UAlg
UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

Universidade do Algarve

Departamento de Ciências Biomédicas e Medicina

Development of Locust Bean Gum Nanoparticles for Protein Delivery

Master dissertation in Biomedical Sciences

Pedro Raimundo

Supervisor: Professora Doutora Ana Margarida Grenha

Faro 2014

UAlg
UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

Universidade do Algarve

Departamento de Ciências Biomédicas e Medicina

Development of Locust Bean Gum Nanoparticles for Protein Delivery

Master dissertation in Biomedical Sciences

Pedro Raimundo

Supervisor: Professora Doutora Ana Margarida Grenha

Faro 2014

Development of Locust Bean Gum Nanoparticles for Protein Delivery

Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Pedro Raimundo

A Universidade do Algarve tem o direito, perpétuo e sem limites geográficos, de arquivar e publicitar este trabalho através de exemplares impressos reproduzidos em papel ou de forma digital, ou por qualquer outro meio conhecido ou que venha a ser inventado, de o divulgar através de repositórios científicos e de admitir a sua cópia e distribuição com objetivos educacionais ou de investigação, não comerciais, desde que seja dado crédito ao autor e editor.

Agradecimentos

Primeiramente queria agradecer à Professora Ana Grenha por me ter aceitado na sua equipa durante quase um ano e pela sua disponibilidade, dedicação, pela transmissão de conhecimentos e na orientação deste trabalho.

Quero agradecer ao Professor Luís Braz pela ajuda na parte prática com especial destaque na síntese dos polímeros, cultura de células e microfotografias TEM.

Quero agradecer à Susana Rodrigues pela sua disponibilidade e paciência em ensinar-me as técnicas essenciais à execução da parte prática deste trabalho e pela ajuda com software e pelo brainstorming de ideias que por vezes surgia.

Agradeço às minhas colegas Ana Dias, Carla Arruda, Filipa Pereira e Ludmylla Cunha pela camaradagem e boa disposição no laboratório ao longo deste ano de trabalho.

Agradeço há minha família pelo apoio importante que me deram não só na execução deste trabalho como em todo o meu percurso académico.

Agradeço há minha namorada Stephany Martins pelo grande apoio que me deu nos momentos mais difíceis e por apoiar e respeitar as minhas decisões.

Agradeço também a todos os amigos e colegas de universidade e residência por terem estado sempre presentes e pelas constantes conversas na tentativa de explicar o trabalho que estava a desenvolver no laboratório.

Resumo

O interesse pela goma de alfarroba tem vindo a crescer ao longo dos últimos tempos. Inicialmente na região do Algarve utilizava-se a goma de alfarroba para alimentar animais de pecuária, contudo, por conter um polissacárido com propriedades importantes em variados setores económicos, passou a ser utilizada na indústria de transformação alimentar como espessante e na indústria farmacêutica como agente gelificante. Além das aplicações referidas anteriormente, a goma de alfarroba pode ser utilizada para muitas mais finalidades. Por se tratar de um polissacárido biodegradável e potencialmente não tóxico, revela-se interessante explorar as suas capacidades como material formador de matriz de sistemas de administração de fármacos e, nomeadamente, de proteínas, por vias mucosas. São vários os sistemas de administração, mas as nanopartículas têm chamado particularmente a atenção no âmbito da administração de proteínas. Para que uma administração por vias mucosas seja efetiva, os sistemas nanoparticulados devem ter um tamanho entre 50 e 500 nm (para maximizar a sua interação), e um potencial zeta positivo (para poderem interagir com o ácido siálico que tem carga negativa e se encontra na superfície das mucosas). Além disso, outras características são requeridas para que se possam utilizar estes sistemas para administração proteica, nomeadamente uma eficácia de encapsulação razoável e ausência de toxicidade.

Este trabalho teve como objetivo o desenvolvimento de nanopartículas de goma de alfarroba, para entrega de proteínas com fins sistémicos, através das vias mucosas. De modo a ir de encontro ao objetivo, o desenvolvimento do sistema nanoparticulado de goma de alfarroba proposto teve por base a utilização de um método de complexação polieletrolítica, segundo o qual as nanopartículas se formam por interação eletrostática entre grupos químicos dos polímeros que apresentam cargas opostas. Dada a neutralidade deste polissacárido, um trabalho anterior do grupo de investigação consistiu numa síntese química para produção de derivados carregados de goma de alfarroba. Foi assim produzido um derivado aminado e um derivado sulfatado, os quais exibem cargas opostas, permitindo a formação das nanopartículas por complexação polieletrolítica. As nanopartículas resultantes foram caraterizadas relativamente ao seu tamanho, índice de polidispersão, potencial zeta, rendimento de produção, eficácia de encapsulação, bem como quanto ao perfil de citotoxicidade no âmbito de administração por vias mucosas.

Para preparar as nanopartículas foram utilizados três rácios de massa de goma de alfarroba aminada (A-LBG) e goma de alfarroba sulfatada (S-LBG), respetivamente 2/1, 1/1 e 1/2. Depois de obter as duas soluções respetivas, o S-LBG é adicionado ao A-LBG, sob agitação magnética moderada, em concentrações diferentes de modo a ter as três razões de massa. A formação das nanopartículas ocorre de forma imediata, mas as suspensões permanecem em agitação durante 10 minutos. Imediatamente após a mistura dos dois polímeros, é possível observar o efeito de Tyndall, que evidencia a formação de nanopartículas. As suspensões destas são centrifugadas a 16000x*g*, a 15 ºC durante 30 minutos, sobre uma camada de 10 μL de glicerol, a qual visa facilitar a ressuspensão das nanopartículas. Após a centrifugação, o sobrenadante é descartado e as nanopartículas são ressuspendidas em 100 μL de água para utilização nos ensaios subsequentes.

A formulação A-LBG/S-LBG = 2/1 apresentou um diâmetro de aproximadamente 560 nm, um índice de polidispersão de 0,432 e um potencial zeta de +43,5 mV. A formulação A-LBG/S-LBG = 1/2 apresentou um diâmetro de aproximadamente 359 nm, um índice de polidispersão de 0,381 e um potencial zeta de -49,7 mV. A formulação A-LBG/S-LBG = 1/1 precipitou no momento em que o S-LBG foi adicionado ao A-LBG e, como tal, esta formulação foi abandonada. Atendendo a estes valores, em termos de diâmetro, a formulação 2/1 apresenta um diâmetro acima do que seria ideal e a formulação 1/2 apresenta um diâmetro entre os valores pretendidos. Relativamente à polidispersão, ambas as formulações apresentam valores abaixo do limite máximo aceite (0,5) no entanto estão acima do valor ideal (0,2). É no entanto importante assinalar que para nanopartículas produzidas com polímeros naturais é muito difícil obter índices de polidispersão abaixo de 0,2. Analisando os valores de potencial zeta, a formulação 2/1 apresenta um potencial positivo, que é mais coincidente com os objetivos da administração transmucosa, e a formulação 1/2 apresenta um potencial negativo. Os valores de potencial observados estão de acordo com a carga exibida pelo polímero predominante em cada formulação. O potencial negativo, apesar de não ser ideal para o objetivo de administração transmucosal, não é desfavorável, porque pode ser utilizado para outro tipo de terapias.

Para o cálculo do rendimento de produção, a preparação das nanopartículas é feita como descrito anteriormente, com exceção do facto de não se utilizar glicerol, nem haver ressuspensão. Após a centrifugação o sobrenadante é descartado e o sedimento de nanopartículas é sujeito a congelação e posterior liofilização. A formulação 2/1 teve um

rendimento de produção de aproximadamente 17% e a formulação 1/2 de 30%, os quais são considerados baixos.

Para testar a capacidade das nanopartículas como transportadores de proteínas, a insulina foi selecionada como proteína modelo. Dado o seu ponto isoelétrico (pI), que é de 5.3, a proteína não dissolve em água. Para a sua solubilização usam-se o NaOH ou o HCl, ambos à concentração de 0,01 M, ficando a proteína com uma carga negativa ou positiva, respetivamente. Na abordagem inicial, tentou-se fazer a associação de uma quantidade de insulina equivalente a 30% do total da massa de polímeros utilizados na preparação das nanopartículas, mas não houve sucesso, já que se verificava precipitação. Após otimização do processo, assumiu-se a quantidade de insulina correspondente a 10% da massa do polímero que contribui com maior massa na preparação de cada formulação. Como a formulação A-LBG/S-LBG = 2/1 tinha mais quantidade de polímero positivo (conferindo um potencial zeta positivo), é maior o número de grupos carregados positivamente que estão disponíveis para interação com outras moléculas, em comparação com os grupos carregados negativamente. Assim, para esta formulação a insulina foi dissolvida em NaOH para assumir uma carga negativa e ter desta forma maiores probabilidades de interação e, consequentemente, de associação às nanopartículas. Após solubilização, a insulina foi adicionada à S-LBG, que tem igualmente carga negativa, evitando-se a interação eletrostática entre ambos os polímeros. Esta solução mista foi posteriormente adicionada à solução de A-LBG para formação das nanopartículas, havendo uma competição entre a insulina e a S-LBG pelos grupos carregados positivamente da A-LBG. Ao contrário, a formulação A-LBG/S-LBG = 1/2 tinha mais polímero de S-LBG, que tem carga negativa, o que proporciona em princípio maior capacidade de interação com grupos carregados positivamente. Neste caso, então, a estratégia consistiu em atribuir à insulina um predomínio de cargas positivas no momento da preparação das nanopartículas. Para isso, a proteína foi dissolvida em HCl e adicionada ao A-LBG, antes da adição desta mistura à S-LBG.

A eficácia de encapsulação de insulina observada foi de 15% para ambas as formulações, um valor considerado reduzido. Com vista a aumentar a eficácia de encapsulação, foi feita uma otimização que teve por base a hipótese de alterar o pH do meio da insulina de forma a ficar próximo do seu ponto isoelétrico. A literatura disponibiliza pelo menos um trabalho que mostra que há uma maior adsorção das proteínas aos polímeros quando estas se encontram em meios com pH perto do seu ponto isoelétrico. No valor de pH

equivalente ao ponto isoelétrico, a insulina ficará com um equilíbrio de cargas positivas e negativas e deixará livres as cargas dos polímeros, para interagirem entre si, maximizando a sua interação e a insulina ficará adsorvida aos polímeros na matriz das nanopartículas formadas. De forma a testar a hipótese anterior, para a formulação 2/1, a solução mãe de insulina foi preparada em NaOH, mas na preparação das diluições de A-LBG e S-LBG a partir das correspondentes soluções mãe, a água utilizada nas diluições foi substituída por tampão citrato (pH 5,0) por ter um pH próximo do ponto isoelétrico da insulina, fazendo com que esta exibisse um equilíbrio de cargas negativas e positivas após solubilização. Para a formulação 1/2 a insulina foi dissolvida em HCl e depois foi inicialmente realizado o mesmo procedimento com o tampão citrato, como para a formulação 2/1, mas ocorria precipitação. A otimização para a formulação 1/2 foi então realizada substituindo a água da solução S-LBG por uma solução de HCl a 0,1 M. As otimizações que foram operadas acolheram sucesso, tendo conduzido a um aumento das eficácias de encapsulação, na formulação 2/1 de 15% para 22% e na formulação 1/2 de 15% para 96%.

As nanopartículas carregadas com insulina foram caraterizadas quanto às propriedades físico-químicas. Para a formulação 2/1 registou-se um diâmetro de 740 nm (PDI de 0,389) e um potencial zeta de +22,8 mV. Para a formulação 1/2 o diâmetro foi de 400 nm (PDI de 0,404) e o potencial zeta -33,6 mV. No entanto, na formulação 1/2 observaram-se alguns agregados de difícil ressuspensão, mesmo após alteração da quantidade de glicerol utilizada.

Dado o objetivo de aplicação das nanopartículas desenvolvidas na administração por vias mucosas, considerou-se relevante avaliar a toxicidade das formulações. Realizou-se assim um ensaio de citotoxicidade em duas linhas celulares epiteliais, Caco-2 e A549. A primeira linha representa o epitélio intestinal, enquanto a segunda representa o epitélio pulmonar e, mais especificamente, alveolar. As concentrações testadas foram de 0,1 mg/mL, 0,5 mg/mL e 1 mg/mL para ambas as formulações. Os tempos de incubação das células com as nanopartículas foram de 3 horas e 24 horas para ambas as formulações, e linhas celulares, bem como para todas as concentrações. Em ambas as linhas celulares a viabilidade ficou acima de 70% para as duas formulações, indicando que as nanopartículas possuem baixa citotoxicidade.

Em conclusão, os derivados de LBG sulfatado e aminado permitem a formulação de nanopartículas com capacidade para associar insulina. No entanto, a formulação 2/1

apresenta um tamanho inadequado para o objetivo de administração por vias mucosas, além de baixo rendimento de produção e eficácia de encapsulação. A formulação 1/2 apresenta um tamanho aceitável para o objetivo descrito, bem como uma eficácia de encapsulação muito significativa. No entanto, o rendimento da formulação é algo baixo e o seu potencial zeta negativo, possivelmente registando uma menor propensão para interação com a superfície epitelial em comparação com uma formulação de carga positiva. Este tipo de nanopartículas, pode ser utilizado em superfícies com carga positiva que requeiram nanopartículas com potencial zeta negativo, ou para encapsular moléculas de carga positiva, maximizando a sua interação.

Palavras-chave: administração de proteínas, complexação polieletrolítica, goma de alfarroba, nanopartículas, polissacáridos

Abstract

The interest of Locust Bean Gum (LBG) for biomedical applications have been increasing in the last few years. Due to its biodegradability and non-toxic characteristics, LBG became an interesting material regarding the production of drug and protein delivery systems. Our aim was to develop a delivery system based on LBG nanoparticles for mucosal protein delivery. Since LBG displays no charged groups, aminated LBG (A-LBG) and sulphated LBG (S-LBG) were synthetized in our laboratory in order to allow the use of a method of polyelectrolyte complexation for the production of the carriers. Using this method enabled forming LBG nanoparticles with distinct properties depending on the weight ratio. A-LBG/S-LBG nanoparticles with three different mass ratios were prepared, namely, 2/1, 1/1 and 1/2. Mucosal delivery of drugs is known to be potentiated by the use of nanoparticles with sizes varying between 50 and 500 nm, in order to maximize the interaction with the epithelium. Moreover, nanoparticles should have positive zeta potential to interact with the negative charges of syalic acid residues present on epithelial surfaces.

In this work, LBG nanoparticles were characterised in terms of size, polidispersion index (PDI), zeta potential (ZP) and production yield (PY). The ability of the carriers to associate the model protein insulin was determined and cell viability tests were also performed to obtain a first indication on the nanoparticle biocompatibility profile. Formulation 1/1 was rejected due to precipitation, the work thus focusing on formulations 2/1 and 1/2. According to the initial objectives, formulation 2/1 showed excessively high size, and low association efficiency (AE, 22%). In turn, formulation 1/2 showed a suitable size and high AE (96%), but it was difficult to ressuspend. Regarding the cytotoxic profile, in a general manner both formulations of LBG nanoparticles showed low cytotoxicity.

In conclusion, after the optimizations, the formulation that appears the most suitable for protein delivery is A-LBG/S-LBG = 1/2, in spite of its negative zeta potential, which does not favour the interaction with epithelial surfaces.

Keywords: locust bean gum, nanoparticles, polysaccharide, polyelectrolyte complexation, protein delivery

List of Abbreviations

AE – association efficiency

A-LBG - aminated locust bean gum

BCA – bicinchoninic acid

DMSO – dimethyl sulfoxide

FBS – foetal bovine serum

G - galactose

LBG - locust bean gum

LC – loading capacity

M - mannose

MTT - Thiazolyl blue tatrazolium bromide

NP's – nanoparticles

PBS – Phosphate buffer solution

PDI - Polydispersion index

PY – production yield

S-LBG - sulphated locust bean gum

SD – standard deviation

SEM – standard error of the mean

SDS – sodium dodecyl sulphate

ZP - zeta potential

Index of contents

Index of figures

Index of tables

1. Introduction

1.1. Nanotechnology applied to biomedicine

In the last few years, the interest in nanotechnology has increased considerably due its potential towards many applications such as informatics, electronics, pharmaceutics and biomedicine, among others. The prefix *nano* refers to a scale in the order of $1x10^{-9}$ m, more precisely between 1-1000 nm. Nanotechnology is now being explored in more depth in many fields and namely in the biomedical area. The creation of nanochips to monitor blood chemistry and the nanocarriers for drug delivery, are valuable and representative examples. Nanotechnologies are very useful in drug delivery because the encapsulation of a drug in a carrier designed at the nanoscale may increase its bioavailability and improve its release profile. These are prominent aspects, as they might reduce the amount of administered drug and the number of needed administrations, thereby reducing the side effects [1].

1.2. Nanoparticles for protein delivery

Designing therapeutic carriers for proteins is a challenging task. Parenteral route is the most commonly employed method of administration, however, requirement of frequent injections due to short *in vivo* half-life results in poor patient compliance. Attending to the previous issues become an urgent scientific challenge being the transmucosal administration the first option for protein systemic delivery [2].

Nanoparticles have been proposed as suitable protein carriers, overcoming many of the limitations of these macromolecules. Proteins show physicochemical characteristics that make them susceptible to degradation and limit their absorption due the high molecular weight and, sometimes, hydrophilicity [3, 4]. In the present work, the chosen protein model was insulin. Like other peptides, insulin shows a poor physical and chemical stability and a relatively short plasma half-time [5]. Per oral bioavailability of insulin is relatively low mainly due to high proteolytic activity in the gut and low permeability of the intestinal epithelium as shown in figure 1.1 [6]. To overcome these barriers, several strategies are underway for peroral administration of insulin, those strategies including the addition of enzyme inhibitors and/ or permeation enhancers, chemical modification, cell penetration peptides, vitamin B12 or cyclodextrin conjugation, polymeric carriers, liposomes or a colon targeting of the drug delivery system where the enzymatic activity is relatively low.

Our aim is to use nanoparticulate systems to avoid the previous issues. A promising strategy is the use of multifunctional polymers exhibiting permeation enhancing and mucoadhesive properties [7].

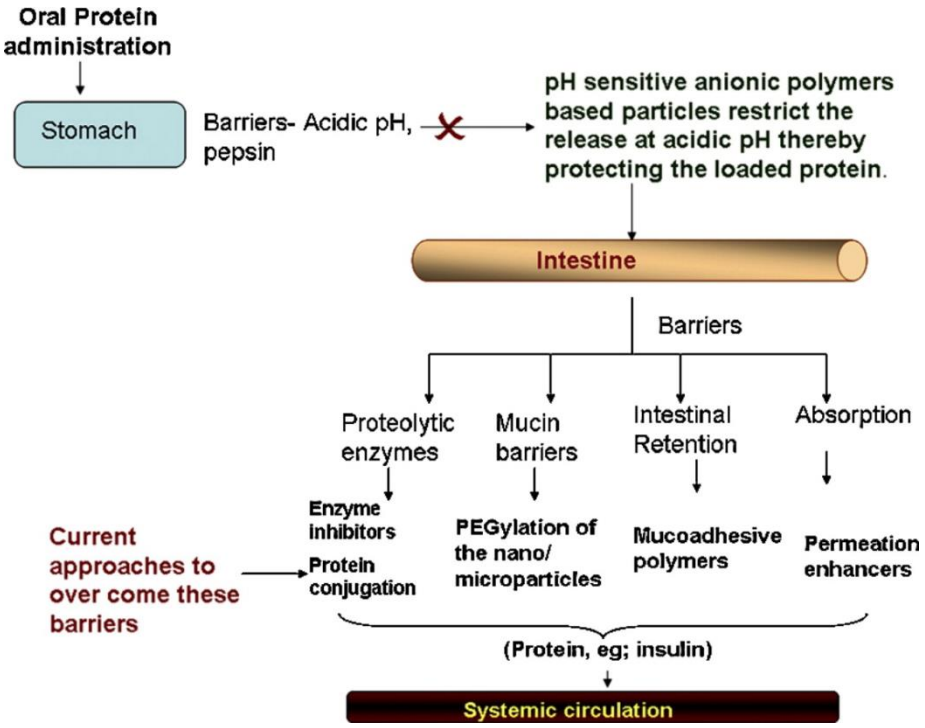

Figure 1.1: Schematic representation of barriers to overcome in insulin delivery by oral route [6].

Previous studies have shown that nanoparticles potentiate the improvement of drug pharmacokinetic profile, providing their stabilisation and, in some cases, permitting controlled release and enhancing drug absorption [8]. Furthermore, the high surface-to-volume ratio displayed by nanoparticles increases the drug loading capacity [9]. Scientists have demonstrated that nanoparticle contact with epithelial surfaces is maximised when their size is between 50 and 500 nm [10, 11], but also when the carriers exhibit a strongly positive zeta potential [12].

1.2.1. Non-parenteral routes of administration

Therapeutic molecules may enter the body via oral ingestion, inhalation, dermal penetration and intravascular injection, among others, being thus distributed through the body and reaching the various organs. Figure 1.2 shows the advantages and disadvantages of each route of administration [13].

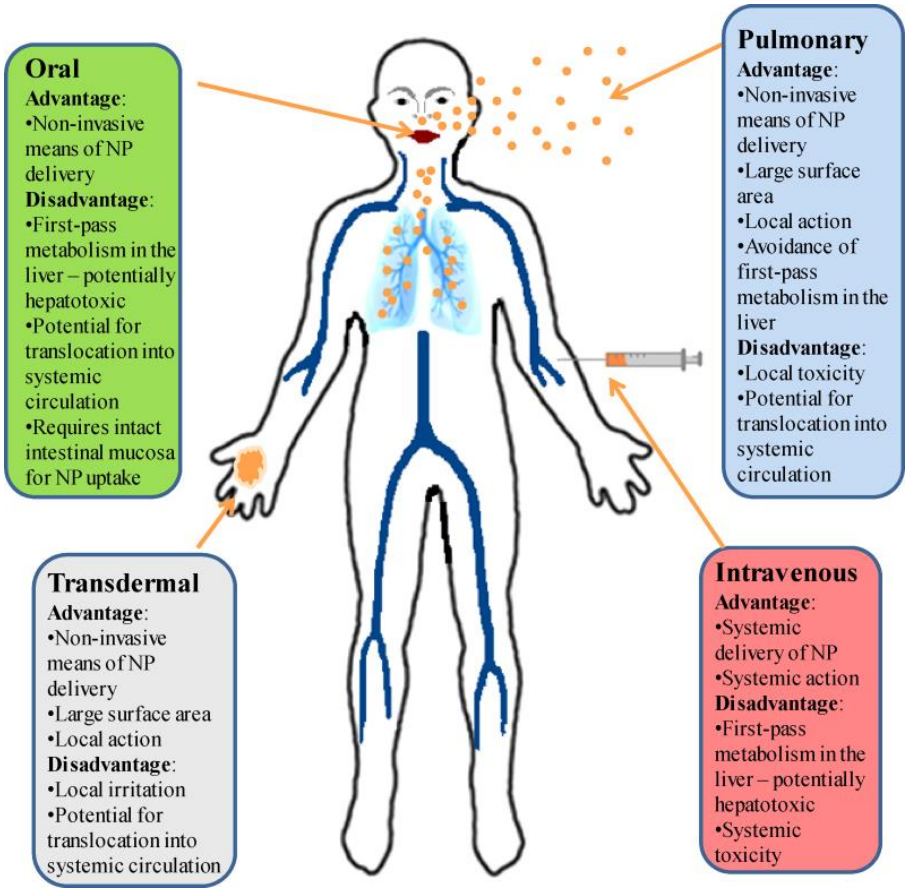

Figure 1.2: Routes of administration of nanoparticles: advantages and disadvantages [9]

Pulmonary, oral and transdermal routes are non-invasive. Transdermal route shows limitations for translocation of the nanoparticles from the dermal surface to the systemic circulation. According to the previous figure, the most suitable routes for protein delivery to the systemic circulation appear to be the pulmonary and oral due to their advantages and disadvantages regarding to the other routes. Nevertheless, other non-parenteral routes are suitable for protein delivery as ocular and nasal [2].

1.3. Polymeric materials as carrier matrix

Different classes of materials are available that permit preparing nanoparticulates, such as polymers, lipids, metals and so on. Nevertheless, polymeric nanomaterial-based therapeutics have been increasingly used in biomedicine, namely in areas such as tissue engineering and drug delivery. In the latter case, cancer, diabetes, and neurodegenerative diseases are examples of diseases that might find great improvements with the application of nanotechnologies. In this context, the main advantages of polymers over other materials for nanomedicine include increased functionality, design flexibility, improved processability and, in some cases, biocompatibility [14].

Polymers can be obtained from different sources. The majority of known polymers are derived from petroleum, which represents a huge environmental problem. Additionally, petroleum sources are finite. Consequently, the use of polymers from other sources, eventually more environment-friendly, became necessary. Polymers from renewable resources can be divided into three major groups, as shown in figure 1.3: natural polymers such as starch and proteins, synthetic polymers from natural monomers such as polylatic acid, and polymers from microbial fermentation such as polyhidroxybutirate [15]. Polymer scientists have performed extensive research in the development of biodegradable polymers. In turn, biopolymer research has found several applications in the area of biomedical science. Despite the apparent proliferation of these materials in the biomedical science, the science and technology of biopolymers is still in its early stages of development. Great opportunities exist and will continue to exist for the engagement of biopolymers in every facet of medical science through intensive research and development [16].

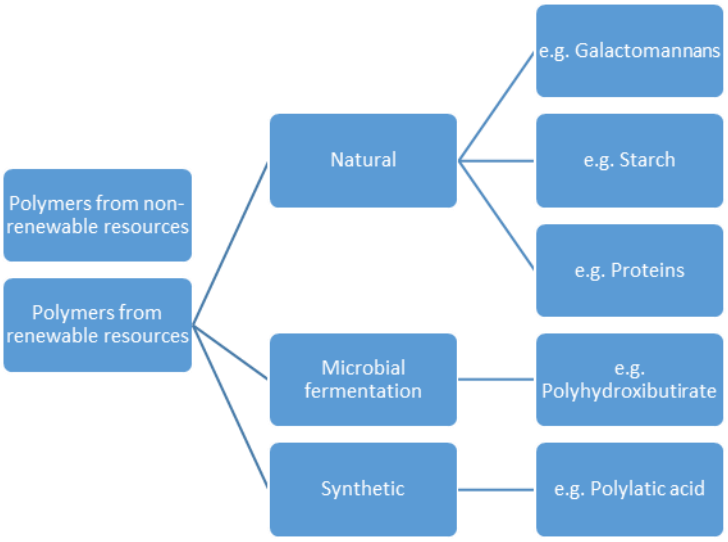

Figure 1.3: Polymers from renewable and non-renewable resources [15].

Natural and synthetic polymers have been used in biomedicine, in pharmaceutical formulations, imaging, drug delivery and targeting, prosthetics and tissue engineering. Synthetic polymers are often used, but most of them lack bioactivity and biocompatibility. Natural polymers have a better profile regarding these features. According to their structure, natural polymers can be ordered into three main classes: polysaccharides; polypeptides and polynucleotides [17]. In the next section a more detailed description of polysaccharides is provided.

1.3.1. Polysaccharides

Polysaccharides can be obtained from different sources: vegetal (i.e. starch), animal (i.e. chitosan) and microbial (i.e. dextran and gellan). These macromolecules present several advantages in therapeutic uses in comparison with the synthetic polymers. Some polysaccharides are very similar with glycosaminoglycans (GAGs) such chondroitin sulphate, dermatan sulphate, keratin sulphate, heparin, heparin sulphate and hyaluroran. The advantage of the chemical similarity of some polysaccharides with heparin, is the fact that those polysaccharides had potential hemocompatibility properties. Another relevant advantage of the polysaccharides are their propensity to be non-toxic and biocompatible and, with some exceptions, their low cost in comparison with other biopolymers like, for example, collagen [18].

Polysaccharides are a class of biopolymers constituted of simple sugar monomers [19]. The monomers are linked together by O-glycosidic bonds that can be made to any of the

hydroxyl groups of a monosaccharide, giving polysaccharides the ability to form both linear and branched polymers. Differences in the monosaccharide composition, chain shapes and molecular weight are important to understand their physical properties including solubility, gelation and surface properties[20].

Owing to the above mentioned properties, polysaccharides are widely used in several fields such as food and cosmetic industries, biomedicine and pharmaceutics, assuming relevant roles that include thickening, gelling, emulsifying, hydrating and suspending actions [21].

1.3.2. Locust Bean Gum as nanoparticle matrix material

1.3.2.1. General features

Locust Bean Gum (LBG) is a neutral polysaccharide composed of mannose and galactose monomers, thus belonging to the category of galactomannans. Besides *Ceratonia siliqua*, other plants might be used as sources of galactomannans, such as *Cyamopsis tetragonoloba* (guar gum) and *Caesalpinia spinosa* (tara gum) [22]. The polysaccharide is obtained from the crush of the endosperm of seeds, which represent 10% of the weight of the fruit of carob tree (*Ceratonia siliqua L.*), has a powdered appearance and assumes a white to yellowish white colour. The seeds have a composition of 80% galactomannan, the remaining 20% are proteins and impurities [23, 24]. The protein content of LBG is represented by approximately 32% albumin and globulin and the glutelin content is approximately 68% [25]. Crude galactomannan can be purified in order to eliminate proteins and other impurities isolating the polysaccharide. The purification process could include one of the procedures: enzymatic and alkaline hydrolysis, precipitation with ethanol or isopropanol, and purification by methanol, or by copper or barium complexes. The performance of purification procedures has shown to result in higher mannose/galactose ratio and decreased amount of proteins and impurities [22].

The Carob tree is very abundant in the Mediterranean region, but it can also be found in North Africa, South America and Asia. The literature provides other expressions referring to locust bean gum, such as carob bean gum, carob seed gum, carob flour and ceratonia [26].

LBG is largely used as a thickener, stabilizer, emulsifier and gelling agent in food and cosmetic industries [27]. The use of LBG in drug delivery is already reported, namely associated with formulations designed for colonic delivery, thus benefiting from the ability of colonic microflora to degrade LBG [28, 29]. Other applications involve topical, ocular and buccal delivery systems. The large majority of formulations are based on tablets, but some hydrogels and multiparticulate systems are described in the literature [22]. LBG use as tablet matrix is related with the fact that polysaccharides are generally considered to play important role in drug release mechanisms from matrixes [30]. It is observed, in most cases, that LBG associated with another polymer, affords an improved effect in controlled drug release systems.

1.3.2.2. Chemical structure, physicochemical properties and enzymatic degradation

As mentioned above, LBG is a galactomannan, evidencing a chemical structure consisting of a (1-4)–linked β-D-mannose backbone with (1-6)-linked side chains of α-D-galactose [21, 26] (figure 1.4). Mannose (M) and galactose (G) are both neutral monomers, thus attributing neutrality to LBG. This polysaccharide has little change in viscosity and solubility in a wide pH range (between 3-11) [31]. Galactomannans differ in their M/G ratio, which in turn depends on the distribution of galactose units over mannose backbone. This ratio is approximately 4:1 for LBG [32], 3:1 for tara gum and 2:1 for guar gum [33]. Substitution patterns of side-chain units and their molecular weight are influenced by harvesting and manufacturing practices, among other factors [34]. Therefore, the previous ratios are approximated and dependent on the material origin and plant growth conditions during production [31]. An important note is the fact that the galactose grafts are not spaced regularly in the mannose backbone but, instead of that, galactose is randomly placed [35]. Importantly, the M/G ratio is the characteristic most affecting galactomannan solubility [21], due to the fact that mannose chains are relatively hydrophobic and galactose units are more hydrophilic. In this manner, LBG has limited solubility, forming aggregates in cold water, as the long segments of unsubstituted mannose are likely to undergo aggregation [34, 36].

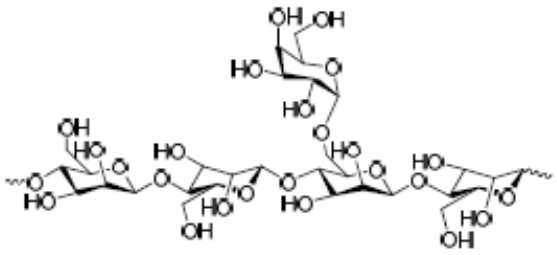

Figure 1.4: Locust Bean Gum Chemical Structure (1-4) – linked β-D-mannose backbone with (1-6)- linked side chains of α-D-galactose (from [31]).

For biopharmaceutical applications, an important issue to assess is the *in vivo* biodegradability of the polymer-based materials. This question is very important since the elimination of the systems by the organism, after administration, is required without the need for additional interventions. The biodegradation of natural polymers occurs by the action of enzymes, microorganisms and pH action, being complex in a biological, physical and chemical way. The mentioned processes lead to a breakdown of polymer chains, resulting in decreased molecular weight and the modification of other properties such as solubility. The biodegradation of LBG is mostly driven by enzymatic processes and there are several enzymes in the human organism with the ability to cleave the LBG molecule. The oral route is the one offering the most effective degradation of the polysaccharide, due to the presence of the enzyme β-mannanase in the human colonic region [22, 37, 38]. This enzyme acts on β-D-(1,4) links of mannose chains converting LBG in three metabolites as shown in figure 1.5. According to this figure, the metabolic processing of LBG is also performed by two other enzymes, β-mannosidase and α-galactosidase, which act on mannose and galactose residues, respectively. These enzymes were also detected in the human colonic region [39].

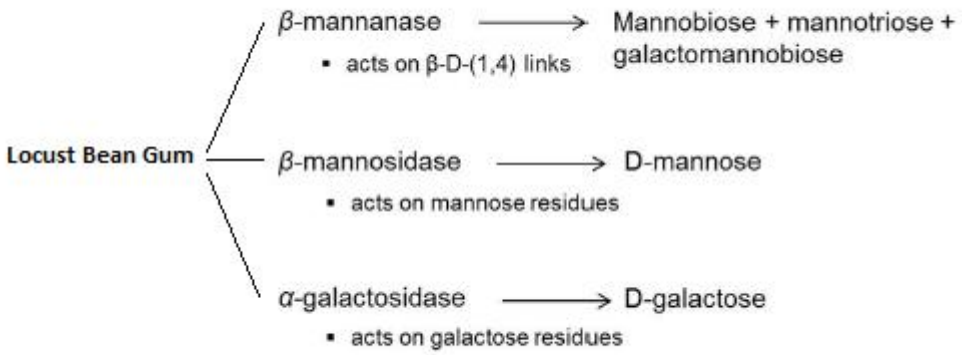

Figure 1.5: Scheme of LBG enzymatic degradation (Adapted from [22]).

The interest of using natural materials as part of drug development has increased in the past two decades [5]. In this work, Locust Bean Gum will be explored as a potential matrix material in the preparation of nanocarriers for insulin delivery.

2. Objectives

The aim of this work was to develop a nanodelivery system based on LBG for transmucosal delivery of proteins. LBG nanoparticles were produced by a method of polyeletrolyte complexation, using two LBG-derivatives previously synthesized by the research group (aminated LBG and sulphated LBG). For the approach of transmucosal delivery to be successful, the nanoparticles should display a size between 50 and 500 nm, apart from a positively charged surface, which permit a more intimate contact between the protein carrier and the epithelial surface, thus contributing for a favourable release of the protein.

The conditions for the preparation of the nanoparticles were optimised, regarding the A-LBG and S-LBG ratios to be used, and the selected formulations characterised in terms of physicochemical properties and production yield. In order to test the ability of LBG nanoparticles as protein carriers, insulin was used as model protein and associated to the nanocarriers, to determine the encapsulation efficiency and loading capacity. Finally, an evaluation of the cytotoxic profile of the developed LBG nanoparticles was performed in two different epithelial cell lines, one representative of the intestinal epithelium (Caco-2 cells) and another one, a model of the respiratory epithelium, namely the alveolar zone (A549 cells). These two cell lines represent the environment of both the oral and the pulmonary route of administration.

3. Materials and cell lines

3.1. Materials

A-LBG and S-LBG were synthesised by our group from LBG, kind gift from Industrial Farense (Portugal). Buffer solution citric acid/sodium hydroxide pH 5.0 was purchased from Fluka (Germany). Hydrochloric acid (HCl) (37%), sodium hydroxide (NaOH) tablets, insulin Mw 5777.6, penicillin-streptomycin solution (10000 units/mL, 10000 µg/mL), trypsin-EDTA solution, Dulbeco's Modified Eagle's Medium (DMEM), trypan blue solution (0.4%), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sodium dodecyl sulphate (SDS) and phosphate buffer saline (PBS) tablets pH 7.4 were purchased from Sigma (Germany). Foetal bovine serum (FBS) from Gibco was purchased from Life Technologies (USA). Ultrapure water (Milli-Q plus, Millipore Iberica (Spain)) was used throughout. Micro BCA (bicinchoninic acid) protein assay kit was purchased from Pierce (USA).

3.2. Cell lines

The Caco-2 and A549 cell lines were obtained from the American Type Culture Collection (Rockville, USA) and used between passages 77-90 and 27-40, respectively. Cell cultures were grown in 75 cm^2 flasks in a humidified 5% CO_2/95% atmospheric air incubator at 37ºC. For both cell lines, cell culture medium was DMEM supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine solution, 1% (v/v) non-essential amino acids solution and 1% (v/v) penicillin/streptomycin. Medium was changed every 2-3 days and cells were subcultured weekly.

4. Methods

4.1. Preparation of A-LBG/S-LBG nanoparticles

Nanoparticles (NPs) were prepared by polyelectrolyte complexation method [40] which consists in the electrostatic interaction between the positive and negative charges of A-LBG and S-LBG, respectively. As LBG has as neutral charge, a previous work of the group consisted in the amination and sulfation of the polymer in order to obtain derivatives with positive and negative charge, respectively. FTIR analysis was performed to production of aminated and sulphated derivatives of LBG (data not shown).

Three mass ratios of A-LBG/S-LBG were used to prepare the NPs by polyelectrolyte complexation, in particular 2/1, 1/1 and 1/2. The stock solution of A-LBG was prepared to reach a final concentration of 1 mg/mL, while that of S-LBG had a final concentration of 2 mg/mL. The solutions were filtered with a 0.45 µm filter prior to use. The formulations 2/1, 1/1 and 1/2 were prepared by adding 1.8 mL of S-LBG to 1 mL of A-LBG, as depicted in Figure 4.1. The concentration of A-LBG was kept constant at 0.5 mg/mL for the preparation of all formulations, but that of S-LBG was modified to obtain different ratios.

The addition of S-LBG to A-LBG was done by dripping during approximately 15 seconds. After that, it was possible to see, almost immediately, the Tyndall effect evidencing the formation of nanoparticles. The suspensions of nanoparticles were mixed by magnetic stirring for 10 minutes and then centrifuged in eppendorfs. Each eppendorf had a layer of 10 µL of glycerol, in order to facilitate the following step of ressuspension. The isolation of nanoparticles was performed by centrifugation (Thermo Scientific-Heraeus Fresco 17, Germany) at 16000xg, for 30 min at 15 ºC. After discarding the supernatants, the nanoparticles were ressuspended with 200 µL of ultrapure water.

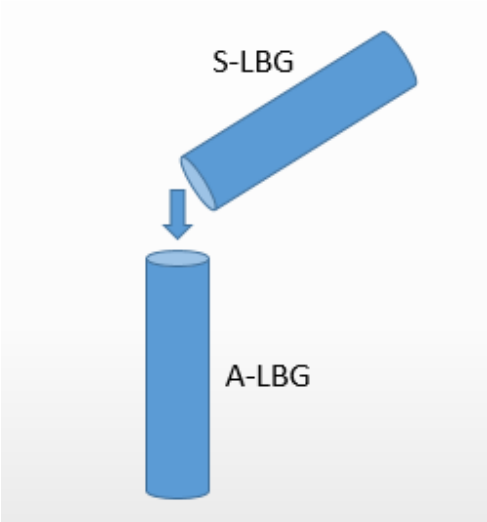

Figure 4.1- Schematic representation of the preparation of A-LBG/S-LBG nanoparticles.

4.2. Characterisation of nanoparticles

The physicochemical characterisation of nanoparticles was performed on freshly prepared samples. Size and polidispersion index (PDI) were measured by dynamic light scattering and zeta potential was measured by laser Doppler anemometry, using a Zetasizer Nano ZS (Malvern instruments, Malvern, UK). To prepare the samples, 20 µL of each formulation were diluted in 1 mL of ultrapure water.

For determination of nanoparticle production yield, the nanoparticles were prepared as described in the previous section but without the use of the 10 µL of glycerol. After discarding the supernatant of each formulation, the pellets were frozen and then dried on a freeze-dryer (Alpha RVC, Christ, Germany). The yield of nanoparticle production (PY) was calculated as follows:

PY = (Nanoparticle sediment weight/Total solids weight) x 100

where nanoparticle sediment weight is the weight after freeze-drying and total solids weight is the total amount of solids added for nanoparticle formation.

The morphological examination of A-LBG/S-LBG nanoparticles was conducted by transmission electron microscopy (TEM; JEM-1011, JEOL, Japan). The samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with carbon films (Ted Pella, USA) for TEM observation.

4.3. Association of insulin to A-LBG/S-LBG nanoparticles

Insulin was chosen as model protein for the association to LBG nanoparticles. Insulin is insoluble in water and in solutions of pH near its isoelectric point (5.0). Two insulin stock solutions were prepared, one with pH < 5.0 (insulin dissolved in HCl 0.01M) and another one with pH > 5.0 (insulin dissolved in NaOH 0.01M).

When insulin is dissolved in NaOH it becomes deprotonated and acquires negative charge, ideal to interact with positively charged groups. In turn, when it is dissolved in HCl, the protonation that occurs attributes positive charges, favouring the interaction with negatively charged groups. In formulation A-LBG/S-LBG = 2/1 the polymer in higher amount was A-LBG which is positively charged. To associate insulin to this formulation, the protein was dissolved in NaOH to display negative charges, thus favouring the interaction with the positively charged amino groups of A-LBG. In contrary, in the formulation A-LBG/S-LBG = 1/2 the polymer in higher amount was S-LBG. Therefore, insulin was dissolved in HCl to be mostly positively charged, favouring the interaction with negatively charged groups of S-LBG.

Objectively, for the preparation of the formulation A-LBG/S-LBG = 2/1, insulin was dissolved in NaOH and the obtained solution was mixed with that of the polymer present in lower amount in the formulation (S-LBG), prior to the addition to A-LBG for the formation of nanoparticles. As such, for the formulation 1/2, insulin was dissolved in HCl and the solution was mixed with that of the polymer in lower amount (A-LBG) prior to the formation of nanoparticles. Insulin stock solutions in NaOH and HCl were prepared at a concentration of 0.9 mg/mL.

The amount of insulin added to the formulations was defined as a function of the mass of the polymers used in the preparation of the nanoparticles. The association of different amounts of insulin was attempted as follows: 1) an amount of insulin corresponding to 30% of the total amount of polymers; 2) an amount of insulin corresponding to 20% of the total amount of polymers; and 3) an amount of insulin corresponding to 10% of the polymer present in higher quantity in each formulation (A-LBG for formulation 2/1 and S-LBG for formulation 1/2).

Insulin was quantified in each sample using the Micro BCA Protein Assay Kit, which provides a colorimetric method optimized to quantify reduced amounts of protein (0.5-20 μg/mL). The method utilizes bicinchoninic acid (BCA) as the detection reagent for

Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment [41]. A purple-coloured water-soluble reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}), which exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations. High absorbance is therefore interpreted as high insulin concentration.

Different calibration curves were performed for each formulation using the adequate solvents (HCl or NaOH). After reacting with the MicroBCA, samples were analysed by spectrophotometry (Infinite M200 Tecan, Austria) at 562 nm.

The protein association efficiency (AE) and the nanoparticle loading capacity (LC) were calculated as follows:

AE (%) = [(Total insulin amount – Free insulin amount)/Total insulin amount] x 100

LC (%) = [Total insulin amount – Free insulin amount)/Nanoparticle weight] x 100

4.4. Evaluation of the cytotoxicity of nanoparticles

The *in vitro* cytotoxicity of A-LBG/S-LBG nanoparticles, as well as that of the raw materials involved in nanoparticle production, was assessed by the metabolic assay thiazolyl blue tetrazolium bromide (MTT) test. Two cell lines representative of pulmonary and intestinal epithelia (A549 and Caco-2 cells, respectively) were used. Caco-2 and A549, used between the passages 77-90 and 27-40, respectively, were seeded at a density of 1×10^4 cells/well in 96-well plates, in 100 µL of the same medium used for culture in cell culture flasks. The cells were grown at 37 °C in a 5% CO_2 atmosphere for 24 h before use.

Three different concentrations (0.1, 0.5 and 1.0 mg/mL) of unloaded nanoparticles, as well as that of raw materials involved in nanoparticle production, were evaluated for cytotoxicity over 3 and 24 h. Sodium dodecyl sulphate (SDS, 2%, w/v) was used as a positive control of cell death. All formulations and controls were prepared as solution/suspensions in pre-warmed cell culture medium without FBS immediately before application to the cells.

To initiate the assay, culture medium of cells at 24 h in culture was replaced by 100 µL of fresh medium without FBS containing the test samples or controls. After 3 or 24 h of

cell exposure, samples/controls were removed and 30 µL of the MTT solution (0.5 mg/mL in PBS, pH 7.4) were added to each well. After 2 h, any generated formazan crystals were solubilised with 50 µL of DMSO. Upon complete solubilisation of the crystals, the absorbance of each well was measured by spectrophotometry (Infinite M200, Tecan, Austria) at 540 nm and corrected for background absorbance using a wavelength of 650 nm [42].

The relative cell viability (%) was calculated as follows:

$$\text{Viability (\%)} = (A - S)/(CM - S) \times 100$$

Where A is the absorbance obtained for each of the concentrations of the test substance, S is the absorbance obtained for the 2% SDS and CM is the absorbance obtained for untreated cells (incubated with cell culture medium). The latter reading was assumed to correspond to 100% cell viability. The assay was performed between three and six occasions with three replicates at each concentration of test substance in each instance.

4.5. Statistical analysis

The t-test and the one-way analysis of variance (ANOVA) with the pairwise multiple comparison procedures (Student–Newman–Keuls method) were performed to compare two or multiple groups, respectively. All analyses were run using the SigmaStat statistical program (Version 3.5, USA) and differences were considered to be significant at a level of $P < 0.05$.

5. Results and discussion

5.1. Preparation and characterisation of A-LBG/S-LBG nanoparticles

Three formulations of LBG-based nanoparticles were produced by polyelectrolyte complexation, according to a procedure detailed in a previous section. Polyelectrolyte complexation is a process that involves electrostatic interaction between oppositely charged groups. This procedure takes the advantage of occurring in a hydrophilic environment with mild preparation conditions, avoiding the use of organic solvents or high shear forces that might compromise the stability of the encapsulated material [40, 43]. LBG is a natural polymer with neutral charge, which hinders the application of polyelectrolyte complexation. In order to overcome that relevant limitation, aminated and sulphated derivatives of LBG were produced in a previous work of the group, thus obtaining positively and negatively charged polymers. The three formulations of A-LBG/S-LBG nanoparticles were designed to have polymeric mass ratios of 2/1, 1/1 and 1/2. After the preparation procedures, the nanoparticles were characterised in terms of size, polydispersion index, zeta potential and production yield. The detailed results are shown in table 5.1.

The formulation A-LBG/S-LBG = 1/1 precipitated immediately upon adding S-LBG to A-LBG. This is probably due to a positive to negative (+/-) charge ratio around 1, which permits a strong interaction between the two LBG derivatives. If anionic charges from S-LBG neutralize A-LBG positive charges, a reduction or elimination of electrostatic repulsion will occur, leading to precipitation [44, 45]. Therefore, this 1/1 formulation was disregarded and the work proceeded only with the two remaining formulations. The formulation A-LBG/S-LBG = 2/1, displayed a size of approximately 560 nm, a polydispersion index (PDI) of 0.432 and a positive zeta potential around +44 mV. The size of the formulation A-LBG/S-LBG = 1/2 was significantly lower ($P < 0.05$), 359 nm, the formulation further registering a PDI of 0.381 and a negative zeta potential of -50 mV.

Table 5.1 – Physicochemical characteristics and production yield of LBG unloaded nanoparticles (mean ± SD, n = 3).

A-LBG/S-LBG (w/w)	Size (nm)	PDI	Zeta potential (mV)	Production yield (%)
2/1	560.2 ± 108.3	0.432 ± 0.050	+43.5 ± 5.8	16.7 ± 3.8
1/1	n.d.	n.d.	n.d.	n.d.
1/2	359.1 ± 69.2	0.381 ± 0.063	-49.7 ± 5.0	30.0 ± 8.6

n.d. - not determined

As was said previously, for the objective of mucosal delivery of proteins, the nanocarriers should exhibit a size between 50-500 nm to permit an intimate contact with the mucosal surface [10, 11]. The nanoparticles of the formulation 2/1 displayed a size slightly above the desired, while those of formulation 1/2 evidenced a suitable size for the objectives. During the experiments, it was observed that A-LBG showed a higher viscosity than S-LBG, which suggests a higher molecular weight. This effect is described in the literature for other polysaccharides [22, 35]. As formulation 2/1 has approximately the double amount of A-LBG comparing to S-LBG, the previous observation might explain the higher size of these nanoparticles. The displayed values of zeta potential corresponded to the expectations. The formulation 2/1 displayed a positive potential, reflecting the higher amount of positively charged LBG, while the formulation 1/2 displayed a negative potential, in line with the higher amount of sulphated LBG. Considering the objective of transmucosal delivery, using carriers with a positive surface charge might be beneficial, as these will have a favoured interaction with the negatively charged epithelial surface. Nevertheless, carriers endowed with a negative zeta potential have also been reported to have successful applications in drug delivery [46, 47]. The polydispersion index was acceptable for both formulations. In this regard, it is important to mention that nanoparticles produced with natural polymers always present some degree of heterogeneity, which naturally reflects on PDI values.

As shown in table 5.1, the production yield was 17% for formulation 2/1 and 30% for the formulation 1/2. Formulation 2/1 has a low production yield, but the size of the nanoparticles is higher than that observed for formulation 1/2. This fact could indicate a stronger interaction between positive and negative charges in formulation 1/2, where A-LBG, which has a higher molecular weight, is present in lower amount.

The morphological analysis of nanoparticles was performed by TEM. Figure 5.1 displays the microphotograph of representative A-LBG/S-LBG nanoparticles. It can be seen that the carriers generally have a spherical shape, apparently corresponding to a compact structure. This morphology is very similar to that normally reported for polysaccharide-based nanoparticles produced by polyelectrolyte complexation [44, 48-51].

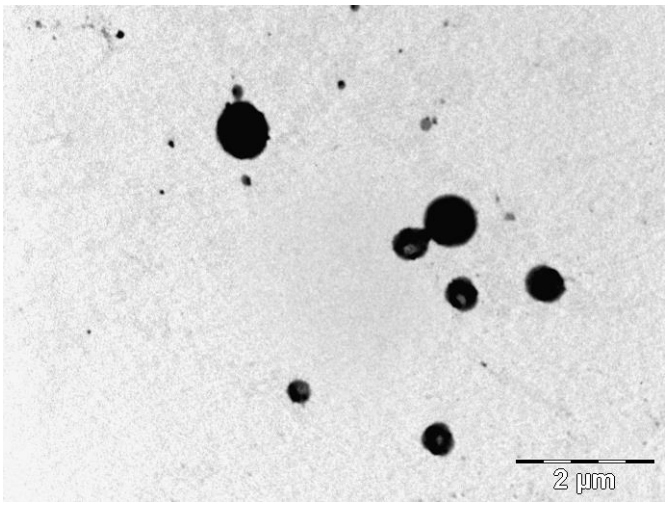

Figure 5.1- TEM microphotograph of representative A-LBG/S-LBG nanoparticles.

5.2. Association of the model protein

Insulin was the selected protein model to test the encapsulation capacity of LBG nanoparticles. As described previously in the methodology section, the association of the protein to the nanoparticles required several optimisations, namely related with the initial amount of insulin to be included in the formulations. The initial approaches included the addition of an amount of insulin corresponding to 30% (at first) and 20% (secondly) of the total amount of polymer, but these options were unsuccessful because severe precipitation was observed. This precipitation was certainly due to the presence of an excess of anionic charges, which neutralize positive charges and, thus, reduce or eliminate electrostatic repulsion, leading to precipitation [44, 45]. The attempt of associating an amount of insulin corresponding to 10% of the polymer present in higher quantity in each formulation was the chosen one, as it led to the production of nanoparticles. However, the encapsulation efficiency was around 15% in both formulations 2/1 and 1/2, which was considered unsatisfactory. In order to improve the encapsulation efficiency, an

optimization was made which relied on changing the pH of the insulin medium so that it was close to its isoelectric point. This hypothesis was constructed based on information available on the literature which refers that there is a higher adsorption of proteins to polymers when proteins are in medium with pH close to their isoelectric point [52]. When the pH is equivalent to the isoelectric point (pI), insulin will display a net neutral charge because the number of positive charges is equal to the number of negative charges. Therefore, the charges of polymers will be free to interact with each other maximizing their interaction and insulin will have a favoured adsorption to the nanoparticles. To test this hypothesis, for the formulation 2/1, the stock solution of insulin was prepared with NaOH and the water used to prepare the nanoparticles (mainly in dilutions of the polymers) was replaced by a citrate buffer solution (pH = 5.0). As insulin pI is 5.3, using a buffer of pH 5.0 will allow a final pH value close to the protein pI. Similarly, for the formulation 1/2, insulin was dissolved previously in HCl and the water used to prepare the nanoparticles was replaced by the citrate buffer solution. However, in the latter case strong precipitation was observed, this formulations demanding further optimisations to improve its encapsulation efficiency. A second approach consisted in substituting the water used to prepare the nanoparticles by HCl solution 0.1 M, which revealed successful. As shown in table 5.2, the association efficiency was thus increased in both formulations. For the formulation 2/1 an improvement from 15% to 22% was observed, while for the formulation 1/2 a very significant improvement from 15% to 96% was verified. The loading capacity was significantly low for both formulations in particular for formulation 2/1. This values are low probably due to a little amount of insulin used for both formulations.

Table 5.2 – Encapsulation efficiency (EE) of A-LBG/S-LBG nanoparticles before and after optimisations and loading capacity (LC) (mean ± SD, n = 3).

A-LBG/S-LBG (w/w)	EE before optimisations (%)	EE after optimisations (%)	LC (%)
2/1	15.0 ± 7.8	22.2 ± 8.1	2.78 ± 1.01
1/2	15.0 ± 5.4	96.1 ± 8.1	12.01 ± 1.02

The insulin loaded nanoparticles were further characterised for their physicochemical properties, the results being shown in the table 5.3. For the formulation A-LBG/S-LBG = 2/1, nanoparticles displayed a size of 740 nm, a PDI of 0.389 and a zeta potential of +23 mV. For the formulation A-LBG/S-LBG = 1/2, nanoparticles displayed a size of 400 nm, a PDI of 0.441 and a zeta potential of -34 mV. Nevertheless, the latter formulation is difficult to resuspend and some large aggregates are seen in suspension after that step.

Table 5.3 - Physicochemical characteristics and production yield of LBG insulin loaded nanoparticles (mean ± SD, n = 3)

A-LBG/S-LBG (w/w)	Size (nm)	PDI	Zeta potential (mV)	Production yield (%)
2/1	740.8 ± 84.3	0.389 ± 0.040	+22.8 ± 2.3	25.8 ± 9.1
1/2	400.0 ± 126.2	0.404 ± 0.135	-33.6± 10.3	51.6 ± 7.9

The production yield was 25.8% for formulation 2/1 and 51.6% for formulation 1/2. The size of both formulations had increased in comparison to unloaded nanoparticles, which is attributed to the presence of insulin in the matrix [52]. Zeta potential decreased for both formulations, resulting in final zeta potential values that are closer to neutrality, as compared with the unloaded formulations. Therefore, it is suggested that in the formulation A-LBG/S-LBG = 1/2 insulin neutralised essentially positively charged groups, while in formulation 2/1 the interaction with negatively charged groups was favoured [44, 52]. Production yield had increased for both formulations, which is explained with the presence of insulin as an extra substance permitting reactions to take place, thus leading to the formation of a higher number of nanoparticles. The registered increase in the size of nanoparticles and their production yield is in concordance with other works available in the literature [44, 51].

5.3. Evaluation of cytotoxicity of the nanoparticles

Assessing the biocompatibility of protein carriers is a major issue in developing protein delivery systems. Therefore, current international guidelines require the contextualization of biocompatibility with a specific route of administration and the amount of the material used [51, 53]. According to the guidelines issued by the International Organization for

Standardization (ISO) 10993-3, a complete set of assays should be performed to test biocompatibility, addressing firstly cellular morphology, membrane integrity, and metabolic efficiency [54].

In this work, the cytotoxicity was assessed by means of the MTT assay, which assesses cell metabolic efficiency, relying on the evaluation of an enzymatic function. To perform the assay, after the exposure to the test formulations and raw materials, cells are incubated with yellow tetrazolium (MTT) salts which are reduced to purple-blue formazan crystals by active mitochondrial dehydrogenase [44, 51]. A higher concentration of the formazan crystals corresponds to a higher amount of metabolically active cells, which is usually interpreted as higher cell viability.

Two cell lines representative of pulmonary and intestinal epithelia (A549 and Caco-2 cells, respectively) were used. Three different concentrations (0.1, 0.5 and 1.0 mg/mL) of both unloaded nanoparticles and raw materials involved in nanoparticle production were evaluated for cytotoxicity over 3 h and 24 h. A statistical analysis was performed in order to compare the obtained values and its meaning in terms of significance.

The results obtained for raw materials in Caco-2 cells are shown in figure 5.2. For both polymers A-LBG and S-LBG, comparing the results obtained for 3 h and 24 h, separately for each polymer, indicates that no significant differences are observed. In turn, comparing each concentration of A-LBG for different exposure times (3 h and 24 h) reveals significant differences ($p < 0.05$), which mainly comprise a decrease of cell viability from 3 h to 24 h. This trend is found in many other works as the prolonged exposure to an aggressive agent usually produces effects at prolonged times [55-57]. On the contrary, this effect was not observed for S-LBG. All concentrations of positively charged polymer reveal values of cell viability below 40%. According to the ISO 10993-5 [58] A-LBG evidences cytotoxic potential as cell viability values below 70% are indicative of toxicity. The influence of charges in cell viability remains largely unresolved, however there are some indications in the literature suggesting some hypotheses [51]. One of the explanations could be the fact that positively charged polymers tend to be more cytotoxic due to a stronger interaction of the positive charges with the negative cell surface charges, sometimes culminating with the internalization of the material and leading to cell death [59-61]. The negatively charged polymer tested in this study (S-LBG) evidences values of cell viability above 70%, which represents low cytotoxicity.

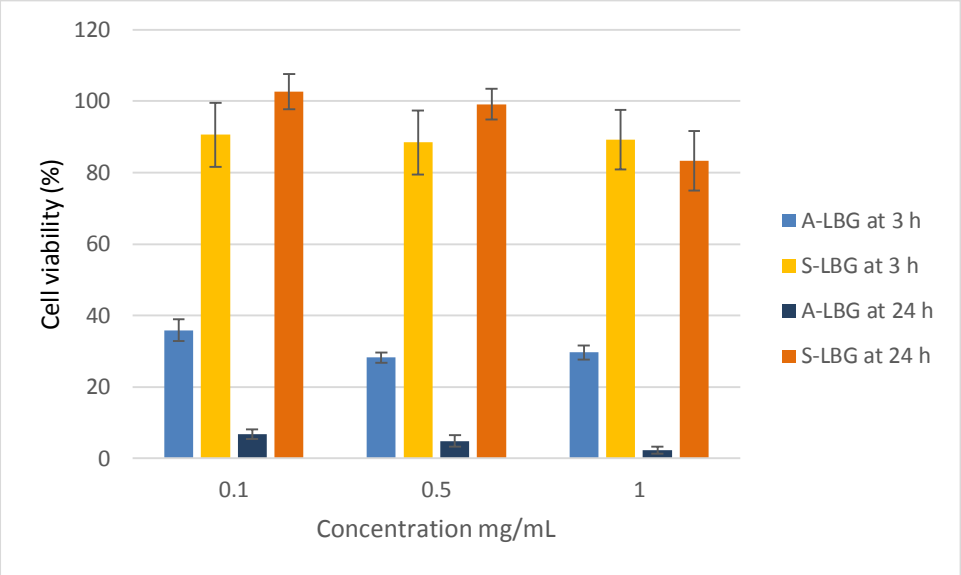

Figure 5.2 –Caco-2 cell viability of raw materials determined by the MTT assay after 3 h and 24 h exposure to increasing concentrations of A-LBG and S-LBG. Data represent mean ± SEM (n = 3, three replicates per experiment at each concentration).

Cell viability of Caco-2 cells upon exposure to nanoparticles is shown in figure 5.3. For formulation A-LBG/S-LBG = 2/1, the comparison of results obtained for 3 h and 24 h, separately, evidenced significant differences ($p < 0.05$) between all concentrations. Comparing the same concentrations for different times (3 h and 24 h) no significant differences are observed. For the formulation A-LBG/S-LBG = 1/2, the comparison of results for the two tested times did not reveal significant differences at any concentration and time. The most remarkable result is that no significant cytotoxicity is observed for both formulations at all concentrations, at both 3 h and 24 h. Actually, the registered viability was over 80% in all cases, which is considered very acceptable according to the ISO10993-5 [58]. Nevertheless, as can be observed in the same figure, the exposure of the cells to the formulation A-LBG/S-LBG = 2/1 resulted in an increase of cell viability with the increase of nanoparticle concentration at 3 h and at 24 h evidencing significant differences ($p < 0.05$) between each concentration for both times. This was unexpected and may be due to the fact that LBG is a polysaccharide with capacity to promote cell proliferation in some cell lines, as reported in literature [62]. Despite formulation A-LBG/S-LBG = 2/1 could improve cell proliferation with the increasing of concentration,

formulation A-LBG/S-LBG = 1/2, induced constant cell viability near 100%, irrespective of the concentration.

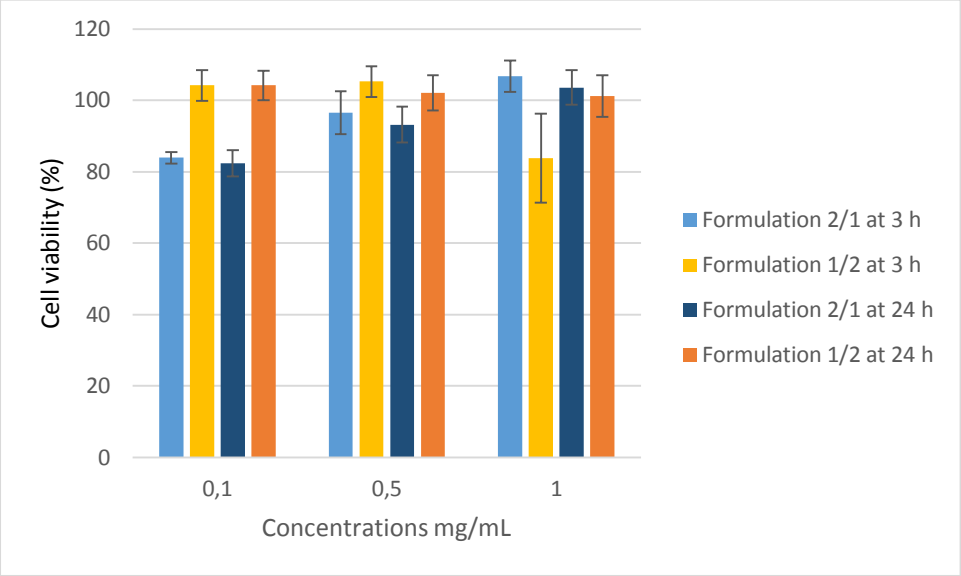

Figure 5.3 – Caco-2 cell viability of nanoparticles determined by MTT assay after 3 h and 24 h exposure to increasing concentrations of A-LBG/S-LBG = 2/1 and A-LBG/S-LBG = 1/2 nanoparticles. Data represent mean ± SEM (n = 3, three replicates per experiment at each concentration).

When the developed nanoparticles were tested on the cell line representing the pulmonary epithelium (A549 cells), the results were somewhat different (figure 5.4). For formulation A-LBG/S-LBG = 2/1, the comparison of results obtained for each concentration at 3 h and 24 h, evidenced a tendency to a slight increase of cell viability with the increase of concentration, although no significant differences were found between the groups. For the formulation A-LBG/S-LBG = 1/2, this comparison did not reveal significant differences for any concentration at 3 h. However, at 24 h significant differences were found between concentrations ($p < 0.05$). Nevertheless, comparison between 3 h and 24 h for same formulation for each concentration revealed no significant differences.

Generally, the tested conditions resulted in viabilities above 70%, with the exception of formulation 1/2 for the concentration of 1 mg/mL at 24 h, which is below 60%. For the formulation 1/2, while the 3 h exposure did not lead to significant differences in viability, at 24 h the differences are significant among nanoparticle concentrations. In that case it

is possible to observe a decrease from 100% for concentration 0.1 mg/mL, to 80% for concentration 0.5 mg/mL and 60% for concentration 1 mg/mL.

A549 cell line seems to be more sensitive than Caco-2 cell line, which is probably due to the origin of the cells. Since Caco-2 are from intestinal epithelium they are exposed to higher mechanical forces and pH changes than A549 cells and, consequently, they present a more resistant pattern.

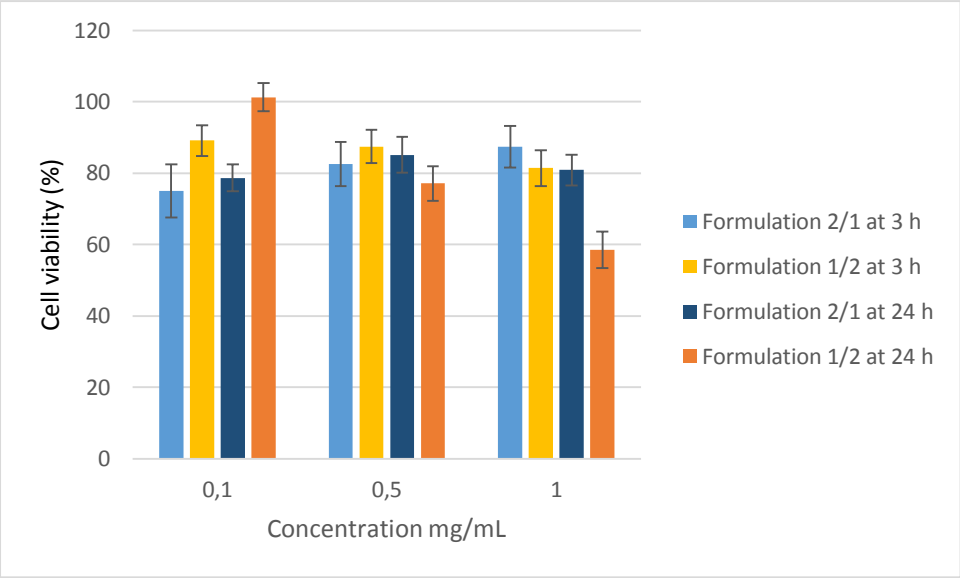

Figure 5.4 – A549 cell viability of nanoparticles determined by MTT assay after 3 h and 24 h exposure to increasing concentrations of A-LBG/S-LBG = 2/1 and A-LBG/S-LBG = 1/2 nanoparticles. Data represent mean ± SEM (n = 3, three replicates per experiment at each concentration).

Taking into account the objectives delineated for the LBG nanoparticles, which concern the transmucosal delivery of proteins, the two developed formulations (2/1 and 1/2) are considered to display adequate cytotoxic profile, as low cytotoxicity was evidenced in cell lines representative of the respiratory and intestinal epithelia. Nevertheless, as proposed by the ISO 10993-5, these results of cytotoxicity should be complemented with those of genotoxicity, acute and sub-acute toxicity to give further indications regarding the biocompatibility profile of the drug delivery systems [58].

6. Conclusion

The combination of the two tested LBG derivatives (sulphated and aminated) enables the formation of nanoparticles by the method of polyelectrolyte complexation. Varying several parameters of the procedure permits obtaining nanoparticles with different physicochemical characteristics (variable size, opposite zeta potential). The developed nanocarriers evidence capacity to associate insulin, which is dependent on several variables of the process, such as the pH of the reaction media. Nevertheless, according to the objectives proposed for the work, which comprise the transmucosal delivery of proteins, formulation A-LBG/S-LBG = 2/1 does not show suitable properties. Formulation A-LBG/S-LBG = 1/2 shows acceptable characteristics, in spite of the evidenced negative charge, which does not favour the interaction with epithelial surfaces in a particular manner. A very positive indication was obtained regarding the cytotoxic profile of the developed carriers, as both formulations evidenced low cytotoxicity upon incubation with two cell lines representative of both the intestinal (Caco-2) and the alveolar (A549) epithelia.

7. Bibliography

1. Ratner, M. and D. Ratner, *Nanotechnology: a gentle introduction to the next big idea*. 2002: Prentice Hall Press. p. 208-210.
2. Patel, A., et al., *Recent Advances in Protein and Peptide Drug Delivery: A Special Emphasis on Polymeric Nanoparticles.* Protein Peptide Letters, 2014.
3. Antosova, Z., et al., *Therapeutic application of peptides and proteins: parenteral forever?* Trends Biotechnology, 2009. **27**(11): p. 628-635.
4. Liu, Z., et al., *Polysaccharides-based nanoparticles as drug delivery systems.* Advanced Drug Delivery Reviews, 2008. **60**(15): p. 1650-1662.
5. Reis, C.P., et al., *Nanoparticulate biopolymers deliver insulin orally eliciting pharmacological response.* Journal of Pharmaceutical Sciences, 2008. **97**(12): p. 5290-5305.
6. Delie, F. and Blanco-Prieto, M.J. *Polymeric particulates to improve oral bioavailability of peptide drugs.* Molecules, 2005. **10**(1): p. 65-80.
7. PATIL, P., et al., *Formulation and in vitro evaluation of mucoadhesive tablets of ofloxacin using natural gums.* International Journal of Current Pharmaceutical Research. **3**(2): p. 93-98.
8. Hartig, S.M., et al., *Multifunctional nanoparticulate polyelectrolyte complexes.* Pharmaceutical Research, 2007. **24**(12): p. 2353-2369.
9. de la Fuente, M., et al., *Nanoparticles as protein and gene carriers to mucosal surfaces.* Nanomedicine (Lond), 2008. **3**(6): p. 845-857.
10. Desai, M.P., et al., *Gastrointestinal uptake of biodegradable microparticles: effect of particle size.* Pharmaceutical Research, 1996. **13**(12): p. 1838-1845.
11. Jani, P., et al., *Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency.* Journal of Pharmacy and Pharmacology, 1990. **42**(12): p. 821-826.
12. Bogataj, M., et al., *The correlation between zeta potential and mucoadhesion strength on pig vesical mucosa.* Biological and Pharmaceutical Bulletin, 2003. **26**(5): p. 743-746.
13. Yildirimer, L., et al., *Toxicology and clinical potential of nanoparticles.* Nano Today, 2011. **6**(6): p. 585-607.
14. Banik, B.L. and Brown, J.L. *Chapter 23 - Polymeric Biomaterials in Nanomedicine*, in *Natural and Synthetic Biomedical Polymers*, S.G. Kumbar, C.T. Laurencin, and M. Deng, Editors. 2014, Elsevier: Oxford. p. 387-395.
15. Yu, L., K. Dean, and Li, L.. *Polymer blends and composites from renewable resources.* Progress in Polymer Science, 2006. **31**(6): p. 576-602.
16. Kariduraganavar, M.Y., Kittur, A.A., and Kamble, R.R. *Chapter 1 - Polymer Synthesis and Processing*, in *Natural and Synthetic Biomedical Polymers*, Kumbar, S.G., Laurencin, C.T. and Deng, M. Editors. 2014, Elsevier: Oxford. p. 1-31.
17. Aravamudhan, A., et al., *Chapter 4 - Natural Polymers: Polysaccharides and Their Derivatives for Biomedical Applications*, in *Natural and Synthetic Biomedical Polymers*, Kumbar S.G., Laurencin, C.T. and Deng, M. Editors. 2014, Elsevier: Oxford. p. 67-89.
18. Cascone, M.G., et al., *Bioartificial polymeric materials based on polysaccharides.* Journal of Biomaterials Science, Polymer Edition, 2001. **12**(3): p. 267-281.
19. Nishinari, K. and Takahashi, R. *Interaction in polysaccharide solutions and gels.* Current Opinion in Colloid & Interface Science, 2003. **8**(4–5): p. 396-400.

20. Malafaya, P.B., Silva, G.A. and Reis, R.L. *Natural–origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications.* Advanced Drug Delivery Reviews, 2007. **59**(4–5): p. 207-233.
21. Rinaudo, M., *Main properties and current applications of some polysaccharides as biomaterials.* Polymer International, 2008. **57**(3): p. 397-430.
22. Dionisio, M. and Grenha, A. *Locust bean gum: Exploring its potential for biopharmaceutical applications.* Journal of Pharmacy and Bioallied Sciences, 2012. **4**(3): p. 175-185.
23. Dakia, P.A., et al., *Composition and physicochemical properties of locust bean gum extracted from whole seeds by acid or water dehulling pre-treatment.* Food Hydrocolloids, 2008. **22**(5): p. 807-818.
24. Bouzouita, N., et al., *The analysis of crude and purified locust bean gum: A comparison of samples from different carob tree populations in Tunisia.* Food Chemistry, 2007. **101**(4): p. 1508-1515.
25. Smith, B.M., et al., *Composition and molecular weight distribution of carob germ protein fractions.* Journal of Agricultural and Food Chemistry, 2010. **58**(13): p. 7794-7800.
26. Rowe R, S.P., Owen S. , *Handbook of Pharmaceutical Excipients*. 5 th ed. 2006, London: Pharmaceutical Press.
27. H. El Batal, A.H., Ouatmane, A., Boulli, A., Dehbi, F., Jaouad A. *Yield and composition of carob bean gum produced from different Moroccan populations of carob (Ceratonia siliqua L.).* Journal of Materials and Environmental Science, 2013. **4**(2): p. 309-314.
28. Hirsch, S., et al., *Lauroyldextran and crosslinked galactomannan as coating materials for site-specific drug delivery to the colon.* European Journal of Pharmaceutics and Biopharmaceutics, 1999. **47**(1): p. 61-71.
29. Raghavan, C.V., et al., *An in vitro and in vivo investigation into the suitability of bacterially triggered delivery system for colon targeting.* Chemical Pharmaceutical Bulletin (Tokyo), 2002. **50**(7): p. 892-895.
30. Hoffman, A.S., *Hydrogels for biomedical applications.* Advanced Drug Delivery Reviews, 2002. **54**(1): p. 3-12.
31. Beneke, C.E., Viljoen, A.M. and Hamman, J.H. *Polymeric plant-derived excipients in drug delivery.* Molecules, 2009. **14**(7): p. 2602-2620.
32. Coviello, T., et al. *Two galactomannans and scleroglucan as matrices for drug delivery: preparation and release studies.* European Journal of Pharmaceutics and Biopharmaceutics, 2007. **66**(2): p. 200-209.
33. Andrade, C.T., et al., *Solution properties of the galactomannans extracted from the seeds of Caesalpinia pulcherrima and Cassia javanica: comparison with locust bean gum.* International Journal of Biological Macromolecules, 1999. **26**(2-3): p. 181-185.
34. Picout, D.R., et al., *Pressure cell assisted solution characterization of polysaccharides. 2. Locust bean gum and tara gum.* Biomacromolecules, 2002. **3**(4): p. 761-767.
35. Mathur, V. and Mathur, M.N. *Fenugreek and other less known legume galactomannan-polysaccharides: Scope for developments.* Journal of Scientific and Industrial Research, 2005. **64**: p. 475-481.
36. Pollard, M., et al. *Investigation of molecular weight distribution of LBG galactomannan for flours prepared from individual seeds, mixtures, and commercial samples.* Food Hydrocolloids, 2008. **22**: p. 1596-1606.

37. Alonso-Sande, M., et al., *Glucomannan, a promising polysaccharide for biopharmaceutical purposes.* European Journal of Pharmaceutics and Biopharmaceutics, 2009. **72**(2): p. 453-462.
38. Nakajima, N. and Matsuura, Y. *Purification and characterization of konjac glucomannan degrading enzyme from anaerobic human intestinal bacterium, Clostridium butyricum-Clostridium beijerinckii group.* Bioscience, Biotechnology and Biochemistry, 1997. **61**(10): p. 1739-1742.
39. Jain, A., Gupta, Y. and Jain, S.K. *Perspectives of biodegradable natural polysaccharides for site-specific drug delivery to the colon.* Journal of Pharm Pharmaceutical Sciences, 2007. **10**(1): p. 86-128.
40. Grenha, A., *Chitosan nanoparticles: a survey of preparation methods.* Journal of Drug Targeting, 2012. **20**(4): p. 291-300.
41. Smith, P.K., et al., *Measurement of protein using bicinchoninic acid.* Anal Biochemistry, 1985. **150**(1): p. 76-85.
42. Carmichael, J., et al., *Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing.* Cancer Research, 1987. **47**(4): p. 936-42.
43. Prego, C., Torres, D. and Alonso, M.J. *The potential of chitosan for the oral administration of peptides.* Expert Opinion on Drug Delivery, 2005. **2**(5): p. 843-854.
44. Rodrigues, S., et al., *Biocompatibility of chitosan carriers with application in drug delivery.* Journal of Functional Biomaterials, 2012. **3**(3): p. 615-641.
45. Crouzier, T. and Picart, C. *Ion pairing and hydration in polyelectrolyte multilayer films containing polysaccharides.* Biomacromolecules, 2009. **10**(2): p. 433-442.
46. Chandratre, S.S. and Dash, A.K. (in press) *Multifunctional Nanoparticles for Prostate Cancer Therapy.* AAPS PharmSciTech, 2014. DOI: 10.1208/s12249-014-0202-z
47. Lee, J.Y., et al., *Nanocomplexes Based on Amphiphilic Hyaluronic Acid Derivative and Polyethylene Glycol-Lipid for Ginsenoside Rg3 Delivery.* Journal of Pharmaceutical Sciences, 2014. **103**(10): p. 3254–3262
48. Goycoolea, F.M., et al., *Chitosan-alginate blended nanoparticles as carriers for the transmucosal delivery of macromolecules.* Biomacromolecules, 2009. **10**(7): p. 1736-1743.
49. Oyarzun-Ampuero, F.A., et al., *Chitosan-hyaluronic acid nanoparticles loaded with heparin for the treatment of asthma.* International Journal of Pharmacy, 2009. **381**(2): p. 122-129.
50. Zorzi, G.K., et al., *Hybrid Nanoparticle Design Based on Cationized Gelatin and the Polyanions Dextran Sulfate and Chondroitin Sulfate for Ocular Gene Therapy.* Macromolecular Bioscience, 2011. **11**(7): p. 905-913.
51. Dionisio, M., et al., *Pullulan-based nanoparticles as carriers for transmucosal protein delivery.* European Journal of Pharmaceutical Sciences, 2013. **50**(1): p. 102-113.
52. Peng, Z.G., Hidajat, K. and Uddin, M.S. *Adsorption of bovine serum albumin on nanosized magnetic particles.* Journal of Colloid and Interface Science, 2004. **271**(2): p. 277-283.
53. Gaspar, R. and Duncan, R. *Polymeric carriers: preclinical safety and the regulatory implications for design and development of polymer therapeutics.* Advanced Drug Delivery Reviews, 2009. **61**(13): p. 1220-1231.

54. ISO, 2003, *Biological evaluation of medical devices Part 3: Tests for genotoxicity, carcinogenicity, and reproductive toxicity.* In: Standardization, I.O.f. (Ed.), 10993-3.
55. Martins, A.F., et al., *Preparation and cytotoxicity of N,N,N-trimethyl chitosan/alginate beads containing gold nanoparticles.* International Journal of Biological Macromolecules, 2014. **72C**: p. 466-471.
56. Kang, T., et al., *In vitro toxicity of different-sized ZnO nanoparticles in Caco-2 cells.* Nanoscale Research Letters, 2013. **8**(1): p. 496.
57. Bhattacharjee, S., et al., *Cytotoxicity of surface-functionalized silicon and germanium nanoparticles: the dominant role of surface charges.* Nanoscale, 2013. **5**(11): p. 4870-4883.
58. ISO, 2009, *Biological evaluation of medical devices Part 5: Tests for in vitro cytotoxicity. In: Standardization.* I.O.f. (Ed.), 10993-5.
59. Bhattacharjee, S., et al., *Role of surface charge and oxidative stress in cytotoxicity of organic monolayer-coated silicon nanoparticles towards macrophage NR8383 cells.* Particle and Fibre Toxicology, 2010. **7**(25) p. 1-12.
60. Ilinskaya, O.N., et al., *Changing the net charge from negative to positive makes ribonuclease Sa cytotoxic.* Protein Science, 2002. **11**(10): p. 2522-2525.
61. Turcotte, R.F., Lavis, L.D. and Raines, R.T. *Onconase cytotoxicity relies on the distribution of its positive charge.* FEBS Journal, 2009. **276**(14): p. 3846-3857.
62. Perestrelo, A.R., et al., *Locust bean gum as an alternative polymeric coating for embryonic stem cell culture.* Material Sciences and Engeneering C: Materials for Biological Applications, 2014. **40**: p. 336-344.