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Faculdade de Ciências e Tecnologia

DEVELOPMENT OF FUCOIDAN/CHITOSAN NANOPARTICULATE SYSTEMS FOR PROTEIN ADMINISTRATION THROUGH MUCOSAL ROUTES

Sara Ataíde Ferreira

Dissertação

Mestrado Integrado em Engenharia Biológica

Dissertação efetuada sob orientação de:

Professora Doutora Ana Margarida Grenha,

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Eu Sara Ataíde Ferreira, declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída

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ABSTRACT

Presently, the administration of therapeutic proteins through non-parenteral routes poses a challenge due to stability problems, mainly attributed to pH and high enzymatic content present in mucosal surfaces. Therefore, the administration of proteins through mucosal routes requires the development of suitable carriers which confer stability and protection against harsh environments of the organism and that further facilitate macromolecule permeation. Polymeric nanoparticles have been proposed as valuable systems to overcome these biological barriers, showing, in some cases, useful properties of controlled release and cellular internalization. In this context, there is also a growing tendency towards the use of natural polymers such as polysaccharides, because of their unique properties and high biocompatibility and biodegradable profile.

In this work, fucoidan/chitosan (FUC/CS) nanoparticles were prepared by polyelectrolyte complexation. The aim lying in the development of these carriers is the expectation that they confer stability and protection to the biomolecules against mucosal environments, such as pH and enzymatic contents, providing a non-parenteral route for the administration of protein-based drugs. In this study, bovine albumin serum, insulin and ovalbumin were used as model proteins. Several FUC/CS mass ratios (4/1 to 1/4) were tested, resulting in nanoparticles with different sizes (338-676 nm) and zeta potentials (+41 a -49 mV). Nanoparticles FUC/CS = 1/4 and 4/1 were proposed for BSA encapsulation and variables such as order of polymer addition over each other and the polymeric solution with which the protein was mixed at first, were tested for their ability to affect the nanoparticles encapsulation efficiency. Efficiencies as high as 100% were registered (FUC/CS = 4/1) and the tested variables were found to have a stronger effect on the formulation FUC/CS = 1/4. The small sizes and high negative and positive charges displayed by the developed nanoparticles, in addition of their ability to associate macromolecules, were considered to hold potential for an application in mucosal delivery.

Keywords: Chitosan, Fucoidan, Mucosal routes, Nanoparticles, Proteins

RESUMO

Presentemente, a administração de proteínas terapêuticas por vias não-parentéricas representa um desafio, devido aos problemas de estabilidade, principalmente atribuídos ao pH e conteúdo enzimáticas em superfícies mucosas. O uso de vias mucosas para a administração de proteínas exige assim, o desenvolvimento de transportadores adequados que confiram estabilidade e proteção contra ambientes agressivos encontrados no organismo e que ainda facilitam a permeação das macromoléculas. As nanopartículas poliméricas surgem então com o fim de ultrapassar estas barreiras biológicas, evidenciando ainda, em alguns casos, propriedades úteis de libertação controlada e internalização celular. Neste contexto, sugere ainda uma tendência crescente para o uso de polímeros naturais, tais como polissacáridos, devido às suas características únicas e propriedades de elevada biocompatibilidade e perfil biodegradabilidade.

Neste trabalho, foram preparadas nanopartículas fucoidan/quitosano (FUC/CS) por complexação polieletrolítica. Ao desenvolver estes sistemas a expectativa é de que eles confiram estabilidade e proteção para as biomoléculas contra ambientes das mucosas, tais como o pH e elevados conteúdo enzimáticos, proporcionando uma rota não-parenteral para a administração de medicamentos à base de proteínas.Neste estudo, a albumina de soro bovino, insulina e ovalbumina foram utilizadas como proteínas modelos. Foram testados vários rácios mássicos de FUC/CS (4/1 a 1/4), que resultaram na criação de nanopartículas com diferentes tamanhos (338-676 nm) e potenciais zeta (+41 a -49 mV). As FUC/CS= 1/4 e 4/1, foram propostas para o encapsulamento da BSA, onde as variáveis tais como a ordem de adição de polímeros (protocolo A e B) e a pré-incorporação da proteína, numa das soluções poliméricas, foram testadas pela capacidade de manipular a eficiência de encapsulação (EE) das nanopartículas. Eficiências de encapsulação de 100% foram registadas (FUC/CS= 4/1) e a as variáveis testadas mostraram ter maior influência nas formulações FUC/CS=1/4. Os pequenos tamanhos e as elevadas cargas negativas e positivas das nanopartículas desenvolvidas, foram considerados adequados para a aplicação na administração de macromoléculas pela via mucosa.

Palavras-chave: Fucoidan, Nanopartículas, Proteínas, Quitosano, Vias mucosas

RESUMO ALARGADO

Das inúmeras doenças que normalmente afetam os seres humanos, várias são causadas ou por uma disfunção fisiológica ou por uma exposição a um fator ambiental. Subjacente a muitas das condições a nível molecular está uma variação na quantidade, função ou atividade de uma ou mais proteínas, que desencadeiam alterações a nível celular, tecidular ou na função de um órgão. Grande parte da atual investigação médica mundial está voltada para a identificação de proteínas-chave envolvidas em mecanismos molecular subjacentes a muitas doenças, a fim de selecionar uma destas proteínas como alvo para o desenvolvimento de um novo medicamento que possa minimizar ou eliminar os sintomas.

Em 1980 a indústria biofarmacêutica tornou-se sinónimo de proteínas terapêuticas produzidas por tecnologia de ADN recombinante, graças ao progresso no campo da biotecnologia que culminou com o surgimento do primeiro organismo geneticamente manipulado. Este progresso permitiu ainda o desenvolvimento de novas terapias bem como a produção em larga escala de bioprodutos que anteriormente só estavam disponíveis em quantidades limitadas, fazendo com que a maior parte das moléculas com potencial terapêutico propostas hoje em dia sejam à base de proteínas.

A formulação de proteínas depende do conhecimento das suas características físico-químicas e biológicas, incluindo a estabilidade química e física, imunogenicidade e perfil farmacocinético. A atividade terapêutica das proteínas é altamente dependente da sua estrutura conformacional. No entanto, a estrutura da proteína é flexível e sensível às condições externas, o que significa que a sua produção, formulação e manipulação requer uma atenção especial na otimização da eficácia e segurança, incluindo a minimização da resposta imune.

Apesar dos progressos da biotecnologia no desenvolvimento de novos medicamentos à base de proteínas, estes continuam a ter indicação para administração parenteral, devido às suas incompatibilidades e especificidades da estrutura química. No entanto, a via parenteral apresenta desvantagens relevantes para o paciente no que respeita o seu conforto e complacência, especialmente no tratamento crónico, onde se verifica uma diminuição na aderência ao tratamento, prejudicando assim o resultado desejado. Muitos esforços de pesquisa estão a ser realizados no sentido de aumentar a complacência do paciente pelo tratamento, quer através da utilização de vias alternativas de administração, ou na redução da frequência das injeções. Hoje em dia, várias superfícies mucosas, tais como a nasal,

pulmonar, oral e cavidade bucal estão a ser amplamente exploradas como vias alternativas para a administração de fármacos e macromoléculas. As nanopartículas emergem então como potencial aplicação na administração de substâncias terapêuticas, a fim de aumentar a eficiência do transporte e melhorar o perfil de libertação do fármaco. As vantagens da utilização de nanopartículas inclui a libertação da substância controlada e/ou prolongada, a redução de efeitos adversos associados com a substância, proteção contra compostos de inativação antes de chegar ao local de ação, aumentando assim a penetração intracelular do principio farmacológico. Alguns destes veículos possuem tamanhos subcelulares que permitem a internalização das partículas, podendo ocorrer a libertação do fármaco dentro da célula. Contudo estes veículos também acarretam desvantagens, como, limitação na capacidade de co associação a outras moléculas igualmente ativas, perfil toxico desconhecido, forma física indefinida. Para além da limitação no que diz respeito a encapsulação do fármaco, estes veículos podem também agregar ou degradar prematuramente devido à sua instabilidade em alguns fluídos biológicos

O sucesso de uma formulação depende da capacidade da proteína em manter a sua estrutura nativa e atividade durante a preparação e a libertação após administração, bem como durante o período de armazenamento. Diferentes métodos são utilizados para a preparação de nanopartículas, que permitem a modulação da sua estrutura, composição e propriedades físico-químicas. Diferentes perfis de libertação do fármaco podem ser conseguidos usando diferentes sistemas nanoparticulados, tais como micro ou nano partículas poliméricas.

A escolha do método de preparação das nanopartículas depende do polímero, da solubilidade do fármaco a ser encapsulado e da função que se quer atribuir ao sistema. A preparação de nanopartículas poliméricas pode envolver o uso de solventes orgânicos e métodos agressivos para biomoléculas. A utilização de polímeros naturais na construção destes veículos de entrega pode ultrapassar estes problemas, pois as nanopartículas podem ser formadas por simples complexação polielectrolítica. Polímeros naturais oferecem a vantagem de serem muito semelhantes, frequentemente idênticos, às substâncias macromoleculares, que os sistemas biológicos estão preparados para reconhecer e metabolizar. Assim os problemas de toxicidade e de estímulo crónico da reação inflamatória, bem como a falta de reconhecimento pelas células, provocados por muitos polímeros sintéticos, podem assim ser suprimidos. Esta semelhança a substâncias naturais ocorrentes, permite a conceção de sistemas de entrega que funcionam biologicamente a nível molecular, em vez de, macroscópico, revelando, por vezes, características únicas capazes de ultrapassar alguns dos problemas inerentes à administração de proteínas por vias não parenterais. Por outro lado, os

polímeros naturais apresentam frequentemente resposta imunológica e a sua manipulação tecnológica é mais complicada do que a dos polímeros sintéticos, devido à sua complexidade estrutural. Industrialmente estas formulações de sistemas de entrega de proteínas não são muito rentáveis devido à baixa eficiências de encapsulação (EE) que ronda normalmente os 10%.

Neste estudo foi então proposto o uso de polímeros naturais para a produção de nanopartículas para administração por vias mucosas, não só por esta classe de materiais apresentar mais possibilidade de cumprir os requisitos de biodegradabilidade e biocompatibilidade, que são obrigatórios em qualquer aplicação biomédica mas também pelas características únicas destes polímeros que podem aumentar a eficácia do tratamento por estas vias. Foram então escolhidos dois polímeros naturais fucoidan e quitosano, respetivamente extraídos de algas castanhas (*Fucus vesiculosus*), com 95% de fucose ésteres sulfatados (polissacárido aniónico), e do exosqueleto de crustáceos através da desacetilação da quitina (polissacarídeo catiónico). O quitosano destaca-se ainda pelas suas propriedades, muco-adesiva e de perturbação transiente das junções célula-célula, aumentando assim a absorção do fármaco.

Embora a técnica de complexação polielectrolítica tenha sido aplicada em outras ocasiões para a obtenção de nanopartículas à base de quitosano por interação com contra-iões, tais como o tripolifosfato ou carragenina, o presente trabalho é um dos primeiros a relatar a produção de nanopartículas resultante da complexação entre o quitosano e fucoidan. Estudos anteriores a este trabalho que relatam a complexação entre fucoidan e o quitosano, descrevem a produção de nanopartículas para encapsulação de curcumina um fármaco anti-tumor, ou o uso de micropartículas vazias (Fucospheres®) para tratamento de queimaduras dérmicas, tornando este estudo o primeiro a relatar a produção de nanopartículas para a encapsulação proteínas.

Neste trabalho, foram preparadas nanopartículas fucoidan/quitosano (FUC/CS) por complexação polielectrólitica. A formação de nanopartículas foi confirmada pela visualização do efeito de Tyndall, característico da formação de suspensões coloidais. O tamanho e potencial zeta das nanopartículas foram medidos por espectroscopia de correlação de fotões e anemometria de laser Doppler, respetivamente, utilizando uma Nanoseries Zetasizer (Malvern Instruments®, UK). Várias razões FUC/CS de massa (4/1 a 1/4) foram desenvolvidas, que resultaram na criação de nanopartículas com diferentes tamanhos (338-676 nm) e potenciais zeta (+41 a -49 mV). Em seguida foram selecionadas as formulações que apresentavam as

características mais apropriadas para a encapsulação de biomoléculas 4/1 e 1/4. As características chave que fazem com que uma formulação de nanopartículas seja adequada para encapsulação de biomoléculas são, um tamanho pequeno e carga elevada (+/-) de modo a promover a interação com as células, bem como um fraco efeito de Tyndall que facilita a posterior resuspensão. Ao encapsular as biomoléculas as nanopartículas tendem a aumentar de tamanho e a perder carga (+/-), pois as cargas superficiais dos polímeros que estariam livres são neutralizadas pelas interações polímero-proteína, resultando num aumento do rendimento de produção e efeito de floculação. Neste estudo, a albumina de soro bovino, insulina e ovalbumina foram utilizadas como proteínas modelos. A BSA foi escolhida para testar as variáveis de encapsulação, pois é muito utilizada em investigação como proteína modelo. Com pI 4.7 de este modelo proteico permite a manipulação da sua carga superficial e consequentemente da interação com os polímeros. Tecnicamente esta manipulação traduz-se na incorporação prévia da BSA numa das soluções poliméricas fucoidan ou quitosano, o que lhe confere carga positiva ou negativa, ou na alteração na ordem de adição dos polímeros o que permite a manutenção de um dado pH por mais algum tempo, visando assim um aumento na EE. A eficiência de encapsulação das nanopartículas foi determinada indiretamente, mediante a quantificação da biomolécula não encapsulada presente no sobrenadante após o procedimento de isolamento das nanopartículas. A quantidade de biomolécula livre foi determinada por Cromatografia Líquida de Alta Pressão HPLC (Agilent ® série 1100, Alemanha) com uma coluna 3,6 Aeris u Widepore colum XB-C18 (Phenomenex ®, EUA). Todas as nanopartículas FUC/CS 4/1 apresentaram um excelente EE de cerca de 100%. A influência das variáveis foi mais pronunciada na FUC/CS 1/4 em que a pré-incorporação de BSA no fucoidan aumentou o EE em ambos protocolos A e B (55 e 87%), relativamente à pré-incorporação no quitosano (11 e 19%). Foi também avaliada a morfologia das nanopartículas FUC/CS 1/4 e 4/1 por TEM, assim como o rendimento de produção das partículas vazias e cheias e capacidade de associação da BSA por gravimetria. As nanopartículas FUC/CS apresentaram algumas variações em termos de tamanho e potencial zeta após 170 dias de armazenamento a 4 º C, mas as variações não comprometeram a aplicação pretendida como portadores de proteína para a administração da mucosa.

INDEX

LIST OF ABBREVIATIONS

Abs	Absorbance
ADN	Ácido desoxirribonucleico
BioM	Biomolecules
BSA	Bovine serum albumin
CkOVM	Chicken ovomucoid
CS	Chitosan
DkOVM	Duck ovomucoid
DNA	Dessoxiribonucleic acid
EE	Encapsulation efficiency
FDA	Food and drug administration
FUC	Fucoidan
FUC/CS	Fucoidan/Chitosan
HPLC	High performance liquid chromatography
*K*Da	Kilo Daltons
LC	Loading Capacity
MW	Molecular weight
NPs	Nanoparticles
P(MAA-g-EG)	Poly(methacrylic acid-g-ethylene glycol)
pI	Isoelectric Point
PY	Production Yield
SD	Standard deviation
TEM	Transmition Electronic Microscopy
TFA	Trifluoroacetic acid
UV	Ultra violet
w/w	Weight/weight

FIGURE INDEX

GRAPHIC INDEX

TABLE INDEX

1 Introduction

1.1 Biomolecule-based therapies

1.1.1 Background

The vastness of diseases that commonly affect humans are caused by either some physiological dysfunction resulting from a gene mutation, incorrect expression of the related protein, or to the exposure to an environmental factor, such as pesticides, diet, bacterial, fungal or viral infection. For many of the conditions at a molecular level underlies a change in the amount, function or activity of one or more proteins which triggers changes in cellular, tissue or organ function [1]. An example of common physiological dysfunction is diabetes, caused by an insufficient activity of insulin or lack of the protein, and often a combination of these two factors [2]. This disease is common throughout the world and reports reveal that in 2011, 366 million people were affected with diabetes and in 2030 this number is expected to rise to 552 million. The highest incidence of diabetes is between 40 and 59 years and 78 000 children develop type 1 diabetes per year [3]. A large part of current worldwide medical research aimed at the identification of key proteins involved in molecular mechanism subjacent to many diseases such as the various forms of cancer and neurological conditions such as Parkinson's disease, motor neuron disease and multiple sclerosis, in order to select one of these proteins as a target for the development of a new drug that can minimize or eliminate the symptoms [1].

1.1.2 Historical frame

During the 1980s biopharmaceutical drugs became synonymous of therapeutic proteins, vaccines, and hormones produced by recombinant DNA technology. A couple of years later, human insulin, developed by Genentech Company (USA), reached the market, stating the industrial application of this technology [4] . Since then, hundreds of research centers and enterprises worldwide have been engaged in research, development and production of biopharmaceuticals. [5] In the 25 years of existence of the biopharmaceuticals market, many

diseases have been and remain the focus of attention both for the development and production of medicines, mainly for cancer, hepatitis, diabetes, growth disorders and hemophilia [6]. In this regard, progress in the biopharmaceutical technologies has created an increasing interest in proteins and peptides due to their role in many pathologies. However, the use of these molecules in medicine has been limited by their low bioavailability, which results from low stability against proteolytic enzymes, hydrolytic degradation, low permeability, and the short half-life in systemic circulation [7]. At the same time, biotechnology appeared as a multidisciplinary science, gathering basic biological sciences such as genetics, microbiology and biochemistry, with chemistry, biological engineering and bioinformatics [8]. This combined expertise led to the development of new therapies and also the large scale production of bio-products that were previously available only in limited quantities. Thanks to this biotechnological breakthrough, most of the molecules with therapeutic potential proposed nowadays are protein-based [9].

1.1.3 Protein formulations

Commercially, most protein-based drugs are formulated as aqueous solutions or suspensions ready for use or as lyophilized powder for reconstitution of the product. The formulation of proteins depends on their physicochemical and biological characteristics, including chemical and physical stability, immunogenicity and pharmacokinetic profile [10]. Therapeutic activity of proteins is highly dependent on their conformational structure. However, the protein structure is flexible and sensitive to external conditions, which means that its production, formulation and manipulation require special attention on optimizing the efficacy and safety, including minimizing the immune response [11]. From a design perspective, proteins are complex and challenging molecules to develop drug delivery systems. The success of a formulation depends on the ability of the protein to maintain its native structure and activity during the preparation and release after administration, as well as during the storage period [12]. Some proteins require sustained release, while others require a controlled, immediate or pulsed release. Different release profiles can be achieved using different particulate systems for drug delivery, such as polymeric micro or nano particles, hydrogels, liposomes and emulsions [13].

In order to develop these new systems several proteins are used as models in research. In the context of this work, it is important to highlight bovine serum albumin (BSA), ovalbumin and insulin. BSA has been primarily used in molecular studies and in the formulation of protein-based drug delivery systems [14]. The extensive use of this protein as model is due to its easy dissolution in water, yet it is relatively resistant to digestion. Presenting an isoelectric point of 4.7 this protein might expose negative or positive charges when in basic or acid environments, respectively. [15]. With a theoretical molecular weight of 69.3 *k*Da this protein has a well studied and documented structure that consists of nine loops connected by 17 disulfide bridges that are protected in the core of the protein. Relatively abundant and cheaper than other proteins, BSA also presents years of stability when stored at 2-8°C. Ovalbumin shares many of these characteristics and, therefore, is also frequently used as model [16]. In parallel, insulin is also used as model peptide, having the great advantage of providing a measurable pharmacological effect and being, thus, used as model therapeutic peptide. After insulin discovery, researchers engaged in the finding of different modes and routes of administration for this protein [12].

1.2 Routes of administration for protein-based formulations

Despite the great biotechnology progress, delivering the new protein-based drugs remains a problem, mainly due to incompatibilities and specific chemical structure [17]. Because of this, most protein-based formulations have an indication for parenteral administration. However, the parenteral route has important disadvantages to the patient, especially in chronic therapy, which decreases therapeutic compliance, thus impairing the expected results [18]. Many research efforts are being made to improve patient compliance, either through the use of alternative routes of administration or by reducing the frequency of injections [19]. The demand for better ways for the administration of proteins has resulted in research for the development of new pharmaceutical technologies. In this regard, the industry interest in developing alternative methods for drug delivery has been growing for years [6]. Nowadays, several mucosal surfaces such as the nasal, pulmonary and oral are being extensively explored as alternative routes for the systemic administration of macromolecular drugs [20].

1.2.1 Gastrointestinal mucosa

The oral route is the preferred for drug administration, being the most widely used. Apart from the simplicity of the administration itself, this approach provides access of the drug to the intestinal epithelium, the greater and the most specific surface area (200 m^2) of absorption existing in the human body (Figure 1.1) [21]. A major limitation in oral protein administration relates to the inherent instability due to inactivation or rapid enzymatic and pH degradation of these molecules in the gastrointestinal tract, in addition to the low permeability through biological membranes due to the high molecular weight and polar surface characteristics [22].

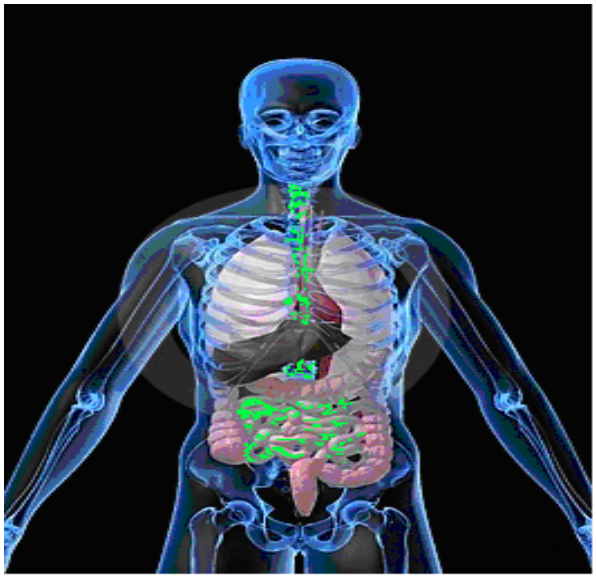


Figure 1.1: Drug (green) distribution in the digestive system trough oral administration. Adapted from [23].

1.2.2 Buccal mucosa

The buccal mucosa has attracted particular attention due to its unique physiological features, such as the avoidance of presystemic elimination, including the first pass effect, although this surface presents a relatively small area available for absorption (50 cm^2) [24]. The oral cavity presents 3 different types of mucosa (Figure1.2), with extremely vascularized epithelium that, combined with a low and very specific enzymatic activity, makes the buccal mucosa a great site for protein absorption [20].

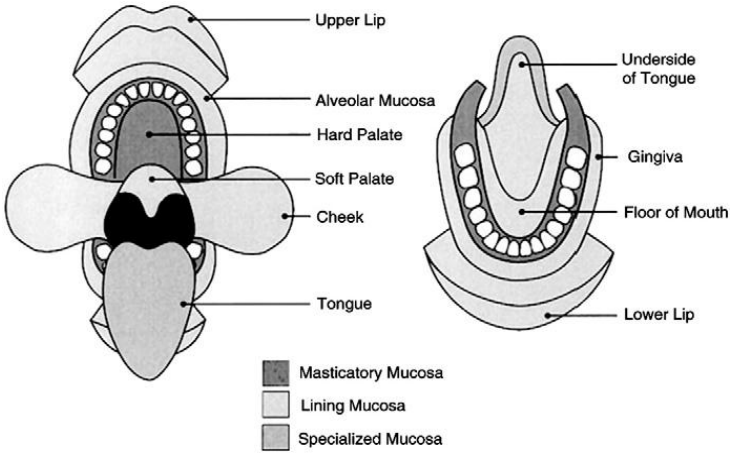


Figure 1.2: Buccal mucosa for drug absorption [20].

1.2.3 Pulmonary mucosa

The large alveolar surface area (100 m^2) suitable for drug absorption (Figure 1.3), presents a low thickness epithelial barrier, extensive vascularization and relatively low proteolytic activity compared to other administration routes. Together with the absence of the first-pass effect, this makes the pulmonary delivery of peptides and proteins an outstanding possibility [25].

Figure 1.3: Drug (green) distribution for pulmonary administration. Adapted from [26].

Insulin is undoubtedly the biopharmaceutical prototype and Exubera® (Pfizer) (Figure 1.4) was the first inhaled formulation approved by the United States Food and Drug Administration (FDA), for pulmonary administration of insulin in a dry powder form. Inhaled

insulin showed to be effective, well tolerated and better accepted in patients with type 1 and type 2 diabetes [27]. However, this technology was removed from the market. The patients claimed that the increased price of Exubera® relatively to the common injectable insulin was worthless and that the needles have gotten so fin that they cause virtually no pain. Moreover the patients also claimed that was not so easy to dose insulin with Exubera® as it was with the inject one. [28]

Figure 1.4: Insulin formulation for pulmonary administration. [29]

1.2.4 Nasal mucosa

The nasal mucosa (Figure 1.5) is also receiving a great deal of attention due to its permeability and easy access to the drug absorption site, although it presents low surface area (160 cm^2). This route is already commonly used for delivery of drugs for treatment of local diseases such as nasal allergy, nasal congestion and nasal infections [30]. A wide range of products has been developed mostly aiming at the advantage of the rapid onset of action for the treatment of pain and erectile dysfunction. Recent developments had brought this route as an alternative for direct administration of drugs in to the brain for the treatment of Alzheimer and Parkinson [31].

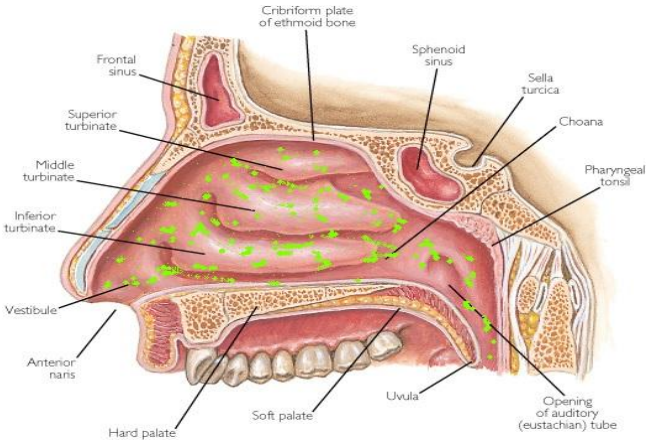


Figure 1.5: Drug (green) distribution in nasal administration. Adapted from [32].

1.3 Transmucosal drug delivery technologies

To overcome the limitations created by the mucosal surfaces, several strategies were developed aiming at improving the bioavailability of therapeutic proteins. The approaches commonly used in formulating mucosal protein delivery systems include specific excipients, such as absorption enhancers, enzyme inhibitors, and mucoadhesive polymers. In addition, formulations are designed in such a manner to provide protection of protein drugs from the harsh human environment [33]. These strategies have proven to improve protein bioavailability and researchers believe that addressing the mentioned drawbacks is possible with the design of a protein formulation that combines all of referred strategies (Table 1.1) [22].

Table 1.1: Main approaches used for mucosal protein delivery. Table adapted from [22].

Approaches	Systems	Advantages	Disavantagens
Absorption enhancers	Bile salts, fatty acids, surfactants, salicylates, chelators, zonular occludens toxin	Increase membrane permeation	Transport of both protein/peptide and undesirable molecules
Enzyme Inhibitors	Sodium glycocholate, camostat mesilate, bacitracin, soybean trypsin inhibitor, aprotinin, CkOVM, DkOVM, polymer–inhibitor conjugates	Resist enzyme degradation	Induced severe side effects in chronic therapy
Mucoadhesive Polymers	P(MAA-g-EG) hydrogel and lectinconjugated alginate microparticles, thiolated polymers, Mucoadhesive patch system mucoadhesive polymer–inhibitor	Site-specific delivery and improve membrane permeation Site-specific drug delivery and resist enzyme degradation	Natural mucus turnover in intestine Extensive costs of certain enzyme inhibitors
Formulation Vehicles	Emulsions Liposomes Microspheres Nanoparticles	Protect drug from acid and enhance permeation through mucosa Improve physical stability and increase membrane permeation Prevent proteolytic degradation . Restrict release of drug to favourable area; Prevent enzymatic degradation and increase intestinal epithelial absorption	Physicochemical instability in long-term storage and requirement for storage at low Temperatures Low stability of liposomes Concerns of protein stability during processing, release and storage Low loading efficiency of hydrophilic drugs, difficulty of precise size control and avoidance of particle aggregation

Abbreviations: CkOVM, chicken ovomucoid; DkOVM, duck ovomucoid; P(MAA-g-EG), poly(methacrylic acid-g-ethylene glycol).

1.4 Polymeric nanoparticles for mucosal administration

1.4.1 Historical frame

Nanoparticles were first developed in the mid 70s in order to carry vaccines and anticancer agents to specific tissues or even cells improving therapeutic efficacy and decreasing the toxic effect of the drugs [34]. Later on, with the growing interest in the therapeutic potential of labile molecules such as protein and peptides, nanoparticles started being explored as vehicles to provide protection, being also proposed for administration through different routes, such as mucosal surfaces (Table 1.2). Nowadays, nanotechnology allows real progress in the achievement of temporal and spatial site-specific delivery [14].

Table 1.2: Advantages and disadvantages of different mucosal routes and administration of nanoparticle through these routes [24] [35] [19].

Administration routes	Advantages	Disadvantages
Oral	• Drug protection against pH and enzymatic damage • Increased permeability across the epithelial membrane	• First-pass metabolism in the liver: potential hepatotoxic effect • Potential translocation into systemic circulation • Requires intact intestinal mucosa for the uptake
Buccal	• Short recovery time of mucosa after stress or damage • Increased permeability to molecular weight and hydrophilic compounds	• Limited to potent molecules • Continuous dilution of drug • Involuntary swallowing of drug
Pulmonary	• Ease of administration • Local action • Rapid absorption and onset of action • Possibility of administering lower doses	• Local toxicity • Potential for translocation into systemic circulation • Airway structure acts as a filter • Mucociliary clearance • Alveolar macrophages • Absorption affected by pathological conditions • Requires complex devices and particles with specific aerodynamic properties • Particles can be exhaled • Many factors affecting reproducibility
Nasal	• Ease of administration • Rapid absorption and onset of action • Fewer side effects • Drug protection from degradation by nasal mucosa and secretion enzymes	• Large interspecies variation, leading to difficult extrapolations of results • Drug diffusion limited by the mucus barrier and mucociliary clearance • Administered volume limited to 25-200 µL • Molecular weight cut-off of ~ 1 *k*Da • Absorption affected by pathological conditions • Limited to potent molecules • Lack of reproducibility

1.4.2 Definition and structural organization

Nanoparticles present variable sizes that range between 10 and 1000 nm, in which the drug can be dissolved, coated, encapsulated or dispersed. Nanoparticulate drug delivery systems can have several terminologies, according to structures and materials composing the systems (Figure 1.6). The use of different production methods can create different and unique systems, which can be used according to the biological interaction necessary for each purpose [36]. In the context of this work, the importance of polymeric nanoparticles will be highlighted, considering their potential for the transmucosal administration of proteins [37].

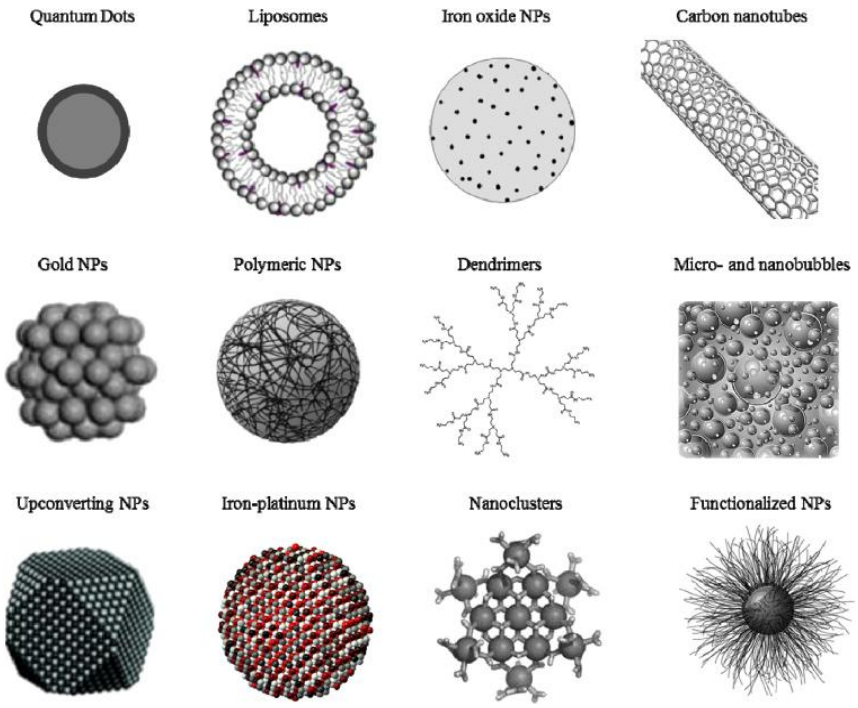


Figure 1.6: Types of terminologies used for nanoparticulate drug delivery systems. (NPs) Nanoparticles [36].

Other systems such as microparticles and hydrogel are also being developed with the same propose of nanoparticles. Table 1.3 describes the advantages and disadvantages of the use of nanoparticles. .Nanoparticles can actually protect labile drugs from the biological barriers and enhance their absorption by optimizing their interaction with the absorption site. Some authors have suggested that nanoparticles may improve the bioavailability of peptide or protein [7].

Table 1.3: Nanoparticles as drug delivery systems, advantages and disadvantages .Adapted from [38] [24].

Advantages	Disadvantages
High surface/volume ratio	Undefined physical shape
Ease of surface modification	Limited capacity to co-associate other functional molecules
Maximized contact with mucosa	Unknown toxicity profile
High drug concentration in desired site	Lack of suitable large-scale production methods
Reduction of adverse drug-associated effects	Low stability in some biological fluids
Intracellular penetration	Tendency for aggregation
Protection of encapsulated molecules	Limited loading capacity (unsuitable for less potent drugs)
Possibility to provide controlled and or/ prolonged release	Small size can provide access to unintended environments
Possibility of targeted delivery	
Enhanced drug absorption	

Polymeric nanoparticles are spherical systems, formed of one or more polymers [39].Polymeric nanoparticles are classified in two categories, nanospheres (Figure 1.7 A) and nanocapsules (Figure 1.7 B), which differ depending on the composition and structural organization. The nanocapsules are vesicular systems in which the drug is within an aqueous or oily cavity surrounded by a polymer membrane, or can also be found adsorbed in the polymer membrane. The nanospheres are formed by a polymeric matrix, where the drug is dispersed or adsorbed [40].

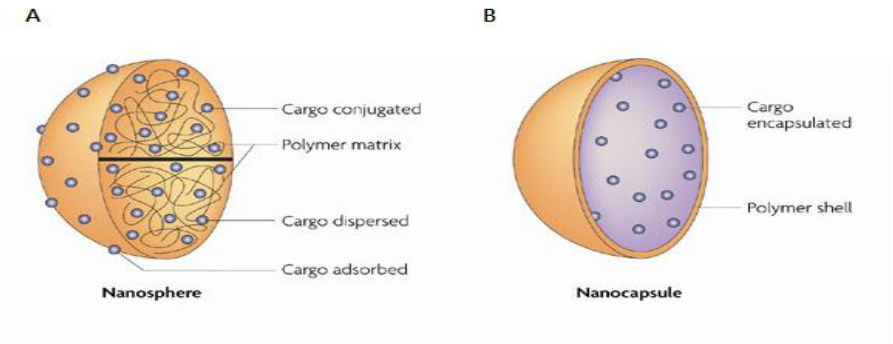


Figure 1.7: Structural differences for polymeric nanoparticles. (A) nanospheres, (B) nanocapsules. Adapted from [41].

1.4.3 Preparation methods

Different methods are used to prepare polymeric nanoparticles, which allow the modulation of their structure, composition and physicochemical properties [42]. The choice of a preparation method depends on the final application of the produced system, type of polymer and the solubility of the drug to be encapsulated. The preparation of polymeric nanoparticles often involves the use of organic solvents and aggressive methods, like ultrasound energy. However, these could affect negatively both the drug/protein to be encapsulated and the organism that will be administered with the nanosystem. Different methods are available which explore different polymer interactions, resulting in techniques such as ionic gelation, polyelectrolyte complexation, emulsification, coacervation and spontaneous self-assembling, among others [43]. Using natural polymers to prepare the nanoparticles permits using methodologies that overcome the mentioned problems regarding aggressive conditions, one of the most used techniques being polyelectrolyte complexation [44].

1.4.4 Characterization

Complete characterization of nanoparticles requires assessment of several parameters: polymer type and concentration, morphology, particle size and zeta potential, production yield, protein encapsulation efficiency, protein loading capacity and type of release profile [25]. Nanoparticle formulation depends on the choice of suitable polymeric systems with high encapsulation efficiency, improved bioavailability and retention time. The desired formulations are generally achieved by trial and error method. Nanoparticle formulations display improved properties as compared with conventional formulations, namely concerning controlled release, targeted delivery and therapeutic impact. These targeting capabilities of nanoparticles are influenced by particle size, surface charge, surface modification, and hydrophobicity [45]. The size of nanoparticles for crossing different biological barriers is dependent on the tissue, target site and circulation. Therefore, size and size distribution determines nanoparticle interaction with the cell membrane and their penetration across the physiological drug barriers (Figure 1.8). In turn, nanoparticle surface charge is important in

determining whether they would cluster in biological fluids or would adhere to, or interact with oppositely charged cells, predicting cellular internalization [39].

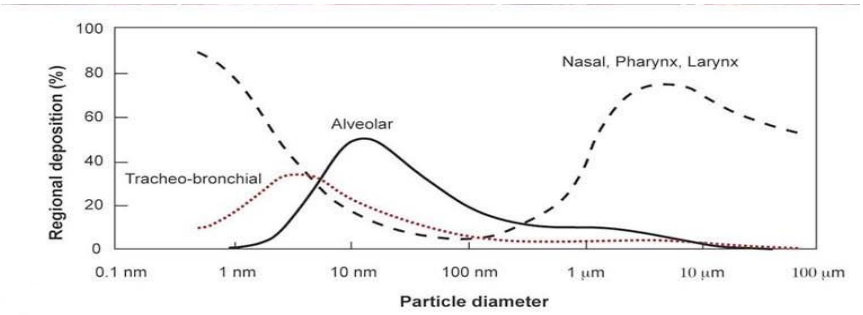


Figure 1.8: Size influence on deposition of inhaled nanoparticles in the human respiratory tract [46].

1.5 Polymers as nanoparticle matrix-forming materials

1.5.1 Definition

A polymer is a high molecular weight molecule composed of repeating small subunits called monomers. The polymers may be classified according to their occurrence as natural or synthetic, as well as for their chain nature, structure, morphology and type of polymerization reaction. Natural polymers have in general more complex structures than synthetic polymers [47]. Both natural and synthetic polymers have been extensively investigated as biomaterials, those with the most frequently reported applications being described in Table 1.4, with their respective advantages and disanvantages.

Table 1.4: Different types of polymers and respective advantages and disadvantages [48].

Occurrence	Polymers	Advantages	Disadvantages
Natural	Proteins Polynucleotides Polysaccharides Gums Resins Elastomers	Biodegradable Biocompatible Nontoxic Function biologically at molecular and macroscopic level. Degradation via natural enzymes; cross-linkers can make less degradable	Biodeterioration Immunological reaction High natural variability Structurally complexity Technological manipulation is more elaborate
Synthetic	Polyamides Polyamine acids Polyalkylated cyanoacrylates Polyesters Poly(ortho esters) Polyurethanes Polyacrylamides	Predictable properties Batch-to-batch uniformity Easy technological manipulation	Too expensive Environmental and human health concerns Lack of recognition by cells Toxicity Stimulation of a chronic inflammatory reaction

1.5.2 Application of natural polymers in nanopharmaceutics

For a polymer to be used as a biomaterial it should not cause inflammatory or toxic reactions at the application site, it must provide the drug with adequate half-life , degradation time should be compatible with the desired application, degradation products cannot be toxic, and should be able to be metabolized and eliminated from the body [49].

Natural polymers may be regarded as the first clinically used biomaterials and, actually, polymers have always been classical excipients in pharmacy. More recently, with the advances in nanotechnology, more sophisticated biodegradable polymers were developed providing new delivery systems for peptides and proteins [48]. However, the development of biodegradable systems requires the control of a great number of variables, since the kinetics of polymer degradation *in vivo* must remain constant, to obtain a controlled release of the substance. Therefore, factors such as pH and temperature, which may promote an increase or a reduction in the rate of degradation of the system, should be evaluated during development [50]. Synthetic biodegradable polymers have shown growing interest in the application as

delivery systems, since natural ones feature, usually, a rapid drug release [51]. The profile and mechanism of drug release depends on the nature of polymer and also the physicochemical properties of the substance incorporated therein [49].

1.5.3 Chitosan

Chitosan is a cationic polysaccharide obtained by the alkaline, partial deacetylation of chitin, the major component of crustacean shells [52]. This linear copolymer consists of β-(1-4)-linked 2-amino-2-deoxy-D-glucose (D-glucosamine) and 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) units, which are displayed in figure 1.9. Due to the content of primary amino groups in the main backbone, this polysaccharide presents cationic character. Chitosan is easily soluble in aqueous acidic solutions, featuring a low solubility at the physiological pH of 7.4 as it is a weak base (pKa around 6.5) [53].

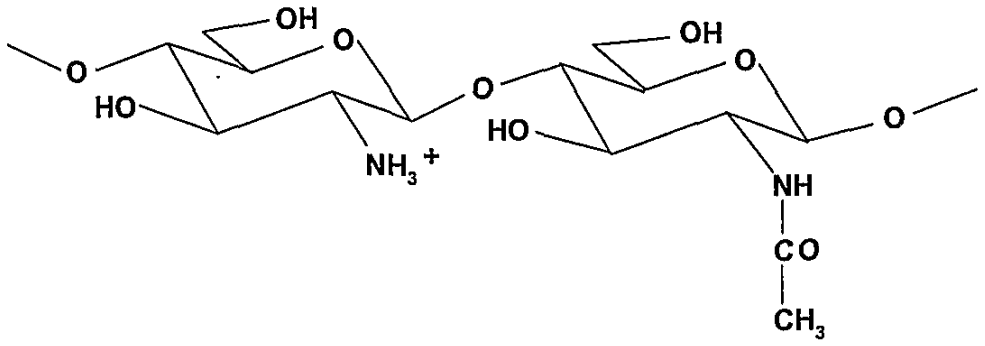


Figure 1.9: Chitosan structure [54].

As the main characteristics justifying its wide use in pharmaceutical applications, it is worth mentioning the biodegradability, biocompatibility and also the strong mucoadhesive properties. Along with the very safe toxicity profile, these make chitosan an exciting and promising excipient for the pharmaceutical industry for present and future applications [55]. These unique features allowed the design of bioadhesive drug carrier systems that have arisen as promising candidates in several mucosal administration routes, thus improving the transport of biomacromolecules such as peptides, proteins, oligonucleotides, and plasmids across biological surfaces [56]. Chitosan particles can also improve drug absorption via the paracellular route, as exemplified in figure1.10. The mechanism of action of chitosan has

been suggested to be a combination of bioadhesion and a transient widening of the tight junctions between epithelial cells [57].

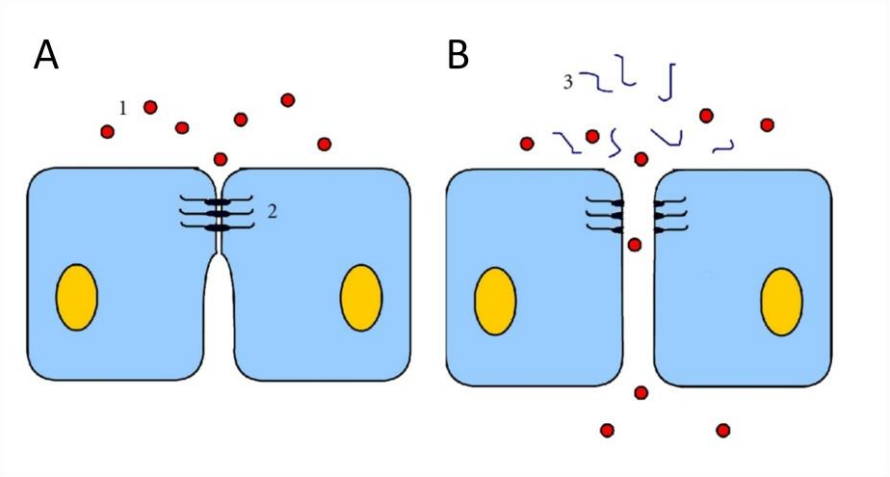


Figure 1.10: Effect of chitosan on the absorption of drugs by the paracellular route. (A) Normal epithelium. (B) Transient disruption of tight junctions by chitosan with enhancement of drug absorption. 1: represents the drug, 2: represents the tight junction. Adapted from [57].

1.5.4 Fucoidan

Fucoidans are anionic polysaccharides containing substantial percentages of L-fucose and sulfate ester groups, extracted from brown algae and some marine invertebrates such as marine cucumber. Commercially available, fucoidan prepared from Fucus vesiculosus contains 44% fucose and 26% sulfate, being water soluble [58]. A structural model in figure 1.12 shows that the core region of fucoidan is primarily α-L-fucose units linked by (1→4) and (1→3) glycosidic bonds, with sulfate groups substituted at the C-4 position on some of the fucose residues. (Figure 1.11) [59].

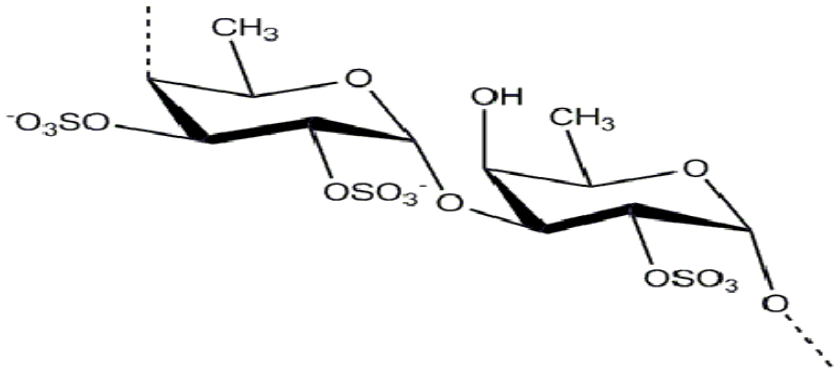


Figure.1.11: Fucoidan structure [71].

For the past decade, fucoidans isolated from different species have been extensively studied due to their varied biological activities, including anticoagulant and antithrombotic, antivirus, antitumor and immunomodulatory, anti-inflammatory, antidislipidemic, antioxidant and anticomplementary properties. Relevant activity was reported against hepatopathy, uropathy and renalpathy, as well as providing gastric protective effects and therapeutic potential in surgery. Fucoidan can showed the ability to sequester toxic heavy metals such as Cd^{2+},Cu^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+}, and Hg^{2+}. This marine biopolymer has shown great properties for drug delivery and has been reported to bind type A I and II transmembrane glycoprotein receptors found in macrophages, this way fucoidan can promote specific interactions of a drug carrier with the macrophages [60].

1.6 State of the Art

Although the technique of polyelectrolyte complexation was applied in other occasions to obtain chitosan-based nanoparticles by interaction with counter-anions, such as tripolyphosphate or carrageenan [61], [52], the present work is one of the first reports on the production of nanoparticles resulting from the complexation between chitosan and fucoidan. Previous reports on fucoidan/chitosan complexation describe the production of nanoparticles to encapsulate the antitumor drug curcumin [62], as well the production of microparticles for protein encapsulation [63] or the use of unloaded microparticles (Fucospheres®) for dermal burn treatment [59], making this study the first that reports fucoidan/chitosan nanoparticles for protein encapsulation. Importantly, the results reported in the present study have already been presented in panel at the 3rd Congress of the Portuguese Society of Pharmaceutical Sciences (Appendix 1) and 9th Central European Symposium on Pharmaceutical Technology (Appendix 2).

2 Objectives

The aim of this work was to verify de ability of chitosan and fucoidan to assemble into nanoparticles which display ability to encapsulate different model proteins, namely BSA, ovalbumin and insulin. The nanoparticles are aimed at an application in systemic mucosal protein administration and, therefore, several specific properties should be evidenced, as follows:

- Size within 50-500 nm to permit a close interaction with the epithelial surface;
- Zeta potential above 30 mV (either negative or positive) to provide adequate stability in aqueous suspension and to maximize interaction with the epithelial surface;
- Adequate protein encapsulation efficiency, preferably above 50%.

3 Materials and Methods

3.1 Reagents

Chitosan (CS) (low molecular weight, deacetylation degree 75–85%), Fucoidan (FUC) from *Fucus vesiculosus*, bovine albumin serum (BSA), insulin and ovalbumin sodium, phosphotungstate dibasic hydrate, sodium hydroxide and glycerol were purchased from Sigma-Aldrich® (Germany). Bradford reagent was purchased from Bio-rad® (Germany) Trifluoroacetic acid (TFA) and glacial acetic acid were supplied, respectively, by Alfa Aesar® (Germany) and Panreac Synthesis® (Germany). Ultrapure water (Integral 3, Millipore®, Portugal) was used throughout. All other chemicals were reagent grade.

3.2 Preparation of fucoidan/ chitosan nanoparticles

Fucoidan/chitosan (FUC/CS) nanoparticles were prepared by polyelectrolyte complexation, in which the negatively charged groups of fucoidan interact with the cationic groups of chitosan, creating electrostatic bonds. Briefly, a 2 mg/mL stock solution of FUC (pH 6.18) was prepared with milliQ water and a 1 mg/mL stock solution of CS (pH 3.17) was prepared with 1% (w/w) acetic acid. Both solutions were filtered before further using (0.2 µm filter, Whatman®, Germany). These stock solutions were then diluted to obtain various concentrations, in order to permit the preparation of nanoparticles with different mass ratios (4/1 to 1/4, w/w). FUC/CS nanoparticles were spontaneously formed by drop wise addition of either 1 mL of FUC into 1 mL of CS solution (protocol A), or 1 mL CS into 1 mL of FUC solution (protocol B), under magnetic stirring for 10 min, at room temperature.

Nanoparticle suspensions were then placed in eppendorf tubes over a layer of 10 µL glycerol that prevents nanoparticle dehydration and aids the subsequent resuspension step. Nanoparticles were isolated by centrifugation at 16000 *g*, for 30 min at 15 ºC (Thermo Scientific®, Germany). The supernatants were discarded and nanoparticles were resuspended in 200 µL of purified water.

3.3 Association of biomolecules to FUC/CS nanoparticles

Three different proteins were associated to the nanoparticles, which were used as models: bovine serum albumin (BSA), insulin and ovalbumin. BSA and ovalbumin were dissolved in milliQ water, while insulin was dissolved in NaOH 0.01 M. The proteins were either associated with FUC or CS prior to the mixture of the polymers, to test the effect of this variable. Along this text, when referring to a nanoparticle formulation loaded with proteins, an asterisk (*) will be placed close to the number representing the polymer with which the protein was mixed prior to nanoparticle formation (example: FUC/CS = *4/1 means that the protein was mixed with the FUC solution prior to pouring of this polymer into the CS solution).

The proteins were associated to the nanoparticles in a concentration of 30% (w/w) respective to the polymer with the higher content in the formulation. Both protocols A and B (described above) were used to prepare protein-loaded FUC/CS nanoparticles, in order to test the effect of the order of addition of polymers on the final characteristics of the nanoparticles. The isolation of nanoparticles was performed as described above.

3.4 Characterization of nanoparticles

3.4.1 Physicochemical properties

The size and zeta potential of nanoparticles were measured by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a Zetasizer Nanoseries (Malvern Instruments®, UK). For the measurements, 20 µL of each sample were diluted with 1 mL purified miliQ water and the suspension placed in an electrophoretic cell. Each analysis was performed at 25 ºC (n = 3).

3.4.2 Morphology

The morphological analysis of FUC/CS nanoparticles was performed by transmission electron microscopy (TEM; Jeol-JEM® 1011, Germany). Concentrated nanoparticle suspensions were obtained upon centrifugation, mounted on copper grids coated with a carbon film (Ted Pella®, USA) and stained with a 2% (w/v) sodium phosphotungstate dibasic hydrate solution.

3.4.3 Determination of nanoparticle production yield

The nanoparticle production yield was determined by gravimetry. For this procedure, nanoparticles were prepared, isolated by centrifugation at 16000 *g*, for 30 min at 15 ºC (Thermo Scientific®, Germany) and the sediments were freeze-dried over 24 h, using a Freeze Dryer (Labconco®, USA) (n = 6). The production yield (PY) was calculated as follows:

$$PY(\%) = \frac{Nanoparticles\ weight}{Total\ solids\ weight} \times 100$$

Where *nanoparticles weight* is the sediment weight after freeze-drying and *total solids weight* is the total amount of solids added for nanoparticle formation (fucoidan and chitosan for unloaded nanoparticles and fucoidan, chitosan, and protein for protein-loaded nanoparticles).

3.4.4 Stability assay

For the assessment of nanoparticle stability, the formulations FUC/CS 1/4 and 4/1 were prepared according to the procedure described above. Aqueous suspensions of nanoparticles were stored at 4 ºC and their size and zeta potential were monitored along time (n > 3).

3.4.5 Determination of BSA encapsulation efficiency and loading capacity of nanoparticles

The BSA encapsulation efficiency was determined indirectly, by quantification of the non-encapsulated protein present in the supernatant after the nanoparticle isolation procedure. The amount of free protein was primarily determined by High Pressure Liquid Chromatography (HPLC, Agilent® 1100 series, Germany) with a Aeris Widepore 3.6 u XB-C18 column (Phenomenex®, USA). The conditions for each run were: temperature 25 ºC; gradient flow (0.1% TFA in water (A), 0.1% TFA in acetonitrile (B); A/B from 95:5 to 35:65 in 15 min) mobile phase, total run time 20 min, UV detection at 280 nm; flow rate 1.0 mL/min; injection volume 20 µL; BSA retention time 8.7 min. A linear calibration curve for BSA in 1% (w/w) acetic acid was obtained over the range 5–120 µg/mL (n = 3) (R^2 = 0.996). Absorbance spectrums of pure solutions of polymers and BSA were run in So Bio UV-Visible spectrophotometer (Varian®, Australia). The nanoparticle protein association efficiency and loading capacity were calculated from Equations indicated below:

$$Encapsulation\ efficiency\ (\%) = \frac{Total\ protein\ amount\ -Free\ protein\ amount}{Total\ protein\ amount} \times 100$$

$$Loading\ capacity\ (\%) = \frac{Total\ protein\ amount - Free\ protein\ amount}{Nanoparticles\ weight} \times 100$$

3.5 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 $P < 0.05$.

4 Results and discussion

4.1 Characterization of FUC/CS nanoparticles

Fucoidan/chitosan (FUC/CS) nanoparticles were successfully obtained, using several concentrations of the two polymers, which resulted in FUC/CS mass ratios of 1/4 to 4/1. The assembly of nanoparticles was mediated by an electrostatic interaction between the negatively charged sulfate groups of fucoidan and the oppositely charged amino groups of chitosan. The order of addition of the polymers was analysed as variable, varying according to Protocols A (fucoidan added over chitosan) and B (chitosan added over fucoidan), as was described in the methodology. Further optimizations of the procedure of nanoparticle preparation are described in appendix 3. Apart from the visible Tyndall effect that proves the formation of a colloidal suspension of FUC/CS nanoparticles, the effect of different mass ratios and order of addition of polymers was evaluated concerning the resultant size, zeta potential and production yield, as described in the following sections.

4.1.1 Morphological and physicochemical properties

Figure 4.1 displays the TEM microphotographs of representative FUC/CS nanoparticles of mass ratios 4/1 and 1/4, which evidence a compact structure and a tendency to a spherical shape.

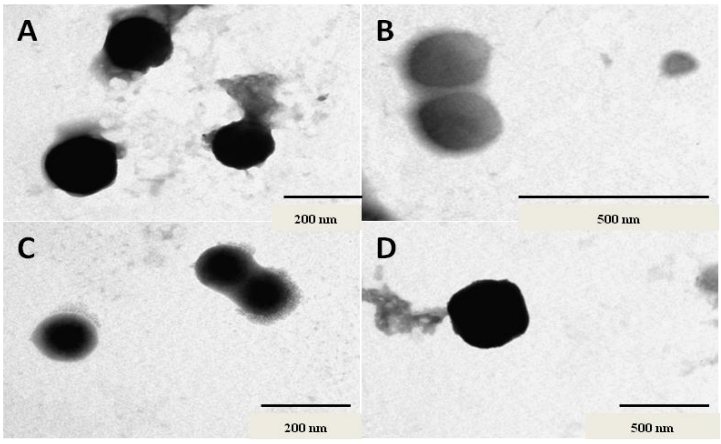
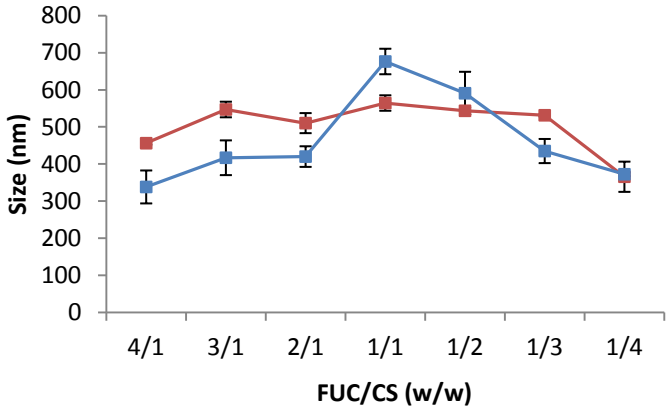


Figure 4.1: TEM microphotographs representative of FUC/CS nanoparticles. FUC/CS = 1/4 (A and B) and FUC/CS = 4/1 (C and D).

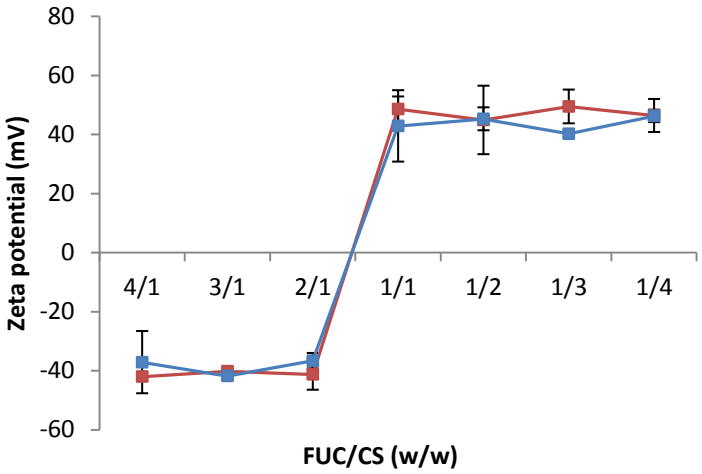
As displayed in Graphic 4.1, the size of FUC/CS nanoparticles varied between 338 and 676 nm. As expected, the variation of mass ratios resulted in different sizes, an observation valid for both protocols A and B, although a particular trend could not be established. Generally, it was observed that the presence of greater amounts of polymer (chitosan and fucoidan) in the formulations resulted in increased particle size of the nanoparticles. Therefore, the formulation FUC/CS = 1/1 presented the highest sizes ($p < 0.05$), 564 and 676 nm for protocol A and B, respectively. Accordingly, the opposite happens when the concentration of the polymers in each formulation reaches the lower limit, with the mass ratios of 4/1 and 1/4 presenting the lowest sizes in each protocol (varying from 338 to 456 nm). This general behaviour can be observed for both protocols, as described. However, size differences were more prominent in protocol B, where nanoparticles displayed the lowest and the highest sizes. As a general trend, the order of addition of one polymer over the other affected the resulting nanoparticle size and, thus, different results were obtained for protocols A and B. When comparing both protocols, statistically significant differences were obtained in all the formulations ($p < 0.05$), except for mass ratios 1/2 and 1/4. For a more direct observation of the size values, a table with that information is available in appendix 4.



Graphic 4.1: Size variation of FUC/CS nanoparticles of different mass ratios, produced using (■) protocol A and (■) protocol B (mean ± SD, n = 3).

In what concerns the zeta potentials of the obtained FUC/CS nanoparticles, a complete shift from strong negative to strong positive charges was observed, depending on the mass ratios (graphic 4.2). Those formulations with higher amount of fucoidan (4/1 to 2/1) resulted in a negative charge, with a maximum of -37 mV registered for FUC/CS = 4/1. On the

contrary, formulations with the higher amount of chitosan (1/2 to 1/4) displayed strong positive charge, reaching a maximum value of +49 mV. Curiously, the formulation with equal mass of both polymers also registered a strong positive surface charge (+43 mV or +49 mV, depending on used protocol), which indicates that chitosan has a higher charge density. The specific values obtained for each formulation and protocol are also available on table x on appendix 4. On the contrary of what was observed for the size, zeta potentials were not affected by the order of addition of polymers tested in protocols A and B. In addition, different mass ratios did not have a pronounced effect on zeta potential either, in contrast with what was observed for nanoparticle size.



Graphic 4.2: Zeta potential variation on FUC/CS nanoparticles of different mass ratios, produced using (■) protocol A and (■) protocol B (mean ± SD, n = 3).

It is a fact that a complete inversion of zeta potential ($p < 0.05$) is obtained when fucoidan changes from the most represented polymer, inducing a strong negative charge, to the less or equal represented polymer, in which cases a strong positive charge is obtained. However, apart from this evident shift, all formulations with higher amount of fucoidan (4/1 to 2/1) displayed a charge around -40 mV and all formulations from 1/1 to 1/4 registered a charge of approximately +45 mV. This effect was reported in several other works concerning chitosan-based nanoparticles, in which similar variations of polymer mass ratios resulted in very small changes of zeta potential, in the order of 4 or 5 mV [64], [65].

Nanoparticles based on chitosan and fucoidan were previously proposed for the treatment of dermal burns [59] and for the encapsulation of stromal cell-derived factor 1 (SDF-1),

which is an important chemokine in stem cell mobilization [66]. In the first case, only positively charged nanoparticles were obtained, inclusive when fucoidan was present in higher amount (FUC/CS = 5/1). The authors justified this effect with the possible formation of an outer layer of chitosan that took place during the nanoparticle assembly [59]. The second work reported very similar results as compared to those described in the present study, with strong negatively charged nanoparticles being obtained when fucoidan is present in higher amount in the formulation (FUC/CS = 5/1 has -49.8 mV). Additionally, FUC/CS = 1/1 also presents a positive charge (+ 24.5 mV), as is reported in the present study [66]. The explanation for the different results found among studies might rely on the use of different chitosan and fucoidan, which is available with very different characteristics and usual very few details are given in the papers.

4.1.2 Nanoparticle production yield

Given the displayed size characteristics, FUC/CS mass ratios of 4/1 and 1/4 were selected to proceed with the studies. At the beginning of this work, only Protocol A was in course for the preparation of nanoparticles and, when protein-loaded nanoparticles were prepared, the protein was always mixed with the polymeric solution corresponding to the lower amount in each formulation (fucoidan in FUC/CS = 1/4 and chitosan in FUC/CS = 4/1). Protocol B was tested later on in an attempt to improve the encapsulation efficiency of nanoparticles with mass ratio 1/4. This is why protocol B was not tested for formulation 4/1 concerning the production yield, as the characteristics of those nanoparticles were satisfactory.

Table 4.1 shows that protocol A tends to provide a higher production yield (PY) for the formulation 4/1 (32%) as compared with the formulation 1/4 (16%), although the difference is not statistically significant due to the high standard deviations. The production yield is a measure of the interactions that take place when both polymers contact with each other. The fact that, in protocol A, formulation 4/1 has a higher yield than formulation 1/4 might indicate that chitosan has a higher charge density. When comparing the yields of 1/4 nanoparticles obtained with different protocols, although protocol B apparently increases the yield, the differences are not significant.

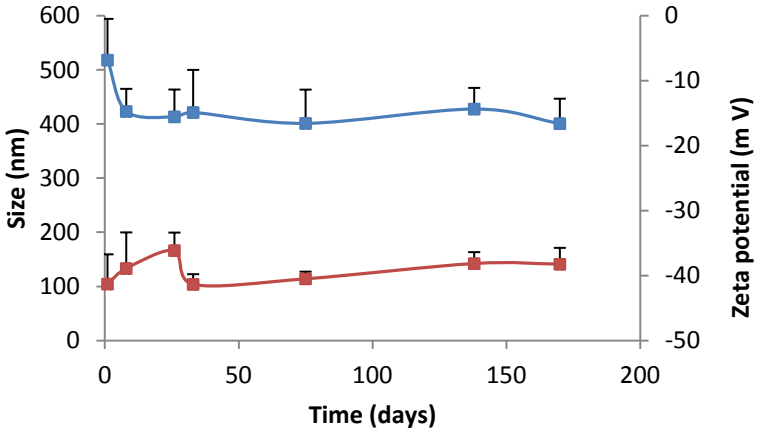
Table 4.1: Production yield of unloaded FUC/CS nanoparticles of mass ratios 1/4 and 4/1 obtained by different protocols (mean ± SD, n = 6).

Protocol	FUC/CS	Production Yield (%)
A	4/1	32 ± 19
	1/4	16 ± 11
B	1/4	26 ± 13

1.1.1 Stability assay

FUC/CS nanoparticles of mass ratios of 1/4 and 4/1 were monitored for their storage stability along time, concerning size and zeta potential. One of the most common problems of colloidal particles relies on their tendency for flocculation [24]. This is frequently accompanied by a decrease in zeta potential to values below 30 mV (in modulus), where the repulsive forces are not enough to maintain nanoparticles separated from each other, leading to aggregation.

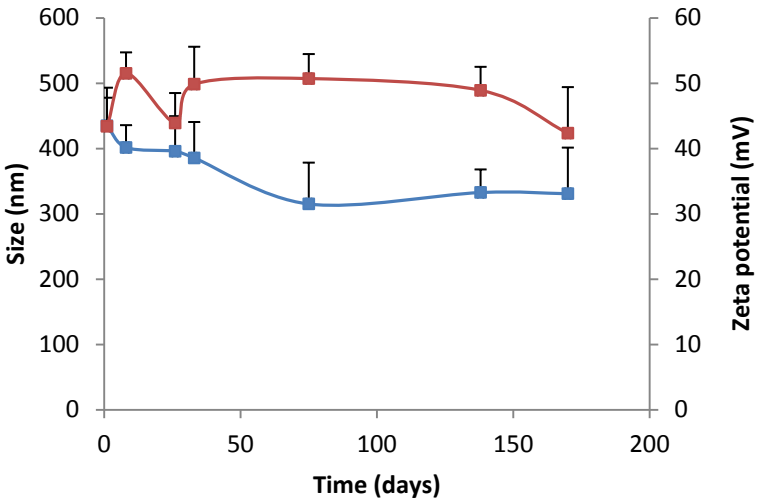
In this study, aqueous suspensions of FUC/CS nanoparticles were maintained at 4 ºC for a period of 170 days. Nanoparticles of formulation 4/1 registered a slight size decrease at the beginning, maintaining stable after that (Graphic 4.3). Nevertheless, as compared with the initial size (518 nm), differences were only statistically significant at the last time point (170 days; $p < 0.05$), in which the registered size was 400 nm. Concerning zeta potential, apart for an apparent instability at the beginning, that was not significant anyway, the zeta potential maintained relatively stable, being -41 mV for fresh nanoparticles and -38 mV after 170 days.



Graphic 4.3: Evolution of (■) size and (■) zeta potential of FUC/CS = 4/1 nanoparticles along time (aqueous suspension stored at 4 ºC), (mean ± SD, n = 6).

FUC/CS = 1/4 nanoparticles exhibited a very similar behavior (Graphic 4.4), with a slight size decrease from 435 to 331 nm in 170 days. Zeta potential also registered an initial variation, but the value registered at the end of 170 days (42 mV) was very similar to the initial (43 mV).

In a general manner, it can be said that nanoparticles register some slight variations in their physicochemical characteristics upon 170 days of storage at 4 ºC, although the variations do not compromise the intended application as protein carriers for mucosal administration.



Graphic 4.4: Evolution of (■) Size and (■) zeta potential of FUC/CS= 1/4 nanoparticles along time (aqueous suspension stored 4 ºC), (mean ± SD, n = 3).

4.2 Association of proteins to FUC/CS nanoparticles

To choose the most suitable formulation for the encapsulation of biomolecules, key features were taken into account, namely concerning particle size, which drives the interaction with mucosal epithelia. FUC/CS nanoparticles 1/4 and 4/1 were selected to undergo protein encapsulation, as they display the smaller sizes and also opposite charges that might further affect cell interaction, which study is of interest in the field. Nanoparticles were tested regarding their ability to associate model proteins of different molecular weights, namely BSA (67 *k*Da), ovalbumin (44 *k*Da) and insulin (5.7 *k*Da). In addition, protein-loaded nanoparticles were characterised for their physicochemical properties. Because of time limitations, nanoparticles encapsulating BSA were the most completely studied and, therefore, only the results regarding these nanoparticles are displayed and discussed in the main document. Results regarding the physicochemical proprieties of ovalbumin- and insulin–loaded nanoparticles are described on appendix 5.

4.2.1 Encapsulation efficiency

The determination of BSA encapsulation efficiency was tested for different formulations of FUC/CS nanoparticles, varying not only the mass ratios (1/4 and 4/1) but also the order of addition of the polymers (protocols A and B) and also the polymer in which BSA is mixed prior to nanoparticle formation. RP-HPLC was used to determine the encapsulation efficiency, a method that is based on the adsorption of hydrophobic molecules onto a hydrophobic stationary phase in a polar mobile phase. BSA has a great affinity to adsorb on hydrophobic surfaces [67], which can be reduced by decreasing the mobile phase polarity by using organic solvents (acetonitrile) resulting in desorption and elution from the reverse phase column. BSA desorption occurred with a gradient flow of water/acetonitrile from 95:5 to 35:65 in 15 min, both solvents contained 0.1% of TFA ,which helped the BSA to desorb. [68]. BSA was eluted at around 8.5 min, and the peak shape and intensity were similar to those of pure BSA samples in the presence of all polymers (figure 4.2 D, E). The retention time of each polymer (figure 4.2 B, C) was determined and revealed to be different from BSA (figure 4.2 A), thus not interfering with BSA determination. To identify each substance, a simultaneous absorbance spectrum was preformed to each peak and compared with the absorbance spectrums of pure BSA, fucoidan and chitosan respectively I, II and III in figure 4.2. However, there was an overlap spectrum of the solvent acetonitrile that was overcome by reading BSA at 280 nm.

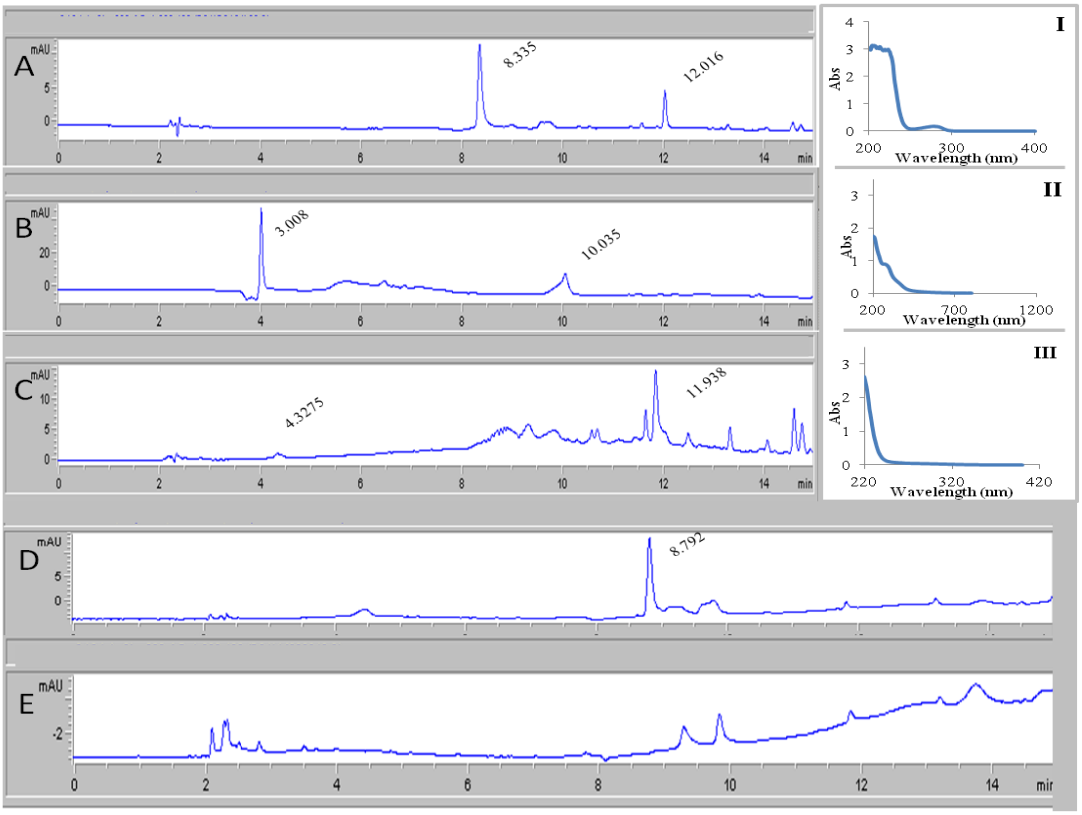
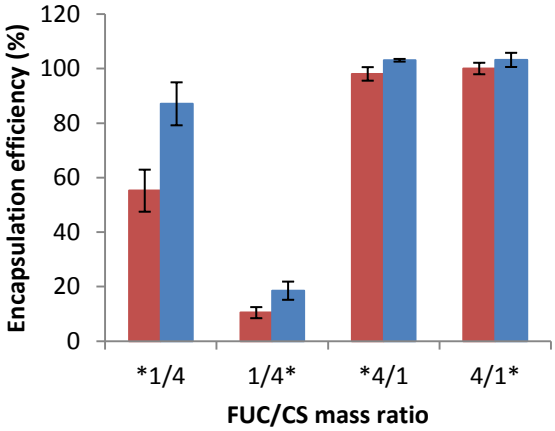


Figure 4.2: RP-HPLC runs of (A) BSA in acetic acid 1% (w/w), (B) fucoidan and (C) chitosan and respective spectrums I, II and III. RP-HPLC runs of (D) low encapsulation efficiency FUC/CS = 1/4, (E) high encapsulation efficiency FUC/CS = 4/1.

The results in graphic 4.5 represent the encapsulation efficiency (EE) of BSA-loaded FUC/CS nanoparticles of mass ratios 1/4 and 4/1 prepared with protocols A and B and with prior BSA incorporation in different polymers solutions. FUC/CS nanoparticles effectively encapsulated BSA. In an initial stage of this study, BSA, insulin and ovalbumin were also associated to nanoparticles and the respective encapsulation efficiencies were estimated using the Bradford protein assay, measuring the absorbance by spectrophotometry at 595 nm (Tecan-Infinite M200, Switzerland). However, it was decided to not proceed with that method of quantification as there chitosan amino groups were interfering in the quantifications. Data obtained with this methodology can be found in appendix 5.

Graphic 4.5 shows a rather different profile of protein association between FUC/CS nanoparticles 1/4 and 4/1 ($p < 0.05$). FUC/CS *4/1 and 4/1* from protocol A displayed, respectively, encapsulation efficiencies of 98% and 100%, while protocol B resulted in a mean of 103% for both formulations. This means that, whatever the variables, the encapsulation efficiency is very high and remarkable as comparing with the majority of

studies regarding protein encapsulation in nanoparticles. In turn, FUC/CS *1/4 and 1/4* from protocol A displayed, respectively, 55% and 11% encapsulation efficiency, while those produced with protocol B resulted in 87% and 19%, respectively. As a general trend, formulations with mass ratio of 4/1 exhibited better ability to encapsulate BSA as compared with 1/4, whatever the tested variables ($p < 0.05$). Additionally, for both FUC/CS mass ratios 1/4 and 4/1 higher encapsulation efficiencies were achieved when BSA was mixed with the polymer with the lowest concentration in each formulation, prior to the addition into the other polymer solution ($p < 0.05$). Regarding the effect of changing the order of addition of polymers, a statistically significant difference was observed for formulations *1/4, 1/4* and *4/1 ($p<0.05$).



Graphic 4.5: Graphic representation of FUC/CS nanoparticles encapsulation efficiency, with protocol A (■) and B (■) and prior BSA (*) incorporation in either polymeric solutions. (Mean ± S.D., n= 3).

A more profound interpretation of the interactions between polymer and BSA in FUC/CS 1/4 and 4/1 nanoparticles, might be achieved by the analyses of the pH of solutions used for nanoparticle assembly, which are expressed in table 4.2. With a pI of 4.7, BSA exposed positive charge when the final pH of its solution was lower than the pI, as happens when it was mixed with chitosan (around 3.3). In turn, when BSA was mixed with fucoidan, the final pH of the solution was 6.8 and, therefore, a negative charge is overall expressed. When the polymeric solutions were mixed together, the final pH of the nanoparticle suspensions were all lower than 4.7, meaning that BSA exposed positive charged at the end of all FUC/CS

nanoparticles preparations. A cross reference of this observation with the graphic representation of the encapsulation efficiency (Graphic 4.5), may explain higher encapsulation efficiency values for FUC/CS 4/1. Since these nanoparticles contain higher amount of fucoidan, the negatively charged polymer, and the BSA was always exposing opposite surface upon polymer mixture, it is quite logical to suggest that these conditions enhanced BSA-polymer interactions, resulting in greater encapsulations efficiencies. The lower EE results displayed by FUC/CS 1/4* are a result of the fact that, in this case, BSA is mixed at first with chitosan and, therefore, it has a positive charge in this solution. When both polymers are mixed, many positive charges (chitosan and BSA) are competing for the negatively charged groups of fucoidan, permitting only a limited interaction that results in a low encapsulation. If the mass ratio remains the same (FUC/CS = 1/4) but BSA is mixed with fucoidan and only after that there is a mixture with chitosan, there is a very significant increase in the encapsulation efficiency ($p < 0.05$), because only many positively charged groups of chitosan will be available to interact with the negatively charged fucoidan and BSA. Anyway, the resulting encapsulations are still lower than those provided by the formulation 4/1. A statistically significant increase in the encapsulation efficiency of formulation FUC/CS *1/4 occurred when protocol was changed from A to B. This difference might be explained by the fact that adding chitosan solution into fucoidan, it was possible to maintain the pH below 4.7 for a longer time, meaning that the protein would be exposing negative charges, thus promoting BSA-polymer interaction.

Table 4.2: pH analyses during the FUC/CS nanoparticle production.

Protocol	**FUC/CS**	**Solutions pH**			
		FUC	CS	BSA+ polymer	(FUC/CS)+BSA
A	*1/4	6.38	3.08	6.80	3.17
	1/4*	6.26	3.97	3.38	3.46
	*4/1	6.28	3.22	6.71	3.48
	4/1*	6.30	4.45	3.26	3.44
B	*1/4	6.38	3.08	6.78	3.13
	1/4*	6.26	3.97	3.34	3.46
	*4/1	6.28	3.22	6.79	3.48
	4/1*	6.30	4.45	3.26	3.49

*Polymer solution to which BSA was added prior to nanoparticle formation

4.2.2 Size and zeta potential

Table 4.3 presents the results corresponding to the unloaded and BSA-loaded nanoparticles. As can be observed, the incorporation of BSA tends to induce an increase on nanoparticles size, but in any case the difference is statistically significant. In turn, the zeta potential of nanoparticles became less negative with the incorporation of the protein in formulation 4/1, whatever the polymer with which BSA was mixed before nanoparticle assembly; while formulation 1/4 exhibits different behaviours depending on the polymer with which the protein is mixed before the formation of nanoparticles. The explanation for these different effects is probably based on different availability of charges to interact among polymers and the protein. To facilitate the interpretation of results Table 4.2 gathers the pH of used polymers, mixtures of polymers with BSA and the final nanoparticle suspension.

The comparison of the zeta potentials of both FUC/CS mass ratios 4/1 and 1/4, of loaded and unloaded nanoparticles, shows a decrease on surface charge of nanoparticles when loaded with BSA, this differences were statistically significant ($p<0.05$). This decrease of the zeta potential is quite logical, and can be easily justified by the interactions polymers-protein, that neutralizes some of the available charges of the nanoparticles. This effect was more pronounced for FUC/CS=1/4 with BSA prior mix in fucoidan, when BSA was exposing negatively charged groups, maximizing the interaction with the available positive groups of chitosan. However, this great decrease on zeta potential may be caused by a rearrangement of nanoparticles structure, where the spatial disposition of the fucoidan chain contributes for this decline of the surface charge. The absence of a decrease on FUC/CS 1/4 with BSA prior mix in chitosan can be explained by the low encapsulation efficiency of this formulation.

Table 4.3: Physicochemical characteristics of unloaded and BSA-loaded FUC/CS (1/4 and 4/1) nanoparticles (mean ± SD, n = 3).

Protocol	FUC/CS	Size (nm)	Zeta potential (mV)
A	**4/1**	456 ± 4	-42 ± 1
	*4/1	521 ± 55	-37 ± 3
	4/1*	525 ± 53	-33 ± 4
B	**1/4**	372 ± 5	+46 ± 2
	*1/4	415 ± 71	+19 ± 5
	1/4*	435 ± 49	+53 ± 5

*Polymer solution to which BSA was added prior to nanoparticle formation

4.2.3 Determination of BSA-loaded nanoparticles production yield and loading capacity

For this study were selected for characterization the FUC/CS mass ratios 4/1 and 1/4 from protocol A and B. As already referred in the above section of unloaded nanoparticles production yield, Protocol B was not trialled for 4/1 nanoparticles. Prior incorporation BSA in either polymeric solutions was not tested. Results of this characterization regarding insulin and ovalbumin can be found in appendix 5 Table 4.4 shows an increase on nanoparticle productions yield upon BSA incorporation, but this differences were only statistically significant for FUC/CS nanoparticles 4/1 and 1/4 for protocol A ($p< 0.050$). The most notorious increase on production yield was observed by FUC/CS 4/1 upon BSA incorporation, where nanoparticles gain about 100% of mass relatively to the unloaded nanoparticles. Showed in table 4.4 are also the loading capacities (LC) of FUC/CS nanoparticles. This characteristic represents the amount of protein relatively to the total mass of nanoparticles, which compose each FUC/CS formulation for example, FUC/CS 1/4 had a 49 % LC, means that about half of this particle is compose of BSA and the other of polymer. Once again the enhancement of the nanoparticle BSA associations was clear in FUC/CS 1/4 when protocol A (36 %) was replaced by B (49%). This shows that protocol B can improve the profitability of these processes, since although PY of FUC/CS 1/4 did not increase significantly when protocol change from A (30%) to B (33%), although cross reference with LC from both protocols (36%) A and (49 %) B shows that the particles can associate more protein, in fact half of the particles mass content was protein.

Table 4.4: Production yield (PY) of loaded and unloaded FUC/CS 1/4 and 4/1nanoparticles (NP) and loading capacities, (mean ± S.D., n> 3).

Protocol	FUC/CS	BSA-loaded NP	
		PY (%)	LC (%)
A	4/1*	65 ± 15	29 ± 7
	*1/4	30 ± 7	36 ± 11
B	*1/4	33 ± 8	49 ± 11

*Polymer solution to which BSA was added prior to nanoparticle formation

5 Conclusions

This work demonstrates that the developed fucoidan/chitosan (FUC/CS) nanoparticles are suitable to be used as protein carriers systems in the ambit of systemic mucosal administration. These nanoparticles are produced in complete hydrophilic conditions, by a very mild procedure of ionic interaction between the negatively charged fucoidan sulphate groups and the oppositely charged amino groups of chitosan. This procedure avoids the use of organic solvents and other aggressive conditions that might be detrimental for the integrity of the drug to be encapsulated. The macromolecules bovine albumin serum (BSA), insulin and ovalbumin, used in this study as model proteins, were efficiently associated to the developed drug delivery systems, as demonstrated by the physicochemical characterization of the systems. The optimization of several variables of the nanoparticle production method allowed obtaining very high encapsulation efficiencies. The small sizes and high negative and positive charges displayed by the developed nanoparticles are considered to hold potential for an application in mucosal delivery of macromolecules. Additionally, FUC/CS nanoparticles demonstrated to be relatively stable in aqueous suspension, only registering some slight variations in their physicochemical characteristics upon 170 days of storage at 4 ºC, although these do not compromise the intended application as protein carriers for mucosal administration. In order to establish a complete characterization of the developed nanoparticulate systems, FUC/CS nanoparticles must undergo further studies, namely the determination of release profile and the toxicity assessment, both *in vitro* and *in vivo*.

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6 Bibliography

[1] K. Wilson and J. Walker, Principles and techniques of biochemistry and molecular biology, Cambrige: Cambridge University Press , 2010.

[2] V. K. Naeayan, D. Williams, E. W. Gregg and C. C. Cowie, Diabetes Public Health, New York: Oxford University press, 2011.

[3] International Diabetes Fedaration, "Diabetes atlas," 2011. [Online]. Available: http://www.idf.org. [Accessed 22 Janeiro 2012].

[4] P. Buckel, "Recombinant proteins for therapy," *Trends in Pharmacological Sciences,* vol. 17, pp. 450-6, 1996.

[5] G. Subramanian, Biopharmaceutical Production Tehcnology, Weinheim: Wiley.VCH Verlag & Co. KGaA., 2012.

[6] W. G., "Biopharmaceutical benchmarks," *Nature Biotechnology,* vol. 24, pp. 769-776, 2006.

[7] C. P. Reis, R. J. Neufeld, A. J. Ribeiro and F. Veiga, "Nanoencapsulation II. Biomedical applications and current status," *Nanomedicine,* vol. 2, p. 53–65, 2006.

[8] R. J. Y. Ho and M. Gibaldi, Biotechnology and Biopharmaceuticals: Transforming Proteins and genes into drugs, new jersey: John Wiiley & sons, Inc., 2003.

[9] G. Walsh, Biopharmaceuticals Biochemistry and biotechnology, Chichester: John and Wiley & Sons Ltd., 2003.

[10] S. Cox, Pharmaceutical Manufacturing Handbook: Production and Processes, New Jersey: John Wiley & Sons, Inc., 2008.

[11] S. Frokjaer and D. E. Otzen, "Protein drug stability: a formulation challenge.," *Nature Reviews: Drug Discovery,* vol. 4, pp. 298-306, 2005.

[12] L. R. Brown, "Commercial challanges of protein drug delivery," *Expert Opinion,* vol. 2, no. Drug Delivery, pp. 29-42, 2005.

[13] L. Jorgensen, E. v. d. W. M. Moeller, H. Nielsen and S. Frokjaer, "Preparing and evaluating delivery systems for proteins," *European Journal of Pharmaceutical Sciences,* vol. 29, pp. 174-182, 2006.

[14] Y. Pathak and D. Thassu, Drug Delivery Nanoparticles Formulation and Characterization, New York: Informa Healthcare USA, Inc., 2009.

[15] K. Murayama and M. Tomida, “Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy,” *Biochemistry,* vol. 43, no. 36, pp. 11526-11532., 2004.

[16] F. Jameel and S. Hershenson, Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals, New Jersey: John Wiley & Sons, Inc., 2010.

[17] S. E. McNeil, "Nanoparticle therapeutics: a personal perspective," *WIREs Nanomed Nanobiotechnol,* vol. 1, pp. 264-271, 2009.

[18] A. Grenha, “Systemic delivery of biopharmaceuticals: Parenteral forever?,” *Journal of Pharmacy and BioAllied Sciences,* vol. 2, p. 95, 2012.

[19] J. L. Cleland, A. Daugherty and R. Mrsny, “Emerging protein delivery methods,” *Current Opinion in Biotechnology,* vol. 12, p. 212–219, 2001.

[20] V. F. Patel, F. Liu and M. B. Brown, "Advances in oral transmucosal drug delivery," *Journal of Controlled Release,* vol. 153, p. 106–116, 2011.

[21] J. B. Dressman and H. Lennernäs, Oral Drug Absorption. Prediction and Assessment, volume 105, New York: Marcel Dekker, Inc., 2000.

[22] K. Park, I. C. Kwon and K. Park, "Oral protein delivery: Current status and future prospect," *Reactive & Functional Polymers,* vol. 71, p. 280–287, 2011.

[23] Webnode, “Anatomia e Fisiologia,” 2009. [Online]. Available: http://anatomiaefisiologiahumana.webnode.com.

[24] L. Braz, M. Dionísio and A. Grenha, “Chapter 7: Chitosan-based Nanocarriers: Effective Vehicles for mucosal Protein Delivery,” in *Chitosan: Manufacture, Proprieties and Usage*, New York, Nova Science Publishers, Inc., 2010, pp. 365-413.

[25] A. Grenha, S. Al-Qadi, B. Seijo and C. Remuñán-López, “The potential of chitosan for pulmonary drug delivery.,” *Journal of Drug Delivery Science and Technology,* vol. 20, no. 1, pp. 33-43, 2010.

[26] Sintomas e Dicas, “Sintomas e Dicas,” 2009. [Online]. Available: http://sintomasedicas.com/.

[27] Nektar Therapeutics, “Pfizer’s Exubera Receives Approval From US FDA; Nektar Co-Developed the Inhalers & Formulation,” *Drug Delivery Technology,* vol. 6, no. 2, p. 12, 2006.

[28] A. Weintraub, “Bloomberg Business week Technology,” 18 October 2007. [Online].

Available: http://www.businessweek.com.

[29] C. Morrison, "The in vivo blog," 2007. [Online]. Available: http://invivoblog.blogspot.pt/.

[30] S. Türker, E. Onur and Y. Özer, "Nasal route and drug delivery systems," *International Journal of Clinical Pharmacy,* vol. 26, no. 3, pp. 137-142, 2004.

[31] L. Illuma, "Nasal drug delivery- possibilities, problems and solutions," *Journal of Controlled Release,* vol. 87, pp. 187-198, 2003.

[32] Bahrian Doctors, "Bahrian Doctors," 2012. [Online]. Available: http://bahriandoctors.com.

[33] J. Shaji and V. Patole, "Protein and Peptide Drug Delivery: Oral Approaches," *Indian Journal of Pharmaceutical Sciences,* vol. 70, no. 3, p. 269–277, 2008.

[34] C. R. Gilbert S. Banker, Drugs and Pharmaceutical Sciences, Modern Pharmaceutics, 4th edition, Basel: Marcel & Dekker, Inc., 2002.

[35] L. Yildirimer, N. T. Thanhb and M. Loizidoua, "Toxicological considerations of clinically applicable nanoparticles," *Nano today,* vol. 6, p. 585—607, 2011.

[36] F. Re, R. Moresco and M. Masserin, "Nanoparticles for Neuroimaging," *jornal of Physics D: Applied Physics,* vol. 45, no. 7, 2012.

[37] K. Y. Leea and S. H. Yuk, "Polymeric protein delivery systems," *Progress in Polymeric Science,* vol. 32, p. 669–697, 2007.

[38] R. C. Pinto, R. Neufeld, A. Ribeiro and F. Veiga, "Nanoencapsulation I. Methods for preparation of drugloaded polymeric nanoparticles," *Nanomedicine,* vol. 2, no. 1, pp. 8-21, 2006.

[39] A. Kumari, Y. S. K. and S. C. Yadav, "Biodegradable polymeric nanoparticles based drug delivery systems," *Journal Colloids and Surfaces B: Biointerfaces,* vol. 75, pp. 1-18, 2010.

[40] W. Abdelwahed, G. Degobert, S. Stainmesse and H. Fessi, "Freeze-drying of nanoparticles: Formulation, process and storage considerations," *Advanced Drug Delivery Reviews,* vol. 58, p. 1688–1713, 2006.

[41] G. Griffiths, B. Nyström, S. B. Sable and G. K. Khuller, "Nanobead-based interventions for the treatment ans prevension of tuberculosis," *Nature Reviews, Microbiology,* vol. 8, pp. 827-834, 2010.

[42] A. Rieuxa, V. Fieveza, M. Garinota, Y.-J. Schneiderb and V. Préata, "Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach," *Journal of Controlled Release,* vol. 116, no. 1, p. 1–27, 2006.

[43] K. S. Soppimatha, T. M. Aminabhavia, A. R. Kulkamia and W. E. Rudzinski, "Biodegradable polymeric nanoparticles as drug delivery devices," *Journal of Controlled Release,* vol. 70, pp. 1-20, 2001.

[44] A. Grenha, B. Seijo and C. R. López, "Microencapsulated chitosan nanoparticles for lung protein delivery," *European Journal of Pharmaceutical Sciences,* vol. 25, p. 427–437, 2005.

[45] A. Eifler and C. Thaxton, "Nanoparticle therapeutics: FDA approval, clinical trials, regulatory pathways, and case study.," *Methods of Molecular Biology,* vol. 726, pp. 325-38, 2011.

[46] G. Oberdorster, "Pulmonary effects of inhaled ultrafine particles," *International Archives of Occupational and Environmental Health,* vol. 74, pp. 1-8, 2001.

[47] M. Elsabahy and K. L. Wooley, "Strategies Toward Well-Defined Polymer Nanoparticles Inspired by Nature: Chemistry versus Versatility," *Journal of polymer science,* vol. 50, p. 1869–1880, 2012.

[48] D. Labarre, G. Ponchel and C. Vauthier, Biomedical and Pharmaceutical Polymers, London: Pharmaceutical Press, 2011.

[49] J. H. Park, M. Ye and K. Park, "Review Biodegradable Polymers for Microencapsulation of Drugs," *Molecules,* vol. 10, pp. 146-161, 2005.

[50] T. Hanemann and D. V. Szabó, "Polymer-Nanoparticle Composites: From Synthesis to Modern," *Journal of Materials,* vol. 3, pp. 3468-3517, 2010.

[51] K. E. Uhrich, S. M. Cannizzaro and R. S. Langer, "Polymeric Systems for Controlled Drug Release," *Chemical Reviews,* vol. 99, pp. 3181-3198, 1999.

[52] A. Grenha, M. E. Gomes, M. Rodrigues, V. E. Santo and J. F. Mano, "Development of new chitosan/carrageenan nanoparticles," *Journal of biomedical materials research. Part A,* vol. 92, no. 4, pp. 1265-72, 2010.

[53] S. A. T. and C. P. Sharma, "Chitosan and Its Derivatives for Drug Delivery," *Journal of Advanced Polymer Science,* vol. 243, p. 23–54, 2011.

[54] G. Dupuis and J. Lehoux, "A simplified method to retrieve chitosan from acidic solutions

thereof". França Patent EP 1701980 A1, 20 setembro 2006.

[55] A. Grenha, "Chitosan nanoparticles: a survey of preparation methods," *Journal of Drug Targeting,* vol. 20, no. 4, pp. 291-300, 2012.

[56] A. B. Schnürch and S. Dünnhaupt, "Chitosan-based drug delivery systems," *European Journal of Pharmaceutics and Biopharmaceutics*, vol. 81, p. 463–469, 2012.

[57] F. Andrade, F. Goycoolea, D. A. Chiappetta, J. Neves, A. Sosnik and B. Sarmento, "Chitosan-Grafted Copolymers and Chitosan-Ligand Conjugates as Matrices for Pulmonary Drug Delivery," *International Journal of Carbohydrate Chemistry,* vol. 2011, p. 14, 2011.

[58] B. Li, F. Lu, X. Wei and R. Zhao, "Fucoidan: Structure and Bioactivity," *Mollecules,* vol. 13, pp. 1671-1695, 2008.

[59] A. D. Sezer, E. Cevher, F. Hatipoglu, Z. Ogurtan, A. L. Bas and J. Akbuga, "The use of fucosphere in treatment of dermal burns in rabbits," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 69, pp. 189-198, 2008.

[60] M. Liraa, N. Santos-Magalhães, V. Nicolasd, V. Marsaude, M. Silvab, G. Ponchele and C. Vauthiere, "Cytotoxicity and cellular uptake of newly synthesized fucoidan-coated nanoparticles," *European Journal of Pharmaceutics and Biopharmaceutics,* vol. 79, no. 1, pp. 162-170, 2011.

[61] W. Fana, W. Yanb, Z. Xub and H. Nia, "Formation mechanism of monodisperse, low molecular weight chitosan," *Colloids and Surfaces B: Biointerfaces,* vol. 90, pp. 21-27, 2012.

[62] Y. C. Huang and U. I. Lam, "Chitosan/Fucoidan pH Sensitive Nanoparticles for Oral Delivery System," *Journal of the Chinese Chemical Society,* vol. 58, pp. 779-785, 2011.

[63] A. D. Sezer and J. Akbuga, "Fucosphere—New microsphere carriers for peptide and proteins delivery," *Journal of Microencapsulation,,* vol. 23, no. 5, p. 513–522, 2006.

[64] S. Rodrigues, A. M. R. Costa and A. Grenha, "Chitosan/carrageenan nanoparticles: Effect of cross-linking with tripolyphosphate and charge ratios," *Journal of Carbohydrate Polymers ,* vol. 89, p. 282– 289, 2012.

[65] A. A. Mahmoud, G. S. El-Feky and G. E. A. Awad, "Chitosan/sulfobutylether-β-cyclodextrin nanoparticles as a potential approach for ocular drug delivery," *International Journal of Pharmaceutics,* vol. 413, no. 1-2, pp. 229-236, 2011.

[66] Y. Huang and T. Liu, “Mobilization of mesenchymal stem cells by stromal cell-derived factor-1 released from chitosan/tripolyphosphate/fucoidan nanoparticles,” *Acta of biomaterialia,* vol. 3, pp. 1048-1056, 2012.

[67] Y. U. Yong, P. Ying and J. I. N. Gang, “Competitive adsortion between bovine serum albumin and collagen observed by atomic force microscope,” *Journal of Chinese Chemical Letters,* vol. 15, p. 1465, 2004.

[68] M. Umrethia, V. L. Kett, G. P. Andrews, K. R. Malcom and D. A. Woolfson, “Selectionn of an analytical method for evaluating bovine serum concentrations in pharmaceutical polymeric formulations,” *Journal of Pharmaceutical and Biomedical Analysis,* vol. 51, p. 1175–1179, 2010.

[69] Z. Liu, Y. Jiao, Y. Wang, ,. C. Zhou and Z. Zhang, “Polysaccharides-based nanoparticles as drug delivery systems,” *Advanced Drug Delivery Reviews,* vol. 60, p. 1650–1662, 2008.

[70] S. A. Agnihotri, N. N. Mallikarjuna and T. M. Aminabhavi, “Recent advances on chitosan-based micro-and nanoparticles,” *Journal of controlled release,* vol. 100, no. 1, pp. 5-28, 2004.

[71] 1999. [Online]. Available: http://glycomix.co.uk/fucus_vesiculosus_fucoidan.htm . .

7 Appendix

7.1 Appendix 1

DEVELOPMENT OF FUCOIDAN/CHITOSANNANOPARTICULATE SYSTEMS FOR PROTEIN ADMINISTRATION THROUGH MUCOSAL ROUTES

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INTRODUCTION

Protein-based biomacromolecules have been used for long in pharmacological therapy, but their administration through non-parenteral routes is a difficult task, due to stability issues mainly attributed to pH and enzymatic contents in mucosal surfaces. It has been a consensus that mucosal routes are valuable alternatives for drug administration, but this demands the development of adequate carriers that confer stability and protection against the mentioned aggressive environments (1). Nanoparticulate systems have proven promising for this end, owing to their high surface-to-volume ratio and capacity for drug encapsulation (2). Using natural polymers for nanocarriers production is advantageous due to the requirements of biocompatibility, biodegradability and absence of toxicity, which are mandatory in any biomedical application. Fucoidan and chitosan are two of these natural polymers, respectively extracted from brown seaweed (*Fucus vesiculosus*) with 95% of fucose sulfated esters (anionic polysaccharide) (3), and the exoskeleton of crustaceans. In the case of chitosan a further step of partial deacetylation of chitin is necessary to obtain the cationic polysaccharide (4). In this work, fucoidan/chitosan (FUC/CS) nanoparticles were prepared by polyelectrolyte complexation (5). The obtained nanocarriers were optimized in terms of several variables, being evaluated for their capacity to associate proteins.

MATERIAL AND METHODS

Preparation of FUC/CS nanoparticles:

Nanoparticles were prepared by a method of polyelectrolyte complexation (6). Briefly, CS was dissolved in acetic acid 1% (w/w) and FUC was dissolved in purified water. FUC/CS nanoparticles were spontaneously formed at room temperature upon incorporation of FUC solution into CS solution (FUC/CS mass ratios between 4/1 and 1/4) and vice-versa. Different mass ratios and the order of addition of polymeric solutions were studied as variables. Protocol A consisted in the addition of FUC over CS and Protocol B was the opposite. Nanoparticles were isolated by centrifugation (16000 x g, 30 min, 15 ºC) and resuspended in 100 µL of milli-Q water.

Model biomacromolecules (BioM) were associated to the nanoparticles, including bovine serum albumin (BSA), ovalbumin and insulin. Proteins were dissolved in appropriate solvents (water or sodium hydroxide 0.1 M) and added to either CS or FUC solution, in order to provide the protein with opposite charge in comparison with the polymer presented at the highest concentration in each formulation. The solution of the second polymer is then poured into the previously prepared polymer/protein solution to produce the nanoparticles.

Nanoparticles characterization:

Nanoparticles production yield was calculated by gravimetry, comparing the real weight of nanoparticles with the initial amount of solids used for their production (n = 3). Nanocarriers' size and zeta potential were measured by photon correlation spectroscopy and laser Doppler anemometry, respectively (Zetasizer® Nano ZS, Malvern Instruments) (n = 3).

Nanoparticles supernatant was assessed to determine the amount of free BioM using the Bradford protein assay and measuring the absorbances by spectrophotometry at 595 nm (Tecan-Infinite M200, Switzerland). A calibration curve was made using the supernatant of blank nanoparticles. BioM encapsulation efficiency (E.E.) and loading capacity (L.C.) were calculated comparing the non-associated protein present in the supernatant with the total amount added for nanoparticles production, as follows:

$$\text{E.E. (\%)} = \frac{\text{Total BSA} - \text{Free BSA}}{\text{Total BSA}} \times 100$$

$$\text{L. C. (\%)} = \frac{\text{Total BSA} - \text{Free BSA}}{\text{Nanoparticle weight}} \times 100$$

Evaluation of nanoparticles stability:

A study of the nanoparticles (FUC/CS = 4/1 and 1/4) stability in water was performed at 4 ºC. Unloaded nanoparticles were isolated by centrifugation and resuspended in water afterwards. Sizes were monitored during 22 days, using the above mentioned techniques (n = 3).

RESULTS AND DISCUSSION

Nanoparticles characterization:

The two variables studied in the production of FUC/CS nanoparticles, which consist in polymer concentration (CS and FUC) and the order of addition of polymeric solutions, resulted in remarkable effects over nanoparticles characteristics. Sizes ranged between 300 and 700 nm (Figure 1) and the highest size corresponded to the highest amount of materials composing the nanoparticles (FUC/CS = 1/1). Moreover, a complete shift of zeta potentials was observed, varying from -42 to +49 mV (Figure 2), which reflects the highest amount of negative or positive polymer composing the nanoparticles.

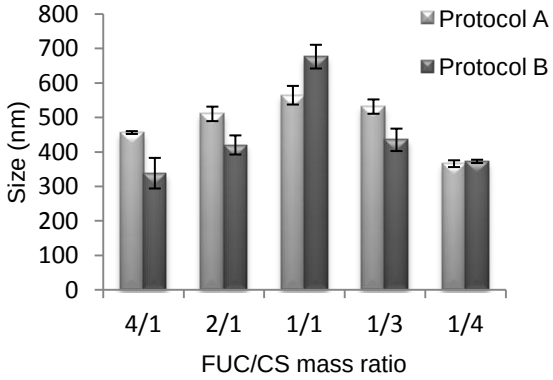

Figure 1 - FUC/CS nanoparticles size corresponding to different mass ratios and preparation protocols (mean ± SD, n = 3).

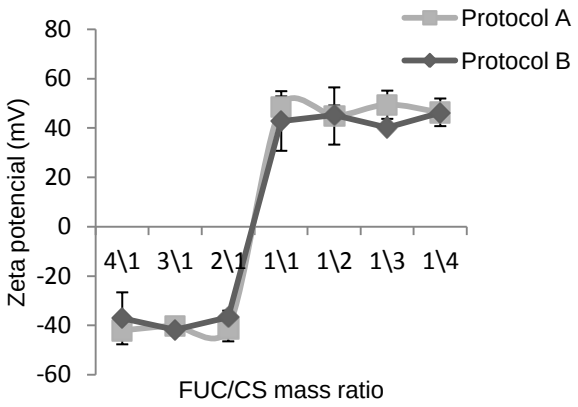

Figure 2 – FUC/CS nanoparticles zeta potential obtained for different mass ratios and preparation protocols (mean ± SD, n = 3).

The most remarkable effect of protocol changes is related with BSA encapsulation. In the formulation FUC/CS 1/4, changing preparation protocol from A to B, it is observed a 60-70% increase in the encapsulation efficiency (from 11-20% in protocol A to 83-85% in protocol B). Formulation 4/1 also shows high BSA encapsulation efficiency, up to 88% with protocol A. Assays regarding ovalbumin and insulin encapsulation show efficiency of 51% and 91%, respectively, in formulation FUC/CS = 4/1 comparatively to the lower efficiency (14%) for both proteins in 1/4 FUC/CS formulation. Release assays are presently being performed.

Evaluation of nanoparticles stability:

The stability assay revealed that, when stored at 4ºC, the nanoparticles (FUC/CS = 1/4 and 4/1) size remains stable for at least 22 days, for formulations obtained by both protocols A and B. Further studies are being performed to assess longer time periods.

CONCLUSIONS

Considering the physicochemical properties, the ability to associate proteins and the demonstrated stability, FUC/CS nanoparticles are considered good candidates for drug delivery purposes. The results indicate that the use of protocol A and B in 4/1 and 1/4 FUC/CS formulations, respectively, provide high BSA encapsulation efficiency.

REFERENCES

[1] Csaba et al., Adv. Drug Deliv. Rev., **61:** 140-157, 2009.

[2] Reis et al., J. Tissue Eng., **1:** 4–24, 2007.

[3] Li et al. Molecules, **13:** 1671-1695, 2008.

[4] Kurita, Marine Biotechnol., **8:** 203–226, 2006.

[5] Berthold et al., J. Control. Release, **39:** 17–25, 1996.

[6] Prestwich et al., J. Control. Release, **53:** 93–103, 1998.

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7.2 Appendix 2

FUCOIDAN/CHITOSAN NANOPARTICLES AS PROTEIN CARRIERS

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KEYWORDS

chitosan, fucoidan, nanoparticles, protein delivery

INTRODUCTION

Fucoidan and chitosan are natural polymers, respectively extracted from brown seaweed (Fucus vesiculosus) and containing 95% of fucose sulfated esters (anionic polysaccharide) [1], and from the exoskeleton of crustaceans. Displaying opposite charges, these polysaccharides enable the production of nanoparticles by polyelectrolyte complexation, occurring under very mild conditions [2]. Nanoparticles have proven to be promising vehicles in protein delivery, due to their high surface-to-volume ratio and capacity for association of macromolecules. In this work, fucoidan/chitosan (FUC/CS) nanoparticles of various mass ratios (1/4 to 4/1, w/w) were prepared by means of an electrostatic interaction, displaying different size and zeta potential. Several conditions were explored to maximise protein incorporation, either varying 1) the order of addition of polymeric solutions (Protocol A: FUC into CS, or Protocol B: CS into FUC), or 2) the polymeric solution (FUC or CS) to which the protein is added prior to nanoparticle formation. Bovine serum albumin (BSA), ovalbumin and insulin were used as model proteins. The use of natural polymers is expected to provide the basis for biocompatibility and absence of toxicity, which are mandatory in any biomedical application [3]. Importantly, the application of fucoidan in the preparation of nanoparticles for drug delivery purposes was never reported before.

RESULTS & DISCUSSION

FUC/CS nanoparticles display sizes of 300 - 700 nm and zeta potential from -42 to +49 mV. In approach 1), BSA was used as model protein, being always incorporated in FUC solution prior to nanoparticle formation. Higher encapsulation efficiencies were obtained for formulations FUC/CS = 4/1 produced according to protocol A (88%) and FUC/CS = 1/4 prepared by protocol B (85%). The approach 2) was tested for BSA, insulin and ovalbumin, and protocols A and B were used for formulations 4/1 and 1/4, respectively. Higher encapsulation efficiency is always observed when the protein is mixed with in the solution of the polymer present in the lower ratio in the final formulation. Release assays are presently being performed. The performed experiments demonstrate the importance of controlling the protein charge in the moment of providing the interaction with polymers. The size and zeta potential of FUC/CS nanoparticles (1/4 and 4/1, w/w) remain stable for up to 6 months, upon storage at 4 ºC. Considering the physicochemical properties and stability, as well as the ability to associate different proteins, FUC/CS nanoparticles are deemed suitable for drug delivery purposes.

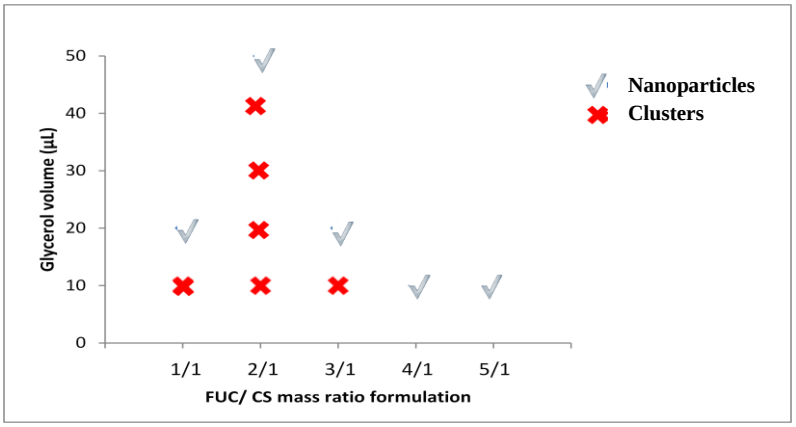
National funding from Portuguese Foundation for Science and Technology (project PTDC/SAU-FCF/100291/2008 and PEst-OE/EQB/LA0023/2011) is acknowledged.

REFERENCES

[1] Li B. et al. (2008) Fucoidan: Structure and Bioactivity. Molecules. 13: 1671-1695
[2] Grenha A. (2012) Chitosan nanoparticles: a survey of preparation methods. J Drug Target. 20: 291-300
[3] Chiellini F. (2008) Micro/nanostructured polymeric systems for biomedical and pharmaceutical applications. Nanomedicine. 3: 367-393

7.3 Appendix 3

The conditions on which nanoparticles formed were optimized. Temperature reveled to have great influence on the nanoparticles formation. During this study nanoparticles were formed at room temperature oscillating between 17-22 ºC, agglomerates were formed above or under this temperature range (data not shown). The Drop wise incorporation of the polymers revealed as a crucial step for nanoparticles formation. A small increase in the rate of addition of polymers causes major changes in terms of size (data not shown). To outcome this problems with nanoparticle resuspension was adopted a strategy that consisted on finding the accurate amount of glycerol for each formulation, by phased additions of more 10 µL glycerol in nanoparticles isolation procedure. The results of this optimization were illustrated in graphic 1. The graphic 7.1 shows the accurate amount of glycerol for FUC/ CS mass ratio formulation 1/1, 2/1, 3/1, 4/1 and 5/1 were 20, 50, 20, 10 and 10 µL respectively. The excess of glycerol usually contributes to nanoparticle aggregation.



Graphic 7.1: Glycerol optimization for each formulation

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The conditions adopted for the nanoparticle isolation procedure, were centrifuge velocity of 16000 G, at 15ºC during 30 minutes that correspond to a standardization used by the research group.

7.4 Appendix 4

FUC/CS nanoparticles size and zeta potential, from protocol A (table 7.1) and B (table 7.2).

Table 7.1: FUC/CS nanoparticles size and zeta potential, from protocol A (Mean± SD, n = 3).

FUC\CS (w/w)	Size (nm)	Zeta potential (mV)
4\1	456±4	-42±1
3\1	547±21	-40±1
2\1	510±27	-41±5
1\1	564±21	49±4
1\2	544±10	45±12
1\3	531±3	49±6
1\4	366±41	46±6

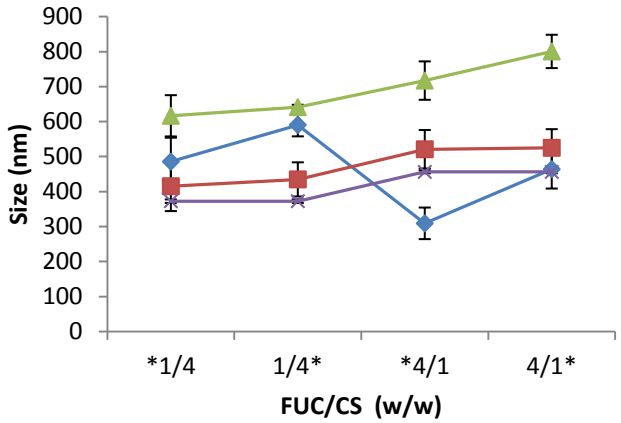
Table 77.2: FUC/CS nanoparticles size and zeta potential, from protocol (Mean± SD, n=3)

FUC\CS (w/w)	Size (nm)	Zeta potential (mV)
4\1	338±44	-37±11
3\1	417±47	-42±1
2\1	420±28	-37±3
1\1	676±34	43±12
1\2	591±58	45±4
1\3	435±33	40±1
1\4	372±5	46±2

7.5 Appendix 5

7.5.1 Proteins association of FUC/CS nanoparticles

BSA, ovalbumin and insulin were dissolved in appropriate solvents respectively in water and sodium hydroxide 0.1 M, and then added to either CS or FUC solution, in order to provide the protein with opposite charge in comparison with the polymer presented at the highest concentration in each formulation. Nanocarriers' size and zeta potential were measured by photon correlation spectroscopy and laser Doppler anemometry, respectively (Zetasizer® Nano ZS, Malvern Instruments) (n = 3).Table x contains size and zeta potential of insulin and ovalbumin –loaded nanoparticles. The graphic 7.2 representation of the tabled size values, plus BSA-loaded and unloaded nanoparticles, reveals the specificity of the interactions between these polymers and each protein, whereas displays different behaviors in terms of sizes.



Graphic 7.2: Representation of FUC/CS mass ratios 1/4 and 4/1 sizes, of unloaded (×), insulin (◆), ovalbumin (▲) and BSA (■) -loaded nanoparticles (Mean ± S.D, n=3).

Nanoparticles production yield (table 7.3) was calculated by gravimetry, comparing the real weight of nanoparticles with the initial amount of solids used for their production (n = 3).

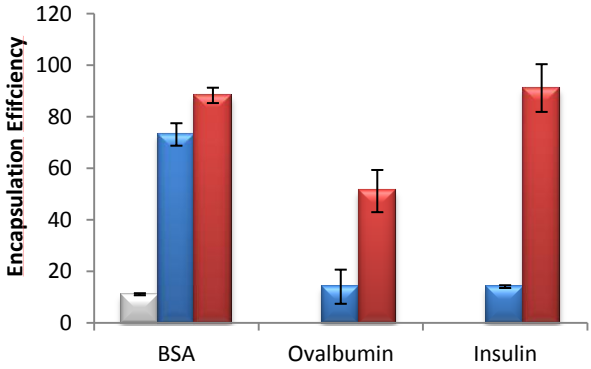
Table 7.3: Production yield and loading capacity of insulin and ovalbumin -loaded nanoparticles of FUC/CS mass ratio 1/4 and 4/1(Mean ± SD, n>3).

Protocol	FUC/CS (w/w)	Insulin-loaded NP		Ovalbumin-loaded NP	
		P.Y. (%)	L.C. (%)	P.Y. (%)	L.C. (%)
A	*4/1	-	-	40±9	21±6
	4/1*	45±13	37±11	46±8	27±5
B	*1/4	33±14	13±6	16±5	13±5
	1/4*	-	-	24±11	0

*Polymer solution to which BSA was added prior to nanoparticle formation

Nanoparticles supernatant was assessed to determine the amount of free BioM using the Bradford protein assay and measuring the absorbance by spectrophotometry at 595 nm (Tecan-Infinite M200, Switzerland). A calibration curve was made using the supernatant of blank nanoparticles. BioM encapsulation efficiency (EE) and loading capacity (LC) were calculated comparing the non-associated protein present in the supernatant with the total amount added for nanoparticles production, as described in Materials and Methods.

These assays with ovalbumin and insulin happened in an early stage of this work, where some characteristic of the polymer were not completely described. An example of this was the fact that using Bradford assay to measure the proteins encapsulations efficiency, not only the proteins were being quantify, but also fucoidan, jeopardizing the results. With this work was evident that each protein has is one unique interaction with this polymers combination. In graphic 7.3 represents the different encapsulation efficiencies for the 3 model proteins used in this study, for nanoparticles FUC/CS 1/4 and 4/1 mass ratios. The most remarkable effect occurs during BSA encapsulation when protocol changes from A to B in FUC/CS 1/4 formulation were observed an increase in 70-80 % the encapsulation ability (EE of 88 %). Still regarding BSA, the 4/1 formulation shows as well a great encapsulation ability (EE of 81-88 %) with protocol A. In ovalbumin and insulin encapsulation, the FUC/CS mass ratio 4/1 had the highest encapsulation efficiencies respectively 51% and 91% that contrast with the very low encapsulation efficiencies in FUC/CS mass ratio 1/4 of 14% for both proteins.



Graphic 7.3: Encapsulation efficiency of FUC/CS nanoparticles 1/4 from protocol A (■) and B (■) and 4/1 for protocol A (■). (Mean ± S.D., n >3)