Polysaccharide-based nanoparticles for drug delivery

(Settore scientifico-disciplinare CHIM/04)
Abstract

Peptide drugs are attracting an increasing interest in line with the better understanding of their role in physiopathology, as well as the continuous progress in biotechnological and biochemical synthesis. However, their delivery by routes other than parenteral delivery remain difficult, and even this route is not fully satisfactory. The production of carriers that allow the administration of drugs though the oral route has been deeply investigated, because they would avoid the disadvantages of parenteral drug delivery such as the low patient compliance, the high cost due to expensive manufacturing of sterile products and the need for qualified personnel administering the drug. In particular, mucoadhesive drug delivery systems give the advantage of improving drug bioavailability by using a lower concentration of the active compound for disease treatment due to the intimate contact with the adsorption site. Furthermore, thanks to the use of specific molecules, it is possible to select the possible target in the gastrointestinal tract and to reduce the costs due to drug localization at the disease site.

In this thesis biodegradable polymeric nanoparticles as mucosal drug carriers for the oral route have been developed and characterized. Chitosan has been selected as the model polymer due to its peculiar characteristics as biodegradability, non-toxicity, tissue-adhesive activity and drug permeation enhancing capability. Moreover, two different classes of derivatives have been synthesized, a trimethylated chitosan and two glycosylated chitosans. The derivatives, as chitosan, have been characterized physico-chemically by NMR, viscometric and SEC studies. Moreover, their mucoadhesivity has been tested with in vitro studies, evaluating their interaction with mucin by turbidity studies, and with ex vivo tests, evaluating the cohesiveness of the binding of polymer tablets to a porcine intestinal mucosa. The chitosan derivatives showed higher mucoadhesive properties compared to native chitosan in both tests.

The preparation method used for particles formation was the ionotropic gelation, where an ionic interaction between the cationic polymer (chitosan or its derivatives) and the anionic tripolyphosphate (TPP) occurred. Since proteins
are very labile molecules, sensitive to several stress factors, this mild preparation method is very suitable to prepare protein loaded nanocarriers. Three model proteins were loaded on nanoparticles, bovine serum albumin (BSA), ovalbumin (OVA) and human insulin (HI), resulting in a good loading efficiency, at least over 50%.

Chitosan nanoparticles were prepared and optimized in terms of particle shape and morphology, size distribution, surface chemistry, biocompatibility, production automatization and storage. The average diameter is 200 nm and surface charge is 25 mV. Particle size distribution curve showed the presence of a bimodal population composed of a fraction of small particles (around 40 nm) and a second population around 250 nm. Analysing the evolution of particles formation, a rearrangement of particles can be noticed after TPP addition, with small particles that aggregated with time to form a more homogeneous population of larger particles.

Particles were stored for 4 weeks to study their stability in time, resulting in a slight increase of size due to a continuous rearrangement of small particles. Moreover, storage of nanoparticles has been investigated using lyophilization and spray-dryer. Three different bioprotectants have been added to chitosan samples, namely trehalose, mannitol and PEG, preventing their aggregation and protecting them from mechanical stress during freezing and drying processes. Trehalose results the best cryoprotectant for both methods.

Following the same procedure, chitosan-derivatives nanoparticles have been also prepared and characterized in size and surface charge. The average size of TMC nanoparticles was about 160 nm with a surface charge of +14 mV. Chitosan-galactose and chitosan-glucose were synthesized and characterized, but only the first one was used for particles preparation. Nanoparticles were prepared using different blends of chitosan and chitosan-galactose and all samples have been characterized in size and surface charge, resulting in size wide-ranging from ca 140 nm to 360 nm and a positive surface charge.

Produced particles have also been associated with other negatively charged polyelectrolytes, with the scope of improving the resistance in the gastro-intestinal tract and the mucoadhesion of the nanoparticles. Three pectins, a model one (polygalacturonic acid) and two commercial ones (one derived from citrus fruit, the other from apples) have been selected and their
interaction with positive charged chitosan nanoparticles has been investigated, either in blended or coated mode. Both methods gave negative charged particles, proof of presence of pectin in the outer shell of particles, having the possibility to obtain the gastro-resistance and mucoadhesivity required.

A characterization of the materials and the nanoparticles by means of spectroscopic methods has been done. All systems and polymers were analyzed using IR-ATR and Raman spectroscopy. Only IR-ATR spectroscopy gave clear results, allowing the possibility of studying the interaction of the polymers in the nanoparticles.

The biocompatibility of polymers, chitosan and chitosan derivatives, pectins, TPP, and the nanoparticle produced has been tested with *in vitro* and *in vivo* tests, confirming their safety. For *in vitro* test the XTT and LDH were performed; for *in vivo* test chick embryos have been used.
Sommario

I continui progressi in ambito biochimico e biotecnologico hanno aumentato l'interesse per le proteine terapeutiche come alternativa ai classici principi attivi. Purtroppo, la loro somministrazione è principalmente parenterale e gli sforzi di diversi team di ricerca mirano a cercare altre vie di somministrazione, quale quella orale, così da evitare gli svantaggi tipici quali il dolore fisico e psichico per il paziente, la necessità di avere personale qualificato e materiale sterile per la somministrazione e gli alti costi di produzione e conservazione. In particolare, i sistemi di somministrazione orale che sfruttano la mucoadesione stanno riscuotendo notevole interesse poiché aumentano la biodisponibilità del farmaco riuscendo a limitarne la dose somministrata grazie allo stretto contatto con il sito di assorbimento. Inoltre, tramite l'uso di molecole adatte, è possibile selezionare l'eventuale sito d’azione all'interno del tratto gastro-intestinale andando a ridurre così il numero delle somministrazioni e la quantità del principio attivo.

In questo lavoro di tesi sono stati sviluppati e caratterizzati nanovettori (carrier) composti da polimeri biodegradabili per la veicolazione di principi attivi a livello della mucosa intestinale.

Il chitosano è stato scelto come polimero modello grazie alle sue caratteristiche peculiari di biodegradabilità, non tossicità, adesività ai tessuti e capacità di favorire l'assorbimento del farmaco. Sono state inoltre sintetizzate due diverse classi di suoi derivati, il trimetil chitosano (TMC) e due diversi derivati glicosilati. Tutti i polimeri sono stati caratterizzati approfonditamente da un punto di vista chimico-fisico con spettroscopia NMR, viscosimetria e studi di cromatografia SEC. Inoltre è stata valutata la loro mucoadesività, sia con test turbidimetrici di interazione con la mucina, che con test ex vivo di forza di adesione alla mucosa intestinale di maiale. Tutti i test effettuati hanno dimostrato la miglior capacità adesiva dei derivati sintetizzati rispetto al chitosano nativo.

Il metodo di preparazione usato per la produzione delle nanoparticelle è la gelazione ionotropica, nella quale si ha la formazione di un nanogel grazie all'interazione ionica tra una specie cationica, il chitosano o uno dei suoi
derivati, ed una anionica, il tripolifosfato (TPP). Siccome i farmaci proteici sono molecole molto labili e sensibili a diversi fattori di stress, questo tipo di preparazione risulta particolarmente adatta grazie alle sue blande condizioni di preparazione.

Tre diverse proteine modello, due tipi di albumina (BSA e OVA) e insulina umana, sono state caricate nelle nanoparticelle prodotte con un’efficienza di caricamento superiore al 50%.

Le formulazioni sono state caratterizzate in termini di dimensioni e carica superficiale, di distribuzione delle dimensioni, di morfologia, di conservazione, e la loro produzione è stata ottimizzata e automatizzata. Il diametro medio è risultato dell’ordine di 200 nm, con una carica superficiale di +25 mV. L’analisi della curva di distribuzione delle dimensioni ha evidenziato la presenza di due popolazioni di nanoparticelle, una con diametro intorno ai 40 nm e l’altra attorno ai 250 nm. Analizzando l’evoluzione della formazione delle nanoparticelle, sia grazie a misure di light scattering che di microscopia in trasmissione, è stato notato come l’aggiunta successiva di TPP favorisca l’aggregazione tra le particelle più piccole, dando origine ad una popolazione di particelle più grandi con una distribuzione complessiva più omogenea.

Le particelle prodotte sono state conservate per 4 settimane e lo studio della loro stabilità nel tempo ha evidenziato un leggero aumento delle dimensioni medie, segno di un continuo riarrangiamento delle residue particelle più piccole. La conservazione dei vettori prodotti è stata studiata anche usando la liofilizzazione e l’atomizzazione, separandoli così dall’ambiente acquoso. Sono stati aggiunti tre bioprotettori per prevenire la possibile aggregazione dovuta a stress da congelamento e rapida disidratazione, PEG, mannitolo e trealosio, risultando quest’ultimo il più efficace in termini di conservazione delle proprietà chimico-fisiche e morfologiche.

Oltre allo studio approfondito sulle particelle di chitosano, anche quelle preparate con i derivati sono state analogamente caratterizzate. Le dimensioni medie delle particelle di TMC è 160 nm, con una carica superficiale di +14 mV. Dei due derivati glicosidici sintetizzati e caratterizzati, il chitosano-glucosilato e il chitosano-galattosilato, solo quest’ultimo è stato usato per la preparazione delle nanoparticelle. Le nanoparticelle prodotte usando diverse miscele di
chitosano-galattosilato e nativo sono state caratterizzate in dimensione, con variazione tra i 140 e i 360 nm, e in carica superficiale, risultando positive.

Inoltre, i sistemi prodotti sono stati associati a un altro polielettrolita carico negativamente, con l'obiettivo di aumentarne la resistenza gastrointestinale e la mucoadesività. I polimeri scelti sono stati tre pectine, una modello (acido poligalatturonico) e le altre due commerciali (una estratta da agrumi, l'altra da mele), e la loro interazione con le nanoparticelle di chitosano cariche positivamente è stata studiata attraverso due diverse metodiche preparative, rivestimento (coating) e miscelazione (blending). Entrambe le metodiche hanno portato alla produzione di particelle cariche negativamente, con la pectina presente prevalentemente nella parte esterna e quindi ottenendo gli effetti di gastroresistenza e mucoadesività ricercati.

La caratterizzazione dei materiali usati e delle nanoparticelle prodotte è stata effettuata tramite due tecniche spettroscopiche, IR-ATR e Raman. Solo la tecnica IR ha dato risultati utili, permettendo di studiare le interazioni tra i polimeri durante la formazione dei carrier.

Infine è stata studiata la biocompatibilità di tutti i polimeri usati, chitosano e suoi derivati, le tre pectine, TPP, e dei carrier prodotti attraverso test in vitro (i saggi XTT e LDH) e in vivo (usando la membrana corioallantoidea dell'embrione di pollo). I test effettuati hanno confermato la biocompatibilità sia delle materie prime che delle nanoparticelle prodotte.
Introduction
1 Drug Delivery

A number of authors have described the ideal medicament as a drug that have the ability to elicit the appropriate response for the disease under consideration, while on the other hand it should possess minimal side effects and under no circumstances cause a new disease.

To make the widespread use of the drug economically feasible, it should be easily administered and production conditions have to be well defined and reproducible. Moreover, it should be administered without the presence of medically trained individuals and, finally, the drug itself must be stable enough to maintain the therapeutic effect before administration without the use of an expensive cold chain.

Peptide drugs are attracting increasing interest with better understanding of their role in physiopathology, as well as progress in biotechnology and biochemical synthesis. However, their use in medicine is limited by low bioavailability, which results from their proteolytic and hydrolytic degradation, low permeability across barriers, and short biologic half-life in the circulatory system. In addition, proteins can undergo inactivation during storage and transport. Despite the advances in biotechnology, these drugs remain difficult to deliver by routes other than parenteral delivery. Obvious disadvantages of parenteral drug delivery are the low patient compliance and high cost due to high manufacturing costs of sterile products and the need for qualified personnel to administer the drug. Consequently, alternative routes of administration are being explored to avoid such disadvantages such as the mucosal route, including nasal, oral and vaginal routes. However, the hydrophilicity and high molecular weights of protein drugs limited their permeation across biological barriers and made them unstable in body fluids due to endogenous processes.

Delivery technologies that facilitate localized delivery to target tissues and improve drug pharmacokinetics strongly improve the efficacy of various therapies. In particular, an increasing number of nanoparticle-based drug delivery systems have been approved for human use or are currently being
evaluated in clinical trials.\textsuperscript{4,5} The development of controlled release systems for drug and gene delivery to mucosal surfaces, such as those of the lung airways, GI tract, female reproductive tract, nose and eye, is of widespread interest.\textsuperscript{6}

1.1 Polyelectrolyte complex for drug delivery

The past development of water-soluble, biodegradable, polymeric, polyelectrolyte excipients has been advantageous due to the biocompatibility and biodegradation of the constituent polymers.

The use of water as a solvent presents a major advantage for products that may be used as drug delivery systems. Both rationalizing the assembly mechanisms and tailoring the size, charge, and loading capability to desirable levels are essential goals needed to advance biodegradable, polymeric, polyelectrolyte nanoparticles as efficient drug delivery vehicles. Polyelectrolyte-based nanoparticles, also termed polyelectrolyte complex dispersions (PECs), are created by mixing oppositely charged molecules. The technology applied in this study produced PECs under the prevailing assembly and complexation theory by employing a four-component system, which provided both versatility and thermodynamic stability.

At the beginning of the previous century, after attending von Weber’s opera “Der Freischutz”, Paul Ehrich hit the idea of magic bullets as ideal drug delivery systems, which preferably recognize specific cells and release the drug at the intended site of action similar to guided missiles. Hundred years later, pharmaceutical technologists are still hunting for appropriate drug delivery systems and in the era of genomics and proteomics, these challenges are extended to drug molecules of large dimension, poor lipophilicity and high susceptibility to inactivation. The aim of this section is to show that PECs are already successfully used in many application and that PEC-based functionalized nanoparticles are the most promising candidates to answer Ehrich’s original idea.
According to literature, there are several PEC characteristics favorable for cellular uptake and colloidal stability, including hydrodynamic diameter less than 100 nm (but larger than 20 nm) and zeta potential -30 to +30 mV. Characterization of PEC properties in terms of size, charge, and morphology, shows the strict dependence on specific molecular parameters of the polyions used, in addition to concentration, ionic strength, pH, and quite important operative mixing conditions (interaction under resting or streaming mixing). Several results show that PECs formulated from precursors with similar, low molecular weights yielded more uniform, attractive size distributions (monodisperse and mean size <200 nm), increased stability in biological and pH environments, and efficient uptake by endothelial cells. Low molecular weight precursors also lead to stability, verified by zeta potential and size measurements, across a wide pH spectrum most likely due to efficient ion pairing.

Indeed, with the primary goal of a PEC system with desirable features that will effectively target and allow uptake by cells. On a separate line, decoration of PECs with suitable pendants has also been investigated. In particular, the effect of passive versus active targeting to endothelial cells for complexes with appropriate properties was demonstrated using fluorescent labeling. Cellular uptake was monitored to further understand the predominant endocytotic mechanisms. The ultimate goal of these studies is to establish the link between physicochemical and biological properties of PEC in order to further these biocompatible products as alternatives to current technologies.

Another task should be stressed, however, before analyzing some effective polyelectrolyte complexes. Although, the objective of drug targeting is to achieve desired pharmacological response at a selected site without undesirable interactions at other sites, the question of fate of carriers is often set aside in the common view of the chemist tailoring nanostructures. The issue cannot be hidden under the carpet, since involves both technological and “ethical” aspects. The easier molecular approach in the construction of suitable nanostructures properly stimuli-responding and releasing drugs has the counterpart of difficulty in transferring the lab experiments to real patient due to intrinsic toxicity of non-biological materials. On the other hand, many biopolyelectrolytes are available and could be used by sacrificing the fine
structural chemistry in favor of less symmetrical objects with higher affinity with cells and tissues.

All bio-based and natural polyelectrolyte, including post-polymerization modified polymers are the elective candidates for the in-vivo application (pharma, food and biomedical).

Several polyelectrolytes are in commerce, including proteins (e.g., gelatin) and polysaccharides (chitosan, hyaluronan, alginate, pectin, gellan, ...). Among the novel biomaterials, chitosan and chitosan-derivatives are being widely used as a pharmaceutical excipient and intensively studied due to their many applications as new pharmaceutical drug carriers.

Chitosan is essentially a polyglucosamine and comprises a series of natural polysaccharide polymers varying in their degree of deacetylation, molecular weight, viscosity, etc. The presence of a variable number of amino groups and substitutions may tune the interaction of this polymer with anionic and polyanionic systems, thereby resulting in a wide range of physicochemical characteristics of such complexes. Hyaluronan is the main component of the extracellular matrix and is a copolymer of glucuronic acid and N-acetyl-glucosamine. Alginate is an unbranched polysaccharide of algal and bacterial origin. It is composed by units of D-mannuronic acid (M) and L-guluronic acid (G) which form homopolymeric block structures along the chains, namely M-blocks and G-blocks interspaced by alternate MG sequences. Pectin is mainly composed by D-Galacturonic acid, which in nature can be methylated in addition to have low amount of other neutral sugar interdispersed in the chain. Several other polysaccharides from microbial (non-pathogenic) sources have been identified and largely employed in pharmaceutics and food uses, such as gellan and xanthan which possess regular structure and efficient properties as viscosity modifiers and gelling agents.

Among the existing preparation techniques, microencapsulation by ionotropic gelation exploits the capability of the biopolyelectrolyte to form hydrogels in presence of proper multivalent counter ions (such as calcium ions for alginate or triphosphate for chitosan), leading to the formation of a 3D-network. In microencapsulation technology, biopolyelectrolytes are often employed together with other materials, in order to combine gelling properties
with other characteristics (deriving from the partner) useful for the final stability of the encapsulating system or to further control the release properties.

In gel particle technology, three methods are commonly described in literature to prepare microspheres by ionotropic gelation: a) dropping the polyelectrolyte solution into a solution of small ions, b) via a w/o emulsification technique and c) complexation of oppositely charged polyelectrolytes by mixing and additional coating procedures. It is also generally accepted that the protein-drug release from hydrogel matrices occurs following two main mechanisms, that is diffusion of the protein through the pores of the polymer network and degradation of the polymer network.\(^7\) In addition, water diffusion (and swelling, when applicable) through the polymeric hydrogel has also been considered as one of the major factors affecting drug release rate.\(^8\)

Some additional thermodynamic issues can be raised inasmuch in the gel formation the overall distribution of binding modes of ions by polyelectrolytes (i.e. non-localized, localized and site-binding) satisfies the electrostatic requirements given by the charge density of the polymer, e.g. alginate. The binding depends on the very polyelectrolyte nature of the polysaccharidic chain [6 and references therein], but it is still subject to phenomena of gel maturation (e.g. syneresis). Due to their polyelectrolyte character, counterions binding is a peculiarity stemming from the intrinsic charge density of the polymers and of the structures induced, making the final state the result of several contributions that can be thermodynamically modelled. The more general observation is that ion-ion interaction occurs with desolvation of the interacting groups and contributes, therefore, to the entropy term of mixing. From the mere macromolecular point of view, the process of ionic gelation occurs with an extensive demixing heterogeneity of chain distribution in solution. Local aggregation of chains is counterbalanced by large domains of low (or null) concentration of polymer. Thus, the physicochemical properties of the gel system are dominated by the presence of an excess of water “trapped” within the porous three-dimensional polymer matrix and may slowly transform by increasing solute-solute interactions.

Since nano-organization of polyelectrolyte-based particles assumes a relevant role in the functional properties, some more general concepts of human uses of “nano-particles” is mandatorily presented.
From the scientific viewpoint, nano- and micro-particles represent the goal of many research projects due to their peculiar properties, not reachable in bulk materials. The distinction must be made between materials composed of patches of two or more different species at nano-level (i.e. nanocomposites) and systems in which the nano-patches have their own structural and dynamic individuality (i.e. nanoparticles). One of the major effect scientists look at is the enhancement of kinetic phenomena at the interphase of nanoparticles.

A general system is that of protein-containing polyelectrolyte submicroparticles that can be easily fabricated as such or eventually loaded into a bulk gel containing another drug for dual-drug delivery or for other nutrient molecule release. However, one of the unique challenges facing nano- and microscaled matrix delivery systems is burst release due to the high surface-to-volume ratio of these particulate systems. Burst release may cause a “dose-dumping” effect and is potentially harmful to patients in clinical applications.

Although not completely understood, burst release has been taken into consideration during the design of delivery matrices as well as in modeling approaches. Several possible parameters affecting burst release have been identified including, in particular, charge density and conformational features in the ionic polymer/drug interactions, fabrication conditions, and sample geometry and/or morphology. Charge and texture of the polymeric matrix tune the actual cross-linking density of the matrix surface and decrease the degree of burst release in addition to exterior coating and internal drug embedding.

Independently of the constitutional characteristics, size effect is ineludible. ‘Particle mass concentration’ has been the most common metric used in all previous drug research. For nanoparticles concentration of particle number and surface area need to be taken into account. Indeed, the use of a metric depends on the specific questions posed, requiring specifically defined measures. Although it is not always possible to predict effects on the basis of size or surface area alone, there is strong evidence of correlation between biological effects and nanoparticles dimensional/surface properties. Changing size of one order of magnitude (e.g., from 100 to 10 nm) gives three order changes in number of particles and one order in total surface. Thus, in many cases the goal of an enhanced interaction with cells is more profitably
achieved by tailoring the appropriate polyelectrolytic surface coating than by reducing particle size.

1.2 Oral route

The development of controlled-delivery systems for drugs has received considerable attention in recent years. To date, oral delivery is still the preferred route of drug administration, especially for chronic therapies where repeated administration is required. Oral administration offers patients less pain, greater convenience, higher likelihood of compliance, reduced risk of cross-infection and needle stick injuries.\textsuperscript{13,14} Thus, formulations of oral drug delivery continue to dominate more than half of the drug delivery market share. Despite these advantages, the oral route is not amenable to the administration of most protein and polypeptide drugs available today, due to their high susceptibility to digestive enzymes in the gastrointestinal (GI) tract, poor absorption, and their limited ability to transport across the intestinal epithelial barrier. Due to this, new strategies of drug delivery have been developed to overcome obstacles encountered by oral delivery\textsuperscript{15}.

The development of protected-delivery systems for drugs has received considerable attention in recent years. The challenge is the design of oral drug delivery vehicles that effectively carry drugs to the intestinal tract. Firstly, they need to remain intact when travelling through the upper GI tract in order to protect the incorporated drugs from chemical and enzymatic degradation. Secondly, they should be able to release the incorporated drugs in the lower GI tract. Furthermore, the released drugs need to be absorbed at an efficient rate in the GI tract in order to be therapeutically effective. In particular, the use of mucoadhesive materials for nanoparticle production attracted a lot of interest because gives the opportunity for a prolonged residence of carriers in the desired target site.
1.3 Mucosal drug delivery

The most significant advance in nonparenteral delivery has probably been the development of drug formulations that can be targeted to the mucosa, typically through oral delivery. The short transit time in the GI tract can be inadequate for particles to release a significant fraction of encapsulated drugs, thereby precluding in many cases the realization of a high local drug concentration over extended periods of time, hence leading to low bioavailability and poor efficacy. To overcome the short transit time, research has largely centered on minimizing the fraction of therapeutics undergoing direct transit and fecal elimination by improving their association to mucus. This phenomenon, known as mucoadhesion, is generally defined as the ability of polymeric systems to adhere to the mucus layer.\textsuperscript{16,17,18} The adhesion is simply defined as the “fixing” of two surfaces to one another.\textsuperscript{19} There are many different terminological subsets of adhesion depending upon the environment in which the process occurs. When adhesion occurs in a biological setting it is often termed “bioadhesion”, while if this adhesion occurs on mucosal membranes it is termed “mucoadhesion”. Bioadhesion can be defined as the binding of a natural or synthetic polymer to a biological substrate. When this substrate is a mucous layer, the term mucoadhesion is commonly used.\textsuperscript{20}

Mucoadhesion slows the particle transit time through the GI tract to the time scale of mucus renewal, thereby promoting an intimate contact with cell surface and, as a consequence, an increase of drug absorption. Undoubtedly as a mean of localising drugs to sites throughout the body, there are several advantages in using bio/mucoadhesive drug delivery systems:

1. As a result of adhesion and intimate contact, the formulation stays longer at the delivery site improving drug bioavailability using a lower concentration for disease treatment.

2. The use of specific bioadhesive molecules allows for possible targeting of particular sites or tissues, for example the gastrointestinal (GI) tract.

3. Increased residence time combined with controlled drug release may lead to lower administration frequency.
(4) The avoidance of first-pass metabolism.

(5) Additionally significant cost reductions may be achieved and dose-related side effects may be reduced due to drug localization at the disease site.\(^ {18}\)

The mucosal route has been used since long time ago for vaccine delivery. It originated with Chinese Medicine, several thousands years ago, when children were allowed to inhale powders made from dried crusts of pox scars. The rational for mucosal immunization is to increase patient compliance, ease of administration, reduced risk of transmission of disease, problems of disposal of contaminated needless and to localize immune response to site of potential pathogen invasion.\(^ {21}\)

Over the last 30 years, the market share of transmucosal drug delivery systems has significantly increased with an estimated value of $6.7 million in 2006. According to a recent report published by Kalorama, worldwide revenue in this area was expected to increase approximately 3.5% a year to reach $7.9bn by 2010. This growth can be related to the ease with which transmucosal products may achieve the therapeutic effect. For example, such dosage forms may be delivered via the nasal route using sprays, pumps and gels, via the oral/buccal route using mucoadhesives, quickly dissolvable tablets and solid lozenge formulations and via vaginal or urethral routes using suppositories, pessaries, vaginal rods and gels.\(^ {22}\) Furthermore, the sustained growth of biotechnology drugs and the inherent need for novel drug delivery technologies that provide easier and more controlled modes of administration has resulted in a dramatic increase in the use of transmucosal systems.\(^ {23}\)

1.4 Mucus: structure, function and composition

Mucus is a viscoelastic gel layer that protects tissues that would otherwise be exposed to the external environment. Mucus is composed mainly of water (>95%) and of crosslinked and entangled mucin fibers secreted by goblet cells and submucosal glands.\(^ {24}\)
Mucins are large molecules, typically 0.5–40 MDa in size \[^{25}\] formed by the linking of numerous mucin monomers, each about 0.3–0.5 MDa \[^{26,27}\], and are coated with a complex and highly diverse array of proteoglycans. At least twenty mucin glycoproteins have been assigned to the MUC gene family \[^{28}\], with several mucin types expressed at each mucosal surface \[^{29}\]. The proteic part of mucus (figure 1) is a high molecular weigh macromolecular system heavily glycosylated, with oligosaccharidic branches that occupy 63% of the proteic core, while the other 37% consists of non glycosylated terminal regions.\[^{30}\]

![Schematic structure of the representative unit of mucin.][1]

Oligosaccharide branches make the mucin more hydrosoluble and also protect the protein core from proteolytic degradation. The proteic core consists of about 800 amino acid residues, 200 of them linked to polisaccharide lateral chains.\[^{32}\] Oligosaccharide chains are normally 2–20 residues long and often fucose, sialic acid, sulfate esters of galactose and N-acetylgalactosamine are the terminal groups.\[^{33}\] The protein nucleus (figure 2) consists of alternate...
sequences mainly composed by serine, threonine and proline. Generally the oligosaccharide chain is linked to the protein core through an ether bond between the a-1 position hydroxyl group from the N-acetylgalactosamine and the hydroxyl group from the serine or threonine amino acids.\textsuperscript{34}

![Diagram](image)

**Fig.2** Schematic representation of structural similarities and differences between a segment of transmembrane mucin (MUC1) and a segment of secreted mucin (MUC2).\textsuperscript{35}

The sugar residues composing the oligosaccharide side chains are galactose, fucose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid, that above pH 2.6 is completely ionized, giving to side chains a negative net charge at physiological pH.\textsuperscript{36} Some authors studied the zeta potential of mucin coated TiO\textsubscript{2} or Al\textsubscript{2}O\textsubscript{3} microparticles, even if mucin is composed of non-compact spherical macromolecular chains, measuring a value of -10 at pH 6, and a positive value under pH 2. This is due to the fact that also basic amino acids are present in lateral chains, even if in small parts.
Different mechanisms have been proposed for mucoadhesion phenomenon. The most important are the wetting and swelling of the polymer that allowed an intimate contact with the tissue, the interpenetration of the polymer chains and entanglement between the polymer and the mucin chains and finally, the formation of weak chemical bonds including ionic bonds, van der Waals interactions and hydrogen bonding.  

It is essential to bear in mind that the mechanisms of mucoadhesion are not completely clear and, moreover, they should be considered more as complementary than alternative mechanisms. In order to develop improved mucoadhesive materials for drug delivery, the chosen polymer should have a high amount of hydrogen-bonding chemical groups, such as hydroxyls and carboxyls, cationic surface charges, high polymer molecular weight, high polymer chain flexibility and surface tensions that will induce spreading into the mucus layer.

The interactions between polymers and mucin chains are related with the ionic strength and pH of the environment, because ionic changes of functional groups or the electrostatic shield effect influence the electrostatic repulsion and the swelling of mucus network. A decrease of environment pH promotes the mucoadhesion of both cationic polymers and anionic polymers that interact with mucin chains with hydrogen bonds. On the other side, an increase of pH promotes an electrostatic repulsion of carboxylated anions, having a swelling of polymer chains with consequent increase of free spaces inside the network. A reduction of polymer chains' density increases single chain mobility and promotes entanglement with mucin chains.

Although mucoadhesion is a promising approach to increase the bioavailability of drugs delivered via mucosal tissues, important fundamental limitations of this approach exist. Since mucoadhesive systems are bound to the mucus layer through interactions with mucin fibers, the transit time of these systems is determined by the physiological turnover time of the mucus layer. For oral delivery, being the intestinal mucin turnover time between 50 and 270 min, mucoadhesive particles are not expected to adhere to mucus for more than 4–5 h. Furthermore, as mucoadhesive systems efficiently adhere to mucus, they are largely incapable of penetrating across the mucus layer and entering the underlying epithelia. Thus, mucoadhesive systems are
especially unsuitable for delivery of drug and gene molecules that require intracellular delivery. To overcome these issues, various attempts have been made to engineer particles that adhere specifically to intestinal cells, as exemplified by the conjugation of tomato lectins to nanoparticles. However, these ligand-bound particles appear to have a limited capacity to diffuse through the mucus layer and, instead, undergo premature adsorption to mucus.\textsuperscript{41,42} Thus, these systems reach the enterocyte surface inefficiently at best, and are instead bound to mucus, transported through the GI tract, and eliminated in the feces in a similar fashion to other mucoadhesive systems. To overcome this problem and achieve longer residence time of particles at mucosal surfaces, the foremost requirement is to engineer particles that can efficiently cross the mucus barrier and subsequently come in contact with the cell surface.

The delivery systems that have shown potential for mucosal delivery of drugs and antigens include biodegradable micro and nanoparticles, liposomes, mucosal adjuvants, bacterial and viral vector systems.

1.5 Micro- and Nano-particles as carriers

Historically, the first report concerning the use of nanoparticles for pharmaceutical applications was made by Birrenbach and Speiser in the seventies.\textsuperscript{43} These early nanoparticles were polyacrylamide nanospheres prepared by inverse emulsification polymerization and were intended to incorporate water-soluble antigenic material for vaccination purposes.

Intestinal enzymes, breakdown by gut microflora, gastric acidity and peristalsis can all degrade drugs and in turn, reduce their bioavailability.\textsuperscript{44} Encapsulation of the active compound in a nanoparticulate carrier provides the drug with protection from the hostile environment in the gastrointestinal tract, the degradation by the low pH of the stomach and the proteolysis from the gut.
Nanoparticles are more stable in the GI tract compared to other colloidal carriers such as liposome, and therefore facilitate the safe passage of antigens through the GI tract.

Drug carriers can be prepared tailoring release properties and site delivery selecting the suitable raw materials. Here is a representative list of polymers that have been investigated for drug delivery applications. They can be broadly classified into natural and synthetic polymers.

**Natural polymers**

- Protein based polymers: collagen, albumin, and gelatin
- Polysaccharides: agarose, alginate, carrageenan, hyaluronic acid, dextran, chitosan, cyclodextrins.

**Synthetic polymers**

- Biodegradable: Polyesters, Polyamides and phosphorous based polymers
- Non- biodegradable: Cellulose derivatives, Silicons and Acrylic polymers

Biodegradable polymers have been the major focus of attempts to develop improved delivery systems for pharmaceutical research. Nanoparticulate systems have been used as an advanced drug delivery carrier due to their unique features, such as capability to protect therapeutic compounds, versatility to control the release profiles of loaded drugs and tuneable surface properties.

In a biodegradable particulate delivery system, the particles are absorbed with greater efficiency than soluble molecules in the mucosal epithelium. It has the potential to reduce the frequency of drug administrations
to establish long-term therapeutic effect. The biodegradable particulate systems can be classified as:

a) Monolithic type, where the drug is evenly dispersed throughout the polymeric matrix;

b) Reservoir type, where the drug is located in solution cavities formed by the polymeric material surrounded by an outer polymer shell.

The advantage of using micro-nanocarriers is that the release properties can be tailored for the drug to produce a sustained therapeutic effect and to protect the drugs from rapid destruction \textit{in vivo}, allowing for presentation of it in its native conformation to the various delivery sites.

The uptake of polymeric nanoparticles by the cells of the mucosal epithelium has been shown in previous studies to be superior to polymeric microparticles. As a matter of that, size is the major factor in the uptake of nanoparticles by M cells. Particles below 1 µm in size are taken up by M cells and delivered to the basal membrane. Particles larger than 5 µm have shown to be taken up by M cells, but remained in the Peyer’s patches. Specifically, the optimal size for nanoparticles to be transcytosed by M cells is 200 nm.

A range of reports have suggested that nanoparticles are capable of entry into intestinal epithelia via M cells on the domes of the Peyer's patches in small animals. Not surprisingly, the Peyer's patches are the intestinal surfaces least protected by mucus and most exposed to chyme. Since no mucus is secreted in the region surrounding these cells, which protrude relatively unprotected into the lumen, the mucus barrier is minimal. Indeed, M cells are positioned as sensory outposts for cellular immune functions, transcytosing particles that impinge on their surface into the interior of the patch.

The dominant opinion is that particulate uptake in GI tract of mammals is principally via the M cells of Peyer's patches, and that uptake by enterocytes plays a minor role. Although this, M cells occupy a relatively small portion
of the total surface area of the GI tract. The potential of drug delivery to the systemic circulation via M cells instead of enterocytes has thus remained debatable. A number of studies have suggested that uptake of nanoparticles may occur through enterocytes as well as M cells;\textsuperscript{45,53} however, as recently pointed out, no satisfying explanation has been advanced to elucidate conflicting reports.\textsuperscript{54} The prospects for using Peyer's patches as a means of delivering peptides and proteins has been said to be "severely compromised" by: (a) the limited efficiency and capacity of the absorption pathway, (b) the time to onset of pharmacological response, due to both the kinetics of processing the particles and the flow rate of the lymph; and (c) the potential loss of drug to local lymphocytes and macrophages.\textsuperscript{55}

Numerous methods exist for the manufacture of nanoparticles, allowing extensive modulation of their structure, composition, and physicochemical properties. The choice of the manufacturing method essentially depends on the raw material intended to be used and on the solubility characteristics of the active compound to be associated to the particles. Regarding the raw material, criteria such as biocompatibility, the degradation behaviour, choice of the administration route, desired release profile of the drug, and finally, the type of biomedical application determine its selection. From these considerations, it is clear that nanoparticle formulation requires an initial and very precise definition of the needs and objectives to be achieved.
2 Nanoparticles formation

In the specific field of biomedicine, one requires natural and synthetic polymers that are biocompatible, biodegradable, capable of binding with proteins, genes, nucleic acids, acidic lipids as well as having the ability of absorption enhancing and mucosal adhesion without toxicity. To use them for the incorporation of bioactive macromolecules and vaccines for therapeutic applications, the design/preparation of these systems must be monitored in terms of particle shape and morphology, size distribution, surface chemistry and polymer nature.

In recent decades, a significant scientific and applicative interest towards non-covalent polymeric gels has grown up, both those of natural or synthetic origin. Microgels are crosslinked hydrogel particles that are confined to smaller dimensions. When the microgel particles are submicron-sized they are known as nanogels. Microgels/nanogels have high water content, biocompatibility, and adjustable chemical and mechanical properties. In addition, they have tunable size from submicrons to tens of nanometers, a large surface area for multivalent bioconjugation, and an interior network for the incorporation of therapeutics. These unique properties offer great potential for the utilization of microgels/hydrogels in applications for tissue engineering, biomedical implants, bionanotechnology, and drug delivery.

The development of various biopolymer-based hydrogels has been explored. They usually have all the properties of the raw materials used as well as being intrinsically biodegradable, nontoxic, and relatively cheap. In addition, biomicrogels/bionanogels possess a high content of functional groups including hydroxyl, amino, and carboxylic acid groups, used in crosslinking with additional functional crosslinkers and furthermore, for further bioconjugation with cell targeting agents. Typical examples of natural biopolymers tested are polysaccharides as chitosan, hyaluronan, dextran, cellulose, pullulan, chondroitin sulfate, and alginate.

Several approaches for aqueous homogeneous gelation have been explored to prepare submicron-sized bionanogels. These approaches include...
a gelation based on (1) chemical and physical crosslinking in water and emulsion and (2) a temperature (or heat)-induced self association in water. Physical crosslinking can be ionic interactions as in ionically crosslinked hydrogels and polyelectrolyte complexes (PEC), or secondary interactions as in chitosan/poly (vinyl alcohol) (PVA) complexed hydrogels, grafted chitosan hydrogels and entangled hydrogels. In this study the use of nanogels prepared by ionic crosslinking and polyelectrolyte complexes (PEC) have been selected.

2.1 Ionotropic gelation

The complexation between polymers and oppositely charged macromolecules can be exploited to prepare micro/nanoparticles suitable for drug delivery. The particles are prepared by ionic crosslinking through self-assembly of chitosan/chitosan derivatives and oppositely charged macromolecules or by addition of a low molecular weight anionic crosslinker, such as tripolyphosphate (TPP), sodium sulfate or cyclodextrin derivatives to chitosan solutions.

The ionic crosslinking methods mentioned above have received much attention in recent years for the preparation of protein formulations because the used processes are simple and mild to proteins, as they do not involve the use of chemical crosslinkers and avoid the use of organic solvents and high temperatures.\textsuperscript{63,64}

Tripolyphosphate (TPP) is a polyanion, which can interact with the cationic chitosan by electrostatic forces. After Bodmeier et al.\textsuperscript{65} reported the preparation of TPP–chitosan complex by dropping chitosan into a TPP solution, many researchers have explored its potential pharmaceutical usage.\textsuperscript{66,67} However, TPP/chitosan microparticles formed have poor mechanical strength thus, limiting their usage in drug delivery.

On the other side, nanoparticles formed by dropping TPP in chitosan solution under constant stirring generate much more interest, due to reduced particle size.
Ionic gelation of chitosan with TPP has been extensively used for the preparation of protein and antigen-loaded nanoparticles. Insulin-loaded chitosan nanoparticles have been prepared by mixing insulin with TPP solution and then adding this to chitosan solution under constant stirring.

Using this method, chitosan nanoparticles loaded with insulin and tetanus toxoid have been prepared and investigated as nasal delivery vehicles. Fernandez-Urrusuno et al. prepared insulin-loaded chitosan nanoparticles with a size 300–400 nm and a positive surface charge. Insulin release in vitro occurred in less than 3 h. However, this relatively fast release is not a disadvantage since the average residence time of nasally administered formulations never exceeds few hours. In another study, tetanus toxoid (TT)-loaded chitosan nanoparticles, with an average size about 350 nm and a positive surface charge, showed a high loading efficiency (around 50–60%). In vitro release studies showed an initial burst followed by a sustained release of antigenically active toxoid for 16 days. Recently, there have been many studies focusing on the mucosal delivery of proteins and vaccines using TMC nanoparticles prepared by ionic gelation.

Chitosan and its derivatives exhibit a pH-sensitive behavior as a weak polybase due to the large quantities of amino groups on their chains. In particular, chitosan dissolves easily at low pH while it is insoluble at higher pH ranges. This property has helped it to be used in the delivery of chemical drugs to the stomach but, for the delivery of protein drugs to the intestine, this property pauses a limitation because, as the matrix gets dissolved in the stomach, the released protein drugs will get denatured. To overcome this, many modifications can be done to improve the stability of chitosan nanogels in the stomach and the subsequent controlled delivery of protein drugs in the intestine such as modify chitosan chemical structure to obtain better behaviour performances or embed/coat gel structure with suitable polymer (as pectin or alginate). In particular, by coating nanoparticles with such polymers, a protection of protein drugs in lower pH of stomach is expected and no release should occur because the hydrogel becomes more compact. When the pH of the medium increases gradually, the hydrogel swells and protein release occurs.
2.2 Polyelectrolyte complexation

Among all methods of development of polymer dispersions for biomedicine, the employment of polyelectrolyte complexes (PEC) represents a very attractive approach, mainly due to the simplicity involved in the preparation.\textsuperscript{76}

The formation of complexes by the interaction of oppositely-charged polyelectrolytes is well known. A variety of polyelectrolyte complexes can be obtained by changing the chemical structure of component polymers, such as molecular weight, flexibility, functional group structure, charge density, hydrophobicity balance, stereoregularity and compatibility, as well as reaction conditions: pH, ionic strength, concentration, mixing ratio and temperature.\textsuperscript{77,78}

Potential fields of application of polyelectrolyte complexes are: as membranes for different end uses\textsuperscript{79} coating on films and fibres,\textsuperscript{79} implants for medical use,\textsuperscript{80,81} microcapsules,\textsuperscript{82} support for catalysis,\textsuperscript{83} binding of pharmaceutical products,\textsuperscript{84} isolation and fractionation of proteins,\textsuperscript{85} isolation of nucleic acid.\textsuperscript{86}

Such a network is formed by ionic interactions as represented in figure 3 and is characterised by a hydrophilic microenvironment with a high water content and electrical charge density. Chitosan and its derivatives have been widely used to form polyelectrolyte complexes. The electrostatic attraction between the cationic amino groups of chitosan and the anionic groups of the other polyelectrolyte is the main interaction leading to the formation of the PEC. It is stronger than most secondary binding interactions,\textsuperscript{91,92} such as those, for example, allowing formation of chitosan/PVA complexes or aggregation of grafted chitosan.

Since chitosan has a rigid, stereo-regular structure containing bulky pyranose rings,\textsuperscript{93} the formation of PEC can induce a conformational change of the other polyelectrolyte, if the latter has a non-rigid structure; e.g. $\alpha$-keratose,\textsuperscript{94} poly (acrylic acid) (PAA),\textsuperscript{95} xylan or collagen. The preparation of a PEC requires, besides chitosan or one of its derivatives, only a polyanionic polymer. No auxiliary molecules such as catalysts or initiators are needed and
the reaction is generally performed in aqueous solution, which represents the main advantage over covalently crosslinked networks and thus favours biocompatibility and avoids purification before administration. The most commonly used polyanions are polysaccharides bearing carboxylic groups such as alginate, pectin or xanthan. Proteins, such as collagen, synthetic polymers, such as PAA, or even DNA have also been investigated.

**Fig. 3** Structure and pH-sensitive swelling of a polyelectrolyte complex containing chitosan; -, negative charge of the additional polymer; +, positive charge of chitosan; —, chitosan; —, additional anionic polymer (adapted from reference 100).

In order to form a PEC, both polymers have to be ionised and bear opposite charges. This means that the reaction can only occur at pH values in the vicinity of the pKₐ interval of the two polymers (the macro pKₐ of chitosan is
about 6.5 \cite{91}. During complexation, polyelectrolytes can either coacervate, or form a more or less compact hydrogel. However, if ionic interactions are too strong, precipitation can occur\cite{93}, which is quite common and hinders the formation of hydrogels. Precipitation can be avoided if electrostatic attraction is weakened by the addition of salts, such as NaCl. Their presence reduces the attraction between the oppositely charged polyelectrolytes by contributing to the counter-ion environment. Hence, no phase separation occurs, and a viscous and macroscopically homogeneous blend is obtained, which may gel as temperature is lowered.\cite{101}

The properties of PEC are mainly determined by the degree of interaction between the polymers. This latter depends essentially on their global charge densities and determines their relative proportion in the PEC. Indeed, the lower the charge density of the polymer, the higher is the polymer proportion in the PEC, since more polymeric chains are required to react with the other polymer. As this proportion and the chemical environment are the main factors influencing swelling, it is possible to modulate the properties of PEC by controlling the complexation reaction.

As PEC hydrogel are formed by ionic interactions, they exhibit pH-, and to a minor extent, ion-sensitive swelling. In addition, they have a high water content and electrical charge density and allow the diffusion of water and/or drug molecules.\cite{102,103} Moreover, chitosan and its derivatives are known for their biocompatibility and for their ability to promote wound healing\cite{104,105} and both properties are maintained after polyelectrolyte complex formation. In addition, depending on the polyanionic polymer used, these systems are generally considered as biodegradable and biocompatible.\cite{106,107} Therefore, trimethyl chitosan (TMC) hydrogels formed by PEC are well tolerated systems\cite{102,103} and can be used in various applications such as drug delivery systems, in cell culture and enzyme immobilisation or for tissue reconstruction and wound-healing management.
3 Biopolymers

3.1 Natural cationic polymers

Among the various bioadhesive materials that have been proposed for mucosal delivery of proteins, cationic polymers have received particular interest. Chitosan, the second most abundant polysaccharide in nature, has attracted particular interest as a biodegradable material for mucosal delivery systems. It has showed favourable biological properties, low toxicity and high susceptibility to biodegradation, mucoadhesive properties and an important capacity to enhance drug permeability and absorption at mucosal sites.

3.1.1 Chitosan

The availability of chitosan, its biocompatibility, and its unique chemical and biological properties make it an attractive biomaterial for a variety of pharmaceutical applications, especially in the areas of wound dressing and drug delivery. It has been used or tested in different forms, such as tablets, matrix, and micro-nanoparticles for the purpose of sustained release, controlled drug delivery, mucosal formulations and, more recently, drug absorption enhancement protein and peptide drug delivery and vaccine development.

Chitosan is a biocompatible and biodegradable natural biopolymer consisting of β-1→4 linked 2-amino-2-deoxy-glucopyranose (figure 4). It is currently manufactured commercially on the large scale by alkaline N-deacetylation of chitin, an abundant biopolymer isolated from the outer shells of crustaceans such as crabs and shrimps. Commercial chitosans have
average molecular weights between 3.8 and 2,000 kDa and are from 66% up to 95% deacetylated.\textsuperscript{115}

![Diagram of chitosan structural unit]

**Fig.4** The repeating structural unit of chitosan.

Chitosan has rather specific solution properties. It is only soluble at acidic pH when the free amino group (-NH\textsubscript{2}) becomes protonated to form cationic amine group (-NH\textsubscript{3}\textsuperscript{+}). At these pHs, chitosan has been identified as a linear polycation with an intrinsic pKa value of 6.5, independent of the degree of acetylation (DA).\textsuperscript{116} The polycationic chitosan molecules have been found to readily adhere to negatively charged surfaces, such as skin, mucus, and proteins.\textsuperscript{117,118} For this reason a lot of interest is growing for using of chitosan as a bioadhesive material. The mucoadhesive properties of chitosan have been illustrated by its ability to adhere to porcine gastric mucosa \textit{in vitro} \textsuperscript{119}, and hence it could be useful for in site-specific drug delivery. Many commercially available chitosans exhibit fairly good mucoadhesive properties \textit{in vitro}.\textsuperscript{117} It has been suggested that residence time of formulations at sites of drug action or absorption could be prolonged through the use of chitosan. It has also been suggested that chitosan might be valuable for delivery of drugs to specific regions of the gastrointestinal tract like the stomach\textsuperscript{120}, small intestine\textsuperscript{121,122}, and buccal mucosa.\textsuperscript{123,124}
H. Ping and colleagues assessed the mucoadhesive property of chitosan onto the small intestine of rats using chitosan microspheres. The result included up to 78% of the particles being adsorbed to the small intestinal mucosa. This mucoadhesion is ideal for increasing the residence time of antigens in the GALT. This study also clearly showed that the charge of the particles had a significant effect on the adsorption of the microspheres, giving rise to electrostatic interactions. A positive zeta potential gave rise to an increase in the amount of chitosan adsorbed to the tissue. On the other hand, particles with a negative zeta potential were not adsorbed onto the intestinal mucosa. Recent studies indicate that the binding of chitosan onto epithelial membranes resulted in cellular F-actin depolymerization and disbandment of the tight junction protein ZO-1. It was in 1994 that Illum et al. showed the permeation enhancing capabilities of chitosan for the first time. This property is currently considered to be the major mechanism by which chitosan enhances drug adsorption.

The integrity of these tight junctions is measured by the Transepithelial electrical resistance (TEER). Chitosan is capable of reducing the TEER, as demonstrated on human colorectal carcinoma Caco-2 cells, where the reduction of TEER by chitosan resulted in the subsequent increase of paracellular transport of inert marker inulin.

Chitosan therefore offers many benefits with regards to improving the uptake of orally delivered drugs in the mucosal tissue through mucoadhesion and opening of epithelial tight junctions. It is important however, to recognize that chitosan is a weak base. In the high pH conditions of the intestine, which could be neutral and even basic (depending on the intestinal tract considered and digestion), chitosan looses its positive charge and precipitates from solution, forming salts with organic and inorganic acids, thus resulting in insufficient enhancement of absorption. At these higher pH values (~pH 7.4), chitosan is unable to decrease the TEER as significantly as observed at lower pH values. At slightly acidic pH values however, i.e. below 6.5, chitosan is protonated and soluble, and it is in this form that the opening of tight junctions can be triggered.

In vivo, chitosan is degraded by several glycosidases, primarily lysozyme and N-acetylglucosaminidase. Chitosan also has stimulatory
effects on macrophages and this activity was suggested as being mainly attributable to the remaining N-acetylglucosamine groups. Activated macrophages were found to be able to accelerate the in vivo degradation of chitosan.\textsuperscript{130}

### 3.1.2 Chitosan derivatives

In spite of its superior properties, chitosan has a major drawback: its solubility is poor above pH 6. At physiological pH, chitosan will lose its capacity to enhance drug permeability and absorption, which can only be achieved in its protonated form in acidic environments.\textsuperscript{131} To solve this pharmaceutical-technological limitation, chitosan has the important property of undergoing chemical modification very easily, helping researchers to improve its chemical and mechanical properties for the purpose of oral protein delivery. This property is attributed to the presence of free amino groups: the C2 primary amino group of chitosan is functionalized to obtain different type of chitosan derivatives, such as carboxymethyl chitosan,\textsuperscript{132,133} chitosan salts, N-trimethyl chitosan chloride, a quaternized derivative,\textsuperscript{128,134} and glycosylated chitosan. Moreover, N-trimethyl chitosan chloride (TMC), and glycosylated chitosans have peculiar characteristics in terms of biohadesivity, enhancement of permeability and absorption resulting more effective that native chitosan.\textsuperscript{135,136}

#### 3.1.2.1 N-trimethyl chitosan (TMC)

N-trimethyl chitosan (TMC) has shown interesting characteristics for its well defined structure (figure 5), for its solubility properties and ease of production.
TMC with a degree of quaternization of 12% was able to increase the transport of several peptide drugs across Caco-2 cell monolayers. Thanou observed that TMC with a degree of quaternization 60% (TMC 60) caused a redistribution of cytoskeletal F-actin. Also, the trans membrane protein occludin displayed a disrupted pattern after incubation with 1% TMC 60. This suggests an interaction of TMC with the tight junction proteins, the mechanism that helps to increase paracellular permeability.

On the other hand, TMC showed good solubility in a large pH range and the capacity of absorption through intestinal epithelial cells. Moreover, TMC is a promoter of big dimension hydrophilic compounds absorption, especially at neutral and alkaline pH, values where chitosan salts do not show that same behaviour.

In neutral and basic environments, where chitosan and chitosan salts are ineffective as absorption enhancers, TMC could considerably contribute to the effective delivery of hydrophilic compounds such as proteins and peptide drugs. TMC is an absorption enhancer as is chitosan, and has shown to increase the transport of inert marker mannitol (32-60 fold), peptide drug buserelin (23-73 fold), and fluorescently labeled dextran 4400 (167-373 fold). TMC is also capable of opening tight junctions of mucosal epithelium.

**Fig. 5** Chemical structure of TMC.
The advantage over chitosan however, is that TMC is able to open these tight junctions at higher pH values (~pH 7.4) at which chitosan is inefficient at opening the tight junctions.\textsuperscript{139}

The TMC synthesis was described by Le Dung et al. in 1994. They obtained a TMC with a degree of quaternarization (Fig. 3) of 53\% using a one step reaction. Starting from this, different types of TMC were obtained: using more steps is possible to obtain a higher degree of quaternarization.

According to this, it is possible to obtain two different TMC using a one step or a two steps reaction: low conversion with one step, high conversion with two steps. For a low conversion TMC, a one step reaction is used, whereby approximately 35 \% of the amine groups are trimethylated; this percentage of conversion is considered fairly low. For a high conversion of TMC on the other hand, a two step reaction is used, whereby at least 60 \% of the amine groups are trimethylated. In this case, the conversion from chitosan to TMC is high.\textsuperscript{128}

\subsection*{3.1.2.2 Glycosylated chitosans}

A new class of chitosan derivatives are glycosylated chitosans, polymers modified by grafting of sugars via a reductive N-alkylation (figure 6).

Hall and Yalpani\textsuperscript{140,141} were the first to report sugar-modified chitosan derivatives by this approach. They synthesized sugar-bound chitosan by reductive N-alkylation using sodium cyanoborohydride and unmodified sugar or sugar-aldehyde derivative. Initially, the sugar-bound chitosans had been investigated mainly for rheological studies; but since the specific recognition of cells, viruses, and bacteria by sugars was discovered, this type of modification has usually been used to introduce cell-specific sugars into chitosan. Morimoto et al. reported the synthesis of sugar-bound chitosans, such as those with D- and L-fucose, and their specific interactions with lectin and cells.\textsuperscript{142,143}
Lectins belong to a group of structurally diverse proteins and glycoproteins that can bind reversibly to specific carbohydrate residues. After initial mucosal cell-binding, lectins can either remain on the cell surface or in the case of receptor-mediated adhesion possibly become internalised via a process of endocytosis. Lectin-conjugated polymers are known as the second generation of mucoadhesives since they are designed to specifically bind to receptors on the epithelial cells. This is the reason why these types of polymers are also called cytoadhesives. Although this property has an advantage over mucoadhesive polymers, we cannot forget that the polymer will have to diffuse through the whole mucus layer before reaching the epithelial cells membrane. As mentioned before, one of the roles of this mucus layer is to act as a shield to protect the cells underneath. Then, cytoadhesive
polymers should not only have the ability to specifically bind the cell membrane but also should be capable of diffusing across the entire mucus layer to find the epithelial surface.

Two different sugar-modified chitosan have been selected for this thesis, synthesized and employed, chitosan-galactose and chitosan-glucose, obtained by grafting of lactose or maltose via a reductive N-alkylation.

Chitosan-galactose showed interesting biological properties. Donati and co-workers synthesized lactose-modified chitosan for a potential application in the repair of the articular cartilage by the same mode. Galactosylated chitosan prepared from lactobionic acid and chitosan with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) showed promise as a synthetic extracellular matrix for hepatocyte attachment.

3.2 Association with other biopolymers: pectins

Pectin is a complex mixture of polysaccharides that makes up about one third of the cell wall dry substance of higher plants. Much smaller proportions of these substances are found in the cell walls of grasses. The highest concentrations of pectin are found in the middle lamella of cell wall, with a gradual decrease from the primary wall toward the plasma membrane.

Commercial pectins are almost exclusively derived from citrus peel or apple pomace, both by-products from juice (or cider) manufacturing. Apple pomace and citrus peel contain 10-15% and 20-30% of pectin on a dry matter basis, respectively.

The structure of pectin is very difficult to determine because pectin can change during isolation from plants, storage, and processing of plant material. In addition, impurities can accompany the main components. Pectin is made up of α-(1, 4) linked D-galacturonic acid units linked in a linear fashion (figure 7).
Pectin molecules also contain rhamnogalacturonan, a neutral sugar, which is responsible for splitting and causing kinks in the galacturonic acid chain. The uronic acids have carboxyl groups, some of which are naturally present as methyl esters and others which are commercially treated with ammonia to produce carboxamide groups. If the percentage of carboxyl groups esterified with methanol is over 50%, the pectin is referred to as pectin with a high degree of esterification. When the percentage of carboxyl groups esterified with methanol is less than 50%, the resultant pectin is said to have a low degree of esterification. As the number of carboxyl groups esterified with methanol approaches 100% (protopectin), pectin begins to lose its solubility in water and its ability to form gel is lost. Most pectins extracted from natural materials, classified as having a high degree of esterification, usually have less than 75% of their carboxyl groups esterified.

One important characteristic of pectin is its ability to form gels. The gelation process can be affected by many parameters such as degree of methylation, distribution of charge along the backbone, average molecular weight, ionic strength, pH, temperature, and presence of soluble solids. Molecular weight of pectin is a key parameter in its ability to form a gel due to the fact that the linear polymer chain must be long enough to accumulate a sufficient number of weak interactions to form stable cross-links.
Gelling can be induced by acid, by cross-linking with calcium ion, through the oxidization of the feruloylester substituents on sugar beet pectin \(^{153}\), or by synergistic reaction with alginate.\(^ {154}\)

In the beginning, pectin was used as gelling or thickening agent in the food industry, and then, as an excipient for pharmaceutical purposes. Pectin-based drug delivery vehicles have been developed, in combination with other polymers. In particular, the interaction with cationic polymer has been investigated due to the pectin property to bind with oppositely charged surfaces and to associate to form complexes: for example, at pH between 4 and 6, pectin, pKa 4.0, is negatively charged while a polymer as chitosan, pKa 6.3, is positively charged. Carriers with a pectin external shield are carriers with the protective and mucoadhesive properties of pectin, solving chitosan particle limitations. In particular, at low pH of stomach, the carboxyl groups of pectin are protonated and become neutral in charge; this allows the chain to adopt a more compact arrangement making the external shield, composed mainly of pectin, harder to water absorption and protecting inner particle structure, especially if made by a gastro-soluble polymer as chitosan.\(^ {155}\) In the intestinal pH environment, the ionization of carboxyl groups of pectin enhances polymer swelling and thus physical entanglement with mucus. Moreover, negatively charged pectin is known for good mucoadhesion related to a balance between an open expanded conformation and available hydrogen bonding sites.\(^ {156}\) Clearly its mucoadhesive capacity depends on environmental pH, which can influence the ionization degree of pectin.

Having pectin as the external shell has a better protective effect for protein drugs in lower pH of stomach, preventing drug release in the stomach (since the hydrogel becomes more compact) and allowing drug release when the pH of the medium increases as in the intestinal tract (the hydrogel swells). Thus it can perform controlled protein release at higher pH of intestine instead of rapidly releasing the protein drugs by rapid dissolution in the stomach.
4 Biocompatibility - Nanotoxicity

4.1 Biocompatibility of nanoparticles as carriers for drug delivery.

The developing of nanotechnology is paralleled by the increasing concern about the safety and the possible toxic effect of the nanoparticles employed in the most various industrial fields. These range from daily applications (e.g. fillers, opacifiers, lubricants, cosmetics, catalysts, electronic devices), to pharmaceutical and biomedical purposes. According to the National Health Information Report, approximately 130 nanoscale materials entered the clinical pipeline in 2006; by 2014 more than 15% of all products on the global market will have some nanotechnology-related products incorporated into their manufacturing process.\textsuperscript{157}

The international regulatory and research institutions are working to develop a common and shared knowledge on the theme of the nanotoxicology. Several platforms have been organised to maximise the synergies between the scientific and industrial groups involved in the nanotechnology research, in order to address all aspects of nanosafety, including toxicology, ecotoxicology, exposure assessment, mechanisms of interaction, risk assessment and standardisation.\textsuperscript{158,159}

Engineered nanomaterials represents a novelty in both form and function, unique chemistry and physics by design, complex interactions with biological and environmental milieu. The ability to manipulate particular nanoparticle features, such as their physico-chemical and biological properties, opens up a lot of possibilities for researchers in rationally designing these nanoparticles for use in drug and gene delivery, as image contrast agents, phototherapy agents and for diagnostic purposes. Drug delivery nanosystems represent a special case: currently, there are in pre-clinical or clinical development several nanoscale material containing drug products such as polymeric nanoparticles, micelles, metal colloids,
nanocrystals, dendrimers, emulsions, liposomes, micelles, nanoshells, gold and multifunctional nanoparticles in nanometer size range. However, the advantageous use of nanoparticles should not lead to harmful side effects.\textsuperscript{160}

Thus, the nanocarriers safety represents a fundamental issue also in the specific sector of oral drug delivery. Gastrointestinal (GI) tract is reached by direct ingestion of the formulation. After oral exposure, nanoparticles distribute to the gastrointestinal tract and eventually to kidneys, liver, spleen, lungs and brain. Some nanoparticles pass through the GI tract and are rapidly eliminated in feces or in urine (indicating that they cannot be absorbed across the GI barrier and into the systemic circulation).

Another aspect to be considered is the blood reactivity of nanoparticles. The evaluation of the hemocompatibility is a requirement of the NP's, especially for those which are designed to be injected, but also for those initially tailored for external applications (oral, nasal administration). It cannot be excluded that these NP’s could finally be transferred within the blood circulation.\textsuperscript{161}

In general, surface charge of nanoparticles influences the uptake and toxicity of nanoparticles through affecting aggregation and agglomeration of nanoparticles. Several studies on the influence of surface charge density (i.e. zeta potential) of negatively charged polymeric nanoparticles showed an increase in plasma protein absorption with surface charge density.\textsuperscript{162} Binding of these proteins has also been shown to correlate with rapid uptake into the liver and the spleen, and clearance of the particles by the Reticulo-Endothelial System (RES).

\section*{4.2 Biocompatibility of biopolymer nanoparticles as carriers for drug delivery.}

Polymeric materials used for preparing nanoparticles for drug delivery should be biocompatible and biodegradable. To this aim, many biopolymeric materials (in particular polysaccharides) have been applied. Moreover such materials take advantage from the reversibility of the processes (mainly
physico-chemical ones) at the base of the NP structure, due to several events which can occur after the administration into the body (removal of cross-linking ions, polymer solubilization or precipitation, ...). This ensures the possibility to work with safe nanoparticles, when safe biomaterials are employed.

Considering polysaccharides in general, they are widely regarded as being non-toxic and biocompatible. Chitosan, the principal biopolymer employed in this Thesis, is approved for dietary applications in Japan, Italy and Finland and it has been approved by the FDA for use in wound dressings. The modifications made to the native polymers could make them more or less toxic and any residual reactants should be carefully removed.\textsuperscript{163}

An important aspect in the use of such biopolymers as drug delivery systems is their metabolic fate in the body and the process of biodegradation. In the case of the systemic absorption of hydrophilic polymers, they should have a suitable molecular weight for renal clearance. If the administered polymer's size is larger than this, then the polymer should undergo degradation.

Biodegradation (chemical or enzymatic) would provide fragments suitable for renal clearance. Chemical degradation in this case refers to acid catalysed degradation, i.e. in the stomach. Although oxidation–reduction depolymerisation and free radical degradation have been reported also for chitosan, these do not represent a relevant mechanism of degradation in vivo. Chitosan can be degraded by enzymes which hydrolyse glucosamine–glucosamine, glucosamine–N-acetyl-glucosamine and N-acetyl-glucosamine–Nacetyl-glucosamine linkages. Chitosan is thought to be degraded in vertebrates predominantly by lysozyme and by bacterial enzymes in the colon. However, eight human chitinases have been identified, three of which have shown enzymatic activity.

After oral administration, chitosan shows some degradation in the gastrointestinal tract. The digestion of chitosan, occurring predominantly in the gut, was found to be species dependent and related to chitosan's NH\textsubscript{2} availability. The chitosan degradation rate can be affected by the polymer's Mw and degree of acetylation. Further, N-substitution may affect enzymatic degradation and this should be considered when new derivatives are suggested for systemic administration. It is also clear that the degradation mechanism of chitosan (and derivatives) is not fully understood when used in
vivo but that there may be adaptive mechanisms which increase its degradation over time.\textsuperscript{164}

From the regulatory point of view, the possibility to use biopolymeric nanoparticles for food and oral drug delivery applications is considered a good opportunity and a safe route to be explored. This because the insoluble but ultimately biodegradable polymeric nanoparticles (chitosan, PLGA, gelatine and modified dextran) will be broken down into their constituent molecules and excreted, at a rate dependent on their physicochemical characteristics. The relative persistence in the body, together with the potential for ultimate total breakdown can be exploited to provide a slow release system, both for delivery of drugs and food components.\textsuperscript{165}

Moreover, also in the food regulatory field, the risk assessment is different for soluble and degradable nanomaterials, which tend to have effects more similar to the original material, than for non degradable nanomaterials, which exhibit different biological properties to ionic, molecular or bulk forms (non-nanoform).\textsuperscript{166}
5 Aim of study

This thesis was developed at the Laboratory of Physical and Macromolecular Chemistry, Department of Life Sciences (DLS), University of Trieste, Italy.

Biodegradable polymers have been the major focus of attempts to develop improved delivery systems for pharmaceutical research.

In the specific field of biomedicine, required natural and semi-synthetic polymers should be biocompatible, biodegradable, capable of binding bioactive macromolecules and vaccines as well as having the ability of absorption enhancing and mucosal adhesion without toxicity.

The purpose of my PhD project was to develop and characterize biodegradable polymeric nanoparticles as mucosal drug carriers for the oral route as well as to promote the rational and optimal design and development of novel carrier systems. Chitosan and chitosan derivatives have been selected for particles preparation and were fully characterized in terms of physico-chemical properties, biocompatibility and mucoadhesivity.

The inotropic gelation was the method used for particles preparation; the design/preparation of these systems was monitored and optimized in terms of particle shape and morphology, size distribution, surface chemistry, biocompatibility, production automatization and storage. The original nanocarriers have been characterized in order to tailor specific properties, including the possibility of tuning the molecular interactions in the polymer assemblies at nanoscale level.

Moreover, the association with other biopolymers has been studied using three different pectins for particles preparation obtaining drug carriers with different surface chemistry and mucoadhesivity. For this study two different methods have been used, the particles coating and the blending, a modified polyelectrolyte complexation.
Materials & Methods
6 Materials

Polymers

- Low Molecular Weight (LMW) Chitosan: Sigma-Aldrich Co. (St. Louis, Mo).

- Very Low Molecular Weight (VLMW) Chitosan: degraded Medium Molecular Weight, Sigma-Aldrich Co. (St. Louis, Mo).

- Modified Chitosans:

  - Trimethilchitosan (TMC) was synthesized as reported in literature [see reference 135]. This derivative possesses mucoadhesive properties superior to those of native chitosan.
  - Chitosan derivatives with galactose (chit-gal) with a low degree of functionalization (50%), synthesized as reported in literature [see references 140 and 167].
  - Chitosan derivatives with galactose (chit-gal) with a high degree of functionalization (65%), synthesized as reported in literature [see references 140 and 167].
  - Chitosan derivatives with glucose (chit-glc) with a low degree of functionalization (42%).

- Polygalacturonic acid, P3889, from Orange, Mw 18 kDa, degree of esterification 10%, Sigma-Aldrich Co. (St. Louis, Mo).

- Pectin from citrus fruit, P9311, Mw 17 kDa, degree of esterification 22%, Sigma-Aldrich Co. (St. Louis, Mo).

- Pectin from apple, 76282, Mw 30-100 kDa, degree of esterification 70-75%, Sigma-Aldrich Co. (St. Louis, Mo).
Proteins

- Albumin from bovine serum (BSA): Sigma-Aldrich Co. (St. Louis, Mo).
- Albumin from chicken egg white (OVA): Sigma-Aldrich Co. (St. Louis, Mo).
- Human Insulin (HI) zinc-free: Novo Nordisk, (Copenhagen).
- Mucin from porcine stomach, Type III, partially purified with sialic acid content approximately of 1%: Sigma-Aldrich Co. (St. Louis, Mo).

Special reagents

- Bicinchoninic Acid Protein Assay Kit: Sigma-Aldrich Co. (St. Louis, Mo).
- QuantiPro BCA Assay Kit: Sigma-Aldrich Co. (St. Louis, Mo).
- Bradford Reagent: Sigma-Aldrich Co. (St. Louis, Mo).
- Cibacron Brilliant Red 3B-A: Sigma-Aldrich Co. (St. Louis, Mo).

Common Reagents

- Sodium Chloride: Sigma-Aldrich Co. (St. Louis, Mo).
- Sodium tripolyphosphate (TPP): Sigma-Aldrich Co. (St. Louis, Mo).
- Acetic Acid: Carlo Erba reagents (Milano).
- Sodium Acetate: Sigma-Aldrich Co. (St. Louis, Mo).
- Hydrochloric Acid: Carlo Erba reagents (Milano).
- Sodium hydroxide: Sigma-Aldrich Co. (St. Louis, Mo).
- Deionized Water.
7 Polymer characterization

7.1 NMR studies

All NMR measurements were measured at 300 K on a Bruker Avance III Ultra Shield Plus 600 MHz spectrometer provided with a two channel BBI probe.

For the monodimensional $^{13}$C spectra has been sampled 65536 points with a window of 200 ppm, while for the bidimensional $^{13}$C spectra has been sampled 512 points with a window of 120.

For the monodimensional $^1$H spectra has been sampled 16384 points with a window of 12 ppm, while for the bidimensional $^1$H spectra has been sampled 1024 points with a window of 12 ppm. The samples has been dissolved in a 10% D$_2$O solution and TSP 1:100 has been used as internal reference. All the experiments has been performed on a volume of 600 µL.

7.2 Size-exclusion chromatography studies

The biopolymer analysis was performed with a triple detection size exclusion chromatography system (SEC$^3$, Viscotek, USA) consisting of an online two channel degasser, a high pressure pump, an autosampler (all parts integrated in the GPCmax, Viscotek, USA), a 0.5 mm stainless steel in-line filter with a nylon membrane, two serially connected ViscoGEL columns (PWXL mixed bed 6–13 µm methacrylate particles, 7.8x300 mm), a temperature controlled triple detector array (TDAmax 305, Viscotek, USA) with a differential refractometer at $\lambda$=660 nm (RID 3580), a right angle (90°) light scattering detector (RALS) and a low angle (7°) light scattering detector (LALS
with a semiconductor laser diode at \( \lambda = 670 \) nm and a four capillary, differential Wheatstone bridge viscometer.

The SEC conditions were as follows: a degassed 0.3 M CH\(_3\)COOH/0.3 M CH\(_3\)COONa buffer (pH = 4.5) with 1% ethylene glycol was used as eluent, the sample concentration was 0.3–1 mg/mL and samples were dissolved for 24 h under shaking, injection volume varied from 10 to 100 \( \mu \)L, flow rate was maintained at 0.7 mL/min, and the column and detectors temperature were kept at 30 °C. Before injection, the sample solutions were filtered through a 0.45 \( \mu \)m cellulose nitrate disposable membrane (Sartorius, Germany). To ensure a low light scattering noise level the eluent was filtrated through a 16–40 \( \mu \)m glass filter. A polyethyleneoxide standard (MW = 22,411, \([\eta] = 0.384 dL/g\), \(M_W/M_N = 1.03\)) was used to normalize the viscometer and the light scattering detectors. Data acquisition and processing were carried out by use of OmniSEC 4.1 software (Viscotek Corporation). A \(dn/dc\) of 0.163 was used for the \(M_w\) calculation.\(^{168}\) The combination of online viscometer and light scattering detector provides essential information on the hydrodynamic size \(R_h\) of polymers in solution after employing Einstein’s viscosity equation:

\[
[\eta] \cdot M_w = \frac{10 \cdot \pi \cdot R_h^3}{3 \cdot N_A}
\]

### 7.3 Viscosity studies

The viscosity of a general solution is proportional to the time of flow of the liquid through the capillary and can be expressed by the following equation:

\[
\eta = A \cdot t \cdot \rho
\]  \hspace{1cm} (1)
where A is a constant that is characteristic of the capillary and \( \rho \) is the density of the solution.

From the measurements of the flow time, made both on polymer solution and on the solvent, the relative viscosity \( \eta_{rel} \) is calculated by the equation:

\[
\eta_{rel} = \frac{\eta}{\eta_0} = \frac{t \cdot \rho}{t_0 \cdot \rho_0} \cong \frac{t}{t_0}
\]

(2)

The approximation is justified because for dilute polymer solutions the densities of the solvent \( \rho_0 \) and of the solution \( \rho \) can be considered equal.

According to the Einstein model, in which the solution is formed by a rigid spherical solute with a specific weight equal to that of the liquid, the viscosity \( \eta \) of the system can be correlate with the volume fraction \( \Phi \) of the spheres themselves:

\[
\eta = \eta_0 \cdot (1 + \upsilon \Phi)
\]

(3)

where \( \upsilon \) is a shape factor related to the geometry of the particles (equal to 2.5 for spherical particles) and \( \eta_0 \) is the viscosity of the solvent.

This equation can be rewritten as:

\[
\left(\frac{\eta}{\eta_0} - 1\right) = \upsilon \Phi = \eta_{sp}
\]

(4)

The specific viscosity \( \eta_{sp} \) can be expressed as a function of the specific volume of suspended particles \( \upsilon \) (mL/g) and their concentration (g/mL) as:

\[
\eta_{sp} = \left(\frac{\eta}{\eta_0} - 1\right) = \upsilon \cdot \upsilon \cdot C
\]

(5)
The limit value for \( C \rightarrow 0 \) of the reduced specific viscosity, \( \eta_{sp}/C \), is called intrinsic viscosity \([\eta]\):

\[
[\eta] = \lim_{C \rightarrow 0} \frac{\eta_{sp}}{C} = v \cdot \nu
\]  

(6)

Where \( C \) is the weight concentration of the polymer.

For the measurement of intrinsic viscosity, a 0.1 g/dL solution of polymer was prepared in a proper solvent. An amount (14 mL) of this solution is transferred in a capillary viscometer (Ubbelohde, Schott Geräte, internal diameter of 0.53 mm).

The flow time (\( t \)) in which the solution flows through the capillary is determined.

Subsequently, the polymer solution is diluted by the addition of different amounts of solvent (1 mL, 2mL, 4 mL, 8 mL, 12 mL), and the series of detection is repeated after each addition. Finally, the flow time of solvent (\( t_0 \)) is registered. Measurements were made at 25 °C. All solutions were filtered with 0.45 microns Millipore filters.

7.4 Mucoadhesion studies

Mucoadhesive properties of the polymers were evaluated using two different methods described previously in literature: an *in vitro* evaluation of the interaction between polymer and mucin in aqueous solution and a biologic approach for adhesiveness using an *ex vivo* evaluation of mucoadhesive strength of polymers on an intestinal mucosa.
7.4.1 **In vitro** evaluation

The in vitro evaluation of the mucoadhesive properties of a polymer is a basic step in the development of a mucoadhesive drug delivery system. Based on the fundamental principles of physical chemistry, mucoadhesive properties have been assessed by evaluation of the interaction between polymers and mucin in aqueous solution.

Both chitosan and its derivatives have been tested: stock solutions of chitosan, TMC, chit-gal and chit-glc have been prepared at a concentration of 2 mg/ml in acetate buffer 0.25 M (pH 4.7). Mucin stock solution has been prepared at the same way, dissolving it in acetate buffer, final concentration 2 mg/ml. Polymer and mucin have been mixed together at different ratios (0.33:1; 1:1; 3:1; 9:1) and kept stirring for 1 hour at room temperature.

Then, the absorbance (turbidity) at 500 nm of all prepared samples has been measured using a UV spectrophotometer. The absorbance of individual polymers and mucin have been measured as controls and used to give the theoretical values for a non-interacting system.

Also the electrostatic shield effect has been investigated using NaCl. Chitosan and chit-gal were mixed with mucin in acetate buffer (pH 4.7) under the addition of NaCl (concentration 1 M) to analyze the shield effect of Na\(^+\) and Cl\(^-\) on mucin/polyelectrolyte electrostatic interaction.

7.4.2 **Ex vivo** evaluation

Thanks to a collaboration with the university of Innsbruck, in order to evaluate the binding to the mucosa as well as the cohesiveness of our polymers, an appropriate method has been adopted. A tablet for each selected polymer has been prepared and then attached to freshly excised intestinal porcine mucosa, which has been fixed on a stainless steel cylinder (diameter: 4.4 cm; height: 5.1 cm; apparatus 4- cylinder, USP XXII) using a cyanoacrylate
glue. Thereafter, the cylinder was placed in the dissolution apparatus (Erweka DT 700) according to the USP containing 1 L of 0.1 M phosphate buffer pH 6.8 at 37 °C. The fully immersed cylinder was agitated with 100 rpm. The experimental set up is illustrated in figure 8. The detachment of the tablets was determined visually from the beginning until the detachment time.

Different polymers have been tested. Tablets of chitosan and its derivatives (TMC, chit-gal and chit-glc) have been prepared, such as polygalacturonic acid and the two commercial pectins (from apple and from citrus fruit).

![Diagram](image)

**c. cylinder; if, intestinal fluid; m, porcine mucosa; t, tablet**

**Fig.8** Experimental set-up for *ex vivo* mucoadhesive strength
8 Particle preparation

8.1 Ionotropic gelation method

8.1.1 Nanoparticles using chitosan as a polycation

The first batch of particles was prepared using the ionotropic gelation method adapted from previous studies\textsuperscript{117}: chitosan was chosen as a polycation, tripolyphosphate (TPP) as anionic molecule. Two different chitosan molecular weight have been tested: 32 kDa (VLMW) and 150 kDa (LMW).

In the formulations the chitosan to TPP mass ratios were varied.

A 0.25% w/v chitosan solution was prepared: 6.25 mg of chitosan were dissolved in 2.5 ml of 0.05% v/v acetic acid solution for 24 hours and the pH was adjusted to 5.5 using a sodium hydroxide solution. The solution was than diluted in deionized water, total volume 5 ml. For the anionic solution, Tripolyphosphate (TPP) was dissolved in deionized water at a concentration of 2.5 mg/ml and subsequent diluted in deionized water. The TPP and chitosan solutions were subsequently filtered through a 0.45 µm membrane (Millipore) to remove any insoluble matter. Then, TPP solution was added to chitosan solution drop wise at different TPP:chitosan ratios under vigorous magnetic stirring at room temperature.

Preliminary experiments were performed with the objective of identifying the optimal concentrations (ratios) of chitosan and TPP for nanoparticle formation (identifiable with the opalescence of the suspension). The formation of nanoparticles was confirmed by particle size analysis (Malvern Zetasizer, Malvern, UK).

To remove the excess of unreacted chitosan, the suspension was centrifuged for 2 hours at 3270 RCF (Relative Centrifugal Force) and the
supernatant was discarded. The particles were resuspended in deionized water, analysed in size and surface charge and lyophilized for further analysis.

8.1.1.1 Determination of chitosan assay

Although there are a large number of studies on chitosan nanoparticle formation in relation of different polymer amounts used, very few of these studies incorporated the yield results. A faster determination of chitosan in aqueous solutions could be obtained thanks to a colorimetric protocol set-up by Muzzarelli in 1998. The method is based on chitosan ability to adsorb dyes: protonated amino groups of chitosan can act as cationic sites for anionic dyes. As dye Cibacron Brilliant Red 3B-A has been selected: after interaction with chitosan at 20 °C a sharp band at 572 nm can be observed. A solution of the dye was prepared by dissolving the powder (150 mg) in unionized water (100 ml). Aliquots of the dye solution (5 ml) were made up to 100 ml with 0.1 M glycine hydrochloride buffer. The final concentration of the dye was 0.075 g/L. The buffer solution was prepared by dissolving glycine (1.87 g) and sodium chloride (1.46 g) and made up to 250 ml with unionized water. Aliquots (81 ml) of this solution were made up to 100 ml with 0.1 M HCl. The final pH was about 3.

Standards solutions of chitosan have been prepared starting from a 0.5 mg/mL stock solution (0.05% acetic acid) adopting the follow procedure: 15, 30, 45, 60, 80, 100, 150, 200, and 250 µL of chitosan solution were introduced into test tubes, followed by different volumes of buffer, to reach 300 µL. Then, aliquots of dye solution (3 mL) were added to each. Then every sample have been shacked using a vortex and subsequently the absorbance were measured with a UV-Vis Cary 4E® spectrophotometer. The chosen wavelength was 575 nm. Buffer (0.3 ml) and dye (3 ml) solutions were used to prepare the reference solution. The absorbance values were measured at 575 nm with a UV-Vis Cary 4E® spectrophotometer provided with Cary Win UV™
software. Buffer (0.3 ml) and dye (3 ml) solutions were used to prepare the reference solution.

8.1.2 Nanoparticles using trimethyl chitosan (TMC) as a polycation

Trimethyl chitosan (TMC) has been chosen to prepare the second batch of nanoparticles using the same method described before. The formulations were prepared by variation of different parameters such as trimethyl chitosan/TPP ratio and TMC solvent used.

The production of TMC involves the trimethylation of the primary amino group in chitosan. With low conversion TMC, a one step reaction is used to convert chitosan to TMC, whereby approximately 35% of the amine groups are trimethylated, and therefore the conversion from chitosan to TMC is fairly low. With high conversion TMC on the other hand, a two step reaction is used to convert chitosan to TMC, whereby at least 60% of the amine groups are trimethylated. In this case, the conversion from chitosan to TMC is high.\textsuperscript{128}

A 0.25% w/v TMC solution was prepared: 6.25 mg of TMC were dissolved in 2.5 ml of 0.05% v/v acetic acid solution for 24 hours and the pH was adjusted to 5.5. The solution was then diluted in deionized water, total volume 5 ml. For the anionic solution, Tripolyphosphate (TPP) was dissolved in deionized water at a concentration of 0.25% and subsequent diluted (final concentration 0.25 mg/ml). The TPP and TMC solutions were subsequently filtered through a 0.45 µm membrane (Millipore) to remove any insoluble matter. TPP solution was added to cationic solution drop wise at different TPP:TMC ratio under vigorous magnetic stirring at room temperature.

According to chitosan nanoparticle preparation, preliminary experiments were performed with the objective of identifying the optimal ratios between TMC and TPP for nanoparticle formation, confirmed by particle size analysis (Malvern Zetasizer, Malvern, UK).
To remove the excess of chitosan, the suspension was centrifuged for 2 hours at 3270 RCF and the supernatant was discarded. The particles were resuspended in deionized water, analysed in size and surface charge and lyophilized for further analysis.

Different TMC solvents (pure water and acetic acid solution) have been tested in order to understand polymer behaviour during nanoparticle formation.

8.1.3 Nanoparticles using chitosan and glycosylated derivatives as polycation

The third batch of nanoparticles was prepared using a blend of chitosan and glycosylated chitosan derivatives as polycation and TPP as polyanion.

The nanoparticles were prepared by variation of formulation parameters such as chitosan degree of substitution, cationic blend composition, cationic blend:TPP ratio and cationic solvent.

The production of glycosylated chitosan consist of a modification by grafting of sugar moieties via a reductive N-alkylation. Different degree of substitution can be obtained.

A 0.25% w/v chitosan solution was prepared dissolving it in 0.05% v/v acetic acid solution for 24 hours; galactosylated chitosan solution was prepared dissolving it in deionized water for 24 hours. Both pH were then adjusted to 5.5 using a sodium hydroxide solution. A blend of them has been prepared and it was then diluted in deionized water, total volume 5 ml. For the anionic solution, Tripolyphosphate (TPP) was dissolved in deionized water at a concentration of 0.25% and subsequent diluted in deionized water. Both solutions were subsequently filtered through a 0.45 µm membrane (Millipore) to remove any insoluble matter before the TPP solution was added to the chitosan. TPP solution was added to cationic blend drop wise under vigorous magnetic stirring at room temperature.

According to chitosan nanoparticle preparation, preliminary experiments were performed with the objective of identifying the optimal ratios between
cationic blends and TPP for nanoparticle formation, confirmed by particle size analysis (Malvern Zetasizer, Malvern, UK).

To remove the excess of unreacted polymer, the suspension was centrifuged for 2 hours at 3270 RCF and the supernatant was discarded. The particles were resuspended in deionized water, analysed in size and surface charge and lyophilized for further analysis.

According to previous TMC studies, only deionized water has been used as solvent, instead of acetic acid solution: this is due to acetic acid interference during spectroscopic analysis, especially Raman analysis.

8.2 Association with other biopolymers

Chitosan exhibits a pH-sensitive behaviour as a weak polybase due to the large quantities of amino groups on its chain. It is insoluble at higher pH ranges while it dissolves easily at low pH. Because the matrix gets dissolved in the stomach, the released protein drugs will get denatured. To overcome this, chitosan modifications can be done to improve its stability or an association with other polymers, as pectin, can be done. Thus it can perform controlled protein release at higher pH of intestine instead of rapidly releasing the protein drugs by rapid dissolution in the stomach.

Two different strategies have been followed: a particle coating and a new particle formation method, the blending.

8.2.1 Particle coating

A coating of produced particles has been done. Different pectins have been used, polygalacturonate acid and two commercial ones, one derived from
citrus fruit and the other derived from apples. The investigated parameter was pectin/nanoparticle mass ratio.

Pectin was dissolved in deionized water for 24 hours, concentration 2.5 mg/ml, and pH was then adjusted at 6.3. The solution was subsequently filtered through a 0.45 µm membrane (Millipore) to remove any insoluble matter. The solution was then diluted in deionized water, total volume 5 ml. Nanoparticles were redispersed after centrifuge in 5 ml of deionized water using ultrasound. Nanoparticles suspension was added to pectin solution drop wise under vigorous magnetic stirring at room temperature. An intense electrostatic interaction between positively charged chitosan nanoparticles and negatively charged pectin will lead to particle coating. The coating will invert the surface charge of the particles from positive to negative values. The present coated nanoparticles presumably have the same surface properties as pectin nanoparticles.

To remove the excess of unreacted polymer, the suspension was centrifuged for 2 hours at 3270 RCF and the supernatant was discarded. The particles were resuspended in deionized water, analysed in size and surface charge and lyophilized for further analysis.

8.2.2 Polyelectrolyte complexation (Blending)

The new batch of formulations was prepared using the blending method described by Goycoolea.\textsuperscript{170}

Chitosan was dissolved in 0.05% v/v acetic acid solution at the final concentration of 2.5 mg/ml for 24 hours, the pH was subsequently adjusted to 5.5 and the solution was then diluted in deionized water. For the anionic solution, a blend of TPP and pectin from apples has been prepared and it was diluted in deionized water, total volume 5 ml. Both tripolyphosphate (TPP) and pectin from apples were dissolved in deionized water at the concentration of 2.5 mg/ml; pectin pH was subsequently adjusted to 6.3. All solutions were previously filtered through a 0.45 µm membrane (Millipore) to remove any
insoluble matter. Chitosan solution was added to anionic blend drop wise under vigorous magnetic stirring at room temperature. The complexation takes place when the constant-positively charged amino groups of chitosan electrostatically interact with the negatively charged carboxylic groups of pectin. Moreover, the presence of TPP should act cooperatively, favouring the incorporation of chitosan in a more compact structure.

To remove the excess of unreacted pectin, the suspension was centrifuged for 2 hours at 3270 RCF and the supernatant was discarded. The particles were resuspended in deionized water, analysed in size and surface charge and lyophilized for further analysis.
9 Particle characterization

9.1 Size and surface charge

The size of the nanoparticles and the value of the surface zeta potential (\(\zeta\)) were determined using a Malvern Zetasizer nano ZS (Malvern Instruments, Worcestershire, U.K.). This instrument is based on the dynamic light scattering with a non-invasive back-scatter (NIBS) technology for size determination and a mixed laser Doppler anemometry and a second generation Phase Analysis Light Scattering (M3PALS) for zeta potential determination.

The diluted samples were then placed in disposable polystyrene cuvettes and DLS measured at 25 °C, 298 K, with a scattering angle of 173°.

The Malvern Zetasizer is capable of measuring particles in the range of 0.6 nm to 6 µm. DLS involves the measurement of the intensity fluctuations in the light scattered from particles. These fluctuations occur due to the random Brownian motion of such colloidal particles.

As the laser beam is focused onto particles suspension, the laser beam is randomly scattered by the particles. The intensity of this beam is measured by a photomultiplier which subsequently produces a single electrical pulse for each photon detected. Due to the Brownian motion of the particles, the intensity of this scattered light fluctuates as the particles move erratically in and out of the beam. Values were represented as z-average diameter (Z Ave), standard deviation (SD) and polydispersity index (PDI).

Particles possess electrical charge due to the ionization of surface groups, or due to the adsorption of ions to the surface. Such a charge results in the production of a potential surrounding the particle which is concentrated nearer the surface, and diminishes with increasing distance into the surrounding dispersion medium. When placed in an electrical field, the charged particle will shift with a characteristic velocity.
Zeta potential measurements involve the determination of this velocity in a known electrical field using the Doppler shift of electrical light. Light from a source is scattered off the particles and detected. If the particle is stationary, which it is not in this case, the scattered light will have the same frequency as the incident light. If the particle is moving, the frequency of the light scattered off the particle is shifted by the Doppler effect.

9.2 Particle morphology: Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM)

Chitosan nanoparticles were observed by transmission electron microscope (TEM) using a Philips EM 208 microscope. All samples for TEM were prepared by putting a single drop onto a carbon-covered 200-mesh copper grid. After air-drying overnight at room temperature, both particle and raw materials were observed.

Spray dried chitosan nanoparticles were observed by scanning electron microscopy (SEM) (Phillips XL30 SEM, Eindhoven, Nederland). The dry powder was placed onto an aluminium specimen stub covered with a double-sided carbon adhesive disc (Taab, Berks, UK) and then analysed using SEM.

9.3 Spectroscopic analysis

9.3.1 IR-ATR studies

IR spectra (range 5000-600 cm\(^{-1}\)) were recorded on lyophilized samples using a Vertex 70 (Bruker Optics GmbH) spectrophotometer (spectral
resolution of 4 cm\(^{-1}\) equipped with a MIRacle\textsuperscript{TM} ATR devices (Pike Optics) with a single reflection diamond crystal (1.8 mm spot size) and using a MCT detector (HgCdTe, mercury-cadmium-tellurium) cooled with liquid nitrogen (Figure 9 a-b).

![Fig.9 (a) ATR-FT IR instrument used for the acquisition of spectra. (b) Graphic representation of MIRacle\textsuperscript{TM} accessory (Pike Optics) with a single reflection diamond crystal.](image)

Both raw materials and formulations have been analyzed. A first attempt has been made using a spectrophotometer FT-IR System 2000R (Perkin Elmer) micronizing the samples into a fine powder and dispersing them in nujol. Considering the difficulty in obtaining an homogenous dispersion, the samples were then analyzed with the technique of attenuated total reflectance (ATR), which gives the possibility to analyze directly solid samples with minimal preparatory operations.
The samples were deposited on the top of a diamond crystal, a material with high refractive index for infrared radiation, and stopped with a high-pressure clamp.

At each reflection, the beam penetrates a fraction of µm in the sample and a small extent of radiation is absorbed. After some reflection, the decrease in the intensity of the beam is sufficient to be detected by the spectrophotometer, giving an IR spectrum in attenuated total reflectance (ATR).

9.3.2 Raman spectroscopy studies

Measurements were performed in collaboration with the micro-Raman spectroscopy laboratory at Department of Industrial and Information Engineering, University of Trieste. The measures were performed by MicroRaman Renishaw InVia. The light source was a Laser High Power NIR 785 nm with power on the sample of 90 mW. The acquisition times were between 10 and 60 seconds. The samples were prepared putting a drop of particles suspension on a fluorite slab and left drying.
10 Particle storage

10.1 Lyophilization

If nanoparticles are stored as aqueous suspensions, degradation and/or solubilisation of the polymers, drug leakage, drug desorption, and/or drug degradation may occur. Lyophilisation probably represents one of the most useful methodologies to ensure the long-term conservation of polymeric nanoparticles. This technique involves the freezing of the suspension and the subsequent elimination of its water content by sublimation under reduced pressure. After the complete desiccation, nanoparticles are obtained in a dried form that is easy to handle and to store. Dried nanoparticles are usually readily redispersible in water without modification of their physicochemical properties.

In some cases, full redispersion of the system may be difficult to achieve: the freezing and dehydration processes induce aggregation and in some cases irreversible fusion of nanoparticles. Furthermore, the crystallization of ice may exercise a mechanical stress on nanoparticles leading to their destabilization.

This problem can be circumvented by desiccating these systems in the presence of an appropriate lyoprotective agent adding it before freezing to protect these fragile systems.\textsuperscript{171}

The most popular cryoprotectants encountered in the literature for freeze-drying nanoparticles are sugars: trehalose, sucrose, glucose and mannitol. Sugars are known to vitrify at a specific temperature denoted Tg.\textsuperscript{172} The immobilization of nanoparticles within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against the mechanical stress of ice crystals. The mechanisms by which sugars protect particles during lyophilisation are frequently referred to as “poorly understood” in literature. However, it is commonly suggested that sugar may interact with the solute of interest though hydrogen-bonding. As a result, the solute might be maintained in a “pseudo hydrated” state during the dehydrating step, and would therefore
be protected from damage during dehydratation and subsequent rehydratation. Such a protective interaction is made possible by the ability of the sugars to remain amorphous during freeze-drying.

All formulations were freeze-dried. In particular, we focus our attention on chitosan NPs analysing the effect of three different lyoprotectants, trehalose, mannitol and PEG on particle size and surface charge. Five different amounts of protectants have been added to particles samples before freezing them; the lyophilized particles were then retained for further sizing and zeta potential measurements.

10.2 Spray drying

Spray drying is a method for collecting particles that is reproducible, rapid and easy to scale up. It is a process that transforms the material from a fluid state into a dried particulate form by spraying it into a hot drying medium. It is a one-step, continuous, particle-drying process. The material could be in the form of a solution, suspension, emulsion, or paste. The resulting product can be powder, granular, or agglomerate particles, depending upon the physical and chemical properties of the feed material, the drier design, and its operation.

Spray drying involves atomization of the suspension into a drying medium, resulting in the rapid evaporation of the solvent and the formation of dried particles. In most cases, air is used as the spray-drying medium. Contact with the spray-drying medium causes evaporation of the solvent from the droplet surface. The evaporation is rapid due to the vast surface area of the droplets in the spray.

A spray dryer consists of a drying chamber with temperature controlled shelves, a condenser to trap water removed from the product, a cooling
system to supply refrigerant to shelves and condenser, and a vacuum system to reduce the pressure in chamber and condenser to facilitate the drying process. The time required to dry the droplets depends on the residence time of the droplet in the gas phase, which, in turn, is determined by the geometry of the chamber, carrier gas flow rates, temperature and pressure. Powder collection can be achieved using a cyclone or filter bag.

In this Thesis a new kind of sprat dryer has been used, the BUCHI Nano Spray Dryer B-90. The peculiar characteristics of this instrument are the droplet generation and the collection of the product.

Regarding droplet generation, atomization is a process that breaks up the bulk liquid into million of individual spray droplets. The energy necessary for this process is supplied by centrifugal force (rotary atomizer), pressure (pressure nozzle) or kinetic (two-fluid nozzle). In the Nano Spray Dryer B-90 (figure 10) the droplet generation is based on a piezoelectric driven actuator: the actuator is driven at an ultrasonic frequency, causing the perforated, stainless steel membrane to vibrate, ejecting millions of precisely sized droplets every second with very narrow droplet size distribution.

![Schematic illustration of the Nano Spray Dryer B-90.](image)

**Fig.10** Schematic illustration of the Nano Spray Dryer B-90.
Different micron-size holed membranes can be used to obtain the desired characteristics of the final product.

As particle collector, an electrostatic cylinder is used instead of classic cyclones. Thanks to this, it can collect fine nanoparticles with high product yields (up to 90 %) allowing to work with small sample quantities.

Also in this case, cryoprotectants are added to the formulation to protect the nanoparticles from drying stress: trehalose, mannitol and PEG are added. The attention was focused on analysing the effect of lyoprotectants on particle size and surface charge. The same amount of protectants has been added to particles samples before spray drying and size has been measured. The dried particles were then retained for further sizing, zeta potential measurements, and SEM analysis.

The spray cap with 4.0 µm mesh hole size has been used in order to obtain the smallest droplets and solid nanoparticles possible. The inlet temperature was set up at 120 °C.
11 Loading capacity

11.1 Loading of nanoparticles with different model drugs

Chitosan nanoparticles have been loaded with three different proteins: bovine serum albumin (BSA), ovalbumin (OVA) and human insulin (HI). Their pI are respectively: 4.8, 4.7 and 5.6.

Different batches were prepared by adding different volumes of a stock solution (4 mg/ml) varying of final protein concentration (200 µg/ml, 400 µg/ml and 600 µg/ml) in order to study the different loading efficiency and characterization value.

A 2.5 mg/ml chitosan solution was prepared dissolving 6.25 mg of chitosan (150 kDa) in 2.5 ml of 0.05% v/v acetic acid solution. The solution was stirred for 24 hours and the pH was adjusted to 5.5 with a sodium hydroxide solution. Selected peptide was dissolved in deionized water and subsequently added directly in the chitosan solution under gently magnetic stirring. The solution, containing chitosan and protein, was then diluted to a total volume of 5 ml in deionized water. For the anionic solution, Tripolyphosphate (TPP) was dissolved in deionized water at a concentration of 2.5 mg/ml and subsequent diluted in deionized water (final concentration 0.25 mg/ml). The TPP and chitosan solutions were filtered through a 0.45 µm mixed cellulose esters membrane (Millipore) to remove any insoluble matter before the drop wise addition of the TPP solution to the chitosan under vigorous magnetic stirring at room temperature. The dispersion was left under constant stirring for about 30 min at room temperature, in order to leave protein to be chiefly loaded on the particles and obtain a homogeneous dispersion.

To remove the excess of chitosan and protein, the suspension was centrifuged for 2 hours at 3270 RCF. The supernatant was collected separately for further investigation, while the precipitated particles were resuspended in deionized water, analysed in size and surface charge and lyophilized for further analysis.
BSA and OVA have been directly added to the chitosan solution, while the HI was added to the TPP solution and then dripped. Indeed, HI has a pI=5.6 and it has a neutral net charge in the chitosan solution (pH 5.5) leading to precipitation. So HI was added to TPP solution (pH 7.5) and then dripped to cationic solution.

### 11.2 Purification protein loaded nanoparticles

The supernatant obtained after centrifugation of the nanoparticles’ suspension was then used to quantity the protein remained in solution and therefore calculate the loading efficiency.

The amount of protein loaded in nanoparticles was calculated as the difference between the total amount initially added to the system and the protein recovered in the supernatant, assuming that the share not found in the supernatant was encapsulated in the particles. The supernatant of unloaded nanoparticles was used as a blank, in order to subtract the interference of the formulation components with the BCA™ and Bradford assays.

### 11.3 Loading efficiency

#### 11.3.1 BCA™ protein assay

The bicinchoninic acid (BCA) assay is a rapid, sensitive and reproducible method of protein determination. The BCA™ allows accurate detection of low protein concentrations between 10 µg/ml up to 2 mg/ml.
Essentially, the assay is based on the reaction of protein with an alkaline copper II (Cu$^{2+}$) reagent (Reagent B) to produce copper I (Cu$^{1+}$) (Fig. 9). This Cu$^{1+}$ species further reacts with two molecules of 4,4’–dicarboxy-2, 2’biquinoline (bicinchoninic acid, Reagent A) to form a copper peptide chelate. The chelation of two BCA molecules with one Cu$^{1+}$ ion results in the colour change from green to purple.$^{173}$ The product of the reaction is water-soluble and has an intense purple colour, which can be quantified by spectrophotometric measurement at a λmax of 562 nm, which does not overlap with the polymer spectra.

![Schematic reaction for BCA protein assay.](image)

**Fig. 11** Schematic reaction for BCA protein assay.

Six BSA standards with different concentrations were prepared using a known quantity of protein. The same procedure was also used with formulations loaded with OVA and HI.

BCA™ Working reagent was prepared according to the manufacturer’s protocol (PIERCE, USA), by mixing 50 parts of BCA™ Reagent A with 1 part BCA™ Reagent B. 25 µl of each standard and sample were placed into a 96-
well microplate and 200 µl of Working reagent was added. Each sample was repeated three times.

The microplate was incubated at 37 °C for 30 minutes after being thoroughly mixed for 30 seconds by a microplate shaker. The microplate was then cooled to room temperature, and the absorbance measured on a plate reader (PIERCE, USA). The absorbance was calculated by subtracting to the average absorbance of each formulation in the BCA-protein assay the absorbance of a blank formulation not loaded with the model protein but prepared in the same way using the following equation:

\[
\text{Sample absorbance} = \text{Ave absorbance of sample} - \text{Ave absorbance of blank}
\]

From the calibration curve generated using the protein standards, the protein concentration of each sample was calculated. The amount of protein loaded to the particles was calculated by subtracting the amount of unloaded protein remained in the supernatant from the total amount of the protein added. The loading efficiency (LE) of the nanoparticles was calculated using the following equation:

\[
\text{LE } (\%) = \frac{(\text{Total amount of protein}) - (\text{unloaded protein})}{(\text{Total amount of protein})} \times 100
\]

### 11.3.2 Bradford assay

Another colorimetric assay to determine the protein concentration is the Bradford assay. The Bradford assay is suitable for determining the protein content of microparticulate formulations: it consists on the formation of a
complex between the acidic dye Brilliant Blue G and proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye with is proportional to the protein present.

The test carries out by mixing 1 part of sample with 30 parts of Bradford reagent, obtaining a final volume of 3.1 ml. Six protein standards with different concentrations were prepared using a known quantity of protein. 0.1 ml of each sample (standard, blank and loaded nanoparticles supernatant) were put in a cuvette and then 3 ml of Bradford reagent was added. Samples have been prepared in triplicate. The samples were incubated at room temperature for 10 minutes to reach the endpoint (cuvettes containing protein shifted to blue) and then the absorbance was measured using a UV-Vis Cary 4E® spectrophotometer.

As for BCA assay, also for Bradford assay the effective absorbance of each sample was calculated by subtracting from its average absorbance the average absorbance of the corresponding blank.
12 Biocompatibility

12.1 *In vitro* tests: XTT / LDH assays

The viability of the Caco-2 cells (ATCC number HTB-37, human epithelial colorectal adenocarcinoma cells, used as model of intestinal epithelium) in the presence of both raw polymer materials (sodium pectate from apple, low molecular weight chitosan and chitosan-galactose) and biopolymeric nanocarriers were evaluated by standard *in vitro* tests. In particular, the XTT assay, a test for the detection of the cell metabolic activity, and the LDH assay, that measures the cell membrane integrity, have been employed.

The XTT test (Tox-2 protocol, Sigma-Aldrich) was used to evaluate the effects of the samples on the mitochondrial enzyme activity of the cells. Cell viability is evaluated by measuring the reduction of the sodium salt of XTT (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxy-anilide inner salt) by the mitochondrial dehydrogenase in living cells. These enzymes reduce the tetrazolium ring of XTT yielding an orange, water soluble formazan derivative (the original XTT solution is yellow). The absorbance of the resulting orange solution is measured by means of spectrophotometry.

Using sterile 96-well tissue culture plates (5000 cells/well), 100 µl of polymer solutions or nanoparticle suspensions in complete Eagle’s MEM cell medium were incubated in triplicate (95% relative humidity, 5% CO₂) for 24 h. The concentration of the samples assayed were: 0,02% and 0,04% (w/v) for both solutions and suspensions (conditions employed for comparison with tests described in literature on similar formulations).¹⁷⁴ The TPP salt used for the preparation of chitosan NPs was also assayed (0,01% w/v). Stock solutions (1% v/v) of polymers were prepared in the suitable buffers (1% v/v acetic acid for chitosan, deionized water for pectate and modified chitosan).

After dissolution, pH was adjusted to 6.5 when needed. The solutions were then diluted to the final concentrations in complete cell medium, heated
and sonicated when necessary, in order to completely dissolve the polymers. Immediately prior to incubation with the cells, the solutions were aseptically filtered. The nanoparticle suspensions have been prepared from sterile formulations diluted in the cell medium.

The XTT solution (20 µl, 5 mg/ml in Eagle’s MEM) was added to each well. The absorbance values were measured at 450 nm using a microplate reader after 4h of incubation at 37 °C. The relative cell viability (%) related to control wells containing untreated cells in culture medium was calculated by equation:

\[
\% \text{ cell viability} = \frac{[A] \text{ test}}{[A] \text{ control}} \times 100
\]

Triton X100 (0,1% v/v) has been also used as positive control.

The viability assay results have been implemented with the lactate dehydrogenase (LDH) release assay, carried out using the same experimental conditions of the XTT assay, according to the Tox-7 procedure (Sigma-Aldrich). The LDH release assay measures the membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The assay is based on the reduction of NAD\(^+\) in NADH by LDH. NADH is then used for the conversion of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically. The LDH release (%) related to control wells containing cells treated with a lysis buffer was calculated by equation:

\[
\% \text{ LDH release} = \frac{[A] \text{ test}}{[A] \text{ control}} \times 100
\]
12.2 *In vivo* test: the chick chorioallantoic membrane (CAM)

One important step before evaluating the efficacy of drug delivery system is the evaluation of the biocompatibility and toxicity of both the carrier and the drug. The biocompatibility of materials used is usually evaluated *in vivo* using mammalian models, such as mice, rats, and dogs, after implantation of the material. An alternative to the use of mammals is the use of flier embryos, in particular chick ones. The use of chick embryo is a promising substitute, even though it can’t be considered an *in vivo* mammalian model.

The chick embryo is a well-known animal model, which has been extensively studied from Aristotle's time until the modern molecular era. Egg embryo had been used for scientific experimentation since almost a hundred years ago for the implantation of tumor cells.\(^{175}\)

The increasing interest in the chick embryo as a model in biological and pharmaceutical research is related to its simplicity and low cost compared with mammalian models. In addition, current laws regulating animal experimentation in the USA, the European Union, and Switzerland allow experimentation with chick embryos without authorization from animal experimentation committees, on the grounds that experiments begin and end before incubation day 12. From these endeavors, the use of chick chorioallantoic membrane (CAM) was developed and validated as an alternative to mammalian models.

Three extraembryonic membranes protecting and nourishing the embryo are formed during development: the yolk sac membrane, the amnion, and the CAM. The latter is a transparent and highly vascularized membrane, formed during the embryo development day 4 to 5 by the fusion of the mesodermal layers of both the allantois and the chorion, resulting in a highly vascularized mesoderm composed of arteries, veins, and an intricate capillary plexus (figure 12 a-b)\(^{176}\) The main function of the CAM is to serve as the respiratory organ for the embryo. The CAM also plays a role in the storage of excretions, electrolyte transport (sodium and chloride) from the allantoic sac, and mobilization of calcium from the shell to start bone mineralization. It is connected to the embryonic circulation by the allantoic arteries and veins, which are associated with lymphatic vessels.\(^{177}\)
There have been several reports on the use of CAM for assessing the effects of different applications in pharmaceutical field: such as angiogenesis and antiangiogenesis\textsuperscript{178}, wound healing\textsuperscript{179}, tissue engineering\textsuperscript{180}, biomaterials and implants\textsuperscript{181}, and biosensors\textsuperscript{182}. The CAM can be used to develop pharmaceutical formulations of various drugs, including testing of biologically
active drugs, investigations on the effects of hydrophilicity and lipophilicity of drugs, drug transport studies and preformulation screenings for toxicity and efficacy of delivery systems.\textsuperscript{183,184}

Toxicity of drugs or carriers on chick embryos can be evaluated in terms of embryo death or adverse effects on the CAM, including inflammation and neovascularization.

Valdes et al. found that both acute and chronic inflammatory responses of the CAM to biomaterials are similar to those found in mammals.\textsuperscript{185}

Chick embryo culture has been developed in two different approaches: “in ovo” method, where the embryos are left inside the eggshell during the assay, and “ex ovo” method, where the embryos are cultivated in recipients simulating the eggshell. The first one has been chosen. Fertilized eggs are first disinfected with alcohol before placing the in the egg incubator at 37.5 °C with 60% humidity. At incubation day 4, a window opening is punctured at the blunt end of the egg; the opening is then covered with a polyethylene film glued with the egg albumen to avoid contamination and the egg is returned to the incubator for further incubation. The incubation day 6 solid samples were applied directly to the CAM and then the egg is incubated again. Raw materials and nanoparticles have been tested. A standardized image procedure has been used to allow subsequent image analysis of sample effect: a stereomicroscope Leica WILD M32 (equipped with lens WILD PLAN 1X) connected to a camera system Leica DFC 320 has been used. The egg was placed at fixed location in an egg holder. The camera and the magnification used have been fixed to provide consistent image capture. Images before and after sample adding have been acquired. After 24 hours all eggs have been examined again and all acquired images have been compared with the previous.
Results & Discussion
Premise

The large variety of systems studied in this work and the many different methodology employed make difficult the presentation of the results without a continuous cross-reference of sections and experimental results. It has been necessary, therefore, to follow an arbitrary order of presentation which proceeds from the simple to the more complex system and to risk therefore some unavoidable repetitions in the particular methods used.

Thus, this chapter of results contains altogether comments and annotations that would have been more correctly allocated in a separate chapter entitled “Discussion”.

The results are presented in the following order. First, the characterization is reported of the chitosan samples and of the new polymer derivatives specifically prepared for developing new nanoparticles. Second, the set-up of conventional chitosan nanoparticles obtained by ionotropic gelation with tripolyphosphate is described, before the characterization of the nanoparticles is analyzed with the goal of fully mastering the process in view also of a scale-up. Then, the preparation of nanoparticles by using chitosan derivatives is described and their basic characterization is given. A further chapter is devoted to the question of the addition of another polyelectrolyte negatively charged, either in blended or coated mode, with the scope of improving the resistance in the gastro-intestinal tract and the mucoadhesion of the nanoparticles.

Finally, the last chapters deal with the characterization of the materials and of the nanoparticles by means of spectroscopic methods and with the assessment of “nano-safety” of the present systems. The first topic has been addressed with the possibility in a near future of mapping the chemical distribution of the polymers in the nano- or micro-particles. The second point is an important issue that needs consideration for both the material used and for the potential hazard of the nanoparticles in terms of size, shape and surface properties.
13 Polymer characterization

13.1 NMR

*Degree of acetylation of unsubstituted chitosan*

The degree of acetylation of chitosan was determined by NMR, according to previously reported methods. The O-Ac group is easily recognizable at 2.1 ppm 1D spectrum of chitosan recorded at room temperature and can be integrated (figure 15). Thus, the chitosan sample used in all the experiments of the present work has a degree of acetylation of 13%, which is slightly higher of that determined in other samples from the same source for which the residual acetylation degree was 11.3%.

*Degree of substitution of glycosylated chitosan*

The glycosylated samples were prepared with a reaction that is considered almost stoichiometric according to the methods described in the literature. The $^1$H,$^{13}$C-HSQC spectrum of glycosylated chitosans recorded at 300 K are reported in figure 13 (a) (left, maltose derivative; right, lactose derivative). The glycosil reaction introduces a pendant made by a glucose (or galactose) unit linked to carbon 4 of the open second sugar (glucose) bound via carbon 1 to the amino group. The spectra show small differences for all signals of the pendant groups excepts for the carbon 4 and the carbon 5, that reflect changes from the glucose to galactose moieties (figure 13 (b)). Also, the substitution of glucose and galactose appears comparable with value of 42.2 % and 49.8 %, respectively. The same spectra provide an average value of residual acetylation of the chitosan of 13.2%, which is consistent with that determined on the parent polymer (13%).
Fig. 13 (a) $^1$H, $^{13}$C-HSQC spectrum of glycosylated chitosans recorded at 300 K (left, maltose derivative; right, lactose derivative); (b) Structure of glycosylated chitosan: the arrows refer to the position that discriminate glucose to galactose moieties.

**Degree of acetylation and methylation of methyl-chitosan**

Some more complexity has been the analysis of the methyl-chitosan sample, since the methylation can occur to a different extent (monomethyl-, dimethyl- and trimethyl- substitution on the free amino groups, but also on the hydroxyl groups at carbons 3 and 6. Indeed, six unknowns are in principle to
be evaluated. First, hydrogen and carbon assignment was accomplished by a series of 2D spectra, namely: 2D-NOESY, 2D-COSY, 2D-TOCSY, $^1$H,$^{13}$C-HSQC, 2D-$^1$H,$^1$H-TOCSY, $^1$H,$^{13}$C HSQC, and 2D-$^1$H,$^{13}$C-HMBC. The complete assignment is reported onto the $^1$H,$^{13}$C-HSQC spectrum (figure 14).

The assignment of the peak at 2.6 ppm/41.2 ppm, placed half-way from what reported in literature for the mono- and di-methylated forms, was based on chemical shift simulation (ChemDraw software) reporting the values of 3.26 ppm/34.6 ppm for the mono-methylated and 2.26 ppm/46.2 ppm for the dimethylated forms. Furthermore, the observed splitting in the proton dimension (but not in carbon) is more likely to arise from two non-equivalent methyls of the N(CH$_3$)$_2$ moiety rather than from two forms of a single N(CH$_3$)$_2$.

**Fig.14** $^1$H,$^{13}$C-HSQC spectrum of methylated chitosan recorded at 300 K.
group. Finally, since a quite strong peak is detected for the tri-methylation, this would imply a larger probability of dimethylation rather than mono-methylation.

The full assignment of the resonances enables us to confidently estimate the degree of acetylation DA and of methylation by the analysis of 1D $^1$H NMR, as all the important signals are well resolved in the proton dimension (figure 15).

**Fig. 15** 1D spectrum of methylated chitosan recorded at 300 K.

By neglecting the small contribution (if any) of monomethylation on the amino group, the degree of acetylation DA, trimethylation TM, dimethylation DM and methylation in position 3 (3-OM) and 6 (6-OM), can be calculated as follows:
The calculation based on this set of equations 1-5 leads to some unrealistic results as the sum of TM, DM and DA is more than 100%. The very different mobility of anomeric protons with respect to methyl protons is in fact expected to influence the relaxation properties underestimating the integral of H1 protons. For this reason we solved the set of equations by relying on the protons of methyl substitution and implying the knowledge of DA. The value of DA was thus introduced as that determined on the parent chitosan, as reported above (% DA = 13 %). By using this value, the methyl substitution values (table 1) show that the reaction yielded a complete methylation of the polymer with a large percentage of tri-methylation (ca 71%). Interestingly, both free OH groups appear methylated with a slightly higher value in favor of the more accessible group on carbon 6.

<table>
<thead>
<tr>
<th>TM %</th>
<th>DM %</th>
<th>3-OM %</th>
<th>6-OM %</th>
<th>DA %</th>
<th>TM+DM+DA</th>
</tr>
</thead>
<tbody>
<tr>
<td>70.9</td>
<td>18.6</td>
<td>41.6</td>
<td>48.9</td>
<td>13</td>
<td>102.5</td>
</tr>
</tbody>
</table>

Tab.1 Acetylation and Methylation degrees in methylated chitosan.
13.2 Intrinsic viscosity

Using the procedure described in "Materials and Methods" section, the values $\eta_{sp}/c$ and $(\ln \eta_{rel})/c$, derived from experimental data in aqueous solution at 25 °C, were plotted as a function of the concentration in g / dL.

In table 2 are reported the values of $\eta_{sp}$ and $\eta_{sp}/c$, that derived from measurements made on chitosan and chitosan derivatives. Last row reports the values of intrinsic viscosity $[\eta]$ that have been calculated from processing the data at infinite dilution. These values are a measure of the hydrodynamic volume and are typical of each polymer, being also related to the molecular weight and to the chain expansion of the polymer in a given solvent and temperature state.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Chitosan LMW</th>
<th>chit-gal</th>
<th>chit-glc</th>
<th>TMC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\eta_{sp}$</td>
<td>$\eta_{sp}/c$</td>
<td>$\eta_{sp}$</td>
<td>$\eta_{sp}/c$</td>
</tr>
<tr>
<td>0</td>
<td>0.305</td>
<td>3.050</td>
<td>0.242</td>
<td>2.435</td>
</tr>
<tr>
<td>1</td>
<td>0.276</td>
<td>2.988</td>
<td>0.232</td>
<td>2.458</td>
</tr>
<tr>
<td>2</td>
<td>0.234</td>
<td>2.922</td>
<td>0.201</td>
<td>2.401</td>
</tr>
<tr>
<td>3</td>
<td>0.179</td>
<td>2.835</td>
<td>0.164</td>
<td>2.385</td>
</tr>
<tr>
<td>4</td>
<td>0.122</td>
<td>2.749</td>
<td>0.115</td>
<td>2.293</td>
</tr>
<tr>
<td>5</td>
<td>0.082</td>
<td>2.674</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>$[\eta] = \lim_{c\to0} \eta_{sp}/c$</td>
<td>2.52</td>
<td>2.16</td>
<td>2.23</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Tab. 2 $\eta_{sp}$ ed $\eta_{sp}/c$ for different modified chitosans.
However, in the absence of a calibration curve, i.e., the parameters of the Mark-Houwink-Sukurada equation, it is not possible to extract the information of the molecular weight from these data. Also for chitosan, the literature contains some controversial parameters, inasmuch both the molecular weight and the degree of acetylation affect the viscosity behavior and more importantly the extent of some molecular aggregation, which in turn jeopardizes the interpretation of the experimental results. Still, with the crude hypothesis of a common viscosity-molecular weight behavior for the four polymers, the results seems to indicate small differences in the two glycosylated chitosan with respect to the parent polymer, while the TMC samples would appear degraded by a factor of about 2. A possible explanation is due to the reaction conditions that are very mild for glycosilation and surely less mild for methylation condition that is carried out in the presence of concentrated NaOH.

13.3 Size and shape by SEC

In order to get more detailed information of both molecular weight and conformation of polymer chains, the chitosan samples have been further investigated by Dr. Mirko Weinhold, at UFT - Centre for Environmental Research and Sustainable Technology, Bremen.

Chitosan sample were analyzed with a “triple detectors” size exclusion chromatography system (SEC³, Viscotek, USA), consisting of a online temperature controlled triple detector array in sequence with two serially connected ViscoGEL columns. The array is made of a differential refractometer, a right angle (90°) (RALS) and a low angle (7°) light scattering detector and a four capillary, differential Wheatstone bridge viscometer. Thus, the final data were Molecular Weigh (M) and Molecular Weight Distribution (MWD) of fractionated polymer samples, including viscosity of each fraction related to a given polymer concentration. The data allow to extract the weight average Mw and the parameters of MHS equation.
Before discussing these results in detail some general comments are necessary to dissipate the problems mentioned in the preceding section. First, the solubility of all samples (recovery between 82 up to 99%) is rather perfect, while usually, chitosan samples experience the opposite problem with low solubility. The good solubility means that the results are representative for the whole sample. Nonetheless, some aggregates may occur since a shoulder is shown sometime in the molecular weight distribution resulting in a non-linear viscosity dependence. Leaving aside the detailed interpretation of these phenomena, the whole set of results is analyzed in order to collect the data that characterize the polymer samples here used, but also to grasp instructive information about the physico-chemical properties of these polymers that may affect nanoparticle formation.

The values of the parameter calculated according the procedures outlined in the literature (see reference 168) are reported in Table 3. In the table only mean values are given and the RSD% column refers to M, as the most important parameter.

<table>
<thead>
<tr>
<th></th>
<th>RSD%</th>
<th>Mw</th>
<th>Mm</th>
<th>Mw/Mn</th>
<th>[α]</th>
<th>R1</th>
<th>R2</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[%]</td>
<td>[kg mol⁻¹]</td>
<td>[kg mol⁻¹]</td>
<td>[dL/g]</td>
<td>[nm]</td>
<td>[nm]</td>
<td>[%]</td>
<td></td>
</tr>
<tr>
<td>Chitosan</td>
<td>9.9</td>
<td>150</td>
<td>50</td>
<td>3.3</td>
<td>2.57</td>
<td>55.8</td>
<td>35.7</td>
<td>82</td>
</tr>
<tr>
<td>TMC</td>
<td>9.9</td>
<td>85</td>
<td>41</td>
<td>2.5</td>
<td>0.82</td>
<td>11.8</td>
<td>21.8</td>
<td>80</td>
</tr>
<tr>
<td>Ch-Gal</td>
<td>2.1</td>
<td>535</td>
<td>42</td>
<td>4.3</td>
<td>1.7</td>
<td>21.1</td>
<td>50.8</td>
<td>98</td>
</tr>
<tr>
<td>Ch-Glc</td>
<td>1.1</td>
<td>535</td>
<td>42</td>
<td>7.3</td>
<td>2.1</td>
<td>22.5</td>
<td>55.7</td>
<td>98</td>
</tr>
<tr>
<td>VLMN Chit</td>
<td>3.1</td>
<td>32</td>
<td>17</td>
<td>1.9</td>
<td>0.6</td>
<td>6.4</td>
<td>-</td>
<td>94</td>
</tr>
</tbody>
</table>

Tab.3 Physico-chemical quantities obtained from the “triple detector” size exclusion chromatography system.

An example of the data sheet of glycosylated chitosan is also given in Figure 16.
Fig. 16 Classical example of a data sheet for a triple detector SEC. In this case, TMC analysis.

The scrutiny analysis of the data of sample 1 reveals typical detector responses (gaussian shape) for a typical commercial sample. Molecular weight
as well as polydispersity is in the expected range (100-500kg/mol and 2-3 Mw/Mn). Upon methylation (sample 2) a slight degradation can be observed as above commented and as it is also reported in literature. In addition, chain compactness increases which can be seen through a decrease of the viscosity. As far as the glycosylated chitosans are concerned, the most evident result is their overall similarity and, more important, the increase (doubling) in the molecular weight while the viscosity does not increase in the same order. This effect is due to fact that with the reaction a pendant with $M \approx 320$ is added on the chain every two monomeric units, therefore almost doubling the original molecular weight in the absence of degradation. As a consequence, a much more dense structure also results for the glycosylated derivatives.

In conclusion, this part of the polymer characterization provided the basic knowledge for understanding the chain expansion behavior of the chitosans used for the preparation of nanoparticles, with the possibility of better tailoring the nanoparticle properties by selecting the suitable starting polymer.

13.4 Mucoadhesion studies

13.4.1 Ex vivo evaluation

The use of ex vivo animal model could be a solution for verifying mucoadhesive properties of polymers in the gastro-intestinal tract. According to the Pharm. Eur. and the USP, this test model can be considered a very reproducible evaluation system to demonstrate improved adhesive properties. The adsorption of prepared tablets on porcine small intestine was tested by counting the residence time of them to the tissue. The results are shown in figure 17.
Two different families of polymers have been tested: chitosan and its derivatives, and three different type of pectins. Chitosan and poly-galacturonic acid have also been used as controls to investigate the behavior of other samples.

Starting from polycationic samples, the main mechanism of action was suggested to be ionic interactions between positively charged amino groups in chitosan and the negatively charged mucus gel layer (mainly the sialic groups). In the interactions between chitosan and mucus, the primary mechanism of action at the molecular level was found to be electrostatic. The interactions are strong at acidic and slightly acidic pH levels, at which the charge density of chitosan is high. In this case, the pH is 6.8, a value where the chitosan has a very low amount of protonated groups (pka ≈ 6.5): there should be a low electrostatic interaction, while a more significant contribute should be from hydrogen bonding interactions.

A better behaviour is expected for TMC, where quaternarized amino-groups can guarantee a higher protonated polymer chain: in fact a moderate increased time has been observed for TMC. On the other side, a strong increase (from 2.6h to 24-26h) in the immobilization time of the glycosylated
Chitosans has been reported. Such behavior could be explained by considering the interactions with the mucus glycoproteins (and not only ionic interactions) by forming physical entanglements followed by hydrogen bonds with sugar residues on chitosan chains, resulting in the formation of a mucus gel network, which allows the mucoadhesive system to remain adhesive for an extended period of time. Mucoadhesion studies revealed that glycosylated chitosans display significantly higher mucoadhesive properties than the corresponding un-glycosided chitosan.

Chitosan dissolves easily at low pH (stomach) while it is insoluble at higher pH range (intestine) and for the delivery of protein drugs to the intestine this property causes a limitation. To overcome this, chitosan modifications can be done to improve its stability, or a coating with different polymers, as pectins, can solve this limitation. Such polymers are characterized by the presence of carboxyl functional groups that give rise to a net overall negative charge at pH values exceeding the pKa of the polymer. The non-ionized carboxylic acid groups bind to the mucosal surfaces via strong hydrogen bonding interactions.

Different pectins have been selected to assess the mucoadhesive performance, with different molecular weight and degree of esterification (DE).

The results showed that mucoadhesive performances of low DE pectins (poly-galacturonic acid and pectin from citrus fruit) are significantly lower than that of high DE pectin (pectin from apples). The mucoadhesive performance of high DE pectin containing low amount of H-bond forming groups was mainly influenced by its high molecular weight, which facilitates coil entanglement. The mucoadhesion of low DE pectins, however, could be explained by a large amount of H-bond forming groups, which promote secondary chemical bond formation in mucoadhesion process. The results showed that a higher degree of esterification and molecular weight of pectin demonstrated a stronger mucoadhesion.

This result, somewhat, differed from those reported earlier, in which low DE pectin demonstrated a stronger interaction with porcine colonic tissues than high DE pectin. Liu et al. suggested that the higher mucoadhesion of low DE pectin was possibly due to its higher net negative charges than those of high DE pectin, when both pectins were similar in the ratio of molecular weight to molecular size. However, in this study, it is likely that the molecular
size of pectin played an important role on the mucoadhesion of pectin, and probably showed a stronger influence than the number of H-bond forming groups (i.e. –COOH) represented by a lower DE.

Finally, a comparison between the cationic polymers and the anionic ones: analyzing the adhesion times, for poly-galacturonic acid and pectin from citrus fruit a detachment time comparable to the control chitosan has been recorded, while a significant increased time has been observed for pectin from apples, although not comparable with glycosylated chitosan.

13.4.2 In vitro evaluation

Following turbidimetric literature studies\(^{121}\), interaction between cationic polymers and mucin has been studied. Thanks to this, it is possible to verify if an interaction between the two species occurs, even if with weak complex formation, giving formation to different size aggregates and variation of refractive index.

The absorbance (A) of the mixture of polymer:mucin and absorbancies of the individual mucin and the different polymers at 500 nm were measured. In absence of aggregate formation (interaction), the absorbance is an algebraic sum of the value for the individual solutions: the theoretical absorbance (A\(_{\text{Theor}}\)) for the mixture of polymer/mucin system was calculated from the individual absorbancies, according to the following equation:

\[
A_{\text{teo}} = \frac{A_{\text{Pol}} \cdot Pol + A_{\text{Mu}} \cdot Mu}{Pol + Mu}
\]

Where

- \(A_{\text{teo}}\) → theoretical absorbance
- \(A_{\text{Pol}}\) → Absorbance of the polymer
- \(A_{\text{Mu}}\) → Absorbance of the mucin
Pol $\rightarrow$ Chitosan fraction (% w/v)
Mu $\rightarrow$ Mucin fraction (% w/v).

The absorbance difference ($\Delta A$) between the measured and theoretical values for the mixture of polymer/mucin system was also calculated (table 4). If no interaction took place, the value of $\Delta A$ should be zero.

<table>
<thead>
<tr>
<th>% mucin</th>
<th>$\Delta A$ Chit</th>
<th>$\Delta A$ TMC</th>
<th>$\Delta A$ Chit-glc</th>
<th>$\Delta A$, Chit-gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0.0680</td>
<td>0.0477</td>
<td>0.0748</td>
<td>0.0346</td>
</tr>
<tr>
<td>50</td>
<td>0.2200</td>
<td>0.1533</td>
<td>0.2577</td>
<td>0.1878</td>
</tr>
<tr>
<td>75</td>
<td>0.3190</td>
<td>0.4342</td>
<td>0.4223</td>
<td>0.3632</td>
</tr>
<tr>
<td>90</td>
<td>0.2782</td>
<td>0.6359</td>
<td>0.4702</td>
<td>0.4814</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Tab.4** Absorbance values at 500nm for mixtures of chitosan/mucin and modified chitosans/mucin, varying the concentration of mucin.

Analyzing all samples singularly, the absorbance differences, measured for all polymer/mucin systems, were much higher than those calculated for the individual solutions. The results are shown in figure 18. These results suggest that there is a strong interaction between tested polymers and mucin giving origin to larger systems that can better scatter the radiation. Moreover, the amount of mucin adsorbed increased with the increasing mucin concentration: results showed that a large amount of mucin was adsorbed on the various batches of polymer giving more scattering samples.
Comparing the results obtained for each polymer a different behaviour can be notice. First of all, TMC has a higher interaction than chitosan. This is mainly due to positive charge on polymer chain, guaranteed by quaternarized amino-groups. As the mucoadhesive properties of chitosan are likely based on ionic interactions of the positively charged polymer with negatively charged moieties of mucin, such as sulfonic and sialic acid substructures, more positively charged chains will consequently be relatively more mucoadhesive.

For glycosylated chitosan a different mechanism occurs: besides ionic interaction, also the interpenetration of the polymer chains and entanglement between the polymer and the mucin chains should be attained and, finally, the formation of weak chemical bonds should be possible, thanks to sugar moieties. Glycosides changed significantly chain molecular weight and hydrodynamic radius compared to chitosan, giving a more random-coil expanse structure.

Fig.18 Turbidimetric measurements of the interaction between mucin and chitosan derivatives. Recording of absorbance at 500 nm.
13.4.2.1 The electrostatic shield effect

In order to study the effect of the buffer ionic strength in the interaction between polymer and mucin, turbidity measurements were performed also in presence of NaCl (1 M). The data concerning the mixtures chitosan/mucin and galactosylated chitosan (chit-gal)/mucin are reported in Table 5.

<table>
<thead>
<tr>
<th>% mucin</th>
<th>ΔA chit</th>
<th>ΔA chit-gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>0.0271</td>
<td>0.0010</td>
</tr>
<tr>
<td>50</td>
<td>0.1027</td>
<td>0.0078</td>
</tr>
<tr>
<td>75</td>
<td>0.1328</td>
<td>0.0212</td>
</tr>
<tr>
<td>90</td>
<td>0.1274</td>
<td>0.0206</td>
</tr>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Tab.5 ΔA of mixtures chitosan/mucin and chit-gal/mucin as a function of mucin concentration. Solutions are made in acetate buffer 0.25 M - NaCl 1 M.

Analysing results for native chitosan (figure 19), increasing the ionic strength of the mixture a reduction of chitosan/mucin interaction occurs, even if not complete inhibition, as can be seen from the values of ΔA (relatively elevated).
In the case of chit-gal (figure 20), on the contrary, when the ionic strength is increased a complete inhibition of the interaction with mucin is observed. This result could be due to a complete shielding of the charges of the glycosylated chitosan (which are lower than the native chitosan) by the ionic species introduced or, alternatively, to an effect on the conformation of the polymer, that can affect the interaction with mucin reducing the entanglement between chains. This aspect should be deepened by specific investigations about the behaviour of these polymers as a function of ionic strength.

**Fig.19** ΔA values (500 nm) of mixtures chit/ mucin as a function of mucin concentration, in presence (pink line) and absence (blue line) of NaCl 1M.
**Fig. 20** ΔA values (500 nm) of mixture chit-gal/mucin as a function of mucin concentration, in presence (pink line) and absence (blue line) of NaCl 1M.
14 Nanoparticles formation and optimization

14.1 Chitosan-TPP nanoparticles preparation

Chitosan nanoparticles have been prepared by ionotropic gelation adding tripolyphosphate (TPP) to a chitosan solution (concentration 1.25 mg/ml) in the drop wise manner using an insulin syringe. Increasing the amount of TPP the chitosan solution became opalescent, sign of nanoparticle formation, and, finally, started flocculating. The suspensions formed in the range between opalescence appearance and flocculation were deeply characterized in terms of size of particles and reproducibility. The best chitosan to TPP mass ratio turn out to be 5:1, giving reproducible nanoparticle with an average size around 200 nm.

A standardized protocol was set-up automating TPP drip: introducing the use of a syringe pump (B. Brown Melsungen) different speeds of dribble were tested and a constant drip (19 ml/h) of TPP solution was selected.

Concerning particle collection, a centrifuge and an ultracentrifuge have been used to separate particles from unreacted polymer and their environment. Different speeds and times of centrifuging have been tested, selecting 3270 RCF (Relative Centrifugal Force) for 2 hours as the best separation condition, giving the higher amount of precipitated particles that was possible to recovery. Increasing speed or time of centrifuge higher amounts of precipitate can be obtained, but recovery of nanoparticles was impossible using different systems such as an ultrasonic bath or an ultrasonic probe. Also the addition of a glycerol bed, a classic solution to prevent particle aggregation due to centrifuge packing, wasn’t suitable for a complete recovery of nanoparticles.

The last step investigated was a scale-up of particles preparation following two different strategies: on one side polymers concentration was kept constant and final volume of samples increased; on the other final volume was
kept constant and polymer concentration increased. Size measurements indicated that tripling total polymer concentration larger particles are obtained, increasing size from 200 nm to 273 nm (SD 17.7). This result is well described in literature. On the other side, even increasing preparation volume of ten times a small variation of dimension was measured, with average size of 237.2 nm (SD 26.5).

14.2 Size and surface charge

Nanoparticles have been characterized in size and surface zeta potential ($\zeta$) using a Malvern Zetasizer nano ZS (Malvern Instruments, Worcestershire, U.K.). The formulations were suspended in water and analyzed, often resulting in bimodal size distributions. Three different types of nanoparticles were prepared, differing for cationic polymer used: chitosan, TMC or a blend of chitosan and chit-gal.

14.2.1 Size of chitosan nanoparticles

The first produced nanoparticles are made using chitosan and tripolyphosphate (TPP) in different ratios. Two experimental parameters were varied throughout this optimization study: chitosan MW and chitosan:TPP ratio. The effects of two chitosan MWs on particle size were studied: a degraded chitosan (32 kDa, VLMW), and a low molecular weight chitosan (150 kDa, LMW).
Initially, VLMW chitosan has been used for nanoparticle production. Different chitosan/TPP ratios have been tested, but only some of them gave reproducible results. First of all, nanoparticle formation, determined by opalescence of solution, starts at selected chitosan to TPP weight ratio of 6:1. Chitosan ability of quick gelling on contact with polyanions relies on the formation of inter- and intramolecular cross-linkages mediated by the anionic molecule. By increasing the amount of TPP, the nanoparticle suspension becomes more and more turbid and even particle aggregation can occur drastically. The explanation of this is related to TPP mechanism of action: when the available quantity of TPP is high the dominantly inter- and intramolecular cross-links are associated with TPP that link nanoparticles to form larger nanoparticles, even flocculating.

As the laser diffraction data show, chitosan/TPP ratio 6:1 and 5:1 are the best formulations: the average size is around 160 nm (164 and 151 nm, respectively).

Regarding the LMW chitosan, the same experimental strategy has been used testing different chitosan/TPP ratios. As shown in table 6 different ratios generate nanoparticles. Also in this case a similar behaviour to VLMW chitosan can be noticed: increasing the total amount of TPP larger particles are obtained, generating aggregates when the chitosan/TPP mass ratio is down to 4:1. However, some of them give unpredictable results in terms of particle formation or flocculation. This is a common behaviour as with polysaccharide preparations batch-to-batch variation appears to be an inevitable consequence of the polydispersity of the macromolecule, since their variation has a determinant impact on physicochemical properties an appropriate selection is required to control the quality of the final product.
<table>
<thead>
<tr>
<th>Chitosan:TPP ratio (w/w)</th>
<th>VLMW Chitosan NPs</th>
<th>LMW Chitosan NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave size (nm)</td>
<td>SD (±)</td>
</tr>
<tr>
<td>3:1</td>
<td>aggregate</td>
<td>aggregate</td>
</tr>
<tr>
<td>4:1</td>
<td>aggregate</td>
<td>aggregate</td>
</tr>
<tr>
<td>5:1</td>
<td>151 ±10</td>
<td>0.185 ±3.5</td>
</tr>
<tr>
<td>6:1</td>
<td>164 ±12</td>
<td>0.198 ±2.9</td>
</tr>
</tbody>
</table>

**Tab.6** Particle size and surface charge of VLMW and LMW chitosan. Different chitosan to TPP mass ratios have been tested.

As with VLMW chitosan, the only reproducible sample is the 5:1 mass ratio. The sample gives nanoparticles with an average diameter of 200 nm and a standard deviation of ±24. These nanoparticles are a little bit larger than VLMW chitosan ones, but size is still in range for drug delivery.

Comparing VLMW and LMW chitosan results, the influence of chitosan molecular mass on particle size can be analyzed. The average particle size increased from 151 nm to 200 nm as chitosan molecular weight increased from 32 kDa to 150 kDa. These results are expected and are consistent with findings that higher molar mass chitosan produced larger nanoparticles.\(^{192,193,194}\)

The polydispersity index (PDI) of particles increased from 0.185 to 0.227 in correspondence to the increase of chitosan molecular mass. However, all of the values of PDI are low indicating that an homogeneous dispersion of them is obtained.

An interesting aspect is the particle size distribution (figure 21). The particle size distribution curve indicates the presence of two different particle size populations.
The bimodal size distribution curve is composed of a fraction of small particles (around 50 nm) and of a second population around 250 nm. The average particle size (200 nm) represents the mean size that even a very few residual large particles strongly influence in final particle size distribution. A bimodal size distribution is also observable with TEM. In the next section a deeper explanation of nanoparticle formation will be given.

Regarding the surface charge, both chitosans give positively charged nanoparticles. When chitosan and TPP are mixed with each other, they spontaneously formed compact complexes with an overall positive surface charge, as obtained by measures of zeta potential values. In accordance with literature, by increasing TPP concentration the zeta potential decreased and for VLMW chitosan particles it shifts from 28.1 mV for 6:1 mass ratio to 23.9 mV for 5:1.

Analyzing the differences between the two chitosans, the same trend as particle size as function of the molecular mass could also be observed: LMW chitosan samples with the same chitosan:TPP mass ratio have higher zeta potential compared VLMW chitosan samples. Those results, similar to those obtained by Gan et al., can be explained because different molecular masses should have different accessibility of cationic groups (although having
the same degree of deacetylation, 87% in our samples) resulting in the higher surface charge.

14.2.2 Evolution of nanoparticles formation

Chitosan formulations can be considered as carrier model type, so most of deeper investigations are performed on them. Regarding particle diameter, average particle size measured for chitosan to TPP mass ratio is 200 nm.

As previously mentioned, size distribution curve of freshly prepared samples presents two populations of particles, one around 50 nm and the other around 250 nm. This typical distribution can be mainly observed in freshly prepared samples; if samples are kept under stirring and analyzed after half a day the smallest particle population disappears. Indeed, instead of the bimodal curve, it became a single Gaussian curve, spread up to a few hundreds of nanometers. This phenomenon could be explained by the fact that the small particles aggregate during stirring to form a more homogeneous population of particles. This phenomenon is well visible in TEM images, where particles ranging from few tens to few hundreds of nanometers are presented. TEM provides the size of almost dehydrated particles, while dynamic light scattering measurements yield an ensemble average of the particle size in solution.

Taking into account dynamic light scattering and TEM results, a mechanism of various steps has been assumed and presented. Chitosan in solution has a random coil conformation. TEM images provide chitosan chain dimension around 20-40 nm, consistent with hydrodynamic radius of 15.8 nm, as previously characterized with a triple detection size exclusion chromatography system (SEC, Viscotek, USA). Moreover, chitosan concentration is 1.25 mg/ml, so next to the limit of critical concentration of coil overlap (C*). This means that chitosan chains are quite close one to another, some of them been statistically in contact with others. By adding a dilute solution of TPP (0.25 mg/ml) a double effect is obtained. On one side, a
dilution of solution generates a more randomly distributed chitosan chains; on
the other, anionic TPP starts interacting with few (single?) cationic groups on
chitosan chains folding over themselves (mainly intramolecular links). Dimension of new entities are comparable to the random coil chain one because are the result of few bent chains grafted together.

Continuing the addition of TPP onto chitosan leads to both the formation
of new particles, supported by the monotonous of size value, and also their
rearrangement towards more compact particle structure. At the end of
polyanion addition, the negative charged TPP interacts with remaining
chitosan chains leading to the primary aggregates (charged chitosan
monomoles exceed TPP by 10 to 1). The partially neutralized positive charges
of chitosan still present in the primary aggregates, favour a rearrangement of
chains and the formations of more compact particles with lower size. A further
rearrangement of nanoparticles is obtained with time by an efficient sharing of
neutralizing anionic charges, producing particles with a Gaussian curve instead
of the bimodal one.

Although such a phenomenon is often described in literature to be a
function of the effective charge density and of flexibility of the polymer, its
quantitative elucidation is still lacking.

The TEM images confirm this hypothesis: they indicate that the particles
appear as small and individual particles, single isolated particles have a
diameter around 30-40 nm and larger particles are due to the aggregation of
single small particles that tend to fuse together generating a larger entity. This
aspect is well shown by subsequent TEM images (figure 22 a-b). Air-dried
samples are not completely desiccated, especially nanogels as chitosan-TPP
particles. During TEM analysis, magnifying as much as possible on a
nanoparticles aggregation, a very fast fusion (a couple of minutes) of single
particles into one entity is proved: the heat of the ray promotes intermolecular
links thanks to the still present aqueous environment of gel-network.
**Fig. 22** (a) TEM images of chitosan nanoparticles. (b) the same image has been taken after few minutes. The aggregative process is well visible.

TEM images about chitosan solution (figure 23) show small particles, around 40 nm diameter as mentioned above, but their behaviour is completely different: magnifying on several close entities, their structure does not change and no fusion occurs even if the ray is kept for long time over them. So the behaviour described before is due to a linking-agent, such as TPP.
Dimension of freshly formed single particles are well visible in TEM images of samples with paraformaldehyde. This agent has been added immediately after TPP dribble, therefore before complete rearrangement of chains and particles. Paraformaldehyde blocks particles interactions and “fixes” them. TEM images show that most particles are single isolated ones and only few of them are grouped together: this is because there was not enough time for a complete TPP redistribution and particle rearrangement.

Since the rearrangement is influenced by stirring, a new batch has been prepared and particle size has been measured immediately, after 2 hours and 24 hours of preparation (figure 24). With stirring, the aggregation level fast increases in the first 120 min and continues after 24 hours: size changes from 226 nm to 302 and 440 nm, respectively. Moreover, the polydispersity index increases after 2 hours (table 7), from 0.282 to 0.456, and decreases after 24 hours, 0.341: initially, most of particles are small single ones, even if average size is 226 nm (dynamic light scattering more easily detects large particles, and even a very small number of them lead to considerable changes in the
obtained size distribution). After 2 hours a partial rearrangement has occurred and a polydisperse suspension is present: volume distribution of laser diffraction size curves shows a fifty-fifty bimodal distribution (the average size is high because even a very few macroparticles will strongly influence it). After 24 hours an equilibrium is found, single particles are arranged together and almost totally absent. At this stage polydispersity starts decreasing.

On the other hand, without stirring, a longer time was necessary for particles to reach the equilibrium at a higher value of size. Without stirring, particle size doesn't increase significantly: it ranges from 208 nm to 217 after 2 hours and 220 after 24 hours. Small particle population is still present after 24 h and polydispersity index is 0.186, lower than samples kept under stirring. Particle size population is less variable because the system is looking for the best equilibrium slowly and few large nanoparticles are present.

![Fig.24 Effect of stirring on particle size.](image)

Fig.24 Effect of stirring on particle size.
<table>
<thead>
<tr>
<th>h after preparation</th>
<th><strong>STIRRING</strong></th>
<th><strong>WITHOUT STIRRING</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave size (nm)</td>
<td>SD (±)</td>
</tr>
<tr>
<td>0</td>
<td>226</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>302</td>
<td>14</td>
</tr>
<tr>
<td>24</td>
<td>440</td>
<td>20</td>
</tr>
</tbody>
</table>

**Tab.7** Effect of stirring on particle size. Average diameter has been measured after 2 and 24 h.

### 14.3 Nanoparticle stability

Size stability as a function of time has been studied also for VLMW chitosan nanoparticle as the important characteristics of colloidal dispersions is their stability upon storage. After preparation, a nanoparticle batch was characterised and then stored at 4 °C and the size was measured after 1 and 4 weeks. All measurements are expressed as Z-average diameter (Z Ave), standard deviation (SD) and polydispersity index (PDI).

All samples presented an increase in size, nevertheless without leading to precipitation (table 8, figure 25). A slight increase of size is measured after 1 week, while a significant increase is shown after 4 weeks: there is a very little increasing between fresh particles, 151 nm, and the 1<sup>st</sup> week, 161 nm, so the diameter can be consider almost the same. Between the 1<sup>st</sup> and the 4<sup>th</sup> week average particle size increase to 187 nm: this means that a rearrangement is still in progress after 4 weeks.

On the other side, the samples left at room temperature tend to aggregate after two weeks. Chitosan total amount is in excess compared to TPP (charged monomole: TPP ratio 10 to 1), and waiting for a more efficient rearrangement of particles leads to obtain big aggregates: in fact, formation of larger nanoparticles is favored at higher chitosan concentration.
### Size stability studies

![Size stability studies](image)

**Fig.25** Particle size as a function of storage time.

### 14.4 Yield results

The amount of chitosan in the supernatant after centrifugation is assays using a colorimetric determination. Keeping constant the chitosan amount, samples with different amount of TPP were prepared following the described protocol (section 8.1.1) and subsequently centrifuged. The supernatants were analyzed following Muzzarelli assay (see reference 169) and the adsorbances
measured. The adsorbance of the sample without TPP was used as a reference (precipitation of aggregated chitosan chains), subtracting its value to the adsorbances of the others samples giving the effective amount of chitosan precipitated as nanoparticles triggered by TPP interaction. The results obtained are plotted in figure 26.

![Graph](image_url)

**Fig.26** Quantity of nanoparticles obtained (in mg) as a function of the quantity of TPP added (in mg).

At the beginning, by adding of TPP to the chitosan solution a slight change in the amount of precipitate can be noticed. As described previously, this could be due to the presence of small nanoparticles that can not be separated from the suspension easily because of the light weight. By increasing the addition of TPP, a sudden increment of precipitate can be observed in correspondence of 3.5 ml of TPP solution (0.875 mg). That volume corresponds to the start of the opalescence of chitosan solution, sign of formation of aggregates of the dimension comparable to incident the light.
The amount of precipitate increases above the addition of 1.25 mg of TPP (5 ml of anionic solution), where larger aggregates are formed. At this point, the amount of precipitated chitosan is of 0.7 mg out of 6.25 mg in the original solution. It is reasonable to assume that some nanoparticles remain in the suspension and are not separated under the mild centrifuge conditions (3270 RFC for 2 hours). This result indicates that the yield of nanoparticles at this stage is low, 9%. However, while increasing TPP amount and/or centrifuge conditions, speed or time, generates flocculation, a recovery of chitosan and further addition of TPP could be explored.

14.5 Loading

14.5.1 Effect of protein concentration on size

Three different proteins have been loaded to chitosan nanoparticles: bovine serum albumin (BSA), ovalbumin (OVA) and human insulin, (HI). As described in the method section, BSA and OVA were added directly to cationic solution and then TPP was dripped onto, whereas HI was diluted with TPP and then added to the cationic solution.

Different amounts of protein were considered by adding an equal volume of protein solution at three different concentration (0.2-0.4-0.6 mg/ml) to the chitosan solution (or TPP solution for HI). The effect on particle size and surface charge was investigated.

As shown in figure 27 (a-b), the presence of a protein has a small influence on particle size, increasing it of few nanometers. This may be ascribed to ionic interaction and ionic cross-linking between negatively charged BSA or OVA (pI = 4.8 and 4.7, respectively) and positively charged chitosan under the preparation conditions (around pH 6). This generates a reduction in the electrical repulsion among biopolymers, where
modifications in the electrical state cause the particle to shrink. Moreover, TPP links intra- and intermolecularly chitosan chains giving a more compact structure.

![Effect of BSA on nanoparticles](image-a)

![Effect of OVA on NPs](image-b)

**Fig.27** Effect of proteins in size and surface charge in chitosan nanoparticles in case of BSA (a) and OVA (b).
Both BSA and OVA are negatively charged and interact with positive groups of chitosan. The decrease of the zeta potential after protein addition (figure 27) is probably due to the partial deposition of the negatively charged protein on particle surface.

Similarly, in case of HI an ionic interaction of chitosan with TPP and HI occurs. The incorporation of HI increases particle size, probably due to concurrent competition between TPP and HI in their interaction with chitosan, thus causing the particle to swell.

When HI is added, being its pI 5.6 very close to pH of the particle suspension (≈ 6), it is negatively charged and interacts with -NH$_3^+$ groups of chitosan, competing with TPP.

After particle formation, its net charge is next to neutrality having, from one side, a weaker interaction with chitosan (a swollen particle) and from the other a less influence on total surface charge (higher zeta potential compared to chitosan/TPP particles).

14.5.2 Particle loading efficiency

Chitosan nanoparticles, loaded with BSA, OVA and HI, have been assayed using the BCA™ protein assay and Bradford assay. The loading efficiency (%) indicates the efficiency of the nanoparticles to incorporate the protein and is reported in figure 28. (Table 9).
BSA and OVA have similar loading efficiency values. Loading efficiency of BSA ranges from 39% to 60%; OVA efficiency is 48%, in case of a starting protein concentration of 0.2 mg/mL, and is 76% increasing protein concentration. The high loading efficiency of both proteins is ascribed to the ionic interaction with chitosan.
The reduction of zeta potential is due to decrease of net charge and can be seen as a proof of protein loading.

Only one amount of HI has been tested considering that high loading efficiency for both model proteins was obtained for all amounts tested (table 10). The loading efficiency is around 55%. The addition of the anionic solution to the chitosan one decreases the strength of electrostatic interaction between insulin and chitosan nuclei, reducing consequently the insulin entrapment efficiency. Therefore, it is expected that increasing pH of the final suspension could promote a higher loading efficiency.

<table>
<thead>
<tr>
<th>Amount of HI (µg)</th>
<th>Ave size (nm)</th>
<th>SD (±)</th>
<th>Z-pot (mV)</th>
<th>SD (±)</th>
<th>L.E. (%)</th>
<th>SD (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>210</td>
<td>18</td>
<td>25.4</td>
<td>2.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>200</td>
<td>330</td>
<td>36</td>
<td>30.2</td>
<td>4.2</td>
<td>55</td>
<td>8</td>
</tr>
</tbody>
</table>

Tab.10 Particle size, surface charge and loading efficiency after HI loading.

14.6 Storage

14.6.1 Lyophilization

The high concentration of particulate system may induce aggregation and in some cases irreversible fusion of nanoparticles. Furthermore, the crystallization of ice may exercise a mechanical stress on nanoparticles leading to their destabilization. The bioprotectants are usually added in order to protect the product from freezing stress (cryoprotectant) or drying stress (lyoprotectant) and also to increase its stability upon storage.
After lyophilization, chitosan nanoparticles have usually problems to redispers: a big aggregate still remain in suspension after long time stirring. With the addition of cryoprotectants a different behavior can be noticed.

First of all, almost all samples redispers properly after lyophilization. Comparing the different protectants (figure 29), trehalose results the best one. Particle size doesn’t increase too much, ranging from 268.3 nm to 341.0 – 497.1 nm, depending from the total amount added. On the contrary, mannitol and PEG samples have a greater increase on size, ranging between 289.6 and 1598 nm. The better result of trehalose could be relate to its peculiar characteristics: less hygroscopicity, an absence of internal hydrogen bounds which allows more flexible formation of hydrogen bonds with nanoparticles during spray-drying, very low chemical reactivity and finally, higher glass transition temperature Tg.196

![Effect of cryoprotectants on size](image)

**Fig.29** Effect of cryoprotectant on particle size.

Moreover, a dependence of stabilization from sugar concentrations can be notice: better results are obtained with higher amounts of cryoprotectants (table 11).
<table>
<thead>
<tr>
<th>Amoun t (w/v)</th>
<th>TREHALOSE</th>
<th>MANNITOL</th>
<th>PEG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave size</td>
<td>SD (±)</td>
<td>PDI</td>
</tr>
<tr>
<td>0.5%</td>
<td>497 19</td>
<td>0.384</td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td>298 59</td>
<td>0.395</td>
<td></td>
</tr>
<tr>
<td>5%</td>
<td>298 80</td>
<td>0.238</td>
<td></td>
</tr>
<tr>
<td>10%</td>
<td>308 24</td>
<td>0.242</td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>279 88</td>
<td>0.232</td>
<td></td>
</tr>
</tbody>
</table>

Tab.11 Particle size of different cryoprotectants: the samples are measured after recovery.

The weight ratio cryoprotectant:nanoparticles is important for stabilizing nanoparticles. A complete redispersion (good average size/poly index) of nanoparticles after freeze-drying could be obtained when sugars were added to the nanoparticles suspension at a concentration of at least 5% w/v.

The crystallization of cryoprotectant and the formation of eutectic with ice can cause phase separation in the cryo-concentrated portion of the frozen nanoparticles suspension with no opportunity for a stabilization interaction with nanoparticles. Individual nanoparticles in the nanoparticles-rich phase can interact and form aggregates. Moreover, the growing crystals of water and sugar may exert mechanical forces on the nanoparticles leading to their fusion. So, any stabilization mechanism requires that at least some of the sugar remain molecularly dispersed in the amorphous nanoparticle phase.197

Another explanation of better results with higher amount of cryoprotectant in mechanism of nanoparticles stabilization during the freezing step is the particle isolation hypothesis. It has been proposed that sugars isolate individual particles in the unfrozen fraction, thereby preventing aggregation during freezing above Tg. In this case, the vetrification is not required for this effect and the spatial separation of particles within the unfrozen fraction is sufficient to prevent aggregation.198
In conclusion, the addition of cryoprotectants before freeze-drying of nanoparticles doesn’t compromise their stability and using the adequate amount of them provide an increased stability to the dried product.

14.6.2 Spray drying

A classic chitosan-TPP formulation was freeze dried after the addition of different bioprotectants. Regarding the size before drying nanoparticles, can be noticed an increase compared to blank particles: particles ranged from 214 nm to 253 nm with trehalose and 279 nm with mannitol, and increased to 397 nm and a polydispersity index of 0.554 with PEG.

After spray drying and subsequent recovery, higher increase can be observed. First of all, blank particles change from nano-sized to micron-sized particles: the average diameter increased of 20 times (table 12).

This is mainly due to particle aggregation during the drying process. This aspect, that had also been observed in previous studies, is well visible in SEM picture (figure 30 a-b), where can be seen nanoparticles fused together generating microparticles.

Fig.30 (a) SEM image of chitosan nanoparticles. (b) A detail of a nanoparticle.
Analyzing the effect of the three protectants (figure 31), different behaviors can be observed. Samples with mannitol cannot be redispersed, a macroaggregation remain in suspension. On the other side, trehalose and PEG permit to obtain smaller particles compared to blank ones, still nanosized: particles ranged from 253 nm to 437 nm with trehalose and from 397 nm to 865 nm with PEG (even if with a very high poly index, 0.753).

Trehalose seems to be a preferable protectant for biomolecules. It has many advantages in comparison with the other sugars as: less hygroscopicity, an absence of internal hydrogen bounds which allows more flexible formation of hydrogen bonds with nanoparticles during spray-drying, very low chemical reactivity and finally, higher glass transition temperature Tg.  \(^{196}\)
The spray-drying of solid nanoparticles demonstrated that the presence of carbohydrates like trehalose provided an increased stability to the spray-dried product, because the sugar layer around the particles prevented the particle coalescence.

**Fig. 31** Effect of the cryoprotectants on particle size
15 Chitosan derivatives nanoparticles

15.1 TMC nanoparticles

The second series comprised particles prepared using trimethyl chitosan (TMC) as cationic polymer in two solvent mixtures and TPP. Two experimental parameters were varied throughout this optimization study: TMC solvent and TMC to TPP mass ratio.

Two different solvents have been tested: deionized water and acetic acid solution. TMC was dissolved in a 0.05% (v/v) acetic acid solution for nanoparticle production, reproducing the same situation of chitosan nanoparticle. Then, due to spectroscopic analysis deionized water has been used, to avoid acetic acid peaks to cover characteristic sample band peaks. Obtained results, both in terms of size and surface charge are comparable, so the use of deionized water as solvent was preferred.

The same experimental strategy used for chitosan nanoparticle production has been followed, testing different TMC/TPP ratios. Different mass ratios have been tested, but only some of them gave reproducible results, as expected. First of all, nanoparticle formation, determined by opalescence of solution, starts at selected TMC to TPP weight ratio of 6:1, as for chitosan formulations. Increasing TPP amount larger nanoparticles are obtained, giving a more turbid suspension, until particle aggregation occurs drastically.

Analyzing particle size data, TMC:TPP ratios 6:1 and 5:1 are the best formulations with the average size around 160 nm (163 and 161 nm, respectively). Larger nanoparticles (846 nm) are obtained for mass ratio 4:1. The nanoparticle formation mechanism is the same proposed for chitosan nanoparticles: TPP interacts with positive charged TMC though inter- and intramolecular cross-links. By increasing the concentration of TPP, links between different particles are promoted, generating larger particles. This can be confirmed by the change in zeta potential and polydispersity index of particles. As shown in figure 32, the zeta potential decreases drastically as
TPP amount increased, which might be caused by the screening of cation on the particle surface during the linkage or aggregation of the nanoparticles. By increasing TPP total amount the same behaviour of higher value for PDI can be noticed, which might be caused by the presence either of aggregates of particles and smaller particles.

![Fig.32 Size and surface charge for the trimethylchitosan nanoparticles.](image)

**15.2 Glycosylated chitosan nanoparticles**

The glycosylated chitosan particles were prepared using a blend of chitosan and chitosan-galactose (chit-gal) as cationic species and TPP as anionic molecule. Experimental parameters varied throughout this optimization study were chitosan degree of substitution, cationic blend composition and cationic blend/TPP ratio.

As for TMC formulation, different solvents have been used to dissolve the two polymers: water and acetic acid solution. We started nanoparticle
production using a 0.05% (v/v) acetic acid solution as solvent; then, due to spectroscopic analysis, deionized water has been used to avoid acetic acid peaks to cover characteristic sample band peaks.

The analysis started using a galactosylated chitosan with a high degree of substitution (HSD, 65%), to obtain a high presence of sugar moieties. The cationic blend:TPP mass ratio 5:1 has been selected, following chitosan and TMC studies. Different cationic blends have been tested to obtain a formulation with the highest amount of chit-gal as possible (table 13).

<table>
<thead>
<tr>
<th>Chitosan:chit-gal HDS ratio (w/w)</th>
<th>0.3:1</th>
<th>0.6:1</th>
<th>1:1</th>
<th>1.3:1</th>
<th>2:1</th>
<th>4:1</th>
<th>5:1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cationic blend:TPP ratio (w/w)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5:1</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td>245/+13.5</td>
</tr>
<tr>
<td>6:1</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td>350/+13.4</td>
<td>220/+20.2</td>
</tr>
<tr>
<td>7:1</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td>no particle formation</td>
</tr>
</tbody>
</table>

**Tab.13** Particle size and surface charge of different chitosan:chit-gal HDS blends and cationic blend to TPP mass ratio 5:1 (w/w). The aggregated formulations are marked with an “x”.

Unfortunately, only samples with a chitosan/chit-gal HDS mass ratio from 5:1 to 2:1 give nanoparticles. For all the others the range between a clear sample and the aggregate generation in too short and variable for a characterization.

Regarding size can be noticed that increasing chit-gal HDS total amount larger nanoparticle are obtained: their diameter ranges from 196 nm to 350 nm, as shown in table 15. The negative aspects are the surface charge and redispersion after centrifugation for particle collection. For surface charge, a good zeta potential was measured for samples with a high amount of chitosan (around +20 mV) and a low value (around +13 mV) for samples with a high...
amount of chit-gal, the samples we are more interested in. This is mainly due to chit-gal structure: protonated amino groups contribute to nanoparticle formation and surface charge but their free number is decreased, because most of them are involved in glycosydic links. Due to this, increasing chit-gal amount a low zeta potential is obtained: this means that the formulations are potentially unstable, because charged particle surface stabilizes nanosuspension by electrostatic repulsion preventing aggregation due to attracting forces between the particles.

Even if a higher zeta potential was measured for other samples, the same problem of particle aggregation after centrifuge occurs: the resuspension become problematic, still after adding glycerol. Due to this, a chit-gal with a lower degree of substitution (LDS, 50%) has been used (table 14).

<table>
<thead>
<tr>
<th>Chitosan:chit-gal LDS ratio (w/w)</th>
<th>Ave size (nm)</th>
<th>SD (±)</th>
<th>Z-pot (mV)</th>
<th>SD (±)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4:1</td>
<td>141</td>
<td>9</td>
<td>12.7</td>
<td>3.2</td>
</tr>
<tr>
<td>2:1</td>
<td>151</td>
<td>13</td>
<td>7.1</td>
<td>2.1</td>
</tr>
<tr>
<td>1.3:1</td>
<td>162</td>
<td>25</td>
<td>10.3</td>
<td>2.3</td>
</tr>
<tr>
<td>1:1</td>
<td>358</td>
<td>60</td>
<td>7.7</td>
<td>2.3</td>
</tr>
<tr>
<td>1:1.5</td>
<td>186</td>
<td>48</td>
<td>10.2</td>
<td>1.2</td>
</tr>
<tr>
<td>1:3</td>
<td>162</td>
<td>37</td>
<td>9.7</td>
<td>1.5</td>
</tr>
<tr>
<td>1:4</td>
<td>156</td>
<td>15</td>
<td>4.7</td>
<td>2.2</td>
</tr>
<tr>
<td>-:5</td>
<td>246</td>
<td>69</td>
<td>11.1</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Tab.14 Particle size and surface charge of different chitosan:chit-gal LDS blends. Cationic blend to TPP mass ratio 5:1 (w/w).
Also in this case the cationic blend/TPP mass ratio 5:1 has been selected, and different cationic blends have been tested (figure 33). All cationic blends generate nanoparticles, giving the possibility to prepare full chit-gal nanoparticles. A lower degree of glycosylation gives more charged chains, allowing better particle formation than high substituted polymer. Particle size varies from 141 nm to 358 nm, reasonably in range for mucosal drug delivery. Analyzing cationic blends, no relation can be found between particle size and chit-gal amount: higher average sizes are obtained increasing its total amount, but standard deviations are so relevant that variation can be considered unimportant. On the contrary, higher amounts of chit-gal LDS generate more polydispersed nanoparticles, aspect emphasized by PDI and standard deviation.

Regarding surface charge (table 16), zeta potential ranges from +12.7 mV to +7.1 mV and gives the same aggregate problem noticed before. The aggregative process is more frequent when chit-gal is the majority part of cationic blend, so aggregation is not only related with surface charge. It has been solved depositing particles on a glycerol bed before centrifuge, giving us the opportunity of a complete redispersion of samples.

![Fig.33](image-url) Size and surface charge for chitosan/chit-gal blends nanoparticles (cationic blend to TPP mass ratio 5:1).
16 Association with other biopolymers

16.1 Size for pectin coated chitosan nanoparticles

Three pectins have been used to coat chitosan nanoparticles: polygalacturonic acid, pectin from citrus fruit and pectin from apples. They differ in Mw and degree of esterification. Those differences could be useful not only for a better particle coating, but mainly for a tailor mucosal delivery carrier.

Different ratios of pectin to nanoparticle have been tested: almost all of them give good results in terms of size (table 15). An increase of chitosan particle size can be noticed for all the three pectins: it increased from an average of 200 nm to a range from 226 nm to 446 nm. This is a proof of particle coating.

<table>
<thead>
<tr>
<th>Pectin:NPs ratio (w/v)</th>
<th>PECTIN FROM APPLE</th>
<th>POLY-GALACTURONIC ACID</th>
<th>PECTIN FROM CITRUS FRUIT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ave size (nm)</td>
<td>SD (±)</td>
<td>Z-pot (mV)</td>
</tr>
<tr>
<td>1.5:1</td>
<td>aggregate</td>
<td>251 68 -36.4 3.2</td>
<td>6953 894 -6.5 4.2</td>
</tr>
<tr>
<td>2:1</td>
<td>331 35 -25.1 2.4</td>
<td>240 24 -44.1 1.2</td>
<td>228 31 -15.6 4.3</td>
</tr>
<tr>
<td>3:1</td>
<td>383 10 -28.5 3.3</td>
<td>252 38 -45.4 4.7</td>
<td>241 10 -24.8 2.4</td>
</tr>
<tr>
<td>4:1</td>
<td>402 21 -35.2 3.1</td>
<td>271 12 -46.7 7.3</td>
<td>226 29 -37.0 5.1</td>
</tr>
<tr>
<td>5:1</td>
<td>446 24 -28.7 2.6</td>
<td>289 28 -43.6 6.2</td>
<td>257 23 -35.0 4.1</td>
</tr>
</tbody>
</table>

Tab.15 Effect of coating on particle size and surface charge with three different pectins.

Thickness depends on pectin used and its amount. Adding the same amount of polymer, pectin from apples produces a thicker coating: this is
mainly due to its high molecular weight. Heavier chains have a higher hindrance compared to smaller ones. Moreover, the presence of a higher amount of polymer increases coating thickness because more chains are available for ionic interaction with positive charged particles resulting in a swollen coating. As a matter of fact zeta potential decreases due to more free carboxylic groups available.

Decreasing pectin amount below 2:1 particle size increases dramatically, giving large particles up to aggregates. This is due to reduction of repulsive electrical charges: zeta potential is next to zero, so the electrostatic repulsion is weak and particle aggregation is favoured. As a matter of fact, the average zeta potential ranges from -15.6 mV to lower values for all the other samples and increase to –6.5 mV for 1.5:1 pectin to particles mass ratio.

As shown in figure 34, for all the other samples the average zeta potential range from -15.6 mV to -46.7 mV and this guarantees particle stability preventing aggregation due to attracting forces between the particles. As can be noticed, coated particles are negatively charged due to the contribution of the pectin: this further demonstrates the coating of particles (also proved in ATR-IR spectra, section 17.2).

![Coating chitosan NPs](image)

**Fig.34** Size and surface charge for chitosan nanoparticles coated respectively with pectin from apple, galacturonate and pectin from citrus fruit.
Surface charge depends from pectin concentration. Increasing pectin total amount surface charge decreased because more carboxylic groups are available resulting is a lower zeta potential. This aspect is well described considering pectin used: sodium polygalacturonate coated particles have lower surface charge value compared to the other pectins because its carboxylic groups are all charged (DE 10.6%,\textsuperscript{199}). On the other side, degree of esterification of the other two pectins reduced the number of available carboxylic groups increasing surface charge.

16.2 Size for blended nanoparticles

The dependence of the nanoparticles size on their composition was studied by varying the amount of pectin and chitosan introduced into the system while the TPP concentration remained fixed.

Samples with different final polymer concentration have been prepared and different chitosan to pectin mass ratios were tested to investigate their behaviour. Varying different size nanoparticles have been produced, ranging from 272 nm to larger particles, up to aggregates. Keeping pectin mass constant, higher chitosan:pectin ratios are invariably accompanied by an overall increase in nanoparticles’ dimension. This aspect is visible in all prepared formulations. This allowed us to establish the dependence of the complexation’s stochiometry on the reaction of the [-COO\textsuperscript{-}] and [-NH\textsubscript{3}\textsuperscript{+}] functional groups of both polyelectrolytes, expressed as the ratio of the chitosan concentration in the nanoparticles relative to the total pectin concentration (table 16 and figure 35).
<table>
<thead>
<tr>
<th>Pectin (mg)</th>
<th>Chitosan (mg)</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Z-pot (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>272</td>
<td>0.194</td>
<td>-21.8</td>
</tr>
<tr>
<td>0.5</td>
<td>1.6</td>
<td>337</td>
<td>0.257</td>
<td>-21.5</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>Aggregate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>277</td>
<td>0.136</td>
<td>-26.85</td>
</tr>
<tr>
<td>1</td>
<td>1.6</td>
<td>314</td>
<td>0.282</td>
<td>-20.0</td>
</tr>
<tr>
<td>2.2</td>
<td>551</td>
<td>0.474</td>
<td>-24.8</td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td>318</td>
<td>0.199</td>
<td>-25.6</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>503</td>
<td>0.597</td>
<td>-24.2</td>
</tr>
<tr>
<td>4</td>
<td>Aggregate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td>344</td>
<td>0.395</td>
<td>-28.4</td>
</tr>
<tr>
<td>3</td>
<td>556</td>
<td>0.478</td>
<td>-23.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Aggregate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td></td>
<td>425</td>
<td>0.440</td>
<td>-29.2</td>
</tr>
<tr>
<td>4</td>
<td>655</td>
<td>0.644</td>
<td>-26.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>551</td>
<td>0.650</td>
<td>-26.8</td>
<td></td>
</tr>
</tbody>
</table>

**Tab.16** Effect of pectin to chitosan mass ratio on particle size and zeta potential.
For smaller and less polydisperse particles a trend can be followed: a concomitant reduction of chitosan and pectin and an increase of TPP amount shares a reduction on particles size. This might be due to the strong effect of TPP that produced a compact structure with chitosan that interact with pectin.

Moreover, pectin total amount doesn’t take effect on particle size (figure 36). Analyzing different batches, comparing same amounts of added chitosan similar average size are obtained. This might be due to intramolecular links of TPP that promote a more compact network reducing available -NH$_3$\(^+\) groups for other interactions with unreacted pectin. Chitosan-pectin-TPP colloidal nanoparticles were formed spontaneously upon the addition of the chitosan solution to the anionic blend solution. It is known that chitosan-TPP nanoparticles are formed by ionic gelation of chitosan, a mechanism that is driven by the cross-linking of chitosan’s -NH$_3$\(^+\) groups with the P$_3$O$_{10}^{5-}$ ionic species of TPP. The intra- and intermolecular linkages created between the negatively charged groups of TPP and a fraction of the positively charged amino groups are responsible for the success of the process. In the presence
of pectin, it is expected that the ionic gelation process occur concomitantly with the complexation of the polyelectrolyte with pectin’s \(-\text{COO}^-\) groups. On the other side, increasing pectin amount more polydispersed particles are obtained. When the concentration of pectin increased, the electrostatic interactions with less available chitosan’s \(-\text{NH}_3^+\) groups results in the formation of more swollen structures due to the increase in the proportion of uncomplexed stretches of the pectin chains.

**Fig.36** Effect of pectin on particle size.

Analysing surface charge, all particles have a negative zeta potential. The negative sign reflect the excess of charged \(-\text{COO}^-\) groups from pectin exposed on the nanoparticles’ surface. Even though the increasing inclusion of chitosan in the systems leads to a reduction in surface charge, the system retains a high negative charged zeta potential.
17 Spectroscopic characterization

17.1 Raman spectroscopy

In nanoparticles spectra (figure 37 (d) and (e)) can be seen clearly the contributions of chitosan and pectin, while the bands of TPP are not visible (perhaps for the relative less amount of this component), but there are not significant shift in the position of the bands. This indicates that the interaction between chitosan and pectin does not perturb the structure or the conformation in such a way to change the structure of the vibrational Raman spectra.

Fig.37 Raman spectra of (a) chitosan, (b) pectin from citrus fruit, (c) TPP, (d) chitosan nanoparticles coated with citrus pectin (e) nanoparticles formed by blending between chitosan and citrus pectin. In nanoparticles spectra, the bands that refers to chitosan are indicated by the dashed arrows, while those due to pectin are indicated with continued arrows. The spectra were obtained with a laser diode at 785 nm, with a power at the sample of 90 mW and exposure times ranging from the 10s and 60s.
The spectra of the polysaccharides are difficult to interpret in molecular terms, but there are some bands markers, such as the one at 853 cm\(^{-1}\) relative of pectin, which should be sensitive to the state of protonation of the pectin. However, it is not been observed a shift of this band. In consideration of the interaction expected between pectins and chitosan, infrared spectroscopy is more suitable to highlight the formation of an interaction between this two polysaccharides.

17.2 FTIR-ATR studies

ATR FT-IR spectra of chitosan nanoparticles and chitosan matrix are shown in figure 38. The broad peak between 3350 and 3270 cm\(^{-1}\) can be attributed to a combination of stretching modes of O-H and N-H bonds in chitosan matrix. In the sample of chitosan nanoparticles this band becomes wider and shifts to lower wavenumbers, indicating an enhancement of the hydrogen bonds system.

In nanoparticles, the 1523 cm\(^{-1}\) peak of \(-\text{NH}_2\) bending vibration shifts to 1533 cm\(^{-1}\). A similar result has been observed in a study of chitosan film treated with phosphate (NaH\(_2\)PO\(_4\)), and attributed to a linkage between phosphoric and ammonium ion. The other peaks observed in the sample of nanoparticles are those of the P=O stretching at 1201 cm\(^{-1}\) and P-O bending at 885 cm\(^{-1}\) of the TPP.
Figure 39 shows the FT IR spectra of polygalacturonate, and citrus and apple pectins. The spectra are reported in the range between 1900 cm\(^{-1}\) and 600 cm\(^{-1}\) because the main changes occurs in this region.

Pectins showed the typical stretching C=O band of methyl ester group and undissociated carboxyl group at 1735 cm\(^{-1}\) (obviously absent in the sample of polygalacturonate) and the asymmetric stretching C=O band of carboxylate at 1598 cm\(^{-1}\). This band in the sample of polygalacturonate is
instead at 1605 cm\(^{-1}\). It can be seen that the peak of stretching C = O of the ester group is more intense in the sample of pectin from apple, while the asymmetric stretching and symmetric stretching bands (at 1408 cm\(^{-1}\)) of the carboxylate group are less marked compared to the sample of pectin from citrus fruit. This is due to the higher degree of esterification of the sample of pectin from apple.

In all three cases the region of 1200 – 1000 cm\(^{-1}\) contains skeletal C-O and C-C vibration bands of glycosidic bonds and pyranoid ring.\(^{204}\)

![FT IR spectra of polygalacturonate, citrus pectin and apple pectin.](image)

**Fig.39** FT IR spectra of polygalacturonate, citrus pectin and apple pectin.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Polygalacturonate</th>
<th>Pectin from Citrus</th>
<th>Pectin from Apple</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\nu$ O-H, N-H</td>
<td>3345</td>
<td>3320-3200</td>
<td>3320-3200</td>
</tr>
<tr>
<td>$\nu$ C-H</td>
<td>2918</td>
<td>2900-2860</td>
<td>2900-2860</td>
</tr>
<tr>
<td>$\nu$ C=O (ester)</td>
<td></td>
<td>1733</td>
<td>1735</td>
</tr>
<tr>
<td>$\nu$ COO$^-$ (as)</td>
<td>1605</td>
<td>1598</td>
<td>1600</td>
</tr>
<tr>
<td>$\nu$ COO$^-$ (s)</td>
<td>1409</td>
<td>1402</td>
<td>1402</td>
</tr>
</tbody>
</table>

**Tab.18** FT IR band wavenumbers (in cm\(^{-1}\)) for polygalacturonate, citrus pectin and apple pectin.
In figure 40 are reported the spectra of chitosan nanoparticles after the coating process with pectins with different degree of esterification. Polygalacturonate is practically a pectin with a degree of esterification equal to zero. In all three cases, the main differences from the starting materials (chitosan and pectin) concerns the peak of asymmetric stretching of the carboxylate (resulting from the polyanion) and the bending peak of the amino groups of chitosan. The shift of those bands indicate a change in the environment of amino and carboxyl groups through the mutual interaction. FTIR band wavenumbers (in cm$^{-1}$) for the C=O stretching band and the amino group bending band are reported in table 19 (a,b,c).

![FT IR spectra of chitosan nanoparticles coated respectively with: polygalacturonate, citrus pectin and apple pectin.](image)

**Fig. 40** FT IR spectra of chitosan nanoparticles coated respectively with: polygalacturonate, citrus pectin and apple pectin.
Table 19 Comparison between the stretching C=O and bending N-H bands wavenumber (in cm\(^{-1}\)) in chitosan nanoparticles coated with: (a) polygalacturonate; (b) citrus pectin; (c) apple pectin.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Chitosan NP</th>
<th>Poligalacturonate</th>
<th>NP coated</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ N-H</td>
<td>1533</td>
<td>1605</td>
<td>1585</td>
</tr>
<tr>
<td>ν COO(^{-}) (as)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Assignment

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Chitosan NP</th>
<th>Pectin from Citrus</th>
<th>NP coated</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ N-H</td>
<td>1533</td>
<td>1596</td>
<td>1589</td>
</tr>
<tr>
<td>ν COO(^{-}) (as)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(c) Assignment

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Chitosan NP</th>
<th>Pectin from Apple</th>
<th>NP coated</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ N-H</td>
<td>1533</td>
<td>1598</td>
<td>1587</td>
</tr>
<tr>
<td>ν COO(^{-}) (as)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The shift observed in the two pectins (table 21, (b) and (c)), do not vary significantly from one to another, probably indicating that in this case the change in the degree of esterification does not affect the number of interactions that are formed with amino groups of chitosan. The effect of the shift is rather more pronounced for the sample coated with polygalacturonate.

In figure 41 is shown the spectrum of the sample of nanoparticles formed by blending between chitosan and pectin from apple. It can be observed a weak peak at 1731 cm\(^{-1}\) corresponding at the C=O stretching band of the ester group of the pectin.

In addition it can be noticed, as in the case of the coated sample, the shift of the asymmetric stretching peak of carboxylate group and the shift of the \(-\text{NH}_2\) bending peak.

In particular, the shift of the \(-\text{NH}_2\) bending peak is greater than what has been observed in the coated samples.
This could be due to the fact that the bending modes are sensitive to the changes in the environment of the group and so are more affected by a perturbative surrounding than the stretching modes.

Therefore this shift of the $-\text{NH}_2$ bending band could be indicative of a greater number of interaction between the amino groups of chitosan and the carboxyl groups of pectin, compared to the coated samples.

![FT IR spectrum of nanoparticles formed by blending between chitosan and pectin from apple.](image)

**Fig.41** FT IR spectrum of nanoparticles formed by blending between chitosan and pectin from apple.

<table>
<thead>
<tr>
<th>Assignment</th>
<th>Chitosan NP</th>
<th>Pectin from Apple</th>
<th>NP coated</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\delta \text{ N-H}$</td>
<td>1533</td>
<td>1598</td>
<td>1556</td>
</tr>
<tr>
<td>$\nu \text{ COO}^-$ (as)</td>
<td>1598</td>
<td>1604</td>
<td></td>
</tr>
</tbody>
</table>

**Tab.20** Comparison between the stretching C=O and bending N-H bands wavenumber (in cm$^{-1}$) in nanoparticles formed by blending between chitosan and pectin from apple.
18 Biocompatibility

18.1 In vitro tests: XTT and LDH assays

Pectins and chitosan are widely regarded as being non-toxic and biologically compatible polymers. Pectins are employed in food industry as gelling agents; chitosan is approved for dietary applications in Japan, Italy and Finland and it has been approved by the FDA for use in wound dressings. The modifications made to the native polymers could make them more or less toxic and any residual reactants should be carefully removed.

From the direct comparison among the toxicological studies reported in literature, a great variability can be found due to the experimental parameters, including the use of different cell lines, the conditions employed, the polymer forms, the molecular weights and suppliers and the differences in the formulations. Very low values of toxicity for chitosan solutions and nanoparticles have been reported in respiratory cell lines; it was also found that application of chitosan solutions to intestinal cell lines was well tolerated and associated with some membrane toxicity only at high concentrations and in a dose-dependent manner.

In this Thesis, the viability of the Caco-2 cells (human epithelial colorectal adenocarcinoma cells, used as model of intestinal epithelium) in the presence of both raw polymer materials (sodium pectate from apple, low molecular weight chitosan and chitosan-galactose) and biopolymeric nanocarriers were evaluated by standard in vitro tests: the XTT assay and the LDH assay.

The XTT test has been used to evaluate the effects of the polymers and the particles on the metabolic activity of the cells. No evidence of cytotoxicity was observed for the polymer solutions or the particle suspensions (figure 40) and a cell viability of around 100% was observed in all test groups. Moreover, the cells resulted to be metabolically active, as already reported for similar formulations.
The results of the LDH assay (figure 43) showed a low release of LDH, if compared to the control used. The application of the nanoparticle suspension equivalent to the raw material concentration doesn’t cause a variation in LDH release: there is no significant difference between nanoparticles and solutions for both the concentrations tested. In conclusion, results are comparable with that of not treated cells used as a control. Thus, no cytotoxic activity could be detected for all the polymers and NP suspensions used.

All these preliminary results are in good agreement with those found by cytotoxicity studies carried out on similar polysaccharidic nanocarriers.\textsuperscript{174}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{xtt-assay.png}
\caption{Results of the XTT assay for the polymer tested}
\end{figure}
The biocompatibility of materials used for drug delivery is usually evaluated in vivo using mammalian models. The chorioallantoic membrane of embryo chicks can be used as an alternative for studying the effects of materials used. After 24 hours from the treatment the samples were analyzed at different magnitude and picture were acquired, analyzing the inflammatory and neo-angiogenic effect (figure 44 a-b). The tablets are partially dissolved and the samples absorbed. In the case of raw materials (chitosan, pectin from apples, trimethyl chitosan), no significant changes occurred after 24 hours of incubation. In fact all embryos were alive and there was no sign of any vascular change in the CAM, e.g. hemorrhaging, hyperaemia (capillary injection) or the occurrence of vessels devoid of blood flow (ghost vessels). This is a good agreement with reported in literature sign that our materials are non-toxic, as
expected and described: chitosan is a polymer approved for dietary applications in Japan, Italy and Finland and it has been approved by the FDA for use of wound dressings, while pectin is commonly used as gelling and thickening agent in the food industry and as an excipient for pharmaceutical purpose.

Moreover, prepared nanoparticles don’t generate death or inflammatory response. This is an important point because, instead of raw materials, it is not always predictable: a lot of attention is put in nanosized carriers concerning the effect (toxicity) of shape/surface structure of the new nanoentities on cells and tissues. These results are in accord with in vitro toxicity test.

The only sample that gives an adverse effect is the TPP tablet. This is an expected result, because the tripolyphosphate salt increases the pH of chorioallantoic membrane drastically, generating an hemorrhagic event and death of the embryo. On the contrary, the amount of TPP presents in nanoparticles is significantly lower and no sign of damages can be noticed. Furthermore, low amounts of TPP are commonly used as food additive to preserve foods such as red meats, poultry, and seafood, helping them to retain their tenderness and moisture.

![Fig.44 CAM image taken from the top of the egg after deposition of prepared tablet. (a) Effect of pectin from apple on chick embryo after 24 h since deposition. (b) Effect of TPP on chick embryo: after 2 h since deposition and hemorrhagic event occurs with death of embryo.](image-url)
Conclusions
Conclusions

During the three years of studies a large variety of systems have been studied and many different methodologies have been employed with continuous cross-reference of experimental results.

Looking at a brief representing conclusion, the main innovative results are:

- Starting from the set-up of conventional chitosan, new chitosan derivatives and different pectins have been prepared for particles with tailored characteristics

- Mastering nanoparticles production and characterizing the evolution of particles formation, the key of isolating a required particle population has been identified.

- The use of several methodologies, including those less conventional for particles characterization, is attractive because of the potentialities both for the chemical distribution of the polymers in the nanoparticles with spectroscopic methods, and for the biocompatibility of polymers and nanoparticles with \textit{in vitro} and \textit{in vivo} tests
References


Atkinson A. and Jacj J. W., “Precipitation of nucleic acids with polyethyleneimine and the chromatography of nucleic acids on immobilized polyethyleneimine”, Biochimica et Biophysica Acta, 308 (1973)


Kertesz Z.I. “The Pectic substances”, on Fermentation of tobacco, tea, cocoa, coffee (1951) 589-590 Intersciences publichers, inc., NY


165 “The Relevance for Food Safety of Applications of Nanotechnology in the Food and Feed Industries”, Food Safety Authority of Ireland, 2008.

166 “Guidance on risk assessment concerning potential risks arising from applications of nanoscience and nanotechnologies to food and feed EFSA Scientific Committee”, European Food Safety Authority (EFSA), 2011.


175 Rous P. and Murphy JB. “Tumor implantations in the developing embryo” J. Am. Med. Assoc., 56 (1911) 741–742.


## Index

Abstract ........................................................................................................... 2

Sommario .......................................................................................................... 5

Introduction ...................................................................................................... 8

1 Drug delivery .................................................................................................. 9
   1.1 Polyelectrolyte complex for drug delivery .............................................. 10
   1.2 Oral route ................................................................................................ 15
   1.3 Mucosal drug delivery ........................................................................... 16
   1.4 Mucus: structure, function and composition ......................................... 17
   1.5 Micro- and Nano-particles as carriers .................................................... 21

2 Nanoparticles formation ............................................................................... 25
   2.1 Ionotropic gelation ............................................................................... 26
   2.2 Polyelectrolyte complexation .................................................................. 28

3 Biopolymers .................................................................................................. 31
   3.1 Natural cationic polymers ..................................................................... 31
      3.1.1 Chitosan ......................................................................................... 31
      3.1.2 Chitosan derivatives ....................................................................... 34
         3.1.2.1 N-trimethyl chitosan (TMC) ...................................................... 34
         3.1.2.2 Glycosylated chitosans ............................................................ 36
      3.2 Association with other biopolymers: pectins ..................................... 38

4 Biocompatibility - Nanotoxicity .................................................................. 41
   4.1 Biocompatibility of nanoparticles as carriers for drug delivery ............ 41
   4.2 Biocompatibility of biopolymer nanoparticles as carriers for drug delivery 42

5 Aim of study ................................................................................................. 45

Materials & Methods ...................................................................................... 46

6 Materials ....................................................................................................... 47

7 Polymer characterization ............................................................................... 49
   7.1 NMR studies ......................................................................................... 49
   7.2 Size-exclusion chromatography studies ............................................... 49
   7.3 Viscosity studies .................................................................................... 50
14 Nanoparticles formation and optimization
14.1 Chitosan-TPP nanoparticles preparation
14.2 Size and surface charge
14.2.1 Size for chitosan nanoparticles
14.2.2 Evolution of nanoparticles formation
14.3 Nanoparticle stability
14.4 Yield results
14.5 Loading
14.5.1 Effect of protein concentration on size
14.5.2 Particle loading efficiency
14.6 Storage
14.6.1 Lyophilization
14.6.2 Spray drying
15 Chitosan derivatives nanoparticles
15.1 TMC nanoparticles
15.2 Glycosylated chitosan nanoparticles
16 Association with other biopolymers
16.1 Size for pectin coated chitosan nanoparticles
16.2 Size for blended nanoparticles
17 Spectroscopic characterization
17.1 Raman spectroscopy
17.2 FTIR-ATR studies
18 Biocompatibility
18.1 In vitro tests: XTT and LDH assays
18.2 In vivo test: the chorioallantoic membrane (CAM) assay

Conclusions

References