de Sousa, 2013). Cellulose glucan chains are parallel and are packed side-by-side, forming microfibrils, stabilizing the structure but reducing its flexibility. The degrees of polymerization and the molecular organization of the chains are the main characteristics affecting chemical and physical properties. Cellulose-based materials are considered biocompatible due to their reduced inflammatory response. Moreover, resorption of cellulose in tissues does not occur since cells are not able to synthesize cellulases (Moura et al., 2013). Due to its hydroxyl groups, cellulose can undergo chemical modifications producing, for instance, cellulose ether derivatives such as methyl cellulose (MC), hydroxypropyl cellulose (HPC), hydroxyethyl cellulose (HEC), hydroxypropyl methyl cellulose (HPMC), and carboxymethyl cellulose (CMC) that can be used to produce controlled delivery systems (Moura et al., 2013; Song et al., 2012). The MW can be varied, making aqueous viscosity and gelation properties tunable, and thus allowing different pharmaceutical and biomedical uses (Moura et al., 2013). MC, HPMC, and CMC are Generally Recognised as Safe (GRAS) excipients by the American Food and Drug Administration (FDA) since 1973 (FDA, 2014).

Microbial cellulose has attracted attention as a novel biomedical material due to its biocompatibility, good strength, and high swelling capacity. Bacterial cellulose is a good candidate for the fabrication of cellulose-based hydrogels, since it is produced as pure cellulose (Mangalam et al., 2009; Müller et al., 2013; Song et al., 2012; Valo et al., 2013). In other words, it does not contain compounds like lignin, pectin, and hemicellulose (Moon, Martini, Nairn, Simonsen, & Youngblood, 2011).

2.2. Pectin

Pectin is a complex polysaccharide also present in plant cell walls. It is an essential component in the initial growth of fruit and is often a waste material from food industry, with consequent high availability (Beneke et al., 2009). Its basic structure is a linear chain of α-(1,4)-linked D-galacturonic acid units, presenting highly branched regions. Like all other galacturonic acid-based polysaccharides, pectin is rich in neutral sugars such as L-rhamnose, L-arabinose, D-galactose, D-xylose, and D-glucose (Beneke et al., 2009; Ridley, O'Neill, & Mohnen, 2001). Nevertheless, the uronic acids have carboxyl groups, providing a negative charge, some of which naturally presented as methyl esters (Rowe, Sheskey, & Owen, 2009). The degree of esterification (percentage of the esterified carboxyl groups),

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is the basis of the classification of pectins (Sungthongjeen, Sriamornsak, Pitaksuteepong, Somsiri, & Puttipipatkhachorn, 2004). Pectin composition is, however, known to vary according to the botanical source, resulting for instance in different amounts of neutral sugars (Beneke et al., 2009).

Pectin has an average MW of about 30–100 kDa (Rowe et al., 2009) and was granted the GRAS statute in 1977 (FDA, 2014). It mainly finds applications as gelling and thickening agent in food industry (Opanasopit, Apirakaramwong, Ngawhirunpat, Rojanarata, & Ruktanonchai, 2008), but applications as pharmaceutical excipient have also been described, as adsorbent, bulk-forming agent, and emulsion stabilizer (Rowe et al., 2009). This polysaccharide shows ability for gel formation, lower degrees of esterification (<50%) leading to the formation of rigid gels by the action of calcium or multivalent cations, which cross-link the galacturonic acid chains (Sungthongjeen et al., 2004). It has been shown particularly suitable for colonic delivery of drugs due to its selective digestion by colonic microflora, alongside with high resistance to proteases and amylases of the upper gastrointestinal tract (Alvarez-Lorenzo, Blanco-Fernandez, Puga, & Concheiro, 2013). A review on the structure and biosynthesis of pectin is available at Ridley et al. (2001).

2.3. Starch

Starch is a plant-derived storage carbohydrate composed of glucose monomers, in the form of both amylose (20–30%) and amylopectin (70–80%; Beneke et al., 2009; Malafaya et al., 2007). While amylose is a nonbranching helical polymer of α-(1,4)-linked D-glucose monomers, amylopectin consists mainly of α-(1–4)-linked D-glucose residues but it is a branched molecule with α-(1–6) branch points every 24–30 glucose residues on average. Most common cereal starches contain 20–30% amylose, as indicated above, but those genetically modified might have up to 70–90% (Beneke et al., 2009). Because starch composition can be considerably variable, depending for instance on the used source (potato, corn, rice, etc.), its MW is also very variable.

Starch is a GRAS excipient since 1979 (FDA, 2014) and is widely used in food and pharmaceutical industries, as thickener, binder, or diluent (Jobling, 2004; Rowe et al., 2009). It is also considered very interesting for biomedical applications, given its inherent biodegradability (it is degraded by amylases), overwhelming abundance and renewability. Additionally, its degradation products are oligosaccharides that can be readily metabolized to produce

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energy, thus not raising particular issues regarding host reaction to degradation products (Malafaya et al., 2007).

Native starch may not be suitable to produce controlled release drug carriers due to substantial swelling and rapid enzymatic degradation, which leads to very rapid drug release. This has led to the use of derivatives that are more resistant to enzymatic degradation, as well as cross-linking and formation of copolymers (Beneke et al., 2009).

2.4. Mannan

Mannan consists of D-mannose molecules linked together by β-(1–4) unions to form a polymer, usually with MW lower than 30 kDa. Mannan derivatives, so called heteromannans, are based on variations of β-mannan backbone, which might be interrupted with D-glucose to form glucomannan and/or branched with α-(1,6)-linked D-galactose to form galactomannan (Chauhan, Puri, Sharma, & Gupta, 2012; Pauly et al., 2013).

Linear chains of β-(1–4) mannan are found in the seed endosperms of certain plant species, like ivory nut and coffee bean (Chauhan et al., 2012; Hägglund et al., 2001; Wolfrom, Laver, & Patin, 1961). The biodegradability of the polymer is ensured by β-mannanase and β-mannosidase, thus providing its elimination (Chen et al., 1995; Dionísio & Grenha, 2012). Mannan is used mainly in food industry as a stabilizer and gelling agent (Pauly et al., 2013) and is being proposed in drug delivery, mainly to compose drug carriers aimed at targeted delivery (Sihorkar & Vyas, 2001; Yu, Liu, Liu, Zhang, & Xu, 2010).

2.5. Glucomannan

Glucomannan is a plant-derived polysaccharide belonging to the mannan family, with frequent application in food industry as thickener and emulsifier. It has a variety of sources, but the most common glucomannan is konjac glucomannan extracted from *Amorphophallus konjac*, known as konjac plant (Xiao, Gao, & Zhang, 2000). Irrespective of its origin, it is composed of β-(1–4)-linked D-mannose and D-glucose monomers, although different ratios of mannose/glucose can be found according to the source. Konjac glucomannan has a mannose/glucose molar ratio around 1.6:1 (Buchala, Franz, & Meier, 1974). The diverse types of glucomannan also vary in the acetylation degree, but the polymer typically presents 5–10% acetylation (Gao & Nishinari, 2004).

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The polymer is water soluble, but its high MW, reported to be up to 1.9×10^6 (Davé & McCarthy, 1997), leads to very viscous solutions. Glucomannan presents gelling properties and is also able to synergistically interact with other polymers, such as carrageenan and xanthan gum, an advantageous characteristic for drug delivery applications (Alonso-Sande, Teijeiro-Osorio, Remuñán-López, & Alonso, 2009).

A comprehensive review on glucomannan potential in drug delivery is available in Alonso-Sande et al. (2009).

2.6. Arabic gum

Arabic gum is also known as Acacia gum and is obtained from the exudates of *Acacia* trees (Beneke et al., 2009), being a GRAS excipient since 1973 (FDA, 2014). Its chemical composition is complex, consisting in a branched molecule with a backbone of (1,3)-linked β-D-galactopyranosyl units, with side chains composed of two to five (1,3)-linked β-D-galactopyranosyl units, joined to the main chain by (1,6)-linkages (Ali, Ziada, & Blunden, 2009). Both the main and the side chains contain other carbohydrates, such as L-arabinose, L-rhamnose, and glucuronic acid (Ali et al., 2009; Beneke et al., 2009). This combination of sugars results in a MW of about 240–580 kDa (Rowe et al., 2009).

As happens with many plant-derived polysaccharides, the chemical composition of arabic gum is reported to vary with its source, the age of the trees from which it is obtained, climatic conditions, and soil environment (Ali et al., 2009).

Arabic gum has wide industrial uses as a stabilizer, thickening agent, and emulsifier, mainly in the food industry (Ali et al., 2009), but it also has an application in pharmaceutical industry as emulsifying and suspending agent, as well as tablet binder (Rowe et al., 2009). A review on the properties and applications of this material is available at Verbeken, Dierckx, and Dewettinckk (2003).

2.7. Tragacanth gum

Tragacanth gum is a plant-derived molecule obtained from the stems and branches of Asiatic species of *Astragalus*, having a MW of about 840 kDa (Rowe et al., 2009; Verbeken et al., 2003). It is a branched and anionic carbohydrate composed of a complex mixture of polysaccharides, being thus very heterogeneous in its structure. It was affirmed as GRAS excipient within the USA in 1972 (FDA, 2014). The composition of the gum shows

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considerable variation according to the *Astragalus* species used as source. Additionally, even when a specific species is used, some variations might be observed deriving from different seasons of collection and also geographical issues (Verbeken et al., 2003), which is typical for natural polymers. Nevertheless, tragacanth gum is composed of two major fractions: one water soluble (tragacanthin) and another that is not soluble but instead water-swellable (bassorin; Ghorbani Gorji, Ghorbani Gorji, Mohammadifar, & Zargaraan, 2014; Rowe et al., 2009). Tragacanthin is composed of (1) tragacanthic acid, which contains residues of D-galacturonic acid, D-xylose, L-fucose, and D-galactose, and (2) an arabinogalactan, containing residues of L-arabinose, D-galactose, and D-galacturonic acid (Aspinall & Baillie, 1963; Fattahi, Sadrjavadi, et al., 2013). Different species of *Astragalus* have been reported to display different ratios of the two fractions, resulting in diverse physicochemical and rheological properties and, consequently, in specific applications and functionalities (Ghorbani Gorji et al., 2014). Its most frequent pharmaceutical application occurs as suspending and emulsifying agent (Rowe et al., 2009). A comprehensive review is available at Verbeken et al. (2003).

2.8. Carrageenan

Carrageenan is an anionic, high MW (well above 100 kDa) sulfated GRAS polysaccharide, obtained from certain species of red seaweed (Beneke et al., 2009; Necas & Bartosikova, 2013).

It is composed of alternate units of β-D-galactose and 3,6-anhydro-α-D-galactose, linked by α-(1,3) and β-(1,4) glycosidic unions. Depending on the method and the algae from which carrageenan is extracted, three main types of carrageenans can be obtained, kappa (κ), iota (ι), and lambda (λ), although several other types are also reported (Alvarez-Lorenzo et al., 2013; Necas & Bartosikova, 2013; Sankalia, Mashru, Sankalia, & Sutariya, 2006). The main difference among subtypes is structural and concerns the degree of substitution (DS) of the sulfate group, the position of the ester sulfate groups, and the presence or absence of anhydrogalactose units (Rowe et al., 2009; Sankalia et al., 2006). The former is one of the features most influencing the properties of carrageenan type (Necas & Bartosikova, 2013). All the described subtypes of carrageenan have between 22% and 35% sulfate groups and it is known that higher levels of ester sulfate result in lower solubility temperature and lower gel strength (Necas & Bartosikova, 2013).

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κ-Carrageenan has an ester sulfate content of 25–30% and an amount of anhydrogalactose units corresponding to 28–35%. ι-Carrageenan has similar ester sulfate content (28–30%) and about 25–30% anhydrogalactose units. In turn, λ-carrageenan has higher sulfate substitution (32–39%) and no anhydrogalactose content (Barbeyron, Michel, Potin, Henrissat, & Kloareg, 2000). From these three types, only κ- and ι-carrageenans evidence gel-forming ability, κ-carrageenan gels being more firm than those obtained with ι-carrageenan, which are more elastic and soft (Beneke et al., 2009; Bixler, 1994).

Carrageenan is susceptible to depolymerization through acid-catalyzed hydrolysis, as glycosidic linkages are hydrolyzed at pH below 3 (Necas & Bartosikova, 2013). Additionally, sulfatases are also involved on its metabolization (Ghosh, 2007). This polysaccharide is widely used in the food industry and also has applications in the pharmaceutical industry, as gel base, emulsifying and stabilizing agent (Rowe et al., 2009).

2.9. Fucoidan

Fucoidan is an anionic polysaccharide containing substantial amounts of L-fucose and sulfate ester groups. It is mainly extracted from brown seaweed, but different algae species can be used as source, such as *Fucus vesiculosus*, *Ascophyllum nodosum*, *Laminaria japonica*, and *Macrocystis pyrifera*, among others, resulting in different polymeric compositions (Berteau & Mulloy, 2003; Li, Lu, Wei, & Zhao, 2008). *F. vesiculosus* is the most common of the species being used and the one providing the simplest polymer of the whole group, with only L-fucose and sulfate units (Li et al., 2008). If other source is used, the structure might comprise units of D-mannose, D-galactose, D-xylose, L-rhamnose, and uronic acid, among others (Anastyuk, Shevchenko, Nazarenko, Dmitrenok, & Zvyagintseva, 2009; Huang & Lam, 2011; Li et al., 2008).

Structurally, the polysaccharide has a backbone of α-(1–3)-linked fucose units or is composed of repeating disaccharide units of α-(1–3)- and α-(1–4)-linked fucose residues with O-2 branches. Depending on the structure of the main chain, fucoidans may be sulfonated at O-4, O-2, or at both positions of fucose units. Moreover, some fucoidans are both sulfated and acetylated (Anastyuk et al., 2009). A wide range of MW has been reported, varying from 5–7 to several hundred kDa. Importantly, low MW fractions are suggested to be more biocompatible (Balboa, Conde, Moure, Falqué, & Domínguez, 2013).

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Fucoidan has been identified to exhibit several interesting pharmacological effects, such as antithrombotic, antitumoral, antiviral, and antiinflamatory activity (Balboa et al., 2013).

2.10. Pullulan

Pullulan is a neutral polymer composed of α-(1,6)-linked maltotriose residues, which in turn are composed of three glucose molecules connected to each other by an α-(1,4) glycosidic bond (Chiellini et al., 2008; Mizrahy & Peer, 2012; Namazi, Fathi, & Heydari, 2011). It is produced from starch by strains of the fungus *Aureobasidium pullulans*, which cultivation parameters strongly affect the resulting MW, usually ranging from 4.5×10^4 to 6×10^5 Da (Cheng, Demirci, & Catchmark, 2011).

Pullulan evidences interesting features regarding drug delivery applications, including water solubility, the production of relatively low viscous aqueous solutions, and adhesiveness (Mizrahy & Peer, 2012; Rekha & Chandra, 2007). Regarding the latter property, it is believed to have a role on the adhesion of the referred fungus to biological surfaces, such as leafs (Pouliot, Walton, Nolen-Parkhouse, Abu-Lail, & Camesano, 2005), indicating potential bioadhesive properties that might be relevant for drug delivery purposes. In fact, pullulan-based nanoparticles were reported to adhere to the nasal epithelium in a study of nasal vaccination (Nochi et al., 2010). More recently, pullulan was described to have a role on hepatocyte targeting, because of its ability to interact with asialoglycoprotein receptors present on these cells, leading to cell internalization (Li et al., 2014; Wang et al., 2013). This ability has increased the interest in the application of pullulan to compose drug carriers specifically targeted to the liver, which is a particularly interesting approach in hepatic cancer.

The polymer is also expected to be biodegradable, being exposed to the hydrolysis of glycoside bonds and the subsequent metabolism of glucose (Teramoto & Shibata, 2006).

2.11. Chondroitin sulfate

Chondroitin sulfate is one of the most physiologically important glycosaminoglycans, appearing in the human organism covalently linked to a protein core and, thereby, forming a proteoglycan (Malafaya et al., 2007) or as a receptor on cell or basement membranes (Alvarez-Lorenzo et al., 2013). It is a component of connective tissues (cartilage, for instance), being therefore of animal origin. The polysaccharide has a linear structure that consists of repeating disaccharide units of (1–3)-β-*N*-acetyl-D-galactosamine and

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(1–4)-β-glucuronic acid, which present sulfate, hydroxyl, and carboxylic acid functionalities, exhibiting a MW of approximately 463 Da. Each monosaccharide might have between zero and two sulfate groups (Alvarez-Lorenzo et al., 2013; Kaneiwa, Mizumoto, Sugahara, & Yamada, 2010), thus providing an overall negative charge. Given the human origin, no biocompatibility issues are expected and biodegradability is also ensured by either bacteria (Chourasia & Jain, 2004; Jain, Gupta, & Jain, 2007) or specific enzymes like hyaluronidases (Kaneiwa et al., 2010).

Considering its nature, the most frequent application of chondroitin sulfate has been in the production of tissue engineering scaffolds and associated materials (Malafaya et al., 2007).

2.12. Heparin

Heparin is an animal polysaccharide exhibiting a linear structure consisting of (1–4)-linked disaccharide repeating units of uronic acid (either D-glucuronic acid or L-iduronic acid) and D-glucosamine residues (Liang & Kiick, 2014; Mizrahy & Peer, 2012). The uronic acid typically comprises an iduronic acid/glucuronic acid proportion of 90–10% (Mizrahy & Peer, 2012). It has a highly anionic charge due to the prevalence of sulfate and carboxylate groups and is characterized by a heterogeneous sulfation pattern (Liang & Kiick, 2014; Mizrahy & Peer, 2012).

With an average MW of 12 kDa (Linhardt, 2003), heparin is produced exclusively by mast cells, where it has a function in storage of granular components such as histamine (Carlsson & Kjellén, 2012), and plays a key role in the regulation of varied biological activities, such as coagulation (Mizrahy & Peer, 2012). As referred above for chondroitin sulfate, considering the human origin of heparin, no biocompatibility or biodegradability issues are expected, heparanases ensuring its decomposition (Rönnberg, Melo, & Pejler, 2012). Heparin has been used as anticoagulant since 1935 (Liang & Kiick, 2014) and has recently been proposed for other applications in drug delivery such as, for instance, the treatment of asthma (Oyarzun-Ampuero, Brea, Loza, Torres, & Alonso, 2009).

3. POLYSACCHARIDE-BASED NANOPARTICLES

3.1. Cellulose

Positively charged cellulose derivatives have been reported as matrix of nanoparticles, mainly aimed at gene delivery. Two types of hydroxyethylcellulose, polyquaternium-4 (PQ-4) and polyquaternium-10

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(PQ-10), were complexed with pDNA, forming nanoparticles of approximately 200 nm. The interactions between pDNA and these cationic celluloses were shown to be stronger than those of pDNA/polyethyleneimine (PEI; Fayazpour et al., 2006). In this context, another work reported the higher ability of strongly quaternized polymers to bind DNA, in comparison with polymers exhibiting lower quaternization degree (Ding et al., 2012). Notwithstanding the strong interaction, the produced complexes exhibited much lower ability for cell transfection compared with PEI complexes, which was attributed to the neutral surface charge in the case of PQ-4/pDNA nanoparticles, as it certainly hinders cell interaction. As for PQ-10/pDNA nanoparticles, in spite of the positive charge, the strong binding of pDNA to PQ-10 possibly impedes DNA release in cytosol. According to the authors, tailoring the nature and extent of cationic side chains may further enhance DNA delivery ability (Fayazpour et al., 2006).

Quaternized cellulose (QC) derivatives with different MW and DS (Song, Sun, Zhang, Zhou, & Zhang, 2008) were also tested for gene delivery. Eight different QC derivatives were used to associate pGL-3 and pEGFP-C1 pDNA and the resulting QC/DNA complexes exhibited effective transfection in comparison to naked DNA. QCs with MW of about 8×10^4 g/mol and DS of about 0.6 displayed relatively higher transfection efficiencies, attributed to the intermediate stability. Furthermore, both QCs and QC/DNA complexes presented low cytotoxicity in human embryonic kidney transformed (293T) cells, wherein it increased slightly with MW and DS increase (Song et al., 2010). The authors further reported cellulose-based polyelectrolyte complex nanoparticles upon mixing negatively charged CMC and positively charged QC. CMC/QC nanoparticles displayed high bovine serum albumin (BSA) encapsulation efficiency, which released steadily from nanoparticles within the first 8 h in PBS pH 7.4. Nanoparticle size and zeta potential could be modulated by adjusting polymer concentrations. CMC/QC nanoparticles exhibited both low cytotoxicity in Caco-2 cells (nanoparticle concentration of 0.8 mg/ml), and significant BSA cellular uptake (Song et al., 2012).

3.2. Pectin

One sole work is available using pectin as nanoparticle matrix material for protein delivery. Pectin was applied to produce a gene carrier by ionic gelation with different cations like calcium, magnesium, and manganese. The association of DNA took place by means of electrostatic interaction as well.

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Only calcium and magnesium permitted the formation of spherical nanoparticles. Moreover, magnesium provided the smaller sizes, from 300 nm to approximately 1 μm, while calcium induced the formation of particles with size between 900 nm and 1 μm. Nevertheless, it was observed that calcium-based particles have a higher ability to associate DNA, possibly due to a better interaction between both the cation and the macromolecule. pDNA encoding the green fluorescent protein (GFP) was incubated with Huh7 cells (a human hepatocellular carcinoma cell line) and it was observed that both calcium- and magnesium-based pectin nanoparticles induced low levels of transfection, as well as low toxicity (Opanasopit et al., 2008). Nevertheless, magnesium nanoparticles permitted higher transfection, possibly due to the lower size and the positive charge of the nanoparticles. The low transfection obtained in general was possibly derived from the high size of nanoparticles, well above 100 nm, which resulted in low ability to reach the intracellular compartment, the first limitation to reach the nuclei.

3.3. Starch

Several starch derivatives were proposed as nanoparticle matrix with the aim of protein delivery. Starch acetate was proposed in the ambit of oral insulin delivery. The acetylation of starch provided a hydrophobic polymer, which was further pegylated. The resultant polymer self-aggregated to form micellar nanoparticles in presence of an aqueous solution of insulin. The nanoparticles have a size around 30–50 nm and negative zeta potential (−4 to −12 mV). The hydrophobic core of starch acetate was suggested to protect insulin from the gastric environment and a release of less than 20% was observed upon 2 h in simulated gastric fluid, as shown in Fig. 2. The nanoparticles were demonstrated to be strongly mucoadhesive as compared with chitosan, which is well known for its mucoadhesion characteristics. Furthermore, depending on the PEG MW, negligible cytotoxicity levels can be achieved in Caco-2 cells (Minimol et al., 2013).

Cationic starch was used to prepare nanoparticles using an emulsification technique. The nanoparticles were loaded with either BSA or bone morphogenetic protein (BMP)-4. Nanoparticle size was found to vary according to the used oil/water ratio, the amount of Span 80 (emulsifier) and starch, and also the amount of phosphoric trichloride ($POCl_3$), used as cross-linker. These variations permitted obtaining nanoparticles with sizes varying within 70 and 500 nm, approximately. Drug loading of both proteins varied within 17% and 27%, the lower value being registered for BMP-4. This protein

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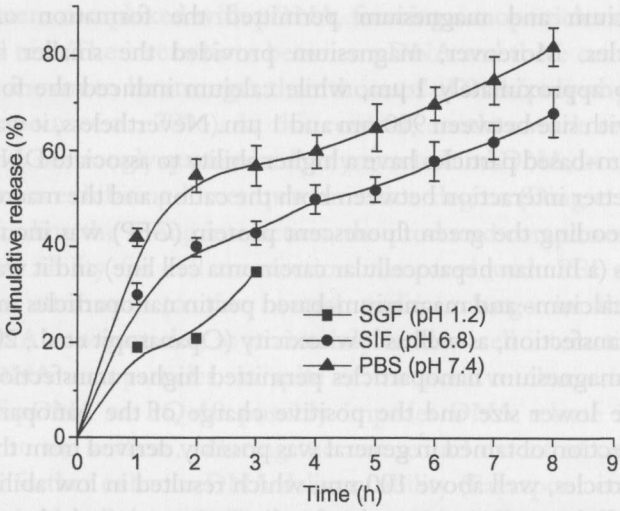


Figure 2 *In vitro* release profile of insulin from nanoparticles of SA-PEG1900 in SGF (pH 1.2), SIF (pH 6.8), and phosphate-buffered saline (pH 7.4). *Reprinted with permission from Minimol, Paul, and Sharma (2013).*

further evidenced a faster release in PBS pH 7.4 (80% in 4 h, in contrast with 58–67% of BSA-loaded formulations), which was justified by a predominant diffusional release of BMP-4, while that of BSA is mostly governed by electrostatic forces. Cytotoxicity was negligible in HeLa cells when these were exposed 24 h to a concentration of up to 0.3 mg nanoparticles/ml (Huang et al., 2013).

In a strategy of gene delivery, the copolymer hydroxyethyl starch (HES)-PEI was used to associate pDNA and form polyplexes. The idea supporting the approach was that HES could provide the controlled shielding/deshielding of polyplexes, due to its biodegradability. Deshielding was catalyzed enzymatically by amylase, which presence in a mixture of polyplexes and erythrocytes led to erythrocyte aggregation, an effect not observed in the absence of enzyme. The aggregation indicates deshielding, which exposes the positive charges of polyplexes and enables the electrostatic interaction with erythrocytes that results in aggregation. Using two distinct cell lines, Neuro2A (murine neuroblastoma) and Huh7 cells, it was demonstrated the ability of HES polyplexes to increase the transfection efficiency by 2 or 3 orders of magnitude when amylase was added to the culture medium (Noga et al., 2012).

In a different approach, nanoparticles comprised of modified starch (no specifications on the derivative were provided) were produced by a

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spray-drying technique using the Nano Spray Dryer from Buchi. This work was one of the first reporting the use of this equipment after its commercialization and was developed to show its potential to produce nanoparticles using different materials. The polysaccharide was sprayed at three different concentrations (0.1%, 1%, and 10%, w/v), resulting in the production of nanoparticles with sizes between 450 and 620 nm, all exhibiting a spherical shape (Li, Anton, Arpagaus, Belleteix, & Vandamme, 2010).

While in all the studies described above starch was used as nanoparticle matrix material, one study reports its application as coating of iron oxide magnetic nanoparticles. These were previously pegylated and functionalized with heparin, and further loaded with protamine. The interest in using starch as coating material relies on its biocompatibility and on the presence of several hydroxyl functional groups, which enable additional conjugations. The resulting nanoparticles (160 nm), demonstrated strong binding affinity and high protein loading, showing promise as carriers of cationic proteins and, thus, as a delivery platform (Zhang et al., 2013).

3.4. Mannan

Mannan residues are known to provide a preferential targeting to certain types of cells, including dendritic cells, macrophages, and epithelial M cells (Gupta, Gupta, & Gupta, 2009). Therefore, this material has been used to decorate drug delivery systems aimed at a specific targeting. Mannan-modified poly(ε-caprolactone)-polyethyleneglycol–poly(ε-caprolactone) nanoparticles encapsulating human basic fibroblast growth factor (bFGF) were demonstrated to induce higher immunization as compared with similar nanoparticles not modified with mannan. As observed in Fig. 3, upon subcutaneous administration to mice, IgG, IgG1, and IgG2a titers were higher, demonstrating the ability of mannan to mediate the specific targeting of dendritic cells, improving humoral immunity (Gou et al., 2008).

A modification with mannan was also used to potentiate the cell targeting ability of solid lipid nanoparticles (SLN), the system being applied in a gene delivery strategy. The nanoparticles were prepared at first and complexed with DNA. The mannan ligand consisted in a graft with phosphatidylethanolamine (PE). SLN–DNA complexes were then modified by adsorption of PE-mannan ligands. Mannan-modified complexes showed agglutination upon incubation with concanavalin A, thus demonstrating the existence of α-mannose residues at the surface of nanoparticles. No cytotoxicity was

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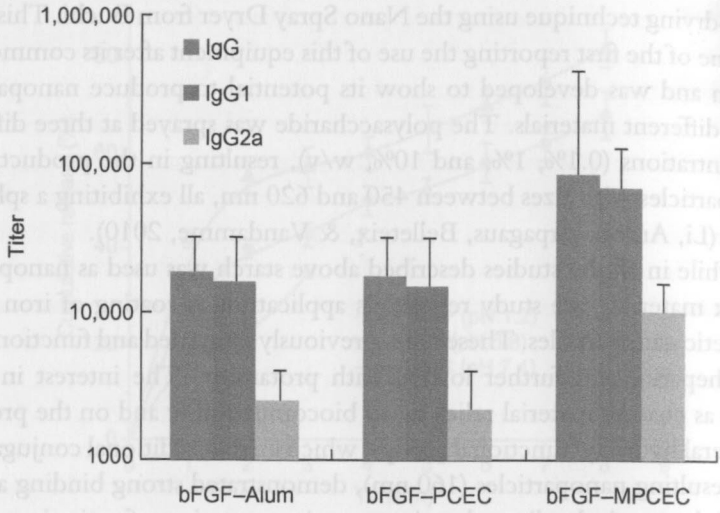


Figure 3 Serum total bFGF-special IgG, IgG1, and IgG2a titer created by bFGF-nanoparticles complex. Mice were immunized subcutaneously on week 0, 1, 2, and 3. The serum was collected on week 4. *Reprinted with permission from Gou et al. (2008).*

observed in RAW 264.7 cells (mouse leukemic monocyte macrophage cell line) and the mannan-modified nanoparticles produced higher gene expression than controls, including lipofectamine, especially *in vivo* in rats (Yu et al., 2010).

More recently, another work reported the preparation of dual-ligand-SLN, one of the ligands being PE-mannan and the other one transferrin-PEG-PE. This composition was aimed at providing carriers with the ability of targeting both cancer cells and macrophages in the liver, in a strategy of liver targeted gene delivery upon systemic administration. Evidencing absence of *in vitro* toxicity in HepG2 and Kupffer cells, the dual-modified vectors complexed with the pGFP (198 nm; gene loading of 89%) were tested in HepG2-induced tumor bearing mice. The transfection efficiency was determined to be approximately 50% higher than that obtained by unmodified SLN (Jing, Li, Liu, Wang, & Sui, 2013).

A different approach regarding gene delivery used mannan as emulsifying wax-nanoparticle coating for macrophage targeting. To demonstrate the system efficiency, nanoparticles (100 nm, positive surface charge) were incubated with J774E cells, a mannose receptor positive mouse macrophage cell line. When the incubation was performed at 37 °C (1 h), the uptake of mannan-coated nanoparticles was 50% higher than that of uncoated nanoparticles. An incubation at 4 °C for the same period resulted in twofold

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higher uptake for coated carriers. It was also shown that this increased uptake of coated nanoparticles was abolished by preincubating the cells with free mannan, confirming that uptake is receptor mediated (Cui, Hsu, & Mumper, 2003).

3.5. Glucomannan

Nanoparticles of carboxymethyl konjac glucomannan–chitosan were prepared by polyelectrolyte complexation. Sizes varied between 50 and 1200 nm, depending on the polymeric ratio, increasing when the concentration of polymers increases. Zeta potential was positive between +15 and +45 mV, being higher for the higher concentrations of chitosan. These physicochemical parameters were modified by changing the pH of the formulation and by adding NaCl to the formulation. The addition of either dilute HCl or NaOH results in size increase and zeta potential decrease. Similarly, adding NaCl to the formulation also leads to an increase in nanoparticle size. This is due to the destabilization caused by increased ionic strength due to electrostatic shielding. On the contrary, zeta potential decreases due to a phenomenon of double layer compression. BSA was successfully associated to these nanoparticles with efficiencies of 30–45%, depending on the specific ratios of chitosan/glucomannan (Du, Sun, et al., 2005). BSA registered a prolonged release up to 24 h in PBS pH 7.4 (80–90% BSA released). Higher concentration of chitosan resulted in slower release, which is explained by the formation of more entanglements between both polymers, resulting in stronger polymeric nets that retard BSA release. The release is much more influenced by the presence of chitosan than by the nanoparticle inner structure, and it was also observed a dependence on ionic strength (pH) and salt concentration (NaCl; Du, Zhang, et al., 2005).

Another work reported the preparation of glucomannan/chitosan nanoparticles, but this time a phosphorylated glucomannan was used as coating of preformed chitosan/tripolyphosphate nanoparticles. Nanoparticles evidenced sizes in the range of 170–300 nm and the zeta potential shifted from positive to negative upon glucomannan coating. The presence of glucomannan contributed to the stabilization of nanoparticles, preventing their aggregation in buffered medium (PBS pH 7.4). Nanoparticles only remained stable along time (up to 120 min) for the highest tested amount of glucomannan (chitosan/tripolyphosphate/glucomannan = 3/1/6.7 (w/w) in comparison with 6/1/23 (w/w)). Nanoparticles further demonstrated ability to associate the protein complex P1 (encapsulation

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efficiency around 25%), a mixture of polypeptides that upregulates natural and specific cell-mediated immune mechanisms (Cuña et al., 2006).

3.6. Arabic gum

Arabic gum–chitosan nanoparticles were proposed as insulin carriers for oral delivery. Taking benefit from its negative charge, arabic gum was complexed with the positively charged chitosan, resulting in nanoparticles of 150–200 nm and strong positive zeta potential, which facilitated the association of insulin (association efficiency of 25–35%). The concentration of the polymers was the parameter most affecting insulin association efficiency. The release was very rapid in pH 1.2 (approximately 90% insulin released in 1 h) and exhibited a more controlled pattern in pH 6.5 or 7.2 (30–60% in 4 h). The authors further suggested that the release resulted from several concomitant mechanisms, such as diffusion, dissolution, and relaxation of polymer chains (Avadi et al., 2010). In a subsequent work, the authors demonstrated a greater *ex vivo* absorption of insulin in rats when loaded in the nanoparticles as compared with the free protein (Avadi et al., 2011).

As described previously for starch, arabic gum nanoparticles were also produced with the Nano Spray Dryer. In this case, the polysaccharide was also sprayed at three different concentrations (0.1%, 1%, and 10%, w/v), resulting in spherical nanoparticles with a size of 350–600 nm (Li et al., 2010).

3.7. Tragacanth gum

The use of tragacanth gum in drug delivery is scarce (Kaffashi, Zandieh, & Khadiv-Parsi, 2006; Siahi, Barzegar-Jalali, Monajjemzadeh, Ghaffari, & Azarm, 2005) and its application as matrix material of nanoparticles aimed at protein delivery was reported only in one occasion, regarding the preparation of a gene delivery carrier. In that work, a water-soluble derivative of tragacanth gum (Fattahi, Petrini, et al., 2013) was complexed with an oppositely charged chitosan oligosaccharide to obtain nanoparticles. These were used to condense DNA, thus acting as gene carriers. After a screening of production conditions, a positively charged formulation (+30 mV) of 130 nm was selected. These chitosan/tragacanth gum nanoparticles evidenced better transfection efficiency in HeLa and HepG2 cells as compared with chitosan nanoparticles formulated in absence of tragacanth derivative; the difference

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being attributed to a weaker complexation of DNA, which facilitated its release and migration (Fattahi, Sadrjavadi, et al., 2013).

3.8. Carrageenan

Our group has recently presented several works on the preparation of nanoparticles based on chitosan and carrageenan (Grenha et al., 2010; Rodrigues, Cordeiro, Seijo, Remuñán-López, & Grenha, 2014; Rodrigues, Rosa da Costa, & Grenha, 2012). The polyelectrolyte complexation between polymers resulted in nanoparticles with a size around 600 nm and ability to associate ovalbumin. As seen in Fig. 4, the protein released according to an approximate zero order kinetics for 3 weeks in PBS pH 7.4 (Grenha et al., 2010).

Nevertheless, as nanoparticle size was out of the optimal interval for transmucosal delivery, tripolyphosphate was added to the formulation to act as cross-linker and decrease dimensions. The strategy was successful, nanoparticles evidencing sizes around 200 nm and strong positive zeta potential (around +50 mV), and the addition of tripolyphosphate was further demonstrated to endow the nanoparticles with increased storage stability (Rodrigues et al., 2012). Recently we have shown the ability of these nanoparticles to associate proteins with different characteristics (insulin and BSA) (Susana Rodrigues and Ana Grenha, unpublished results). An absence of toxicity was demonstrated in two cell lines representative of the respiratory epithelia (Calu-3 and A549 cells). This conclusion was drawn based on the results of a cytotoxicity test (MTT), the monitorization of transepithelial

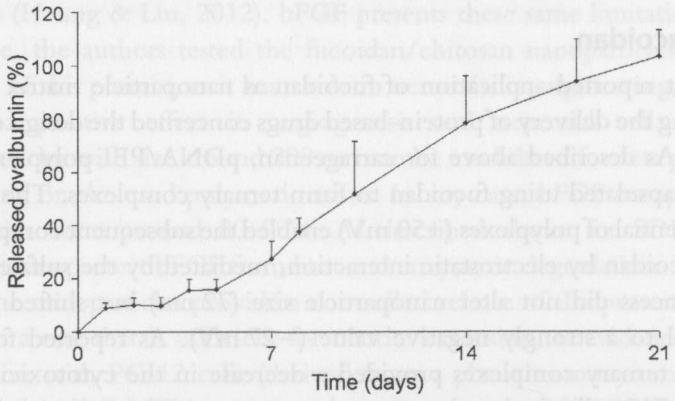


Figure 4 Ovalbumin release profile from chitosan–carrageenan (CS/CRG = 3.5/1) nanoparticles in PBS pH 7.4 at 37 °C (ovalbumin = 30% (w/w) based on chitosan; mean ± SD; $n \geq 3$). *Reprinted with permission from Grenha et al. (2010).*

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electrical resistance and the study of the generated inflammatory response (Rodrigues et al., 2014). Based on these results, the nanoparticles were proposed as candidates for nasal and pulmonary transmucosal delivery of proteins.

Chitosan–carrageenan nanoparticles obtained by the same method were applied to encapsulate recombinant human erythropoietin. Depending on the process conditions (concentration of polymers, polymer mass ratio, pH of reaction medium), nanoparticles with size from 200 up to 1000 nm and zeta potential of +40–55 mV were obtained. Response surface modeling was applied to optimize nanoparticle characteristics and an encapsulation efficiency of approximately 50% was achieved. The protein released according to a controlled pattern in PBS pH 7.4, releasing 50% in 2 weeks (Bulmer, Margaritis, & Xenocostas, 2012).

Carrageenan was also used to encapsulate previously formed pDNA/PEI polyplexes. Upon encapsulation, the new carriers were called ternary complexes. The positive zeta potential of the polyplexes (+50 mV) enabled an electrostatic interaction with the sulfate groups of carrageenan, resulting in a shift of the zeta potential to −36 mV and a final size of 92 nm. Possibly because of the newly exhibited negative zeta potential, the ternary complexes provided a decreased in the cytotoxicity effect on B16-F10 cells (skin melanoma cell line). They further demonstrated to prevent the agglutination with erythrocytes that was observed for the original polyplexes. However, carrageenan-based ternary complexes have shown very little capacity for cell uptake and transfection, thus limiting the interest in the use of these carriers for gene delivery purposes (Kurosaki et al., 2009).

3.9. Fucoidan

The first reported application of fucoidan as nanoparticle matrix material regarding the delivery of protein-based drugs concerned the design of a gene carrier. As described above for carrageenan, pDNA/PEI polyplexes were also encapsulated using fucoidan to form ternary complexes. The positive zeta potential of polyplexes (+50 mV) enabled the subsequent complexation with fucoidan by electrostatic interaction, mediated by the sulfate groups. This process did not alter nanoparticle size (72 nm) but shifted the zeta potential to a strongly negative value (−27 mV). As reported for carrageenan, ternary complexes provided a decrease in the cytotoxicity effect on B16-F10 cells, further demonstrating to prevent the agglutination with erythrocytes that was observed for the original polyplexes. However, the ternary complexes have shown very little capacity for cell uptake and

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transfection, limiting their interest in gene delivery applications (Kurosaki et al., 2009).

Another group studied the use of fucoidan–chitosan nanoparticles in tissue engineering applications, acting as carriers of stromal cell-derived factor 1 (SDF-1), which is an important chemokine in stem cell mobilization, further playing a critical role in the biological and physiological functions of mesenchymal stem cells (MSC). Chitosan/fucoidan, chitosan/tripolyphosphate, and chitosan/tripolyphosphate/fucoidan nanoparticles were prepared. All the formulations evidenced tunable physicochemical properties (size and zeta potential) and efficiently associated the protein (encapsulation efficiency between 60% and 100%). Although chitosan/tripolyphosphate/fucoidan nanoparticles were those associating the lower amount of protein (60–68%), they evidenced the most favorable release profile in PBS pH 7.4. A burst release of approximately 20% was observed for the first 12 h, while a controlled release was then obtained up to 7 days with an accumulated release of 30%. In turn, the other formulations enabled the release of significantly lower amounts of protein (less than 10%) in the same period. SDF-1 released from these nanoparticles also evidenced higher mitogenic activity as compared with free SDF-1. In fact, MSC showed a much more intense growth upon 1 or 3 days of incubation with SDF-1-loaded nanoparticles. Moreover, the migration of MSC in response to the controlled release of SDF-1 closely follows the kinetics of SDF-1 release from nanoparticles. The carriers further protected SDF-1 against proteolysis and heat treatment, providing the means to overcome some of the major drawbacks of this protein, which are the short half-life and easy enzyme degradation (Huang & Liu, 2012). bFGF presents these same limitations and, therefore, the authors tested the fucoidan/chitosan nanoparticles for the delivery of this protein, as it has a role in nerve tissue engineering, namely having a positive effect in angiogenesis and neuronal cell survival. Nanoparticles with size around 200 nm can be modulated for zeta potential depending on the used polymeric ratios. In any case, bFGF encapsulation efficiencies were around 80–90%. As described above for SDF-1, the nanoparticles protect bFGF from heat and enzymatic degradation. Furthermore, the nanoparticles provide a controlled release of the protein (4% in 24 h), in a rate that is sufficient for neurite extension, as evidenced upon incubation with PC-12 cells (derived from a pheochromocytoma of the rat adrenal medulla). This permits using a low amount of bFGF for neurite extension and also a low concentration of nanoparticles. In fact, a cytotoxicity test in this cell line evidenced the absence of toxicity of all the

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nanoparticle formulations (either negatively or positively charged) up to 72 h, which is possibly due to the low concentration of nanoparticles used in the study (125 ng/ml; Huang & Yang, 2013). Fucoidan/chitosan nanoparticles thus showed potential for both stem cell mobilization and nerve tissue engineering.

Finally, the most recent report involves the preparation of fucoidan/chitosan nanoparticles by means of electrostatic interaction, as reported in the other cases, but assisted by ultrasonic energy. From a total of nine tested conditions, only in one case submicron particles were obtained, displaying a size of 920 nm. BSA was easily encapsulated in the nanoparticles (encapsulation efficiency ranging between 46% and 89%) and released according to a controlled profile, reaching 80% cumulative release in 96 h (Liu et al., 2014). Although the association of the protein and the release profile is interesting, these nanoparticles present the great disadvantage of requiring the use of aggressive conditions that are not applied in a simple polyelectrolyte complexation. Additionally, notwithstanding the higher energy that was used, the nanoparticles are almost out of the size range to be classified as such, with the consequent loss of favorable properties, such as for instance the ability to have an intimate interaction with mucosal epithelia or tissues.

3.10. Pullulan

Pullulan has been applied as nanocarrier matrix material regarding varied objectives, including immunization, gene delivery, and also the design of imaging tools. Pullulan exhibits high flexibility in aqueous solutions and its chemical structure can be modified by hydrophobic molecules, such as cholesterol (Rekha & Sharma, 2011). Cholesterol-bearing pullulan self-aggregates in nanoparticles (Akiyoshi et al., 1998; Lee & Akiyoshi, 2004) which have been reported as protein carriers. Insulin was tested in this regard, producing stable complexes (1 week, room temperature) which provide protection to the protein, preventing enzymatic (α-chymotrypsin) and thermal (90 °C) denaturation. Although no significant difference was found on glycemic levels upon IV administration to rats, in comparison with free insulin, it was demonstrated that insulin retained better its biological activity when administered in encapsulated form (Akiyoshi et al., 1998). The referred nanoparticles, however, evidence low intracellular uptake due to the neutral charge of pullulan. To improve this ability, cholesterol–pullulan was functionalized with ethylenediamine, endowing the final nanoparticles with positively charged surface (nanoparticle size was <50 nm). Different

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amounts of amino groups were tested and fluorescently labeled BSA (FITC-BSA) was used as model protein. Upon incubation of protein-loaded nanoparticles with HeLa cells, the uptake of FITC-BSA increased over 6 h, in a direct proportion of the content of amino groups. Importantly, no cytotoxic effect was observed at final nanoparticle concentration of 100 nM for up to 24 h (Ayame, Morimoto, & Akiyoshi, 2008). The same nanoparticles were used to associate quantum dots (QDs) to provide the release of those as cell imaging tools. Effective intracellular delivery systems of QDs are needed to enable cell application without interference with cellular functions. The association of a protein to QDs and the encapsulation of this QD–protein complex in ethylenediamine-functionalized cholesterol–pullulan nanoparticles permitted cellular internalization of QDs. In fact, nanoparticles remained detectable inside rabbit MSC for at least 2 weeks, with little effect on their *in vitro* chondrogenic ability. These systems were thus proposed as candidate cell tracers in tissue engineering (Toita et al., 2008).

The cholesterol–pullulan nanoparticles were also proposed for nasal vaccination, using *Clostridium botulinum* type-A neurotoxin (BoHc/A) as antigen. Nanoparticles prepared with conventional cholesterol–pullulan demonstrated no improved *in vivo* results (mice) as compared with naked antigen and, thus, the authors synthesized a cationic derivative containing 15 amino groups per 100 glucose units. Upon successful *in vitro* (HeLa cells) internalization of nanoparticles prepared with this derivative, an *in vivo* imaging study showed the effective intranasal delivery of nanoparticles carrying $T^{18}FU$-labeled BoHc/A which remained continuously retained by the nasal mucosa, while in the absence of nanoparticles, the antigen disappeared from the nasal cavity within 6 h. IgA titers were only detected upon treatment with antigen-loaded nanoparticles and not with non-encapsulated antigen. Additionally, it was shown that no transition into the olfactory bulbs or brain occurs over a 2-day period post-administration, indicating the safety of the carrier regarding dissemination to the central nervous system (Nochi et al., 2010).

Cholesterol–pullulan-based nanoparticles were also proposed in the ambit of cancer immunization. The overexpression of HER2 oncoprotein has been related with several adenocarcinomas (breast, ovarian, lung, gastric) and HER2-based peptides are known to induce CD8+ cytotoxic T lymphocytes (CTLs). These cells are the major *in vivo* effector cells for the rejection of HER2+ tumors. As evidenced in Fig. 5, HER2-loaded cholesterol–pullulan nanoparticles demonstrated *in vivo* ability to induce

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CD8+ CTLs against HER2+ tumors and the complete rejection of tumors was observed when nanoparticles were administered early after tumor implantation in mice (up to day 3; Shiku et al., 2000).

Pullulan–PEI nanoparticles were proposed as gene carriers (size < 100 nm), benefiting from the positive charge attributed by PEI. Different degrees of PEI conjugation were tested, but only the lowest degree permitted acceptable HepG2 cell viability (above 80%). The conjugation of pullulan with PEI did not hinder the plasmid nuclear localization of the latter. Additionally, using pGFP and pGL3, the transfection efficiency provided by pullulan–PEI nanoparticles was similar to that obtained for PEI, but the former evidenced improved hemocompatibility (Rekha & Sharma, 2011).

Our group has also proposed pullulan nanoparticles as protein carriers for nasal and pulmonary transmucosal delivery. Upon synthesizing charged derivatives of pullulan, the aminated derivative was complexed with carrageenan and the sulfated derivative with chitosan. Positively charged nanoparticles (180–270 nm) were obtained which evidenced ability to associate BSA (34–44%). The protein showed a burst release of 30% in PBS pH 7.4, which maintains for up to 24 h. No cytotoxic effect was observed for nanoparticles upon exposure to Calu-3 cells (cell viability around 80–90%), a respiratory cell line representative of both pulmonary and nasal epithelia (Dionísio et al., 2013).

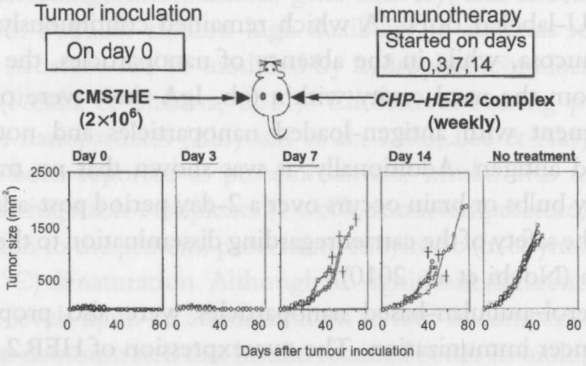


Figure 5 Therapeutic effects of CHP–HER2 vaccine. BALB/c mice were challenged with CMS7HE 2×10^6 sc and weekly received CHP–HER2 complex containing protein 20 μg starting on the day of challenge, or 3, 7, or 14 days after. Each group consisted of four mice. Each line represents a single mouse. *Reprinted with permission from Shiku et al. (2000).*

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3.11. Chondroitin sulfate

The pDNA/PEI polyplexes referred above in the sections addressing fucoidan and carrageenan were also tested with chondroitin sulfate to obtain different ternary complexes. The polyplexes interacted with the sulfate groups of the polymer, inducing a change of the zeta potential to −29 mV. Nanoparticle size is 77 nm. The high agglutination activity with erythrocytes and the cytotoxicity observed for pDNA/PEI complexes in B16-F10 cells were reversed upon formation of this ternary complex. Actually, from all the tested materials, which include not only carrageenan and fucoidan reported herein, but also alginate and hyaluronic acid, only the ternary complex formed with chondroitin showed comparable transgene efficiency to the pDNA/PEI complex. The uptake and gene expression of this ternary complex were significantly inhibited by hypothermia and the addition of chondroitin sulfate, suggesting that the carrier was taken by chondroitin sulfate-specific receptor-mediated energy-dependent process (Kurosaki et al., 2009).

The first report to chitosan/chondroitin sulfate nanoparticles prepared by a simple polyelectrolyte complexation appeared in 2011, proposing these nanoparticles as protein carriers. FITC-BSA was the model protein (association efficiency up to 90%) and the nanoparticles exhibited sizes between 150 and 500 nm, depending on the polymer ratios and the amount of protein initially added. The protein released progressively in PBS pH 7.4, reaching approximately 70% release in 90 min. Uptake studies performed in Caco-2 and HEK293 cells indicated the occurrence of nanoparticle endocytosis while, as shown in Fig. 6, cytotoxicity tests in Caco-2 cells evidenced absence of toxicity after 24 h incubation, up to a concentration of 0.1 mg/ml (Yeh et al., 2011). However, this might be considered a low concentration to test.

A similar work also used FITC-BSA as model protein and demonstrated that varying polymer mass ratios provided different sizes and zeta potentials, the latter suffering a complete shift from positive to negative when chondroitin sulfate was present in higher amount. Nanoparticles of 250 nm and around −20 mV were selected for subsequent assays. A cytotoxicity assay performed on Caco-2 cells led to viabilities around 95% after 72 h incubation with FITC-BSA-loaded nanoparticles, but again the maximum concentration tested was only 0.1 mg/ml, which is considerably lower than usual concentrations reported in other studies available on the literature. Transport studies performed in the same cell line revealed the transport of

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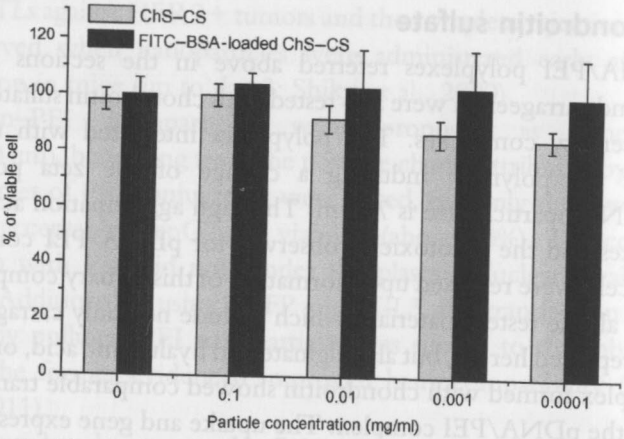


Figure 6 Cytotoxicity profile of ChS–CS and FITC–BSA-loaded ChS–CS NPs after 24 h incubation with Caco-2 cell. Percent viability of fibroblasts is expressed relative to control cells ($n = 6$). *Adapted with permission from Yeh, Cheng, Hu, Huang, and Young (2011).*

nanoparticles into the cells by a mechanism of endocytosis. It was further observed that positively charged nanoparticles had a more favorable transport than those exhibiting a negative charge (Hu, Chiang, Hong, & Yeh, 2012).

Similar nanoparticles were used for the delivery of platelet lysates in bone regenerative medicine. With a size around 200 nm, a first approach consisted in using BSA as model protein, which exhibited a controlled release in PBS pH 7.4 for up to 4 weeks (30–50% release, depending on formulation). The incubation with human adipose-derived stem cells (hASCs) evidenced an absence of toxicity and also demonstrated the ability of nanoparticles to be taken up by the cells. The latter effect is proportionally dependent on nanoparticle concentration and was maximized at 24 h, decreasing after that. Platelet lysates were successfully adsorbed onto the surface of nanoparticles. hASCs cultured in presence of these carriers showed enhanced cell viability, proliferation, and calcium deposition, suggesting increased osteogenic differentiation. The authors further proposed the possibility of using the carriers to replace conventional serum used in cell culture, as platelet lysate is as an autologous source of proteins, thereby permitting avoiding the risk of immunogenic responses (Santo, Gomes, Mano, & Reis, 2012).

Chondroitin sulfate was also used to form a nanocomplex (150–250 nm) with BMP-2. The nanocomplex was then incorporated in titanium-hydroxyapatite surface. Immobilized BMP-2 evidenced a controlled release

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up to 4 weeks. Titanium-hydroxyapatite surfaces provided faster proliferation of mouse osteoblast cells when the nanocomplex was present (Fig. 7). Additionally, the expression of osteocalcin and type I collagen, bone-specific markers, was significantly upregulated by the use of the nanocomplex. It was thus demonstrated that titanium surfaces can hold both osteoconductive apatite and osteoinductive growth factor and, therefore, the authors proposed the application of the system in dental and orthopedic implants (Bae et al., 2012).

3.12. Heparin

Heparin was associated to chitosan to produce nanoparticles by polyelectrolyte complexation. These were used as carriers of bFGF and were further incorporated inside a collagen membrane, in a strategy for tissue repair. Although bFGF was the target molecule for encapsulation and design of the system, human serum albumin (HSA) was also tested as model biomolecule, being used to perform the release assay. Nanoparticles displayed a size around 150 nm and a negative zeta potential (−39 mV). HSA exhibited a controlled release profile in PBS pH 7.4 for up to 20 days, in an assay performed directly from nanoparticles in absence of the membrane (Li et al., 2011). Although this is a work involving heparin nanoparticles, most of

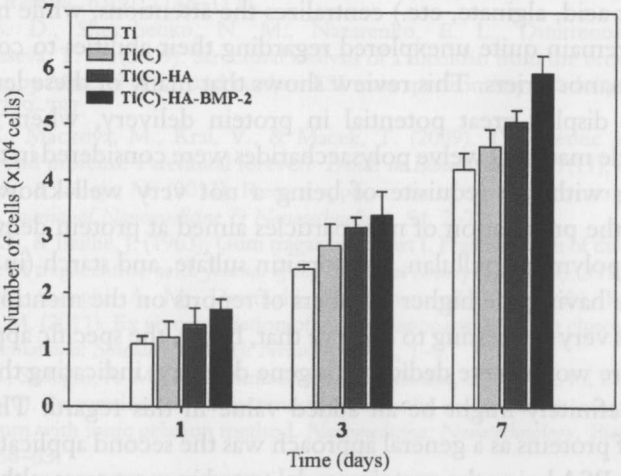


Figure 7 Cell proliferation on the different Ti substrates. As the number of cells was increasing with time, they were more proliferative on the Ti(C)-HA-BMP-2 than those on the other substrates. *Adapted with permission from Bae, Choi, Joung, Park, and Han (2012).*

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the observed effects are ascribed to the nanoparticle associated collagen membranes.

Only one other work reports the use of heparin in the nanoparticle matrix, as a functionalization of magnetic nanoparticles. Owing to heparin negative charge, it acts as a linking moiety for cationic molecules, as is the case of protamine. Heparin-functionalized magnetic nanoparticles were pegylated and coated with starch, being proposed as a protein delivery platform. Protamine was used as model protein and a loading of 22.9 μg protamine/mg of Fe was achieved. These nanoparticles have shown a half-life of 9.37 h, 37.5-fold higher than commercially available pegylated magnetic nanoparticles, and they further demonstrated a superior tumor targeting ability (Zhang et al., 2013).

4. CONCLUSIONS AND FUTURE PERSPECTIVES

In the past decades, excipients have been assuming relevant roles on pharmaceutical formulation, endowing drug carriers with specialized functions and leaving aside the traditional concept that describes excipients as inert substances that simply aid on the formulation preparation. Polysaccharides are one of the material classes finding a greater number of applications in drug delivery and they are particularly envisaged in nanoparticle formulations. Nevertheless, a restrict number of these polymers (chitosan, hyaluronic acid, alginate, etc.) centralizes the attentions, while many other polymers remain quite unexplored regarding their abilities to compose the matrix of nanocarriers. This review shows that many of these less explored substances display great potential in protein delivery, when integrating nanoparticle matrix. Twelve polysaccharides were considered in this review, complying with the requisite of being a not very well-known material regarding the preparation of nanoparticles aimed at protein delivery. From this set of polymers, pullulan, chondroitin sulfate, and starch (in this order) were those having the higher numbers of reports on the mentioned objective. It was very interesting to observe that, by far, the specific application to which more works were dedicated is gene delivery, indicating that polysaccharides definitely might be an added value in this regard. The systemic delivery of proteins as a general approach was the second application in line, in this case BSA being the protein model tested in most cases, although insulin was used in several works as well. Some works approached strategies on tissue engineering, regarding the delivery of growth factors. Other applications gathering attention were immunization and the specific targeting of

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cells (cancer cells, macrophages, dendritic cells, etc.) that might be mediated by polysaccharides like pullulan or mannan.

As a whole, it is suggested that several of the presented polysaccharides worth a deeper exploration, particularly when specific niches of application can be found, which is the case for instance of cell targeting. Nevertheless, it is obviously considered that substantial amount of research remains to be conducted to unveil the real potentialities.

ACKNOWLEDGMENTS

The authors acknowledge the Fundação para a Ciência e Tecnologia through research projects PTDC/SAU-FCF/100291/2008 and PTDC/DTP-FTO/0094/2012, and strategic project PEst-OE/EQB/LA0023/2014.

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