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FORMULATION AND EVALUATION OF FUCOIDAN/CHITOSAN NANOPARTICLES AS A DELIVERY SYSTEM FOR PROTEINS

IZDELAVA IN VREDNOTENJE NANODELCEV IZ FUKOIDANA IN HITOSANA KOT DOSTAVNEGA SISTEMA ZA PROTEINE

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CONTENT

ABSTRACT

The administration of therapeutic proteins through non-parenteral routes has been widely investigated over the last few years. It represents a challenge due to stability problems, mainly attributed to pH and high enzymatic content in mucosal surfaces, and therefore, the development of suitable carriers which would be able to provide stability and protection against harsh conditions in the organism is a logic consequence. Polymeric nanoparticles proved to be a useful tool for therapeutic purposes, since they have advantages connected with high surface to volume ratio (which enables increased drug loading), they offer protection to the encapsulated molecules and also provide intimate interaction with mucosal surfaces, increasing drug absorption.

While chitosan (CS) is the most used of polysaccharides in drug delivery, fucoidan (FUC) is another polysaccharide with limited applications reported in the field. Using both of the mentioned polymers, the purpose of our study was to create fucoidan/chitosan (FUC/CS) nanoparticles by a mild polyelectrolyte complexation at FUC/CS mass ratios of 1/4 and 4/1 and bovine serum albumin (BSA) used as model protein.

Nanoparticles size varied from 164 to 461 nm, being either positively (+36 mV) or negatively charged (-32 mV), depending on the formulation. The efficiency of BSA association to nanoparticles was 100% for FUC/CS = 4/1 (method A) and 37% for FUC/CS = 1/4 (method B). In addition, a higher percentage of released protein in nanoparticles formed by method A was detected, incubating them in the solution of HEPES buffer. A stability study in the presence of lysozyme was also made, showing a slight change in nanoparticles size, more obvious with the nanoparticles produced by method A and with the higher concentration of lysozyme. The cytotoxic profile of both nanoparticles and raw materials in concentrations raging between 0.1 and 1 mg/ml, was determined (MTT assay) in an alveolar cell line (Calu-3 and A549). A three-hour exposure to the highest concentration of NP resulted in cell viability of 66-82%. Nevertheless, the 24h contact resulted in a strong decrease in cell viability. There was no inflammatory response detected after the exposure to nanoparticles.

Keywords: Nanoparticles, Chitosan, Fucoidan, Bovine serum albumin, Mucosal routes

RAZŠIRJENI POVZETEK

Številne bolezni, ki so danes prisotne med ljudmi, so običajno posledica fiziološke disfunkcije ali izpostavljenosti škodljivim okoljskim dejavnikom. Večina nepravilnosti na molekularni ravni je je posledica nihanja količine in izgube funkcije oz. aktivnosti enega ali več proteinov, to pa povzroča motnje delovanja celice, tkiva ali organa. Velik del najnovejših medicinskih raziskav je zato usmerjen v identifikacijo ključnih proteinov, ki so vključeni v molekularne mehanizme in so vzrok številnim boleznim. Te proteine je smiselno izbrati kot tarčo za razvoj novega zdravila, ki bi bilo zmožno izključiti ali pa vsaj zmanjšati simptome.

Leta 1980 se je biofarmacevtska industrija osredotočila na proizvodnjo terapevtskih proteinov, izdelanih z rekombinantno tehnologijo, zaradi katere je lahko prišlo do pojava prvega manipulativno gensko spremenjenega organizma. To odkritje je omogočilo razvoj novih terapij in masovno proizvodnjo bioloških molekul, ki so bile prej dostopne le v omejenih količinah. Postalo je jasno, da predstavljajo največji del terapevtsko obetavnih molekul prav proteini.

Formulacija proteinov je odvisna od poznavanja njihovih fizikalno-kemijskih in bioloških karakteristik, vključno s kemijsko in fizikalno stabilnostjo, imunogenostjo in farmakokinetičnim profilom. Terapevtska aktivnost proteinov je zelo odvisna od njihove konformacije. Ker pa je struktura proteinov gibljiva in občutljiva na zunaje dejavnike, moramo biti pri njihovi produkciji, formulaciji in manipulaciji še posebej pozorni na optimizacijo učinkovitosti in varnosti.

Kljub napredku na področju razvoja novih zdravil na osnovi proteinov v biotehnologiji so ti zaradi njihove nekompatibilnosti in kemijske specifičnosti še vedno v večini administrirani parenteralno. Znano je, da parenteralana aplikacija predstavlja veliko slabost za paciente, kar vpliva na njihovo komplianco, predvsem kadar gre za kronično zdravljenje. Veliko truda se vlaga v iskanje alternativnih možnosti aplikacije proteinov oz. zmanjševanje pogostosti vbrizgavanja teh zdravil. Nekatere biotehnološko pridobljene učinkovine, kot so peptidi, proteini in nukleinske kisline, delujejo znotraj celic, zato morajo prispeti tja, da dosežejo farmakološki učinek. Zaradi omejene permeabilnosti in

stabilnosti biofarmacevtikov je le-te za učinkovito dostavo potrebno vključiti v dostavne sisteme, ki omogočajo ciljanje na mesto delovanja.

Nanodelci se pojavljajo kot možni dostavni sistemi za aplikacijo učinkovin, saj povečujejo učinkovitost transporta in vplivajo na profil sproščanja učinkovin. Njihova velika difuzivnost in aktivno ciljanje izboljšata učinkovitost zdravljenja, saj se poveča obseg privzema v tarčne celice in podaljša čas zadrževanja dostavnega sistema znotraj celic, hkrati pa aktivno ciljanje zmanjša potencialne neželene učinke, ki so posledica neselektivnega vnosa in delovanja zdravilnih učinkovin na netarčna tkiva. Zaradi izredno majhne velikosti teh delcev je njihova celokupna površina zelo velika, kar pripomore k povečani topnosti in hitrosti raztapljanja, poleg tega pa je učinkovina, ki je vgrajena v nanosistem, zaščitena pred snovmi, ki bi lahko povzročile njeno deaktivacijo, še preden bi prispela do tarčnega mesta. Prav njihova majhnost in posledično velika specifična površina pa vodita do intenzivnih intereakcij z biološkimi sistemi in omogočata hitre terapevtske učinke pri nizkih odmerkih.

Naštete prednosti pa spremljajo tudi slabosti, ki omejujejo njihovo uporabo. Nanodelci so zmožni sovezave na molekule, ki so prav tako aktivne, imajo veliko težnjo po agregiranju/aglomeriranju, njihov toksikološki profil je pogosto nepoznan. Nekatere razikave namreč kažejo, da je toksično delovanje nanodelcev lahko bistveno drugačno kot pri večjih delcih kemijske sestave.

Uspeh formulacije nanodelcev z enkapsuliranim proteinom je odvisen predvsem od zmožnosti proteina, da obdrži svojo nativno strukturo in aktivnost med izdelavo, in od profila sproščanja proteina iz nanodelcev po aplikaciji. Profili sproščanja je odvisen od uporabljenega materiala in načina izdelave. Različne metode priprave tako omogočajo modulacijo strukture, kompozicije in fizikalno-kemijskih lastnosti.

Izbira metode za pripravo nanodelcev je odvisna od lastnosti uporabljenega polimera, topnosti učinovine, ki jo želimo enkapsulirati, in njene funkcije v celotnem nanosistemu. Priprava nanodelcev lahko vključuje uporabo organskih topil in agresivnih metod za biomolekule, kar lahko vodi do izgube aktivnosti teh molekul. Uporaba naravnih polimerov kot dostavnih vehiklov pa odpravi te težave, saj omogoča tvorbo nanodelcev s

polielektrolitsko kompleksacijo. Naravni polimeri ponujajo številne prednosti, med njimi tudi to, da so zelo podobni, včasih celi identični makromolekulam, ki so jih biološki sistemi zmožni prepoznati in metabolizirati. Tako se izognemo težavam toksičnosti in stimulaciji vnetnega odziva, kot tudi celičnemu neprepoznavanju, ki je posledica sintetičnih polimerov. Te unikatne karakteristike omogočajo odpravo nekaterih težav, povezanih z neparenteralno aplikacijo proteinov, po drugi strani pa naravni polimeri lahko izzovejo imunski odziv, saj je včasih njihova tehnološka manipulacija zaradi njihove strukturne kompleksnosti bolj kompleksna od tiste, ki je uporabljena v sinteznih polimerih.

V naši študiji smo za pripravo nanodelcev uporabili naravna polimera, hitosan in fukoidan. Ne samo da sta ta dva polimera biorazgradljiva in biokompatibilna, kar je obvezno pri katerikoli biomedicinki aplikaciji, ampak sta izredno uporabna tudi zaradi njunih unikatnih karakteristik, ki lahko povečajo učinkovitost zdravljenja. Fukoidan je ekstrahiran iz rjavih alg (Fucus vesiculosus) in predstavlja anionski polisharid, medtem ko hitosan, pridobljen iz skeleta rakov po deacetilaciji hitina, služi kot kationski polisaharid. Hitosan izstopa tudi po svojih lastnostih, kot sta mukoadhezivnost in povečanje prehodnosti medceličnih stikov, saj na ta način poveča absorpcijo učinkovine.

Čeprav je tehnika polielektrolitske kompleksacije pogosto uporabljena pri pripravi nanodelcev na osnovi hitosana, ki reagira z anioni, je ta raziskava med prvimi, v katerih je izvedena kompleksacija hitosana s fukoidanom. Predhodne študije, v katerih je bila izvedena polielektriolitska kompleksacija med hitosanom in fukoidanom, so bile usmerjene v zmožnost teh nanodelcev kot dostavnega sistema za kurkumin v terapiji raka. Raziskave so potekale tudi že na praznih nanodelcih z namenom zdravljenja dermalnih opeklin. Naša študija pa je med prvimi študijami, ki nanodelce iz hitosana in fukoidana obravnajo kot dostavni sistem za proteine.

Nanodelce smo pripravili po dveh metodah, A in B, ki sta se razlikovali po masnem razmerju uporabljenih polimerov. Metoda A je predstavljala masno razmerje FUC/CS=4/1, metoda B pa masno razmerje FUC/CS=1/4. Tako pripravljenim delcem smo izmerili velikost (164-461 nm) in zeta potencial (-32-36 mV), ki je bil odvisen od naboja polimera, prisotnega v višji koncentraciji. V nadaljevanju poskusa smo na

nanodelce vezali protein goveji serumski albumin (BSA), ki je zaradi svojih lastnosti pogosto uporabljen v različnih raziskavah. Z izoelektrično točko pri 4,7 dovoljuje manipulacijo površinskega naboja in interakcijo s polimeri, kar vodi do visoke učinkovitosti vgrajevanja (EE). Ta je bila določena indirektno, in sicer z določanjem količine nevezanega proteina s tekočinsko kromatografijo visoke ločljivosti (HPLC), in je bila najvišja (100 %) za metodo A ter precej nižja pri nanodelcih, izdelanih z metodo B (37 %). Stabilnost nanodelcev smo preizkusili ob prisotnosti lizocima, ki je le blago vplival na spemembo velikosti delcev, nekoliko bolj očitna je bila sprememba pri delcih, pripravljenih po metodi A. Citotoksičnost nanodelcev in fukoidana pri različnih koncentracijah (med 0,1 in 1 mg/ml) smo preverili s citotoksičnim testom MTT na alveolarnih celičnih linijah Calu-3 in A549, ki sta bili delcem oz. polimeru izpostavljeni 3 ure in 24 ur. 3-urna izpostavitev celic najvišjim koncentracijam nanodelcev je pokazala 66 do 82 % preživetje celic, medtem ko se je ta po 24-urni izpostavljenosti precej znižala. Vnetnostnega odziva celic po izpostavitvi nanodelcem nismo opazili.

LIST OF ABBREVIATIONS

Abs	Absorbance
BSA	Bovine serum albumin
CS	Chitosan
EE	Encapsulation efficiency
FUC	Fucoidan
FUC/CS	Fucoidan/Chitosan
HPLC	High performance liquid chromatography
IL-6	Interleukin 6
IL-8	Interleukin 8
kDa	Kilo Daltons
LC	Loading Capacity
LPS	Lipopolysaccharide
MW	Molecular weight
NPs	Nanoparticles
PNPs	Polymeric nanoparticles
PY	Production yield
SD	Standard deviation
UV	Ultra violet
w/w	Weight/weight

1 INTRODUCTION

1.1 BIOMOLECULE-BASED THERAPIES

1.1.1 BACKGROUND

The absorption of protein-based macromolecules administrated by pulmonary route has gain a growing interest over the last decade. Although many biomolecules seem therapeutically promising, their administration could be difficult due to their physicochemical and biopharmaceutical features. Instability is also the main reason why parenteral delivery is usually the only option for patients, but since it is invasive and painful, it often leads to therapeutic incompliance (1). Since the lung is capable of absorbing pharmaceuticals either for local or systemic delivery, pulmonary delivery of drugs has gained a big scientific and biomedical interest in the health care research area. It is an outstanding target for the peptide and protein delivery, mostly because of the major alveolar surface area appropriate for drug absorption. Thin epithelial barrier, vast vascularisation, absence of first pass effect and relatively poor proteolytic activity in comparison to other administration routes are also the advantages worth mentioning.

The precondition for a dependable and targeted protein delivery is of course a development of the appropriate carrier (2).

1.1.2 PROTEIN FORMULATIONS

Structural complexity and instability are the main reasons that make protein drug formulation extremely challenging. Those two features are also the main reasons why most protein-based drugs, are designed as aqueous solutions or suspensions ready to use as lyophilized powder for the reconstruction of the product. The 3-dimensional structure of the majority of recombinant proteins is responsible for their biological activity and needs to remain unaltered throughout the shelf-life of the product. As a result of either cleavage or aggregation, a reduction in efficacy and adverse immunologic effect appear. Combining all those facts together shows how important the evolution and assessment of prosperous drug delivery systems is (3). Considering the fact that some proteins demand

sustained release, while others demand controlled, immediate or pulsed release, different release profiles have to be achieved by using diverse particulate systems for drug delivery (4).

Various proteins have already been used as models by different research groups in order to develop new systems. In our work, bovine serum albumin (BSA) has been utilized as a model protein. Bovine Serum Albumin is a massive, stable, soluble, monomeric, globular protein which presents about half of all the serum proteins. Thanks to its ligand bonding capacity, it is known as a transporter molecule for a wide ambit of metabolites, drugs, nutrients, metals and other molecules, frequently used in clinical, pharmaceutical, and biochemical applications (5). The extensive use of this protein as a model is the result of its easy dissolution in water, yet it is relatively resistant to digestion. It represents an important part in stabilizing extracellular fluid volume and sustaining osmotic balance, and it is also a promising binder of harmful toxins and free radicals, often used to provide nutritions and bind toxins in microbiological, cell and tissue culture media (6).

With its isoelectric point at 4.7, this protein can expose negative or positive charges when in basic or acid environments, respectively (7). It consists of nine loops connected by 17 disulphide bridges that are protected in the core of protein. Its theoretical molecular weight is 69.3 kDa and it is stable for years when stored at 2-8°C (8).

1.2 ROUTES OF ADMINISTRATION OF PROTEIN-BASED FORMULATIONS

The application of macromolecules in therapy remains a problem due to stability and/or permeation issues. These limitations represent a challenge for the pharmaceutical industry, which is working hard on the development of appropriate non-injectable drug carriers. Because of many disadvantages connected with parenteral route of drug delivery (especially with patients in chronic therapy), many research efforts have been made to improve patients compliance, either by using other routes of application or by minimizing

the injecting intervals (1). Nasal, pulmonary and oral mucosal surfaces are investigated as potential substitute routes for the systemic administration of protein-based drugs (9).

1.2.1 GASTROINTESTINAL MUCOSA

There are various routes of drug administration, each of them having advantages and disadvantages. Being simple and convenient, the oral route of drug administration is preferred to any other. On the other hand, oral application of protein drugs presents a great difficulty because of their poor bioavailability and also because of the unpredictable nature of gastrointestinal absorption. While developing oral protein formulations, some barriers such as weak permeability of major molecules, absence of lipophilicity and inactivation by rapid enzymatic and pH degradation in the gastrointestinal tract have to be taken into account (10). Apart from the simplicity of administration itself, this approach ensures drug's access to intestinal epithelium, being known as the greatest and the most specific surface area (200 m^2) of absorption that exists in the human body (11).

1.2.2. BUCCAL MUCOSA

The buccal part of the oral mucosal cavity also proposes a promising route for systemic drug delivery. It is a great site for protein absorption due to its rich blood supply, relatively good permeability and the physiological features such as the avoidance of presystemic elimination, including the first pass effect (12). However, its surface presents a relatively small area available for the absorption (50 m^2).

1.2.3. PULMONARY MUCOSA

Since lungs are capable of absorbing pharmaceuticals for local deposition and systemic delivery, pulmonary delivery of drugs has gained an extensive scientific and biomedical interest (13). Properties such as large alveolar surface area, thin epithelial barrier, vast vascularisation and poor proteolytic activity (in comparison to other application routes) in combination with the avoidance of the first pass effect, make the pulmonary delivery of protein-based drugs an excellent objective (2). Particles targeting the deep lung have to be limited in size. An aerodynamic diameter between 1 and 5 μm (as shown in Figure 1)

should be reached, so the particles can pass through the mouth, throat and conducting airways, but they should not be too small, so they cannot fail to deposit and be breathed out. However, numerous particles will certainly be removed from the lung by mucociliary clearance. Once the nanoparticles reach the deep areas of lungs, they have to face many defence mechanisms, among which alveolar macrophages and enzymatic activity are just the two most common ones (2).

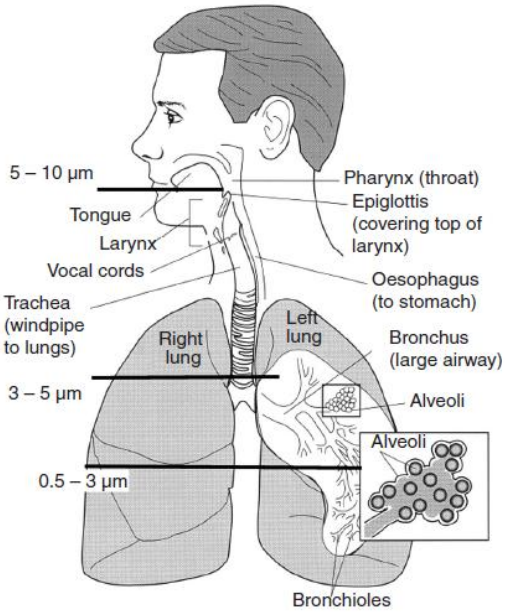

Figure 1: The respiratory tract and areas targeted by different sized particles (14).

1.2.4. NASAL MUCOSA

The nasal mucosa is receiving a great deal of attention as an alternative route for systemic delivery of the drugs which are now limited to intravenous application. It presents a great surface area (160 m^2), porous endothelial membrane, extensive total blood flow, has the advantage of avoiding the first-pass metabolism, and it is easily accessible to drug absorption site. Drugs are rapidly cleared from the nasal cavity after intranasal application, having a quick systemic absorption as a result (15).

1.3. TRANSMUCOSAL DRUG DELIVERY TECHNOLOGIES

Mucosal surfaces are without any doubt the most frequently used and also the most suitable routes for drug delivery. Nevertheless, macromolecular drugs like peptides and proteins are not able to conquer all the mucosal barriers and/or are often degraded before getting into the blood stream. To overcome these limitations, notable strategies have been devoted to examine new routes (alternative to injections) for the systematic delivery of such macromolecules, aiming the transmucosal routes such as nasal, pulmonary and oral routes as the most promising ones. The knowledge about the mechanisms of interactions between nanomaterials and biological surfaces, together with the development of nanotechnologies and characterization techniques, brought to a new approach, using nanocarriers for transmucosal drug delivery of macromolecules (16).

Different strategies were developed aiming at the improvement of bioavailability of therapeutic proteins. The techniques frequently utilized in developing mucosal protein delivery systems contain unique excipients, like absorption enhances enzyme inhibitors and mucoadhesive polymers (17). Considering their colloidal size, the nanosystems are capable of crossing and transporting the associated drug through the mucosal barrier, functioning as transmucosal macromolecular nanocarriers (16).

1.4 POLYMERIC NANOPARTICLES FOR MUCOSAL ADMINISTRATION

1.4.1 HISTORICAL FRAME

The idea of nanoparticles connected to drug targeting was inspired by Paul Ehrlich, after having visited an opera performance called "Der Freischütz" (Greilig 1954), where "Freikugeln" created by calling the spirit of the devil played an important part. The fact that these bullets constantly reached their target, even if the rifleman did not aim

correctly or if the target inaccessible, inspired him and thus the idea of nanoparticles and drug targeting was born (18).

First nanoparticles were developed in the mid-seventies so as to carry vaccines and anticancer agents to specific tissues or even cells in order to improve therapeutic efficacy and to decrease the toxic effect of the drugs (19).

The number of nanoparticles used for pharmaceutical and medical application has been on the increase since the earliest commercial nanoparticle drug-loaded product became available on the market in 2005 (Abraxane®, human serum albumin nanoparticles containing paclitaxel) (18).

1.4.2 DEFINITION AND STRUCTURAL ORGANIZATION

Micro- and nano-technologies are sophisticated technologies, developed with the intention of meeting unique requirements in the field of drug administration (20). Great effort has been made in order to present a comprehensive definition of nanoparticles. A definition for pharmaceutical need (that has now entered relevant specialised Encyclopaedias') is as follows (Kreuter, 1994b, 2004): "Nanoparticles are solid colloidal particles ranging in size from 10 to 1000 nm (1µm). They consist of macromolecular materials in which the active principle (drug or biologically active material) is dissolved, entrapped, encapsulated and/or to which the active principle is adsorbed or attached." (18).

Several terminologies are used when talking about nanoparticulate drug delivery systems, according to structures and materials that compose the systems. Usage of different production methods can create different and unique systems shown in Figure 2, which can be used in a biological interaction necessary for each purpose (21).

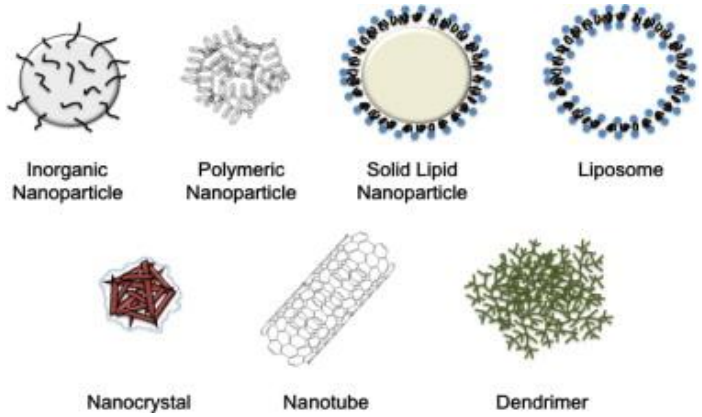

Figure 2: Different nanoparticulate drug delivery systems (22).

Regarding the fact that the application of macromolecules depends of their stability and permeation issues, nanoparticles have appeared as one of the most promising tools, mainly because of the increased surface-to-volume ratio, which ensures a close interaction with epithelial surfaces. They give the possibility to the encapsulated molecules to maintain their biological activity, from the production act to the terminal release (1). The advantages and disadvantages of the use of nanoparticles are described in the Table I.

TABLE I: Advantages and disadvantages of nanoparticles as drug delivery systems. Adapted from (23).

ADVANTAGES	DISADVANTAGES
High surface/volume ratio	Undefined physical shape
Surface easily modified	Limited capacity to co-associate other functional molecules
Potential contact with mucosa	Unknown toxicity profile
High drug concentration in desired site	Lack of suitable large-scale production methods
Decrease in adverse drug-associated effects	Low stability in some biological fluids
Intracellular penetration	Tendency towards aggregation
Protection of encapsulated molecules	Limited loading capacity
Possibility of reaching controlled and/or prolonged release	Small size providing access to unintended environments
Possible targeted delivery	
Enhanced drug absorption	

In the context of our work, the importance of polymeric nanoparticles (PNP) will be highlighted, especially considering their potential for the transmucosal delivery of proteins through pulmonary mucosa (24). Polymeric nanoparticles are classified into two categories, nanospheres and nanocapsules, with the following definition (Rao, Geckeler, 2011): "Nanospheres are matrix particles, whose entire mass is solid and allow molecules to be adsorbed either on the sphere surface or encapsulated within the particle. They are usually spherical, but also “nanospheres” with a nonspherical shape are possible. Nanocapsules are vesicular systems, a kind of reservoir, in which the substances are entrapped in a liquid core (either oil or water), surrounded by a solid material shell." An illustration of PNPs is shown in Figure 3 (25).

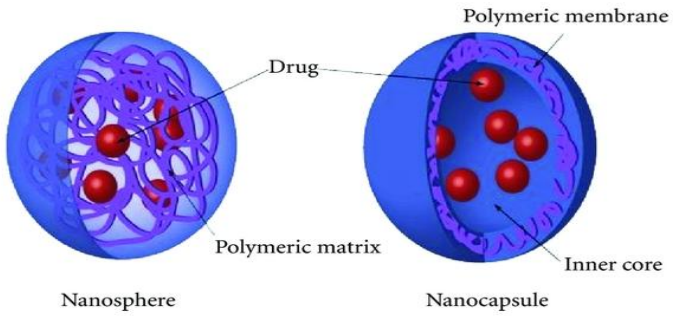

Figure 3: Two main types of polymeric nanoparticles, a nanosphere and a nanocapsule (26).

1.4.3 PREPARATION METHODS

Different techniques have been developed to synthesize polymeric nanoparticles, mainly classified into two categories, depending if the formulation demands a polymerization reaction or the formulation is reached straight from a macromolecule or performed polymer (27). A schematic representation of diverse preparation methods for PNP is given in Figure 4 (25).

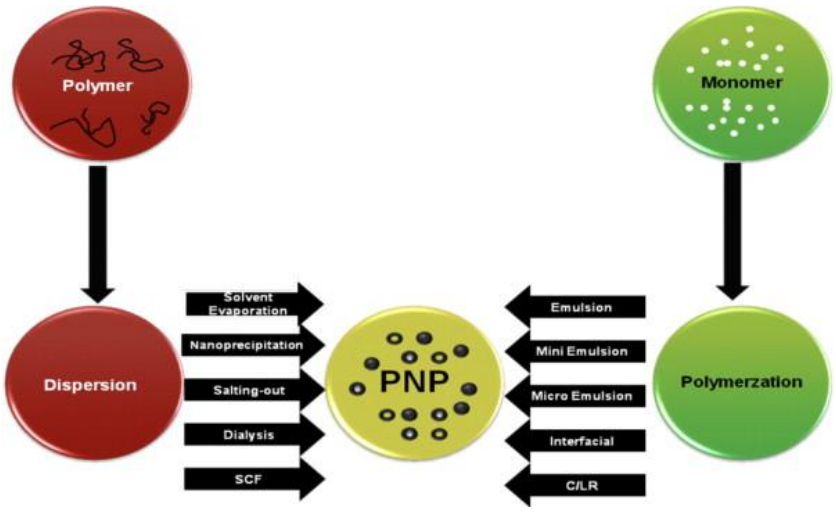

Figure 4: Different preparation techniques of PNP (25).

Usage of different methods to prepare polymeric nanoparticles allows the modification of their structure, composition and physicochemical possessions (28). This is the reason why the selection of production technique is based on a numerous elements, for example the sort of polymeric system, area of administration, size demands and so forth. (25). Organic solvents and aggressive methods such as ultrasound energy are frequently used for the preparation of a suitable nanosystem. The aggressive conditions could have a negative effect on both, the drug/protein to be encapsulated, and the organism to which the nanosystem will be administrated (29). The use of natural polymers allows using methodologies that overcome the before mentioned problems (2).

Chitosan nanoparticles have been produced to encapsulate different proteins, from bovine serum albumin to tetanus and diphtheria toxin, vaccines, anticancer agents, insulin, and nucleic acids (27). Using chitosan, many methods have been developed, mainly involving emulsification, different types of coacervation or even slight modification of these. The methods include emulsion droplet coalescence, emulsion solvent diffusion, reverse micellar method, ionic gelation, desolvation or polyelectrolyte complexation, a method that was also used for the preparation of our nanoparticles (1). Ionic gelation is a term, used when the chitosan gelation is induced by small anionic molecules (phosphate,

sulphate, citrate), while polyelectrolyte complexation is a term used when anionic macromolecules are used instead of small molecules (1).

1.5 POLYMERS AS NANOPARTICLE MATRIX-FORMING MATERIALS

1.5.1 DEFINITION

A polymer is a macromolecule composed of either many repeating units of one type (homopolymers) or many repeating units of several types (copolymers) (30). Different classifications of polymers are known, according to their occurrence as natural or synthetic, and also their chain nature, structure, morphology and type of polymerization reaction (31). Synthetic polymers are in most cases obtained by linking together a large number of small molecules (monomers), while the structure of natural polymers is usually more complex (30, 31). Both, natural and synthetic polymers have some advantages and disadvantages that are described in Table II.

TABLE II: Advantages and disadvantages of different types of polymers. Adapted from (23).

OCCURRENCE	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,	Predictable properties Batch-to-batch uniformity	Too expensive Environmental and human

Polyamine acids, Polyalkylated cyanoacrylates, Polyesters, Poly(ortho esters), Polyurethanes, Polyacrylamides)	Easy technological manipulation	health concerns Lack of recognition by cells Toxicity Stimulation of a chronic inflammatory reaction

1.5.2 APPLICATION OF NATURAL POLYMERS IN NANOPHARMACEUTICS

Being biocompatible and biodegradable, some polymers have been utilized extensively as drug delivery systems. They have been used as carriers for controlled delivery of low molecular weight drugs and also as bioactive proteins. If we want to use either a synthetic or a natural polymer as biomaterial, it is important that it does not cause inflammatory or toxic reactions at the application sites- Instead, it has to provide appropriate half-life, degradation time has to be compatible with the desired application, there cannot be any toxic degradation products, and it should also have the capability of being digested and easily eliminated from the body (32).

To obtain a controlled release of the substance, the kinetics of polymer in vivo degradation has to remain constant. Therefore, a great number of variables must be monitored. Factors such as pH and temperature should be evaluated during development, since they may cause an increase or a decrease in the rate of degradation of the system (33). Because of the fact that natural polymers usually show a rapid drug release, synthetic biodegradable polymers are more frequently used in the application as delivery systems (34). The nature of a polymer and the physicochemical properties of an incorporated substance have the biggest influence on the profile and the mechanism of the drug release (32).

1.5.3 CHITOSAN

Different polymers have been used as a vehicle to prepare nanoparticles, usually preferring those of natural origin, since they agree to the demands of biocompatibility, biodegradability and lack of toxicity in comparison to synthetic ones. (35).

Chitosan is a polymer of natural origin, composed of repeating units of N-acetyl-D-glucosamine and D-glucosamine as shown in Figure 5. It is obtained by alkaline, partial deacetylation of chitin, the major component of crustacean shells (36). It presents a cationic character, due to the content of amino groups in the main backbone. Chitosan is easily soluble in aqueous acidic solutions, featuring poor solubility at the physiological pH of 7.4 as it is a weak base (pKa around 6.5) (37).

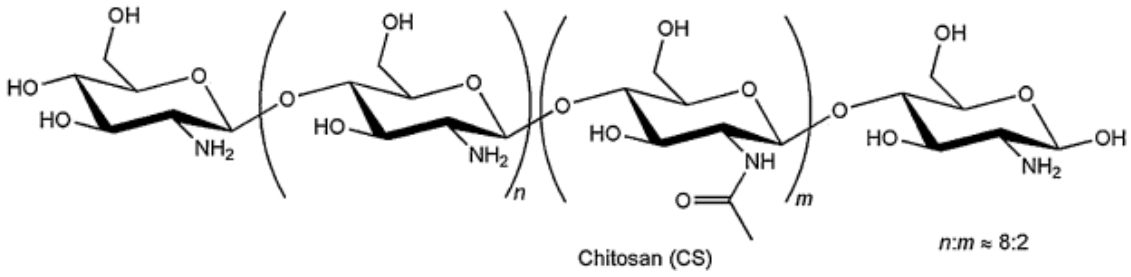

Figure 5: The structure of chitosan (36).

When it comes to the production of nanomedicines, it is one of the most frequently used natural polymers due to its attractive characteristics for drug delivery when formulated in nanoparticulate form. Its most attractive property is the ability to adhere to mucosal surfaces, which leads to a prolonged residence time at drug absorption sites and enables higher drug permeation (1). Chitosan particles can also improve drug absorption via the paracellular route as shown in the Figure 6. The mechanism of action of chitosan is probably a compound of bioadhesion and a temporary widening of the tight junctions among epithelial cells (38).

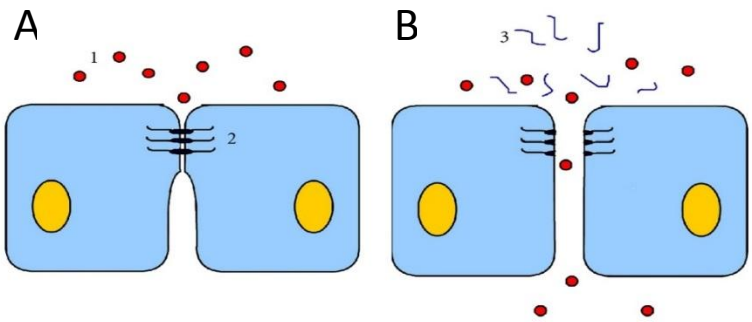

Figure 6: The consequence of chitosan on the absorption of drugs by the paracellular route. (A) Normal epithelium. (B) Temporary interference of tight junctions by chitosan with enhancement of drug absorption. (1: the drug, 2: the tight junction, 3: chitosan molecules). Adapted from (23).

Commercially, chitosan is accessible in a range of different types, mainly differing in the molecular weight and degree of deacetylation, which have an influence on features such as solubility and mucoadhesivity. With a p*K*a of about 6.5, chitosan is soluble in acidic solutions because of the protonation of the amino groups constructing the polymeric chain at this pH. Highly deacetylated chitosan (85%) is also soluble in solutions of pH up to 6.5, but with the decreasing deacetylation degree, the solubilisation is decreased also. The mucoadhesive capacity of the polymer raises with the raise in the deacetylation degree, as this contributes to more positively charged amino groups accessible for the interaction with negatively charged mucus residues (sialic acid) (20). This characteristic makes it appropriate for interacting with negatively charged fucoidan to form nanoparticles (39).

1.5.4 FUCOIDAN

Fucoidan (FUC) is an anionic sulphated polysaccharide extracted from brown seaweed and some invertebrates such as marine cucumber. It is formed mainly of L-fucose and the sulphate ester group as shown in the Figure 7. It can also contain uronic acids, protein and other monosaccharides such as mannose, glucose and galactose (39). Fucoidan used in our study was prepared from *Fucus Vesiculosus*, containing 44% fucose and 26% sulphate and being water soluble (40). Due to its various biological activities such as

anticoagulant, antithrombotic, antivirus, antitumor, anti-inflammatory and antioxidant features, it has been examined intensively over the last few years.

Its molecular weight, structure and composition (particularly the position and quantity of its sulphate groups), have a great impact on its bioactivity. Some studies have shown that with the higher molecular weight, the capability to act as an anti-inflammatory agent increases. On the contrary, the lower the molecular weight is, the more it is effective as an anticoagulant and antithrombotic substance. It is capable of stabilizing the growth factor, improving the binding with cell surface receptors and acting as an antithrombotic inhibitor for clinical therapy. Given the fact that it comes from natural nontoxic algae means that its potential of being a biomaterial is extremely high (39).

Fucoidan has also showed the capability to isolate toxic heavy metals like $Cd^{2+,}$ Cu^{2+}, Zn^{2+}, Pb^{2+}, Cr^{3+} and Hg^{2+}, and also to bind type A I and II transmembrane glycoprotein receptors discovered in macrophages, promoting particular interactions of a drug carrier with the macrophages (41).

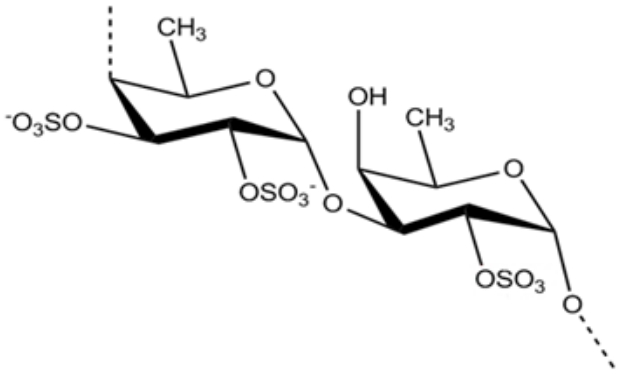

Figure 7: The structure of fucoidan (39).

1.6 CHITOSAN/FUCOIDAN NANOPARTICLES

The technique of polyelectrolyte complexation to obtain chitosan based nanoparticles by interaction with counter-anions has already been mentioned in several reviews, reinforcing the potential of these carriers in the biopharmaceutical and biomedical fields (1). Chitosan nanoparticles were first mentioned in 1994. They were obtained by emulsification and cross-linking, used for the intravenous delivery of 5-florouracil. Since that time, chitosan has been in the centre of attention of various studies for drug delivery purposes (1). However, the present work is one of the first reports describing the formation of nanoparticles after the complexation of chitosan and fucoidan, encapsulating bovine serum albumin as a model protein.

In previous reports the fucoidan/chitosan nanoparticles are mentioned as a carriers used to encapsulate the antitumor drug curcumin (39), as well as the material used in the production of microparticles for protein encapsulation (42) or the use of unloaded microparticles for dermal burn treatment (43). One of the newest reports (made in Portugal) also describes the development of fucoidan/chitosan nanoparticulate systems for protein administration through mucosal route, associating bovine serum albumin (BSA), ovalbumin and insulin, but without making the cytotoxical studies.

2 OBJECTIVES

The aim of our work was to verify the possibility of chitosan and fucoidan to form nanoparticles that show ability to encapsulate bovine serum albumin. We characterized the particles for their size, zeta potential, production yield, encapsulation efficiency, loading capacity and stability in the presence of lysozyme. The toxicity studies of unloaded nanoparticles were made on the Calu-3 and A549 cell line and the inflammatory response generated by the exposure to nanoparticles was also evaluated on the Calu-3 cell line.

The nanoparticles are aimed at an application in systemic mucosal protein administration and should therefore evidence the following specific properties:

- Size within 50-500 nm to allow close interaction with the epithelial surface;
- Zeta potential above 30 mV (negative or positive) to provide adequate physical stability in aqueous dispersion as well as to maximize interaction with the epithelial surface;
- Adequate protein encapsulation efficiency, preferably above 50%
- Viability of the cells after exposure to nanoparticles higher than 50%
- Low inflammatory response of the cells after exposure to the nanoparticles (23).

3 MATERIALS AND METHODS

3.1 MATERIALS

- Sigma-Aldrich® (Germany):
  - Chitosan (CS) (low molecular weight, deacetylation degree= 75-85%),
  - Fucoidan (FUC) from *Fucus vesiculosus*,
  - albumin from bovine serum (BSA),
  - lysozyme from hen egg white,
  - pentasodium tripolyphosphate,
  - phosphate buffer saline (PBS) tablets pH 7.4,
  - Dulbecco's modified Eagle's medium (DMEM),
  - non-essential aminoacids (100%),
  - penicillin/streptomycin solution (at +10,000 units/ml/+10,000µg/ml),
  - L-glutamine 200mM,
  - trypsin–EDTA solution (2.5 g/l trypsin, 0.5 g/l EDTA),
  - trypan blue solution (0.4%),
  - thiazolyl blue tetrazolium bromide (MTT),
  - sodium dodecyl sulphate (SDS),
  - dimethyl sulfoxide (DMSO),
  - glycerol,
  - glacial acetic acid.
- Gibco® (USA): Fetal bovine serum (FBS).
- JT Baker® (Netherlands): Acetonitrile (HPLC grade).
- Alfa Aesar® (Germany): trifluoroacetic acid (TFA).

Ultrapure water (Milly Q Plus, Millipore Iberica®, Spain) was used throughout.

3.2 PREPARATION OF FUCOIDAN/CHITOSAN NANOPARTICLES

Chitosan/fucoidan nanoparticles were prepared according to a procedure that had been previously developed in the laboratory, based on the polyelectrolyte complexation of chitosan with fucoidan. The positively charged amino groups of chitosan interact with the negatively charged sulphate groups of fucoidan, creating electrostatic interactions. BSA was used as a model protein.

Briefly, chitosan was dissolved in 1% (w/w) acetic acid and fucoidan was dissolved in Milly Q water in order to obtain solutions of concentration of 1 mg/ml respectively to reach final theoretical FUC/CS ratios 4:1 (method A) and 1:4 (method B). Both solutions were filtered before further use (0.2 μm filter, Whatman®, Germany).

Spontaneous formation of nanoparticles occurs upon dropping of 1 ml of solution containing the polymer present in the lower amount to 1 ml of the solution containing polymer present in the higher amount, in the duration of approximately 10 minutes of mild magnetic stirring at a room temperature.

Method A (FUC/CS = 4/1) corresponds to dropping 1ml of CS solution (0.3 ml CS+0.9 ml H_2O) into 1 ml of FUC solution (1 mg/ml) under magnetic stirring at the room temperature. On the contrary, in method B (FUC/CS = 1/4) 1 ml of FUC solution (0.3 ml FUC+0.9 ml H_2O) was dropped in 1 ml of CS solution (1 mg/ml).

Nanoparticle dispersions were later put in eppendorf tubes over a layer of 10 μl glycerol that prevents nanoparticle dehydration and aids the subsequent resuspension step. After the centrifugation at 16.000 g for 30 minutes at 15°C (Thermo Scientific®, Germany) the supernatant was discarded and 100 μl of Milly Q water was added to each eppendorf in order to resuspend the nanoparticles (Figure 8).

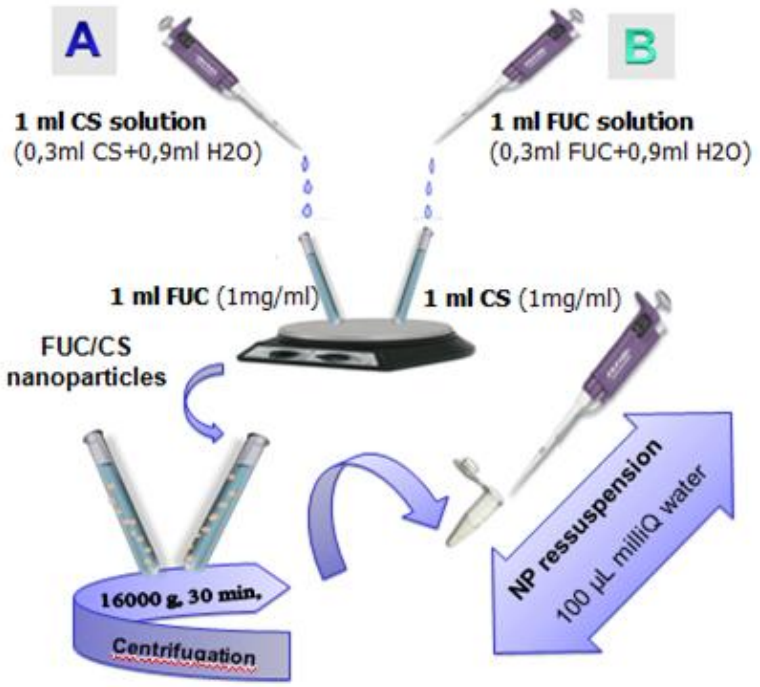

Figure 8: Preparation technique of the nanoparticles. Adapted from (23).

3.3 ASSOCIATION OF BIOMOLECULES TO FUCOIDAN/CHITOSAN NANOPARTICLES

To prepare the protein-loaded FUC/CS nanoparticles, the theoretical content of protein (BSA) was 30% (w/w), respective to the polymer present in the higher concentration in each formulation (CS or FUC). BSA was dissolved in water to obtain a solution of 1.5 mg/ml (solution pH = 6.51), and further dropped in CS solution (method A: 0.30 ml CS + 0.240 ml BSA + 0.660 ml H_2O) or FUC solution (method B: 0.30 ml FUC + 0.240 ml BSA + 0.660 ml H_2O) (Figure 9).

The isolation of protein-loaded nanoparticles was performed by the same procedure as described above.

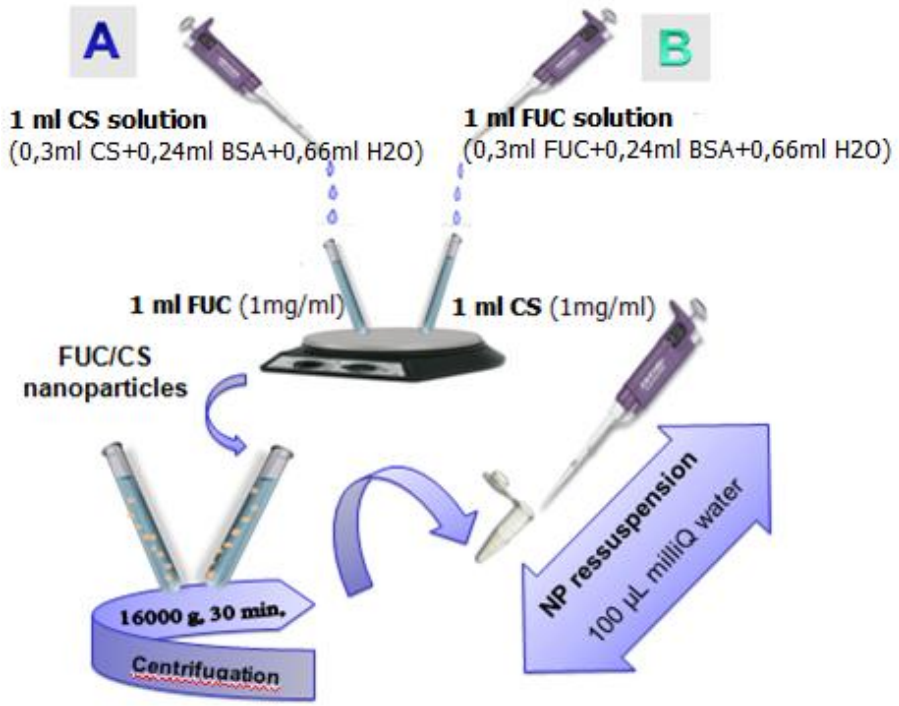

Figure 9: Preparation technique of the protein-loaded nanoparticles. Adapted from (23).

3.4 CHARACTERIZATION OF NANOPARTICLES

3.4.1 PHYSICOCHEMICAL PROPERTIES

The size and zeta-potential of nanoparticles were measured on freshly prepared samples by photon correlation spectroscopy and laser Doppler anemometry, respectively, using a Zetasizer Nano ZS90 (Malvern instruments®, Malvern, UK) (Figure 10). For the particle size and zeta potential analysis 20 µl of each sample was diluted in 1 ml of purified Milly Q water and the dispersion was placed in an electrophoretic cell. Each particle size analysis lasted for 120 s and was performed at 25°C with the detection angle at 90°.

Figure 10: The Zetasizer Nano ZS90 used in our study.

3.4.2 MORPHOLOGICAL EXAMINATION OF FUCOIDAN/CHITOSAN NANOPARTICLES

The morphological examination of nanoparticles was performed by transmission electron microscopy (TEM) (JEM- 1011, JEOL, Japan). Concentrated nanoparticles were obtained upon centrifugation, samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with carbon films (Ted Pellla, USA) for TEM observation.

3.4.3 DETERMINATION OF NANOPARTICLES PRODUCTION YIELD

The nanoparticles production yield was determined by the means of gravimetry. Six tubes of dispersions (2 ml) were prepared for each method. First, 1 ml of the suspension was put in an eppendorf (previously marked and weighted, without glycerol bed) and centrifuged for 30 min (16.000g, 15°C). After the first centrifugation the supernatant was discarded and a second 1 ml of dispersion was added to the same eppendorf, over the former pellet in order to be centrifuged again. After the second centrifugation, the

supernatant was discarded again and the sediment was freeze-dried at -80°C using a Freeze Dryer (Labconco®, USA).

The production yield (PY) was calculated by the following equation:

$$\text{PY (\%)} = \frac{NP\ weight}{total\ solid\ weight} \times 100$$

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

3.4.4 DETERMINATION OF ENCAPSULATION EFFICIENCY AND LOADING CAPACITY OF BSA IN NANOPARTICLES

The encapsulation efficiency of BSA in nanoparticles was determined indirectly, by quantification of the non-encapsulated free protein in supernatant after centrifugation of nanoparticle dispersion (16.000 g, 30 min, 15°C). The amount of free BSA in the supernatant was determined by reverse-phase High Pressure Liquid Chromatography (HPLC, Agilent® 1100 series, Germany). The method is based on the adsorption of hydrophobic molecules onto a hydrophobic stationary phase in a polar mobile phase. The affinity of the BSA to adsorb on hydrophobic surfaces can be reduced by decreasing the mobile phase polarity. This can be achieved with the use of organic solvents (acetonitrile) resulting in desorption and elution from the reverse phase column. 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. The retention time of each polymer revealed to be different from BSA, not interfering with BSA determination.

The following chromatographic conditions were used: mobile phase consisting of acetonitrile and 0.1% TFA aqueous solution initially set in the ratio 30:70 (v/v), which was linearly changed to 40:60 (v/v) over 5 minutes. From 5 to 10 minutes the ratio 40:60 (v/v) was kept constant. Eluent was pumped at a flow rate of 1 ml/min, the injection

volume was 20 µl and detection wavelength was 280 nm. A calibration curve of the protein was made in PBS (pH 7.4).

The encapsulation efficiency (EE) and loading capacity (LC) of BSA in nanoparticles were calculated by the equation:

$$\text{Encapsulation efficiency (\%)} = \frac{Total\ amount\ of\ protein - Amount\ of\ free\ \ protein}{Total\ amount\ of\ protein} * 100$$

$$\text{Loading capacity (\%)} = \frac{Total\ amount\ of\ protein - Amount\ of\ free\ protein}{Nanoparticles\ weight} * 100$$

3.4.5 MTT ASSAY

Cell viability was assessed in Calu-3 and A549 cell lines using the thiyzolyl blue tetrazolum bromide (MTT) assay. The unloaded nanoparticles and the FUC were assayed for the cytotoxicity over 3h and 24h. Cell cultures were grown using 75 cm^2 flasks in a atmospheric air incubator at 37°C, 5% CO_2 and 95% relative humidity. The cell culture medium was 500 ml DMEM, with 50 ml FBS, 5 ml non-essential amino acid solution (non essential Amino acid solution contains the standard non-essential amino acids found in MEM culture media, each at a 10mM concentration), 5ml L-glutamine solution (200 mM) and 5 ml penicillin/streptomycin. Medium was renewed every 2–3 days.

At the beginning of the assay the cells were first counted and the number of cells needed was calculated. The cells have been seeded in the 96 well plate a day before and incubated at 37°C, 5% CO_2 and 95% relative humidity (Figure 11).

Figure 11: The 96 well plate.

For the MTT assay the solution of FUC (2.5 mg/ml) and the unloaded nanoparticle dispersions were prepared using both methods: A (7 formulations) and B (9 formulations). Nanoparticle dispersions prepared by method B (FUC/CS = 1/4) were diluted with 100 µl of water (50 µl per eppendorf), because of lower production yield. 10% SDS was used as a positive control (Figure 12).

The following dilutions were made:

FUCOIDAN:

0.1 mg/ml V (FUC)= 0.16 ml V (medium)= 3.84 ml	0.5 mg/ml V (FUC)= 0.8 ml V (medium)= 3.2 ml	1.0 mg/ml V (FUC)= 1.6 ml V (medium)= 2.4 ml

NANOPARTICLES METHOD A (FUC/CS = 4/1):

0.1 mg/ml V (NP)= 0.08585 V (medium)= 1.9142 ml	0.5 mg/ml V (NP)= 0.4292 ml V (medium)= 1.5708 ml	1.0 mg/ml V (NP)= 0.8585 ml V (medium)= 1.1415 ml

NANOPARTICLES METHOD B (FUC/CS = 1/4):

0.1 mg/ml V (NP)= 0.546 ml V (medium)= 0.9454 ml	0.5 mg/ml V (NP)= 0.237 ml V (medium)= 0.727 ml	1.0 mg/ml V (NP)= 0.546 ml V (medium)= 0.454 ml

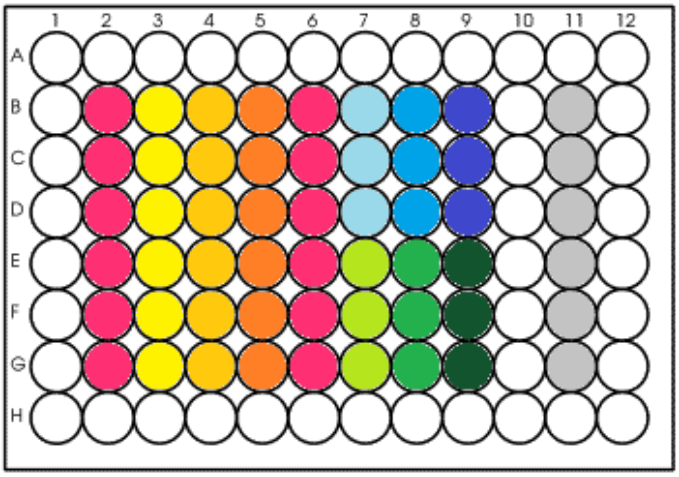

Figure 12: The dilutions and the distribution of the materials used for the MTT assay.

After 3 or 24h of cell incubation with the formulations the test solutions were removed by tapping on a towel and 50 µl of the MTT solution (0.5 mg/ml in PBS, pH 7.4) was added to each well. After 2h, any formazan crystals generated were solubilised with 100 µl of DMSO and after complete solubilisation of the crystals, the absorbance of each well was measured by spectrophotometry at 540 nm, for background at 650 nm (Infinite M200, Tecan, Austria).

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

$$\text{Viability (\%)} = \frac{(A-S)}{(CM-S)} * 100$$

In the equation *A* is the absorbance obtained for each of the concentrations of the test substance, *S* is the absorbance obtained for the 10% SDS and *CM* is the absorbance

obtained for untreated cells (incubated with CCM). The latter reading was assumed to correspond to 100% cell viability.

3.4.6 DETERMINATION OF INFLAMMATORY RESPONSE

The inflammatory response generated by the exposure to nanoparticles was evaluated on Calu-3 cells. The cells were seeded in 96 well plates ($2x10^4$ cells/well). After 24 hours of incubation they were exposed to nanoparticles (1.0 mg/ml), dispersed in cell culture medium. Lipopolysaccharide solution (LPS) in the concentration of 10 µg/ml was used as a positive control, while the incubation with only culture medium was used as a negative control. After 24h of incubation, the cell supernatants were collected and centrifuged. The levels of IL-6 and IL-8 were determined by quantitative ELISA (IL-6 and IL-8 Quantikine ELISA kits, R&D Systems, USA).

3.4.7 STABILITY OF NANOPARTICLE DISPERSIONS IN THE PRESENCE OF LYSOZYME

Lysozyme was dissolved in PBS pH 7.4 resulting in a pH of 6.8-7.0, which is close to the lung pH and to the optimal pH for enzymatic activity (pH 6.4) (2). Nanoparticles without protein were prepared using methods A and B.

Following the calculations of concentration, nanoparticle dispersion prepared by method A (3 formulations) was diluted with 200 µl of Milly Q water (100 µl per each eppendorf) and the nanoparticle dispersions prepared following the method B (6 formulations) were diluted with 100 µl of Milly Q water (50 µl per each eppendorf). Stock solution 1 mg/ml of lysozyme was prepared. The stability of the nanoparticles was analysed following their incubation in solutions of lysozyme (0.2 mg/ml and 0.8 mg/ml) at 37°C under mild horizontal shaking for 90 min. The samples were collected every 15 min, and size and zeta potential were measured.

Solutions:

Method A (FUC/CS = 4/1)	**Method B (FUC/CS = 1/4)**
0.2 mg/ml lysozyme:	**0.2 mg/ml lysozyme:**
0.2 ml ly+ 0.429 ml NP+ 0.3708 ml H_2O	0.2 ml ly+ 0.546 ml NP+ 0.3708 ml H_2O
0.8 ml/ml lysozyme:	**0.8 mg/ml lysozyme:**
0.4 ml ly+ 0.429 ml NP+ 0.171 ml H_2O	0.4 ml ly+ 0.546 ml NP+ 0.055 H_2O

3.4.8 RELEASE STUDIES

The release of BSA was determined by incubating the nanoparticles in the solution of HEPES, NaCl and glucose (pH=7.4) with horizontal shaking at 37°C. The number of samples needed was calculated from the encapsulation efficiency, which was 100% for the method A (FUC/CS = 4/1) and 37% for the method B (FUC/CS = 1/4). Following these calculations, 4 formulations for method A and 6 formulations for method B were prepared. At appropriate time intervals (15 min, 30 min, 60 min, 120 min, 240 min and 24 h) samples (1 ml) were collected, filtered (0.2 µm filter, Whatman®, Germany) and the amount of protein released was evaluated by HPLC.

3.4.9 STATISTICAL ANALYSIS

The t-test and the one-way analysis of variance (ANOVA) with the pair wise multiple comparison procedures (Student-Newman-Keuls method) were performed to compare two or multiple groups, respectively. All analyses were run using the SPSS statistical programme and differences were considered significant at a level of $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 CHARACTERIZATION OF FUCOIDAN/CHITOSAN NANOPARTICLES

4.1.1 PHYSICOCHEMICAL PROPERTIES

FUC/CS nanoparticles were prepared by a very mild polyelectrolyte complexation between the cationic chitosan and anionic fucoidan, at FUC/CS mass ratios 1/4 and 4/1. When the CS and FUC are mixed, the inter- and intra-molecular electrostatic interactions take place between anionic sulphate groups from fucoidan and cationic amino groups of chitosan. These attractions could force the macromolecular chains of chitosan and fucoidan curl up, leading to an insoluble chitosan- fucoidan complex formation. Those unloaded nanoparticles (without BSA), produced by method A (FUC/CS = 4/1) display a particle size in the area of approximately 150-170 nm (average size 164 nm) and zeta potential with average -34 mV, while nanoparticles produced by method B (FUC/CS = 1/4) have the average size of 461 nm and zeta potential 60 mV.

Concerning the zeta potentials of the obtained FUC/CS nanoparticles, a complete shift from strong negative (method A) to strong positive (method B) values could be observed. The zeta potential variation clearly indicates a predominant composition of either the positively or negatively charged polymer in each formulation.

The difference in size and zeta potential between the nanoparticles prepared using method A and the ones prepared using method B is statistically significant ($p < 0.05$).

Evaluating the size and zeta potential of FUC/CS nanoparticles in previous studies, with mass ratios different from the ones we used, show that even when the formulation with equal mass of both polymers is prepared, the nanoparticles display a strong positive surface charge, which indicates a higher charge density of chitosan. The FUC/CS nanoparticles had previously been proposed for the treatment of dermal burns (43) and for the encapsulation of stromal cell-derived factor 1 (SDF-1), an important chemokine in stem cell mobilization (39). Only positively charged nanoparticles were obtained in the

first study, even if the FUC was present in higher amount (FUC/CS=5/1). That effect was explained as a possible occurrence of an outer layer of chitosan among nanoparticle formation (43). In the second study, the nanoparticles with a strong negative charge were obtained when fucoidan was present in higher amount in the formulation. These differences could be ascribed to the use of different chitosan, which is available with very different characteristics.

Incorporation of BSA shows statistically significant differences among the formulations. After adding BSA as a model protein, an electrostatic interaction occurs between the positively charged amino groups of CS, the negatively charged sulphate groups of FUC and negatively charged BSA, respectively resulting in the nanoparticle formation of 187 nm in size on average and zeta potential -32 mV using the method A, and 302 nm and 36 mV using method B (Table III).

In method A (FUC/CS = 4/1) the size of the particles increased due to the lower amount of anionic charges needed to counterbalance CS amino groups. A decrease in size with method B (FUC/CS = 1/4) is related to the cross-linking effect, which includes the condensation of polymeric chains, outcomming in smaller particles. The decrease in zeta potential is a logical result, not only because of the inclusion of the negatively charged BSA in the matrix of nanoparticles, but also because of the general size reduction, which exposes a smaller number of charged groups due to the diminished surface.

TABLE III: The size and zeta potential of unloaded and protein-loaded nanoparticles.

Nanoparticle Formulation	**Size (nm)**	**Zeta potential (mV)**
FUC/CS = 1/4 Unloaded	461 ± 49	+ 60 ± 3
FUC/CS = 1/4 BSA-loaded	302 ± 49	+ 36 ± 6
FUC/CS = 4/1 Unloaded	164 ± 9	- 34 ± 3
FUC/CS = 4/1 BSA-loaded	187 ± 25	- 32 ± 3

4.1.2 MORPHOLOGICAL PROPERTIES OF FUCOIDAN/CHITOSAN NANOPARTICLES

The TEM microphotographs of FUC/CS nanoparticles prepared by both methods evidenced a compact structure and spherical morphology, as determined in Figure 13.

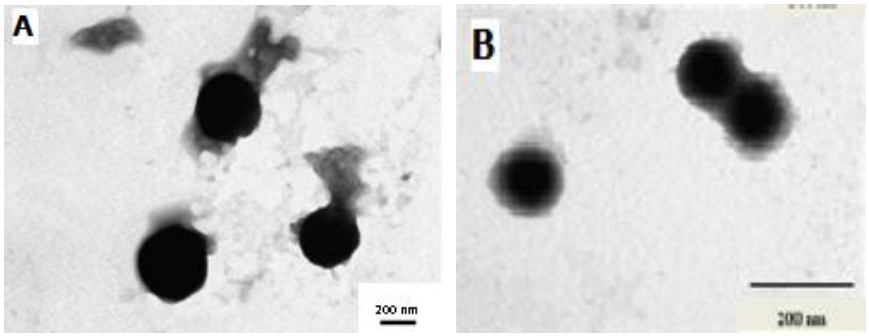

Figure 13: Transmission electron micrograph of FUC/CS nanoparticles prepared by (A) method A (FUC/CS=4/1) and by (B) method B (FUC/CS=1/4)

4.1.3 PRODUCTION YIELD

The production yield was calculated upon the lyophilisation of the nanoparticle dispersions (Table IV). It was higher in the nanoparticles prepared by method A (FUC/CS = 4/1), higher in protein loaded particles than with the unloaded ones. That is probably a consequence of the incorporation of the BSA in the matrix of nanoparticles, providing a cross-linking effect.

The process yield was much smaller in formulations produced by method B (FUC/CS = 1/4), what could be explained by the mechanism of the nanoparticles formation. By adding the protein, the number of negative charge increases, reacting with positive CS charge.

The differences between the production yield of nanoparticles prepared by method A and B were statistically significant ($p < 0.05$), while the differences between the unloaded and

BSA loaded nanoparticles in each formulation did not lead to a statistically significant difference due to the high standard deviations.

TABLE IV: The production yield of blank and protein-loaded nanoparticles.

Nanoparticle Formulation	Production Yield (%)
FUC/CS = 1/4 Unloaded	18 ± 4
FUC/CS = 1/4 BSA-loaded	13 ± 3
FUC/CS = 4/1 Unloaded	27 ± 2
FUC/CS = 4/1 BSA-loaded	47 ± 6

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4.2 DETERMINATION OF ENCAPSULATION EFFICIENCY AND LOADING CAPACITY OF BSA IN NANOPARTICLES

The encapsulation efficiency of BSA in nanoparticles prepared by method A was 100%, in contrast to only 37% for the nanoparticles prepared by the method B, which makes the difference between the methods statistically significant. The fact that the nanoparticles prepared by method A encapsulate the highest amount of protein (Table V), without showing any alterations in their physicochemical properties could be justified by an effective encapsulation, meaning that the protein is not located on outer surface of nanoparticles.

TABLE V: The encapsulation efficiency and loading capacity of BSA in nanoparticles prepared by methods A and B.

Nanoparticle Formulation (BSA-loaded)	Encapsulation Efficiency (%)	Loading Capacity (%)
FUC/CS = 1/4	37 ± 0.9	25 ± 6
FUC/CS = 4/1	100	50 ± 6

That can be explained by the differences in charge of BSA (isoelectric point= 4.7) at the moment of interaction with the polymers. In the formulation A, BSA is first mixed with CS (pH=3.3), thus mainly presents positive charge. When it is further mixed with FUC, to form the nanoparticles, the final pH of the dispersion is around 3.5. Therefore, the protein is still positively charged, which favours the interaction with the negatively charged sulphate groups of FUC, the polymer predominantly composing these nanoparticles. On the contrary, in formulation B, BSA is first mixed with FUC, at pH of approximately 6.8. In this case, the protein is negatively charged. However, when dropping the mixture into CS solution, the final pH becomes around 3.5 and a protein shifts to a predominant positive charge, which does not favour the interaction with the chitosan, which also exhibits a positive charge. Therefore, as chitosan is the polymer present in the higher amount, a strong interaction is not expected, leading to lower encapsulation.

4.3 CYTOTOXICITY OF FUCOIDAN/CHITOSAN NANOPARTICLES

In our work, the cytotoxicity of FUS/CS nanoparticles was evaluated using the metabolic assay MTT, which constitute the formation of a dye performed by cells with active mitochondrial activity. It is based on the fact that only cells that remain viable after exposure to the test materials are capable of metabolizing the yellow tetrazolium salts,

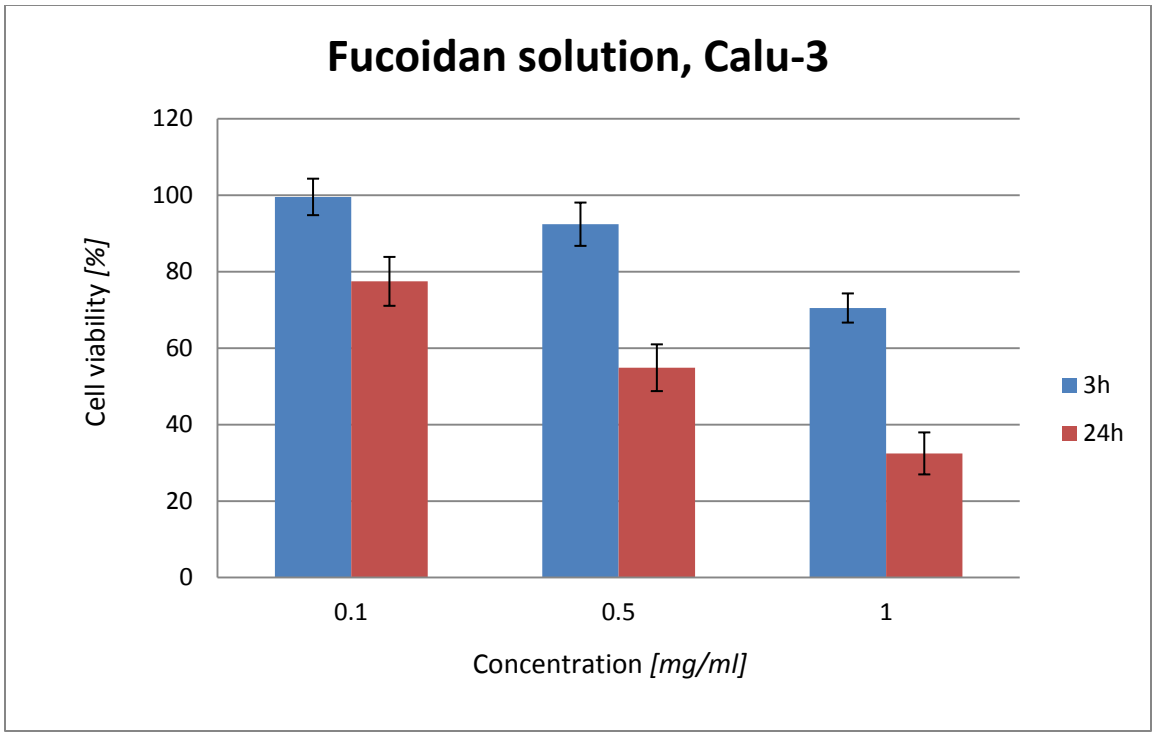
reducing it to purple-blue formazan crystals, which cannot be dissolved in water. Formazan crystals are later dissolved after adding a detergent and quantified spectrophotometrically. In this assay, a disturbance of mitochondrial activity is used as an indicator of disrupted cell function. The higher the concentration of a dye is, the higher is the amount of metabolically active cells, which is customary explained as higher cell viability (20).

Cells were cultured and exposed to different concentrations of fucoidan and nanoparticles for 3h or 24h. Calu-3 and A549 cell lines were used to proceed the MTT test, and the 10% SDS was used as a positive control. Calu-3 and A549 did not give evidence to be sensitive either to the nanoparticles or to the fucoidan if they were exposed for a short time (3h) and at lower concentrations (0.1 mg/ml and 0.5 mg/ml). After 24h, the viability was much lower even for the lowest concentration.
First, only a study of the fucoidan effects on the Calu-3 cell culture was made and it showed that the viability at the concentration 0.1 mg/ml and 0.5 mg/ml was more than 80% in the 3h well plate, but much lower in the 24h well plate (50%).

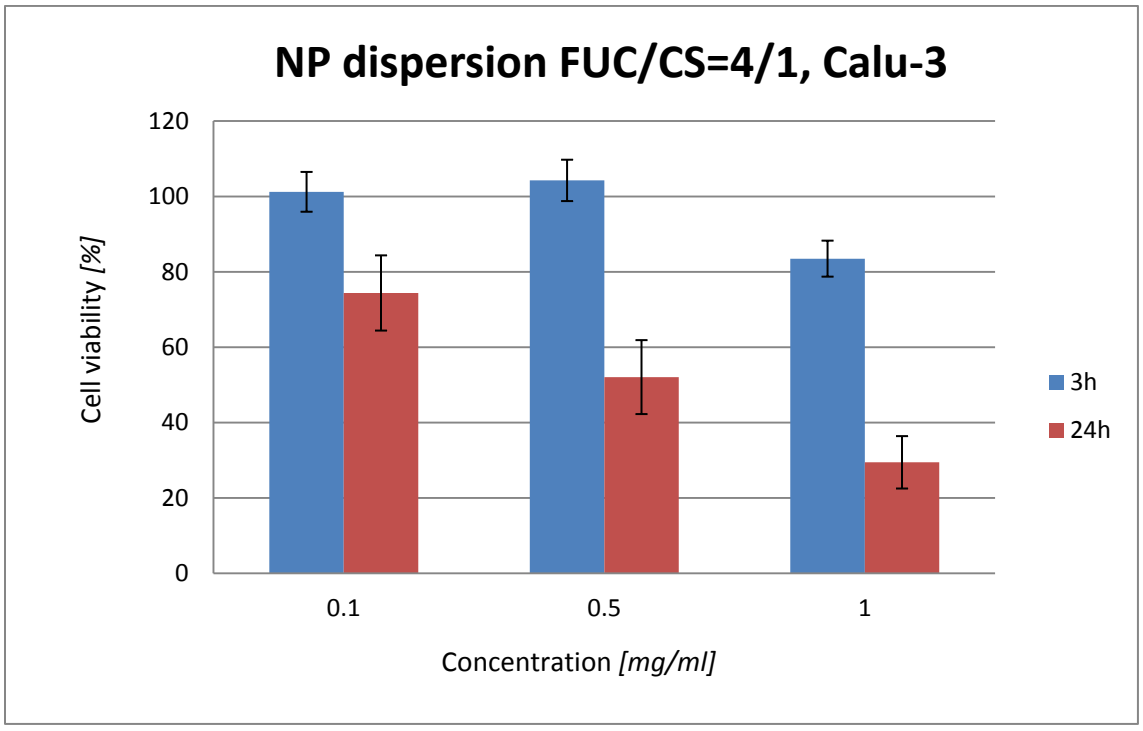
When the nanoparticles were tested, they did not show toxic effects in the 3h plates, the viability was 100% in both, samples prepared by A and B methods for the concentrations 0.1 and 0.5 mg/ml. Lower viability was seen after 24 h exposure, even with the lowest concentrations of nanoparticles (Figure 14).

The fact that molecular chains modify the conformation when they are formulated as carriers explains the difference in behaviour between fucoidan molecules and fucoidan carriers. That happens, because after the formation of nanoparticles the exposure of many of the groups, which are in charge for interaction when in solution, is not possible. The same phenomenon can also be observed with chitosan molecules.

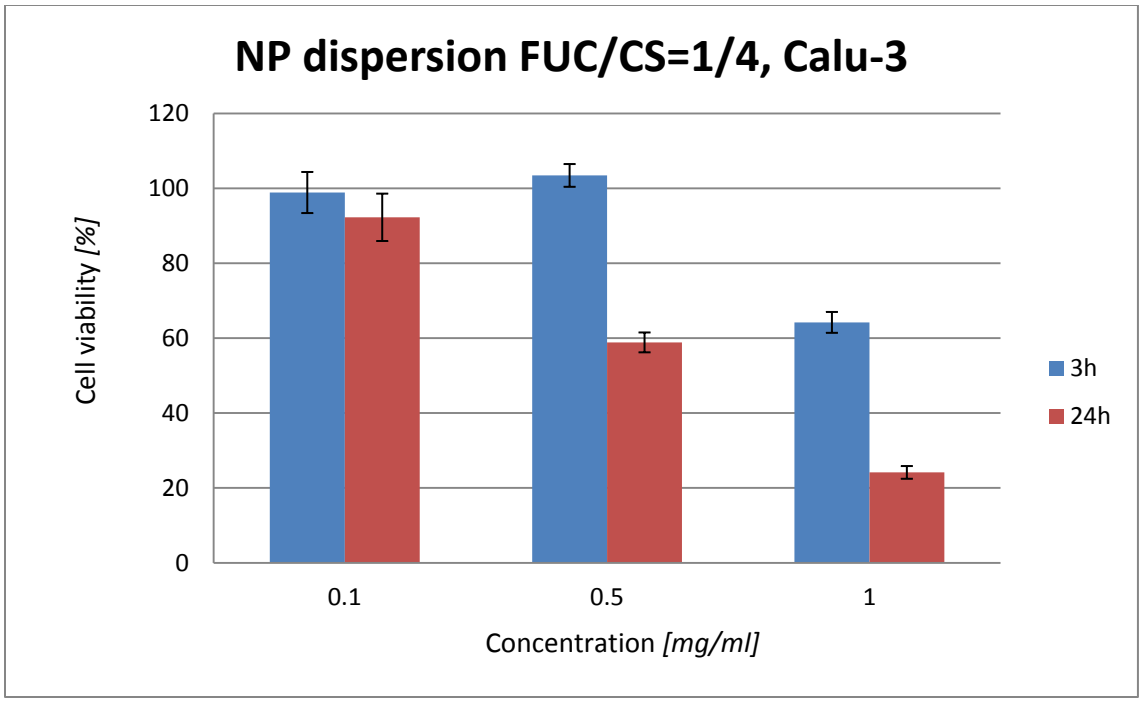
The results on A549 cell line were similar ($p > 0.05$) viability was a bit lower when incubating with fucoidan solutions in both, 3h in 24 well plate (Figure 15). That demonstrates that A549 cells are more sensitive to stimulation than the Calu-3 cells.


Fucoidan solution, Calu-3
120
100
80
60
40
20
0
Cell viability [%]
0.1
0.5
1
Concentration [mg/ml]
3h
24h

a)

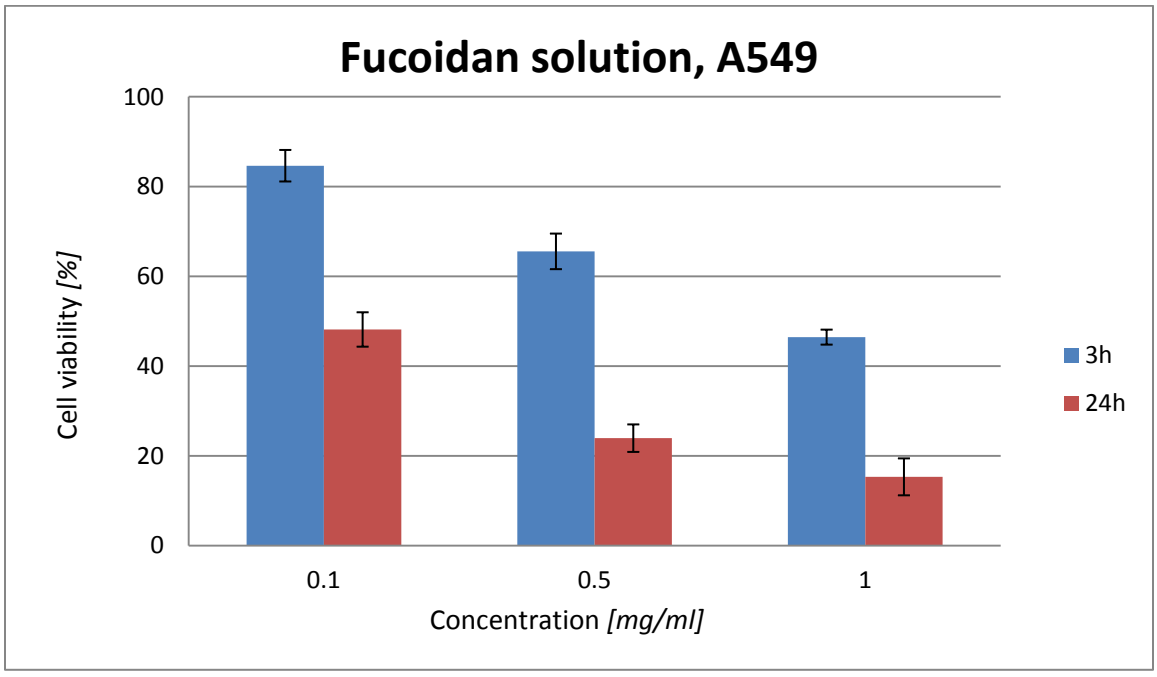

NP dispersion FUC/CS=4/1, Calu-3
120
100
80
60
40
20
0
Cell viability [%]
0.1
0.5
1
Concentration [mg/ml]
3h
24h

b)

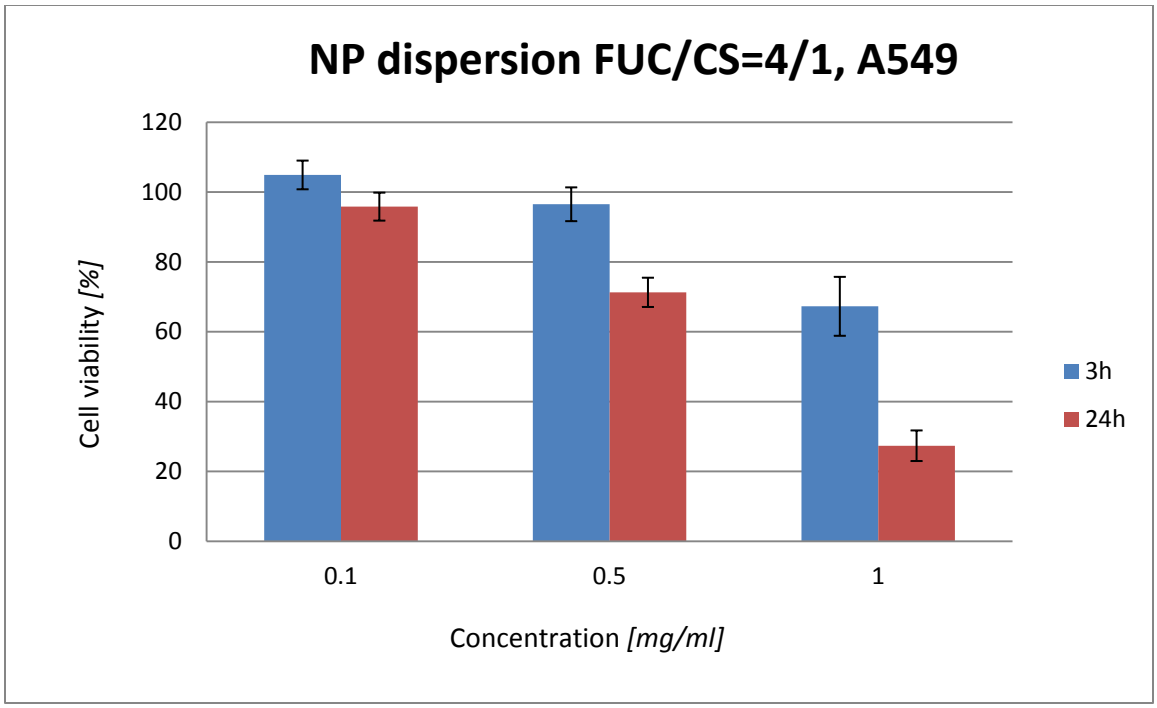


c)

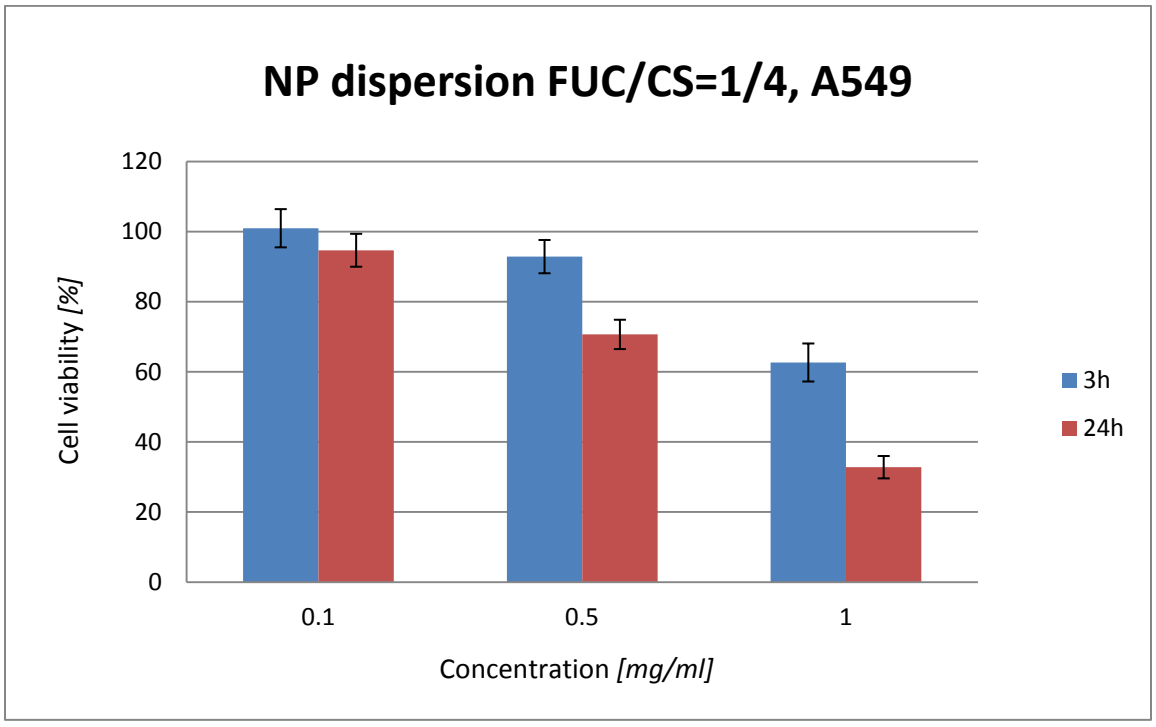
Figure 14: The Calu-3 cell viability determined by the MTT assay after 3 and 24 hours exposure to a) fucoidan solution, b) NP dispersion FUC/CS=4/1 (method A) and c) NP dispersion FUC/CS=1/4 (method B)



a)



b)



c)

Figure 15: A549 cell viability determined by the MTT assay after 3 and 24 hours of exposure to a) fucoidan solution, b) NP dispersion FUC/CS=4/1 (method A) and b) NP dispersion FUC/CS=1/4 (method B).

In our work only the evaluation of cytotoxicity of fucoidan as a polymer was done, because chitosan had already been studied several times by various conditions applied. There is a great number of various cells used with various material concentrations applied taking into account different contact times. Even more, the intrinsic variations of chitosan as a polymer also have to be considered (different molecular weight, deacetylation degree ...).

We focused just on the studies that were related to the drug delivery, considering only the MTT assays made on Calu-3 cell line in our laboratory. We could conclude that in lots of situations chitosan solutions exhibited a particular degree of toxicity, mainly influenced by the dose. Apart from that, toxicity was also connected to the polymer characteristics, for instance molecular weight, degree of deacetylation, the pH of the incubation medium and the length of incubation. It could also be observed that when using a polymer as matrix material of a drug there is no toxicity in most cases in concentrations ranging up to 1 mg/ml. Elevated concentrations were also sometimes referred to increase cell viability (20).

4.4 DETERMINATION OF INFLAMMATORY RESPONSE OF THE CELLS EXPOSED TO FUCOIDAN/CHITOSAN NANOPARTICLES

The inflammatory response of the Calu-3 cells after being exposed to nanoparticles was evaluated by the quantitative ELISA to find alterations in the proinflammatory cytokines IL-6 and IL-8. Since the Calu-3 cells are developing in an air-liquid interface, they show numerous properties of the bronchiolar epithelium, which is in vivo a barrier layer between inspired gas and other visceral tissues. This advantage can also be used when evaluating the airway inflammation (44).

It was demonstrated that no inflammatory response was detected after the exposure to nanocarriers (Figure 16), as the IL-6 and IL-8 levels remained similar to those of unexposed cells.

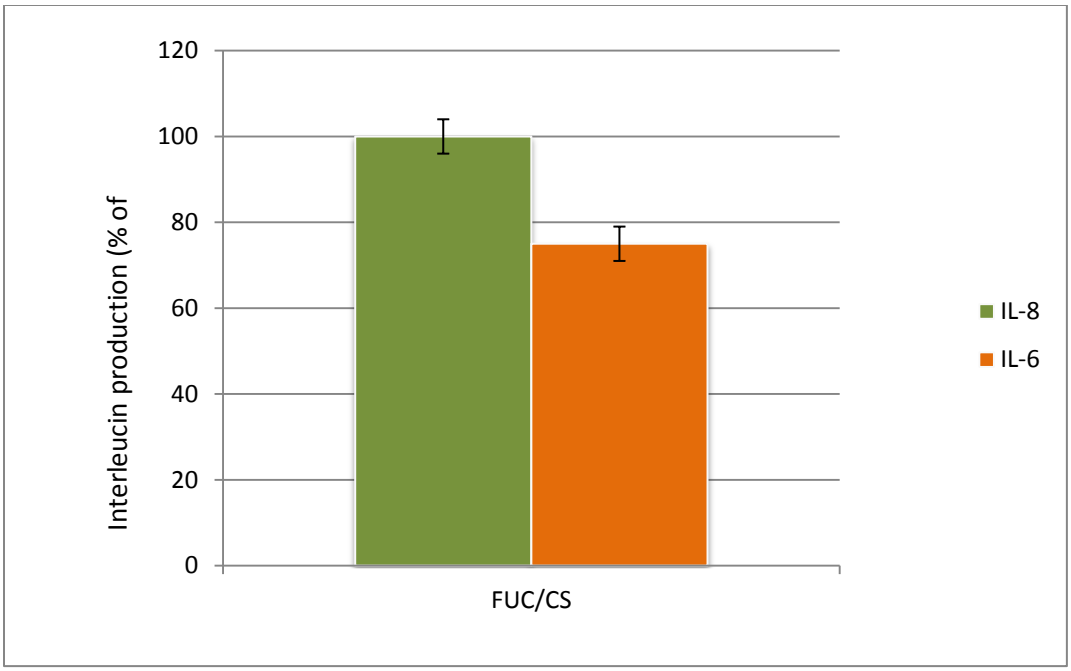


Figure 16: IL-6 and IL-8 secretion by Calu-3 cells exposed to nanoparticles and Lipopolysaccharide for 24 hours.

4. 5 STABILITY OF THE NANOPARTICLE DISPERSIONS IN THE PRESENCE OF LYSOZYME

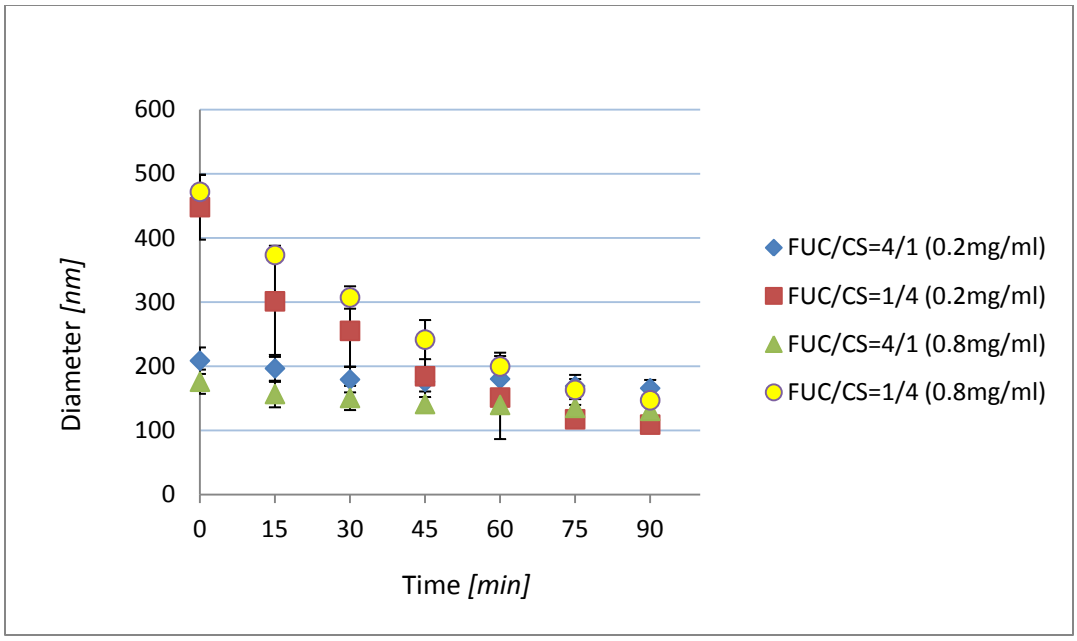
Nanoparticle dispersions were incubated with lysozyme at 37ºC. The size and zeta potential of the nanoparticles were measured every 15 min in the formulation of nanoparticles without BSA in the concentration of 0.2 mg/ml and 0.8 mg/ml of lysozyme. The enzyme concentration of 0.2 mg/ml was used, based on the studies carried out by Konstan et al., who discovered that this was the highest lysozyme concentration in human tracheo-bronchial secretions. In addition, we selected the 0.8 mg/ml concentration in order to examine the nanoparticles behaviour in radical conditions (2).

The size of the nanoparticles prepared by method A (FUC/CS=4/1) was decreasing until the 45 min of incubation, but after that time, it tended to remain more or less constant till the end of experiment. At the time of 60 min it even increased a little bit, probably due to aggregation phenomenon.

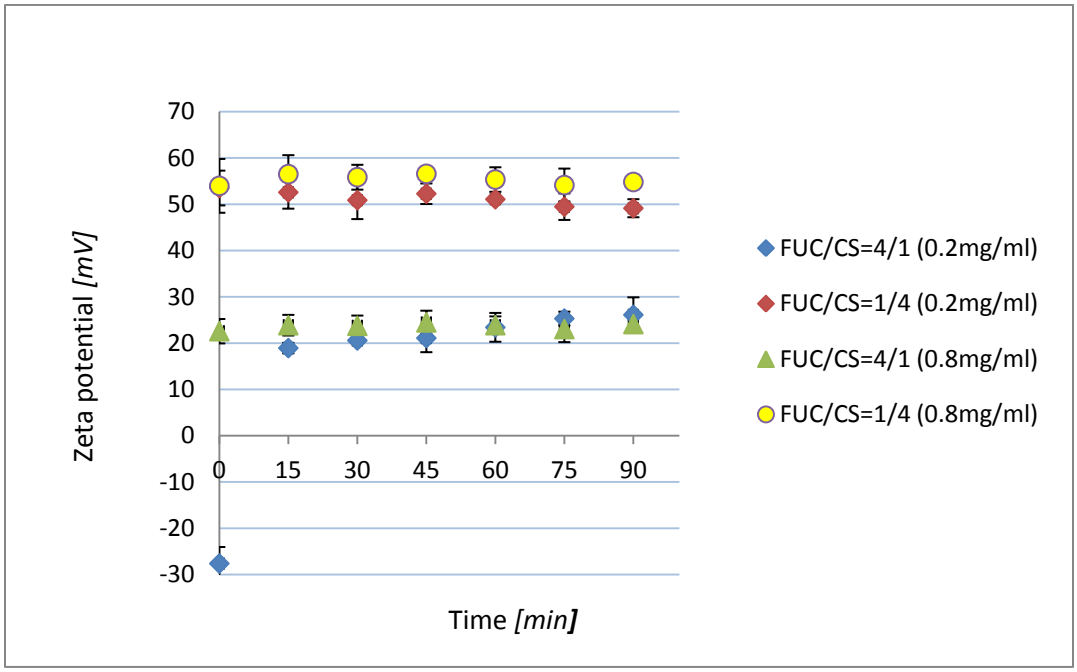
The size of nanoparticles prepared by method B kept decreasing at all times, and the change in size was more obvious than in nanoparticles prepared by method A, probably because of the higher content of chitosan in the formulation B. As we predicted, the size decrease was higher when the particles were incubated with higher concentration of lysozyme (0.8 mg/ml) (Figure 17).

Pulmonary lysozyme is capable of degrading chitosan (formed of D-glucosamine and N-acetyl glucosamine units) and has been reported as capable of breaking CS nanoparticles by hydrolyzing the glycoside bonds between the acetyl glucosamine units (2). It interacts with the acetamide groups but it does not so with free amino groups. The chitosan that we used in our experiment had a deacetylation degree of approximately 85%, so only a mild decrease in size was expected after the incubation with lysozyme. But even so, this minor degradation might be enough to generate some polymer fragments, which could eventually detach from the nanoparticles (48).

The incubation of the particles also did not lead to a significant modification in zeta potential in method B, while the zeta potential in formulation A became positive due to the predicted attack on chitosan. This could be explained by the fact that lysozyme is a cationic protein and its interaction with the nanoparticles does not affect its surface charge.



a)



b)

Figure 17: Changes of the a) size and b) zeta potential of FUC/CS nanoparticles in the presence of lysozyme.

4.6 RELEASE STUDIES

The release of BSA loaded nanoparticles was evaluated in the solution of HEPES buffer (pH=7.4) at 37°C and it is presented in Figure 18.

The different release profile is probably related to the amount of protein encapsulated in the nanoparticles, which is almost three times higher in method A (100 %) than in method B (37 %). The agglomeration of the nanoparticles occurs in both formulations in the HEPES buffer because of the chitosan, which is not soluble at the pH 7.4 and it precipitates.

A higher percentage of released protein after 24 hours in nanoparticles formed by method A can be explained by the fact that those nanoparticles have a lot of protein entrapped and any derangement will force some protein to leave the particle. As the nanoparticles prepared by method B have less protein entrapped, there is probably less protein on the surface and can remain entrapped longer.

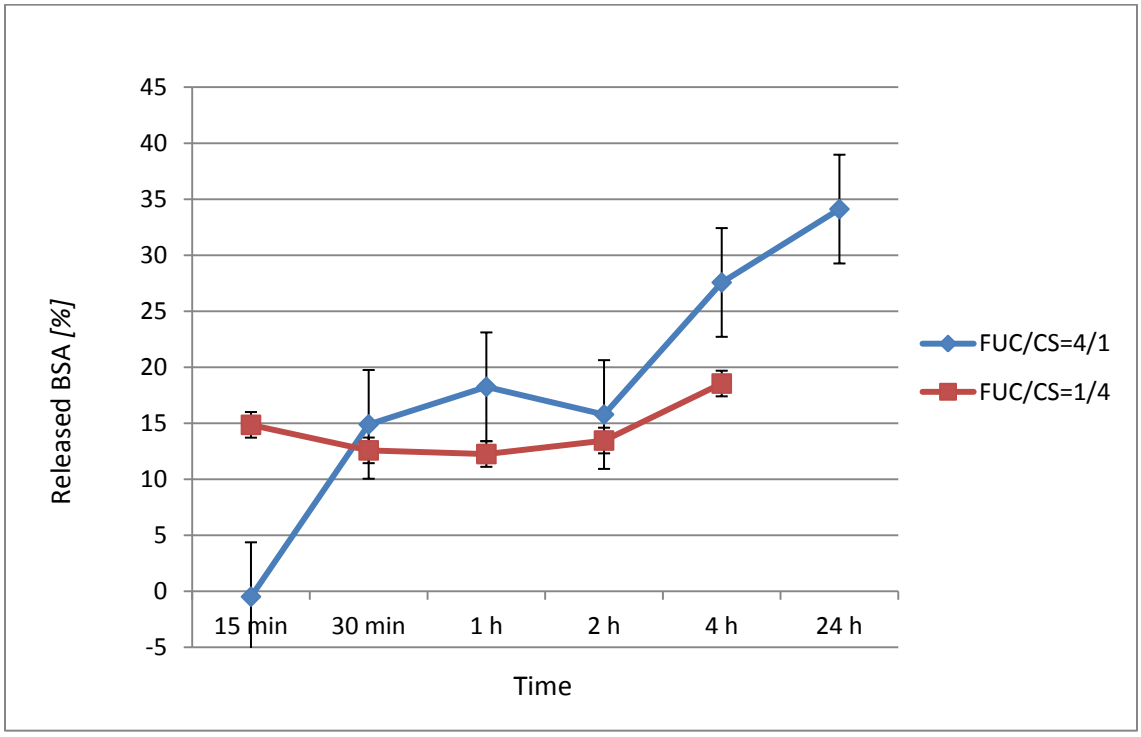


Figure 18: Release profiles of bovine serum albumin from the nanoparticles in the solution of HEPES.

5 CONCLUSION

Our work shows that the prepared fucoidan/chitosan (FUC/CS) nanoparticles could be used as a protein delivery system in the field of systemic mucosal administration. The formation of nanoparticles based on chitosan and fucoidan occurs due to ionic interaction between the negatively charged sulphate groups of fucoidan and the positively charged amino groups of chitosan. This procedure is made in a complete hydrophilic medium, avoiding the usage of organic solvents and aggressive preparation conditions which could be determined for the integrity of the encapsulated drug. The produced nanoparticles are aimed at application in transmucosal delivery of therapeutic macromolecules, namely proteins. Bovine serum albumin was associated to the nanoparticles and the cytotoxicity and inflammatory response of the nanocarriers in an alveolar cell line was created.

The small sizes and high negative and positive charges (varying with the polymeric composition) which were obtained after the procedure, are believed to carry potential for application in mucosal delivery of macromolecules. BSA, used in this study as a model protein, was effectively associated to the developed nanocarriers, the encapsulation efficiency reaching 100% for the formulation A, though encapsulation efficiency was much lower in the particles produced by method B (37%). Because of that, the release profile of BSA in the solution of HEPES buffer shows that the release of protein from the particles made by the method A is different due to a different polymeric composition of the formulation.

Overall, the presented results encourage the application of these nanoparticles as protein carriers, but subsequent assays should focus on biocompatibility, using other relevant cell lines and different tests to solidify the potential of the formulations.

BIBLIOGRAPHY

(1) A. Grenha: Chitosan nanoparticles: a survey of preparation methods. *J Drug Target 2012;* 20, (4), 291-300.

(2) A. Grenha, B. Seijo and C. R. López: Microencapsulated chitosan nanoparticles for lung protein delivery. *Eur J Pharm Sci 2005;*25, 427–437.

(3) http://www.project-pharmaceutics.com/services/formulation-science/

(4) L. Jorgensen, E. W. M. Moeller, H. Nielsen and S. Frokjaer: Preparing and evaluating delivery systems for proteins. *Eur J Pharm Sci 2006;* 29, 174-182.

(5) K. A. Majorek, P. J. Porebski, A. Dayal, M.D. Zimmerman, K. Jablonska, A. J. Stewart, M. Chruszcz, W. Minor: Strucural and immunologic characterization of bovine, horse, and rabbit serum albumins. *Mol Immunol 2012*, 75, 174-182.

(6) http://www.tcsbiosciences.co.uk/bovine_serum_albumin.php

(7) K. Murayama and M. Tomida: Heat-induced secondary structure and conformation change of bovine serum albumin investigated by Fourier transform infrared spectroscopy. *Biochemistry 2004;* 43, (36), 11526-11532.

(8) F. Jameel and S. Hershenson: Formulation and Process Development Strategies for Manufacturing Biopharmaceuticals. New Jersey: John Wiley & Sons, Inc., 2010.

(9) V. F. Patel, F. Liu and M. B. Brown: Advances in oral transmucosal drug delivery. *J Control Release 2011;*153, 106–116.

(10) K. Park, I. C. Kwon, K. Park: Oral protein delivery: Current status and future prospect. *React Funct Pol* 2013; 72, 280-287.

(11) J. B. Dressman and H. Lennernäs: Oral Drug Absorption. Prediction and Assessment, 105, New York: Marcel Dekker, Inc., 2000.

(12) A. H. Shojaei: Buccal Mucosa As A Route For Systemic Drug Delivery: A Review. *J Pharm Pharmaceut* 1988; 1, 15-30.

(13) http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3276033/

(14) http://anatomiaefisiologiahumana.webnode.com.

(15) S. Türker, E. Onur and Y. Özer: Nasal route and drug delivery systems. *Int J Clin Pharm 2004;* 26, (3), 137-142.

(16) C. Prego, M. Garcia, D. Torres, M. J. Alonso: Transmucosal macromolecular drug delivery. *J Control Release* 2005; 101, 151–16.

(17) J. Shaji and V. Patole: Protein and Peptide Drug Delivery: Oral Approaches. *Indian J Pharm Sci 2008;* 70, (3), 269–277.

(18) J. Kreuter: Nanoparticles - a historical perspective. *Int J Pharm* 2007; 331, 1–10.

(19) C. R. Gilbert S. Banker, Drugs and Pharmaceutical Sciences, Modern Pharmaceutics, 4th edition, Basel: Marcel & Dekker, Inc., 2002.

(20) S. Rodrigues, M. Dionisio, C. R. Lopez, A. Grenha: Biocompatibility of Chitosan Carriers with Application in Drug Delivery. *J Funct Biomat 2012; 3*, 615-641.

(21) F. Re, R. Moresco and M. Masserin: Nanoparticles for Neuroimaging. *J Phys D: Applied Physics 2012;* 45, (7).

(23) S. Ataide Ferreira: Development of fucoidan/chitosan nanoparticulate systems for protein administration through mucosal routes; Faro (CBME), 2013.

(24) K. Y. Leea and S. H. Yuk: Polymeric protein delivery systems. *Prog Polym Sci 2007;* 32, 669–697.

(25) J. P. Rao, K. E. Geckeler: Polymer nanoparticles: Preparation techniques and size control parameters. *Prog Polym Sci* 2011; 36, 887-913.

(26) http://www.hindawi.com/journals/mi/2012/126463/fig3/

(27) C. P. Reis, R. J. Neueld, A. J. Ribeiro, F. Veiga: Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. *Nanomedicine: Nanotechnology, Biology, and Medicine* 2006; 8–21.

(28) 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. *J Control Release 2006;* 116, (1),1–27.

(29) K. S. Soppimatha, T. M. Aminabhavia, A. R. Kulkamia and W. E. Rudzinski: Biodegradable polymeric nanoparticles as drug delivery devices. *J Control Release 2001;* 70, 1-20.

(30) D. Labarre, G. Ponchel and C. Vauthier, Biomedical and Pharmaceutical Polymers, London: Pharmaceutical Press, 2011.

(31) M. Elsabahy and K. L. Wooley: Strategies Toward Well-Defined Polymer Nanoparticles Inspired by Nature: Chemistry versus Versatility. *J Polym Sci 2012;* 50, 1869–1880.

(32) J. H. Park, M. Ye and K. Park: Review Biodegradable Polymers for Microencapsulation of Drugs. *Molecules 2005;* 10, 146-161.

(33) T. Hanemann and D. V. Szabó: Polymer-Nanoparticle Composites: From Synthesis to Modern. *J Mater 2010;* 3, 3468-3517.

(34) K. E. Uhrich, S. M. Cannizzaro and R. S. Langer: Polymeric Systems for Controlled Drug Release. *Chemical Reviews 1999;* 99, 3181-3198.

(35) S. Rodrigues, A. M. Rosa da Costa, A. Grenha: Chitosan/carrageenan nanoparticles: Effect of cross-linking with tripoliposphate and charge ratios. *Carbohydrate Polymers* 2012; 89, 282-289.

(36) A. Grenha, M. E. Gomes, M. Rodrigues, V. E. Santo and J. F. Mano: Development of new chitosan/carrageenan nanoparticles. *J Biomed Mater Res A 2010;* 92, (4), 1265-72.

(37) S. A. T. and C. P. Sharma: Chitosan and Its Derivatives for Drug Delivery. *J Adv Polym Sci 2011;* 243, 23–54.

(38) 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. *Int J Carbohydr Chem 2011;* 2011, 14.

(39) Y. Cheng Huang, U. Lam: Chitosan/Fucoidan pH sensitive Nanoparticles for oral delivery system. *J Chin Chem Soc 2011*; 58, 779-785.

(40) B. Li, F. Lu, X. Wei and R. Zhao: Fucoidan: Structure and Bioactivity. *Mollecules 2008;* 13, 1671-1695.

(41) 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. *Eur J Pharm Biopharm 2011;* 79, (1), 162-170.

(42) A. D. Sezer and J. Akbuga: Fucosphere—New microsphere carriers for peptide and proteins delivery. *J Microencapsulation 2006,* 23, (5), 513–522.

(43) 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. *J Pharm Biopharm 2008*, 69, 189-198.

(44) Y. Zhu, A. Chidekel, T. H. Schaffer: Cultured human airway epithelial cells (Calu-3): A model of human respiratory function, structure and inflammatory response. *Crit Care Res Pract 2010*, 2010, (394587), 1-8.