Tomas Bata University in Zlín
Centre of Polymer Systems

Doctoral Thesis Summary

Biopolymer-Based Nanocomposite as Drug Delivery System

Nanokompozit na bázi biopolymeru jako systém dodávání léčiv

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Dedication

This doctoral thesis is dedicated to my beloved brother, **Vahid Yadollahi,** who was battling cancer during the period of my PhD study. He provided me with inspiration and support, but he unfortunaly did not see it completed. Vahid, your memory will remain forever in my heart.

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I would also like to remember and spiritually thank my beloved late mother, Soghra Yadollahi, my late brother, Vahid Yadollahi, and my late sister – in – law, Asiye, for their blessings and support. Their memories will always be in my heart.

Contents

Abstract

Whey protein isolate (WPI), employed as a carrier for a wide range of bioactive substances, suffers from a lack of colloidal stability in physiological conditions. To address this issue, stabilized PolyElectrolyte Nanoparticles (PENs) have been developed using two techniques. The first technique involved polyelectrolyte complexation of negatively charged WPI and positively charged chitosan (CS), and the second used ionic gelation in the presence of polyanion tripolyphosphate (TPP).

Considering the effect of pH and concentration on the self-assembly of nanoparticles (NPs), the pH and nanoparticle concentration had to be optimized first. Thus, the WPI-based core was coated with a CS-based shell and stabilized by TPP at pH=8. The resulting stabilized nanostructures were characterized using various physiochemical methods (DLS, SEM, FT-IR, TGA), and their encapsulation efficiency and in vitro release were evaluated. The spherical NPs with an average size of about 248.57 ± 5.00 nm and surface charge of +10.80 ± 0.43 mV demonstrated high encapsulation efficiency (92.79 ± 0.69) and sustained release of a positively charged chemotherapeutic drug such as doxorubicin, which contributes to increasing the stability of WPI/CS complexes. The Z-average size and size distribution showed negligible increases in size and aggregates over three weeks.

The results obtained confirm the effectiveness of the suggested methods in improving the colloidal stability of PENs making them a promising carrier for nanoparticle cargo delivery.

Abstrakt

Syrovátkový proteinový izolát (WPI) je nosič využívaný pro širokou škálu bioaktivních látek, který má za určitých fyziologických podmínek nedostatečnou koloidní stabilitu. Pro zlepšení byly navrženy dva postupy pro stabilizaci polyelektrolytových nanočástic (PEN). První postup byl pomocí polyelektrolytové komplexace záporně nabitého WPI a kladně nabitého chitosanu (CS). Druhý byl postup pomocí iontové gelace v přítomnosti polyaniontu tripolyfosfát (TPP).

Na stabilitu samouspořádání nanočástic má vliv pH a koncentrace nanočástic, proto musí být nejprve optimalizovány. Proto bylo jádro WPI pokryto vrstvou chitosanu a následně stabilizováno pomocí TPP s pH=8.
Vzniklé stabilní nanostruktury byly charakterizovány fyzikálně-chemickými metodami DLS, SEM, FT-IR a TGA. Hodnocena byla úroveň zapouzdření a uvolňování nanočástic in vitro. Sférické nanočástice s průměrnou velikostí asi 248,57 ± 5,00 nm a povrchovým nábojem +10,80 ± 0,43 mV prokázaly vysokou účinnost enkapsulace (92,79±0,69) a kontinuální uvolňování pozitivně nabitého chemoterapeutika doxorubicin, které přispívá ke zvýšení stability WPI/CS komplexů. Velikost Z-average a distribuce velikostí vykazovaly v průběhu tří týdnů zanedbatelný nárůst velikosti a tvorby agregátů.

Získané výsledky potvrzují vhodnost navržených postupů pro zlepšování koloidiální stability PEN, což z nich činí slibné nosiče pro řízené dodávání nanočástic.

1. Introduction

Cancer is the second most widespread and dreadful disease after cardiovascular issues, killing millions of people annually worldwide. Despite significant advances in medicine, there are still considerable obstacles that need to be addressed in order to enhance cancer treatment. Nanobiotechnology is a new discipline connecting physical and biological sciences to create new tools for comprehending biological systems and disease treatment and diagnosis [1, 2]. Drug delivery systems in nanobiotechnology are essential for prevention and disease treatment, especially in cancer [3]. A significant variety of drug delivery vehicles have been investigated to improve the safety and efficacy of anticancer medications [2, 4]. Therefore, natural food-grade materials such as whey proteins can be utilized as promising and versatile nanodelivery systems (NDS) [5, 6].

Whey protein (WP) obtained from whey is a by-product of cheese production and has gained popularity as NDSs due to low cost, low toxicity, biocompatibility, biodegradability, and a variety of functionalities including high encapsulation efficiency, sustained release behaviour, and rapid absorbance across biological membranes [7-11]. Whey protein isolated (WPI) contains higher than 90% protein quantities (i.e., α-lactalbumin and β-lactoglobulin), less than 1% lactose, and 1% milk fat [12]. Due to the amphiphilic component, WPI is utilized for food emulsion stabilization, bioavailability and stability improvement of hydrophobic nutrients [7, 12-14]. Despite the vulnerability to thermal processing, pH, and ionic strength [15-17], this component has also demonstrated the potential in delivery systems to carry a wide range of bioactive substances through the functional groups on the primary structure and release them at particular target sites [6, 18]. This study also aims to fabricate polyelectrolyte nanoparticles. (PENs) by integrating WPI with a biodegradable polymer such as chitosan (CS) as a drug delivery system. CS is a biopolymer of N-acetyl glucosamine and D-glucosamine units obtained by deacetylation of chitin. This cationic polysaccharide is widely employed in medicine and food technology due to its mucoadhesive, biocompatibility, biodegradability, non-toxicity, and antibacterial properties [19-22]. CS shows a positive charge below its pka of 6.5 through the protonation of amino residues, which is the basis of immobilization of negatively charged molecules such as proteins, medicines, and other cargos [23, 24]. CS forms polyplex aggregation or coacervation in the presence of polyanions such as tripolyphosphate (TTP)[25]. TTP is a nontoxic, weak polyprotic acid and multivalent anionic cross-linker with a pKa of 0.89. It can improve the quality of

electrostatic interactions, which leads to the formation of PENs and a sustained release rate [26, 27]. Moreover, pH considerably impacts the electrostatic interaction of CS with the negatively charged molecules [28]. When these interactions were established, anionic residues/cationic chitosan aggregation [29] or coacervation [30] complexes are formed, which can be employed for various purposes, including the micro and nano-encapsulation of pharmaceuticals and chemicals (28, 29).

There are studies on polyplexes such as WPI/CS film in food packaging [31, 32] and nanoparticles as delivery systems [33, 34]. They utilized the nano-precipitation technique for loading a trypsin inhibitor [33, 35, 36] and oil-in-water (O/W) emulsion to encapsulate α-tocopherol [34]. In other studies, Lin et al. co-assembled CS with WP to fabricate nanoparticles (NPs) for loading Ophiopogon japonicus [37], and Xu et al. demonstrated the physiochemical stability of co-assembled WPI-CS NPs under adverse environmental stresses [38]. Nonetheless, this study aims to design an innovative WPI-CS NP as a nano drug delivery system through two techniques, including polyelectrolyte complexation and ionic gelation by self-assembly of an anionic WPI core at a pH value higher than its pKa value of 5.5 [39] with a cationic CS shell in the presence of a negatively charged cross-linker such as *tripolyphosphate* (TPP). TPP, with its polyanionic properties, forms polyelectrolyte nanoparticles to improve sustained release performances [25]. Finally, the nanoparticles are utilized for loading doxorubicin (DOX) as a model drug. DOX with the pKa value of 9.93 is in the cationic form at pH 7.4 [40], which predicts driving forces such as electrostatic and hydrophobic interactions for loading into the core. These driving forces arising from the opposite charges of the functional groups can stabilize the polyelectrolyte complexes, and steric stabilization protects WPI and DOX from the harsh environment of cancerous tissue (i.e., pH and thermal conditions)[41].

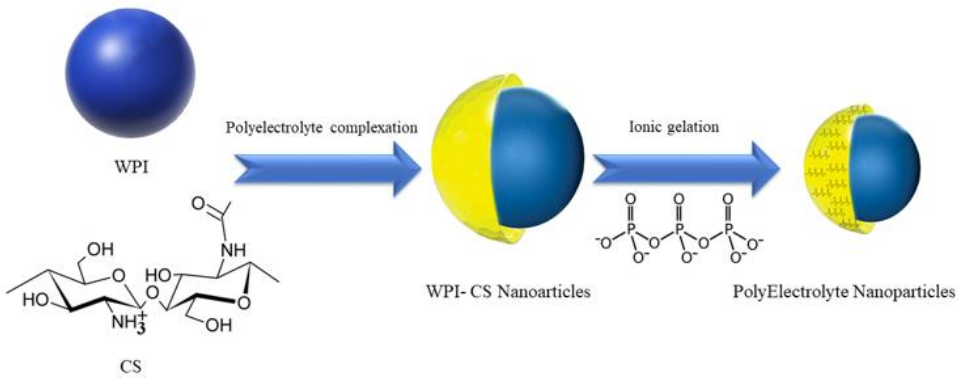

Figure 1 Schematic of WPI-CS PENs as drug delivery system[42].

2. Polyelectrolyte nanoparticles

Natural and synthetic polyelectrolytes have gained great interest in the last years for various applications. Polyelectrolyte complexes (PECs) are micro-/nanostructured units with exceptional properties obtained via the polymeric interaction between cationic and anionic polyelectrolytes. PECs as safe drug delivery carriers are spontaneously formed by mixing the oppositely charged polyelectrolyte solutions in water without using organic solvents nor chemical cross-linker or surfactant, leading to interpolymer ionic condensation. Intensifying attention on the PECs study is aroused in academia and industry since the fabrication process of PECs is mild and they are ideal vectors for the delivery of susceptible drugs and macromolecules. The formation of colloidal PECs is a safe and green process for manufacturing materials for drug delivery applications. Hence, the fabrication process is simple to implement such as one-shot addition and it is also energy efficient with a low shear rate at room temperature [43]. The properties are affected by the conditions of the liquid medium, the order of addition of the polyelectrolytes, the mass ratio, and the polyelectrolyte concentration, among others. The driving force results mainly from the release of molecular counterions into a polyelectrolyte-depleted solution phase, which increases the entropy of the system. In addition, inter-macromolecular interactions such as Van der Waals forces, hydrogen bonding, hydrophobic interactions are involved in the formation of PEC structures as well [44]. The PEC formation generally involves two or three steps. The first step is instantaneous and leads to the formation of a random primary complex with significant distortions of the configuration of polymer chains. Then, the secondary complex is formed by rearrangement of existing linkages within intra-complexes. It involves the formation of new bonds, e.g., electrostatic bonds, hydrogen bonds, hydrophobic interactions, etc. Finally, under certain conditions, primary and secondary complexes can aggregate (probably by hydrophobic interactions) and lead to various stable structures: entangled aggregates, fibrils, ordered networks, etc [45].

The emergence of nanotechnology in the biomedical field has opened up new opportunities especially for research on the vectorization of drugs, as the nanocarriers could protect drugs from premature degradation, improve their bioavailability, and prolong the residence of drugs in desired anatomic sites, thus reducing required doses and prolonging administration intervals. A myriad of versatile colloidal carriers with complex manufacturing procedures were designed for delivering therapeutic molecules to the target sites, nevertheless, colloidal vectors with facile and green fabricating processes are still of utmost interest for pharmaceutical researchers as well as enterprises. PolyElectrolyte Nanoparticles (PENs) obtained by layer-by-layer self-assembly of polycations/polyanions have the advantage of being easily prepared and they display favourable physicochemical features of different polymers. Nevertheless, under physiological circumstances, PENs are unstable, suffering from a lack of colloidal stability in physiological conditions. Due to their potent self-assembly of reversible electrostatic interaction between oppositely charged polymers, which are advantageous for the controlled release and encapsulation of charged cargos, PENs have gained interest. The controlled assembly of polycation and polyanion may lead to the formation of nanoparticles, macro-hydrogels, or films. It is imperative to strictly control the manufacturing conditions that can obtain colloidal PENs instead of forming macrocomplexes. As a matter of fact, the structure of the colloidal PENs is highly impacted by the intrinsic parameters, particularly the characteristics of the polyelectrolytes (e.g. molar mass (M_w), the nature of the ionic groups, charge density) as well as the extrinsic parameters, such as the polyelectrolyte concentration, the charge mixing ratio, the pH, ionic strength of the mixing medium and the mode of addition (dropwise or one-shot addition). The use of polysaccharides constitutes a supplementary strength of PENS formation since they are generally regarded as safe and biodegradable with no toxicity. In addition, abundant resources in nature and low production cost offer them great potential to be developed. Particularly, most natural polysaccharides have hydrophilic groups such as hydroxyl, carboxylic acid and amino groups, which could form noncovalent bonds with biological tissues (mainly epithelial and mucous membranes), forming bio-adhesion.

2.1 Protein /polysaccharide in drug delivery system

Natural biopolymers, including proteins and polysaccharides, have become an interesting alternative to synthetic ones in the fabrication of different NDS. The investigation of the synergistic effects between the unique properties of proteins (silk, gelatine and whey protein) and polysaccharides (alginate, chitosan, cellulose, starch, pectin and carrageenan) and the presentation of NPs has greatly advanced for the development and application of NPs in the field of drug delivery [46]. Complex nanosystems fabricated by hybridization of different types of materials such as lipids, proteins and polysaccharides are usually superior to simple ones in terms of features and applications. Proteins and polysaccharides hold great potential for development of nanocarriers for drug delivery purposes based on their unique biocompatibility, biodegradability, ease of functionalization, improved biodistribution and minimal toxicity profiles. Protein-polysaccharide nanohybrids have gained a lot of attention in the past few years, particularly for drug delivery applications [47]. The advanced progress of research in the fabrication of different nanosystems for drug delivery applications gives an insight into their added benefit in that area. The scope of benefits includes enhanced drug solubility, modulated drug release, enhanced targeting efficacy and better uptake by cells, enhanced drug stability and reduced toxicity, and enhanced biocompatibility, among others. A variety of materials, including natural and synthetic ones, have been used in the fabrication of these nanosystems[48]. Proteins and polysaccharides are the major components in biological systems with different roles ranging from structural ones to enzymatic activity, or energy production [49]. Many of them can be even found interacting specifically *in vivo* such as the interaction between lysozyme and glycosaminoglycans in cartilages, or the interaction between heparin and antithrombin in the inhibition of coagulation. These natural interactions inspired the fabrication of protein-polysaccharide nanohybrids which are superior to their individual components showing features not usually attained individually. Thus, the choice of the right components for the fabrication of protein-polysaccharide nanohybrids is important in creating certain sizes, hydrophilicities, surface charges, release patterns etc. [50]. Many factors affect the electrostatic complexation and physiochemical properties of protein-polysaccharide nanohybrids resulting in the formation of coacervates or soluble complexes [51]. The pH of the complexation medium influences almost all of the physiochemical properties of the produced nanocomplex, as it is the primary factor in the creation of the ionic complex. Both the polysaccharide and the protein must have opposing

surface charges for the ionic combination to form. Because polysaccharides only contain negative or positive ionizable groups, the protein will be forced to carry a charge. Given that proteins have a zwitterionic nature, their charge can be either positive or negative depending on their pI; negatively charged proteins will be found above their pI and positively charged ones below it [48]. It has been revealed that the stability of protein-polysaccharide nanohybrids is enhanced when sensitive drugs are encapsulated within their matrix. Chemotherapeutic drugs cause extreme toxicity to healthy tissues. To lessen their toxicity, many anti-cancer medications were added to protein-polysaccharide nanohybrids. When doxorubicin (DOX) was encapsulated into albumin-dextran nanohybrids, the average body weight of the hepatoma H22-bearing mice increased significantly (from 20.7 to 24.4 g after 7 days) and their survival rate increased by 35.4 %. In contrast, when DOX was free, the mice's body weight changed from 20.7 to 16.1 g and their survival rate increased by only 1.24%. Additionally, the DOX dose was increased to 8 mg/kg thanks to the nanohybrids, which raised the survival rate to 55.7% [52]. Researching the interactions between proteins and biopolymers, such as polysaccharides is crucial since there are many uses for them, including the encapsulation and stabilisation of active substances in complex assemblies. When biomaterials such as proteins and polysaccharides are combined to encourage the trapping of active chemicals, complexation occurs. The miscibility of the parts of the complex system is made possible by the contact forces between the charged groups in the polymeric chains. When building delivery systems, it's critical to comprehend the interactions that occur between the polymers and between the wall material and the active chemical. However, when employed in encapsulation applications, certain characteristics of the biopolymers, such as structure, functional groups, or electrical charge, as well as external factors such as pH or ratios, can affect the performance and structure of the complex system [48]. The molecules' affinity, which enables them to form compatible structures through electrostatic interactions, is primarily responsible for the complexation process. Controlling physicochemical factors such as pH, temperature, concentration, and proportion is essential when using these biopolymers in a complexation system as it triggers a phase phenomenon that leads to a stable system. The electrostatic interactions (Fig. 2), the interfacial phenomena generated between polymeric systems, and the retention characteristics of these materials when interacting with hydrophobic or hydrophilic chemicals have all been explained by a number of physicochemical parameters. These kinds of things could prevent the complicated system from assembling properly, which could lead to phase separation, immiscibility, or the development of multiple-gel

formations. Comprehending the stabilisation of protein-polysaccharide systems necessitates multiple investigations and sufficient knowledge regarding the molecular conformation and physicochemical changes occurring throughout the procedure. A rise in temperature can denaturize proteins, which can result in the creation of covalent bonds mostly through disulfide bridges. Moreover, complexation may happen if components are introduced to create multiphase structures or if the pH changes the electrostatic charges of the biopolymers. It is crucial to stress that the assembly, complexation, and obstruction to the ordering of the complex system are determined by the features of the biopolymers, such as the molecular weight of the polysaccharide or the amphiphilic nature of proteins.

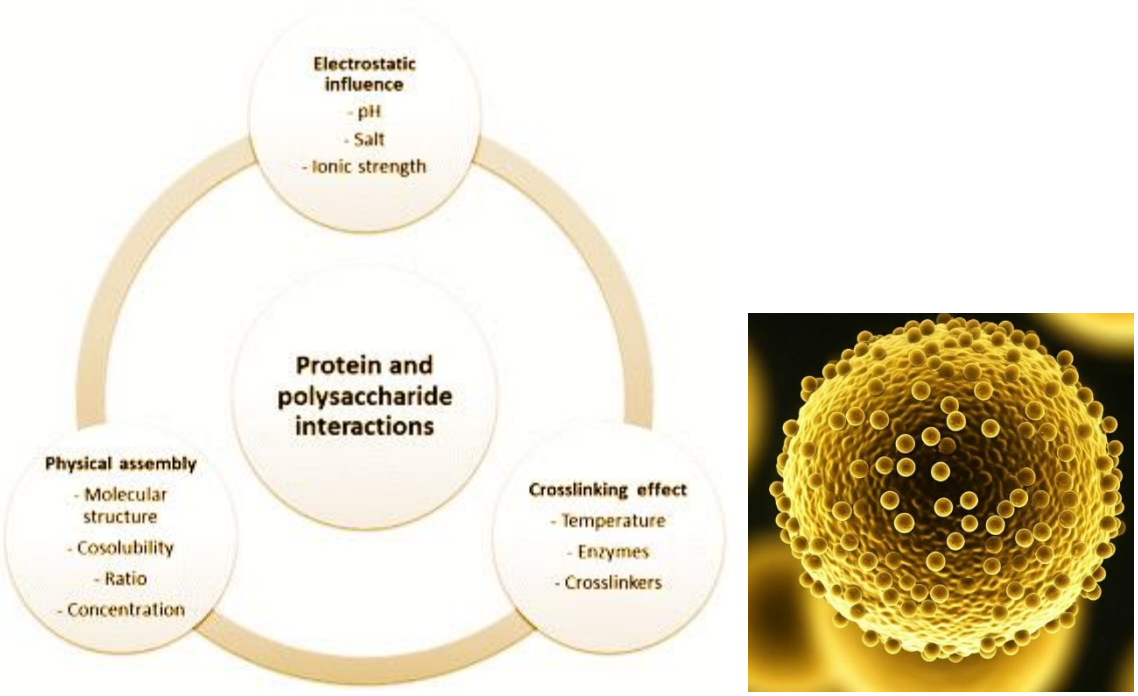

Figure 2 Parameters promoting the protein-polysaccharide interaction[53].

The functional groups at the ends of polymeric chains determine how polysaccharides bind to other molecules. Chemical alterations can be used to alter the functional groups in order to enhance their characteristics and augment their capacity for interaction. Because of electrostatic interactions, modified polysaccharides with positive or negative groups can interact with water to increase their solubility and either prohibit or promote intermolecular connection [54]. On the other hand, peptide bonds bind the amino acids that make up proteins. A vast range of functional groups are present in amino acids, allowing them to interact with other molecules in solution as well as solvents [55]. Moreover, the

polar and non-polar groups of amino acids give proteins their amphiphilic behaviour, and interaction forces such as hydrogen bonds, disulfide bridges, and attraction-repulsion interactions are necessary for the stabilisation of complexes. The solvent (water) and other elements in the solution may compete with one another as a result of these interactions. Extrinsic variables including temperature, ionic composition, and ionic strength affect the solubility of all biopolymers[56], but pH is the primary determinant of protein solubilization. The pH of proteins controls their alterations and the nature of their interactions with other molecules and solvents. The isoelectric point (pI), which denotes the insoluble state of a protein at which protein-protein interactions are enhanced and the protein's net charge is equal to zero, is also significant. Because the protein molecule and the solvent are incompatible, the closer the protein is to the pI, the more insoluble it is. Proteins in the solution precipitate at the pI. At the pI of the protein and the pKa of the polysaccharide, the complex fully occurs[57]. However, depending on the isoelectric point (pI), pH can alter how some proteins behave. The net charge is positive for pH<pI and negative at pH>pI. Because single bond COOH becomes single bond COO^- groups (increased negative charge) and single bond NH_3^+ becomes single bond NH_2^+ groups (reduced positive charge) when pH rises, proteins become less charged [58].

2.1.1 Whey protein isolate

WPI is made from milk, which contains two key proteins that make up 65 % of the weight of whey protein: β-lg and α-la. The globular shape of whey protein is primarily composed of β-lactalbumins (12–25 %) and β-lg (35–65 %). Whey protein hydrolysate (WPH) and WPI (90 percent protein) are crucial parts of whey protein. Combinations of globular proteins with diverse compositions and functional characteristics make up whey protein. WPI has extensive control over this protein's functional characteristics and uses it as a Drug delivery mechanism. In WPI, the β-lg is a prominent globular protein. With an isoelectric point of 5.2, β-lg has a molecular weight of 18.4 kDa. There are two disulfide bridges and one free cysteine among the 162 amino acids that make up each monomer. These proteins share characteristics with other common surfactants in that they are amphiphilic. Directly linked to the impact of WPIs, the amino acids and disulfide bonds are hidden away in the structure of proteins. The primary factor influencing increased adsorption on the drug surface is the denaturation of these nonpolar and disulfide links within the protein. The schematic representation of whey protein-

based DDs. The NPs synthesized from WPI biomaterials have demonstrated promise because of their superior biocompatibility, high drug encapsulation efficiency, and lack of toxicity to healthy tissues. The NPs made from whey protein are quickly absorbed via biological membranes, have a regulated release behaviour, and are safer to produce. Taking into account WPI can bind to hydrophobic components, it is a great option for creating new DDS for lipophilic drugs. [59].

2.1.2 Chitosan based PENs

CS is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine residues and N-acetyl-D-glucosamine units considering the fact that it is typically made by alkaline deacetylation of chitin derived from crab exoskeletons [31]. A high molecular weight can cause chitosan's solubility and viscosity to decrease, while a low degree of deacetylation increases the solubility and viscosity of the gel that forms [32]. Besides possessing the merits of most natural polysaccharides, such as high biocompatibility, excellent biodegradability, and abundant availability, the positive ammonium groups of CS (arising from the protonation of the glucosamine residues in weakly acidic conditions) endow its good bioadhesivity as well as anti-tumour activity [60]. Amino groups help the polymer become soluble in a slightly acidic solution and are helpful for chemical modification and electrostatic interactions in drug delivery systems. Furthermore, as compared to synthetic cationic polymers, CS exhibits relatively little immunogenicity despite the amino groups and positive change. It can be utilised as a bioadhesive, adhering to negatively charged mucosal cells and improving retention and transit into cells. Molecular weight, the degree of acetylation (DA), and even the pattern of acetylation (the distribution of glucosamine and N-acetylglucosamine units along the chitosan chain) will affect its chemical and biological properties. The degree of deacetylation (DD = 100 − DA) of chitosan is about 50% or higher. In dilute aqueous acid solutions, the amino groups of CS become protonated, allowing for its dissolution. In fact, the solubility of chitosan in 1 % or 0.1 M acetic acid is a simple and practical criterion used to differentiate it from chitin. However, CS solubility depends on its DD, the ionic concentration, the pH, and the distribution of acetyl groups along the chain, as well as the conditions of isolation and drying. If deacetylation of chitin is performed under homogeneous conditions chitosans with a DD of about 50% might dissolve, but if deacetylation is carried out under heterogeneous conditions, DD of 65% or higher is usually needed to achieve dissolution [61]. Also, because it has free –OH and $–NH_2$ groups in its structure,

it is amenable to chemical modifications that can potentiate some of its properties for certain applications (Fig. 3). CS possesses also mucoadhesive and absorption-enhancing properties. It opens the tight junctions between cells, allowing drugs to traverse the mucosal cells. These properties also make CS an ideal candidate for delivering drugs and bioactive molecules in general. Numerous reports demonstrate the various applications of CS in drug delivery, with several reviews on the subject.

CS nanoparticles are applied in drug delivery, not only through the traditional administration routes (e.g., oral and parenteral), but also via mucosal (nasal, pulmonary, vaginal) and ocular routes [62]. CS is freely soluble in an acidic solution, releasing a free amino group and developing a positive charge over the polymeric chain. CS had poor solubility at pH above 6.5. Generally, the chitosan nanoparticle can be formed by incorporating a polyanion like TPP (tripolyphosphate) into CS with constant stirring [63]. Given its positive charge, chitosan facilitates medication administration, especially for hydrophilic and high molecular weight compounds, to target tissues by improving the opening of epithelial tight junctions and enlarging paracellular channels. Furthermore, the quaternary ammonium groups improve adherence and uptake through the mucosa by providing a fixed charge that is pH-independent. Many procedures for obtaining CS nanoparticles have been proposed. Particularly, the introduction of hydrophobic moieties into chitosan molecules by grafting to generate a hydrophobic-hydrophilic balance promoting self-assembly is a current and appealing approach. The grafting agent can be a hydrophobic moiety forming micelles that can entrap lipophilic drugs or it can be the drug itself. Another suitable way to generate self-assembled chitosan nanoparticles is through the formation of polyelectrolyte complexes with polyanions [64]. Given their dual nature as both macromolecules and electrolytes, polyelectrolytes are very complicated materials. By adjusting production parameters, including molar masses, charge density, charge mixing ratio, pH value, ionic strength etc., chitosan, a flexible cationic polyelectrolyte, can bind with polyanions to generate colloidal PECs. When compared to low molar mass chitosan, high molar mass chitosan typically generated larger particle sizes. Choosing two oppositely charged polyelectrolytes with significant differences in Mw or degree of polymerization (about 30) could significantly increase the colloidal stability of PECs. Additionally, a non-stoichiometric mixing ratio is required in order to build colloidal PECs since an excess of chitosan chains, which act as the outer stabilising shell at the PEC interface and prevent particle coagulation, must be

provided. The addition of salt has a significant effect on the structure of chitosan-based nanoPECs. Excessive salt concentrations have the potential to separate the polyelectrolytes and compromise the integrity of the PENs.

Chitosan-based PENs are especially suitable for the mucosal delivery of sensitive biological molecules like vaccines and proteins like insulin because of chitosan's advantageous properties, particularly its mucoadhesive nature and absorption enhancement capability, as well as the safe and environmentally friendly manufacturing process of colloidal PENs. CS exhibits cationic behaviour, which enables it to bind with negatively charged protein residues. Based on specific research, chitosan is effective as a protein-synergistic wall material [65].In order to formulate PECs for use in gene delivery, mucosal delivery, anti-HIV therapy, and cancer therapy, CS is primarily combined with polyanions[65].

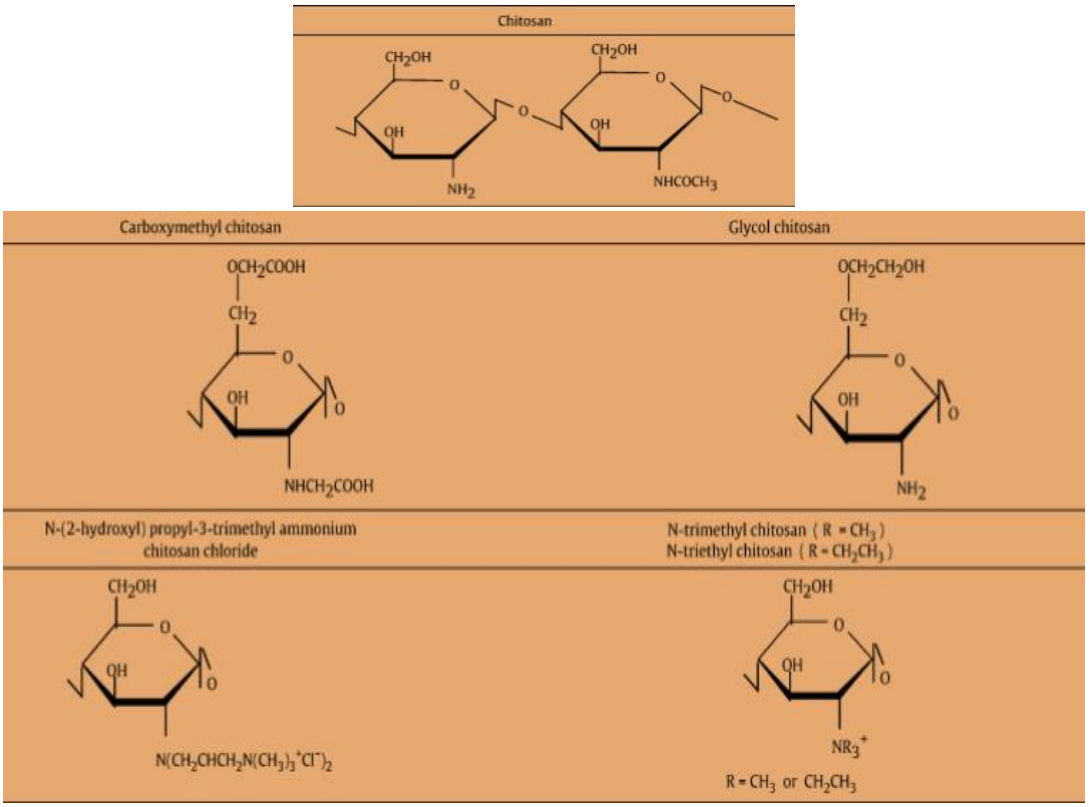

Figure 3 The chemical structure of chitosan and chitosan derivatives [66].

3. Literature review on CS-WPI structure

Whey protein isolate (WPI) has emerged as a promising material for nanoparticle-based drug delivery systems due to its biocompatibility and functional properties. Whey protein-based nanoparticles can be used as a delivery system for bioactive compounds. 1) Whey proteins have several functional properties that make them an ideal delivery material for nano-delivery systems (NDSs). 2) The physicochemical properties of whey protein-based NDSs can be modulated by various manufacturing processes to impact the bioavailability of encapsulated bioactive compounds. 3) Whey protein-based NDSs can enhance the stability and bioavailability of encapsulated bioactive compounds. The formation of stable complexes between WPI and CS has been investigated under various conditions. Optimal complex formation occurs at specific pH ranges and concentrations, with particle sizes around 200-220 nm and positive zeta potentials (Xu et al., 2020; Zhao & Xiao, 2017). These complexes exhibit improved stability under environmental stresses, including temperature, salt, and sugar concentrations (Xu et al., 2020). Complexation with chitosan enhances the heat stability of WPI solutions, preventing denaturation and aggregation (Zhao & Xiao, 2017). The interaction between WPI and CS is primarily driven by electrostatic attractions, forming a porous network structure (Xu et al., 2020; Fekih Ahmed et al., 2018). Complexes demonstrate stability over extended storage periods and can be used to stabilize water-in-water emulsions (Xu et al., 2020; Khemissi et al., 2018)[67]. These findings provide insights into the industrial production of chitosan-protein complexes for various applications, including microencapsulation and beverage formulation (Xu et al., 2020; Zhao & Xiao, 2017) [67].

Recently Xu et al established a stable complex formed from whey protein isolate and chitosan, and evaluated its stability under various environmental stresses. The optimal conditions for forming a stable WPI-chitosan complex are 0.2 % (wt/wt) WPI, 0.05% (wt/wt) CS, and pH 5.7, resulting in a complex with a particle size of 217.8 ± 11.3 nm and a zeta potential of 16.7 ± 0.92 mV. The complex is formed through electrostatic interactions between the amine groups of chitosan and the carboxyl groups of WPI, and has a porous network structure with heterogeneously sized vacuoles. Determination of the optimal conditions for forming the stable complex (0.2 % whey protein, 0.05% chitosan, pH 5.7). Assessment of the complex's stability under environmental stresses (NaCl, sugar, heat) and during storage (4°C for 30 days, 25°C for 20 days) [67]. Fekih Ahmed et al. explored in their studies that stable dispersions of chitosan-protein particles can be formed by

complexation of fractal whey protein aggregates and chitosan. Stable dispersions of protein-polysaccharide particles were formed when the pH was gradually increased between 4.1 and 6.8, whereas without CS the protein aggregates precipitated between pH 4.1 and 5.4. The size of the protein-chitosan complexes changed with pH, first increasing sharply between pH 4.1 and 4.5, then remaining constant up to pH 5.6, and then increasing again. A minimum amount of CS was needed to form stable complexes at pH 5.0, and the size of the complexes decreased with increasing chitosan concentration [68].

Chitosan can form stable complexes with whey protein isolate and improve its heat stability, depending on the pH. At pH 4.0, a small amount of chitosan can prevent heat-induced denaturation and aggregation of whey protein. At pH 5.5, the formation of chitosan-whey protein complexes improves the heat stability of the dispersion, preventing precipitation for up to 20 days. A medium ratio of chitosan to whey protein (1:5) produced the most stable particles in terms of size and surface charge [69]. Furthermore, Khemissi et al. explored that Stable complexes of whey protein microgels and polysaccharides (κ-carrageenan or chitosan) can be formed and used to stabilize water-in-water emulsions. Formation of protein microgels by heating whey protein isolate solutions. Mixing the microgels with anionic (κ-carrageenan) or cationic (chitosan) polysaccharides. Characterization of the molar mass and size of the microgel-polysaccharide complexes using light scattering techniques, as a function of pH and composition.Visualization of the structure and stability of the complexes at higher microgel concentrations using confocal laser scanning microscopy [70].

Zhuzhu Liu et al. examined the Cold-gelation whey protein-chitosan complex hydrogels that can be used to encapsulate and deliver bioactive substances or drugs. Cold-gelation WPI-CS complex hydrogels were fabricated using genipin as a crosslinking agent. The study investigated the effect of varying CS concentrations on the properties of the WPI-CS complex hydrogels. The WPI-CS complex hydrogels showed a controlled release of curcumin, with lower release rates in simulated gastric and colon environments. The WPI-CS complex hydrogel had higher thermal stability compared to WPI alone[71]. M. Semenova et al in their paper examined the impact of covalent and non-covalent interactions between chitosan and whey protein isolate on the structure, thermodynamics, and functionality of their complexes with essential lipids. The covalent (WPI–CS) conjugate had a greater degree of protein unfolding, a more open architecture of a random coil, and a lower density compared to the electrostatic (WPI–CS) complex. The structural features of the covalent conjugate contributed to the

formation of ternary (WPI–CS) + (PC−ALA) complex particles with higher molar mass, density, and more open architecture compared to the electrostatic ternary complexes. Differential scanning calorimetry and laser light scattering to characterize the structural properties of covalent and electrostatic complexes between WPI and CS. Formation of ternary complexes between the WPI-CS conjugates and phosphatidylcholine (PC) liposomes enriched with α-linolenic acid (ALA), and characterization of their structural properties. Characterization of the size, solubility, surface charge, and oxidative stability of the ternary complexes, as well as the microviscosity of the encapsulated PC-ALA liposomes using electron paramagnetic resonance spectroscopy[72].

Loleny Tavares et al. studied the Complex coacervation between chitosan and whey protein isolate to form a stable composite edible film with improved physicochemical properties compared to individual biopolymer films. The incorporation of chitosan (CS) into a whey protein isolate (WPI) matrix resulted in a composite film with improved mechanical properties, including higher tensile strength, lower deformation, and increased malleability and flexibility, compared to the individual CS and WPI films. The composite CS/WPI film had lower solubility and water vapour permeability compared to the mono-component CS and WPI films. The addition of CS to the WPI film led to a decrease in the equilibrium moisture content, and the sorption isotherm of the composite film followed a type II isotherm. Analysis of the microstructure of CS/WPI coacervate and individual biopolymer dispersions using cryo-SEM[73]. Peifeng Lv et al. evaluated whey protein isolate and chitosan can form stable complex particles that effectively stabilize Pickering emulsion gels. The addition of chitosan to WPI gel particles resulted in the formation of larger complex particles (WCCPs) with a positive surface charge. The WCCPs were more effective as Pickering emulsifiers compared to the pure WPI gel particles, as indicated by the decrease in the three-phase contact angle. The Pickering emulsion gels stabilized by the WCCPs exhibited rheological properties that were affected by the concentration of the particles, with higher concentrations leading to smaller droplet sizes, reduced creaming, and stronger gel formation[74].

Other studies have explored the interactions between WPI and CS or chitosan oligosaccharide (COS). These interactions can significantly affect WPI digestibility, with both CS and COS reducing intestinal digestibility by over 40 % (Wang et al., 2022). pH and preheating conditions influence the interaction between CS/COS and WPI, with CS showing greater efficacy in preventing WPI denaturation and aggregation at pH 4.0 (Ma et al., 2023)[75]. WPI-CS complexes

demonstrate stability under various environmental stresses, including temperature and salt concentration (Xu et al., 2020)[67]. Furthermore, the nature of the associative interactions (covalent or non-covalent) between WPI and CS affects the structure and functionality of their complexes with essential lipids, influencing parameters such as particle size and oxidation protection (Semenova et al., 2020)[72].

Linlin Wang et al realized that chitosan and chitosan oligosaccharide (COS) reduce the digestibility of whey protein isolate through electrostatic interaction. Both CS and COS significantly reduced the digestibility of WPI, with decreases of 43.33 % and 52.31 % respectively. The reduced digestibility was due to an electrostatic interaction between the amine groups of CS and the carboxyl groups of WPI, which altered the stability and structure of the WPI - CS affected WPI digestibility by precipitating the protein and enzyme, while COS affected it by decreasing or inactivating the enzyme activity[76]. Ninging Ma et al. observed that Chitosan and chitosan oligosaccharide interact with whey protein isolate, affecting its heat stability and in vitro gastric digestive behaviour. CS was more effective than chitosan oligosaccharide (COS) in preventing the denaturation and aggregation of WPI when heated at pH 4.0. Changes in electrostatic interactions and protein structure affected the formation of the CS/COS-WPI complexes as the pH increased from 4.0 to 6.0. The inhibition of WPI digestion in the simulated gastric environment had opposite trends as the ratio of CS or COS to WPI increased[75]. Zhengtao Zhao et al. explored in their studies Chitosan can improve the heat stability of whey protein solutions by forming complexes at specific pH values. At pH 4.0, a small amount of chitosan can prevent heat-induced denaturation and aggregation of whey protein. At pH 5.5, the formation of chitosan-whey protein complexes improves the heat stability of the dispersion, preventing precipitation for up to 20 days. A medium ratio of chitosan to whey protein (1:5) produced the most stable particles in terms of size and surface charge[69].

Recent studies have explored the potential of WPI and CS composite particles for various applications. These particles exhibit a core-shell structure, with WPI forming the core and CS the shell (Xu et al., 2022; Pang et al., 2024) [67]. Particle size, surface hydrophobicity, and morphology are influenced by preparation conditions, such as temperature [77]. These composite particles show promising tribological properties, with potential applications as fat replacers in low-fat emulsions[77]. Similar core-shell structures formed with WPI and xanthan gum also exhibit interesting lubrication behaviours, although particle size and

clustering issues need further investigation [78]. Overall, WPI-CS composite particles offer versatile functionality in food and pharmaceutical applications.

Fei-Yue Xu et al. studied that Succinylated whey protein isolate-chitosan core-shell composite particles can effectively encapsulate and protect water-insoluble compounds like curcumin. Combining WPI or succinylated whey protein isolate (SWPI) with CS resulted in changes to the zeta-potential, surface hydrophobicity, and secondary/tertiary structures of the composite particles.The SWPI-CS composite particles (H-CS) exhibited a soft core-rigid shell morphology due to various intermolecular interactions. The H-CS composite particles had a higher binding constant and encapsulation effectiveness for the compound curcumin (CUR), and were more effective at stabilizing CUR against photodegradation and thermal degradation. Furthermore, Zhihua Pang et al. observed that whey protein/chitosan composite particles show potential as fat replacers and biolubricants. WPI/CS composite particles had larger particle sizes than pure WPI particles, with WPI/CS-95 being the largest. WPI/CS-75 particles had a distinct core-shell structure. WPI/CS-75 demonstrated superior lubricating effects compared to pure WPI particles, and could be used as a fat replacer in low-fat emulsions while maintaining low friction [78].

In this current research, whey protein isolate-chitosan polyelectrolyte nanoparticles were developed as a drug delivery system with high encapsulation efficiency and sustained release. We developed innovative stabilized polyelectrolyte nanoparticles (PENs) by combining whey protein isolate (WPI) and chitosan (CS) through polyelectrolyte complexation and ionic gelation with tripolyphosphate (TPP). The PENs showed high colloidal stability for up to 3 weeks, with negligible increases in size and aggregation. The PENs demonstrated high encapsulation efficiency and sustained release of the model drug doxorubicin, which was attributed to strong electrostatic and hydrogen bonding interactions between the drug, WPI, CS, and TPP. The primary outcomes measured in our study were the physicochemical properties of the PENs, including size, polydispersity, zeta potential, surface morphology, functional groups, and thermal stability, as well as the drug loading efficiency (encapsulation efficiency and loading capacity) and sustained release of the PENs with the chemotherapeutic drug of doxorubicin [42].

4. Aim of the doctoral thesis

The doctoral thesis seeks to explain the development of biopolymer-based nanocomposite in biomedical applications. This prospective research was designed to investigate the development of Novel PENs that offer stable drug delivery with sustained release capabilities, and the improved colloidal stability enhances systemic circulation and drug effectiveness.

This goal was achieved by stepwise accomplishment of the following objectives:

- The research seeks to fabricate innovative stabilized PolyElectrolyte Nanoparticles (PENs) using whey protein isolate (WPI) and chitosan (CS) as a drug delivery system.
- The study focuses on enhancing the colloidal stability of WPI by creating PENs through two techniques: polyelectrolyte complexation and ionic gelation to self-assemble WPI with CS in the presence of tripolyphosphate (TPP).
- The goal is to coat the WPI core with a CS shell and stabilize it with TPP as crosslinker, leading to high encapsulation efficiency and sustained release of the chemotherapeutic drug doxorubicin (DOX).
- By integrating WPI with CS, the research aims to utilize the positive charge of CS for immobilizing negatively charged molecules, forming PENs that can be employed for drug delivery purposes, and further nanostructure physiochemical characterization.

5. Methodology and results

5.1 Materials

WPI (protein >71.0 % and Ash < 6.0 %) was supplied from MEGGLE (Wasserburg am Inn, Germany). Low molecular weight CS (Mw of 50−190 kDa, degree of deacetylation (DD) ≥75 %), DOX, Polysorbate 80 (PS 80), TPP, dimethyl sulfoxide (DMSO), and dialysis tubing with cut-off 12 kD MWCO were purchased from Sigma Aldrich (St. Louis, MO, USA). The other chemicals, including acetic acid, sodium chloride, di sodium hydrogen phosphate, potassium chloride, and potassium dihydrogen phosphate, were provided by VWR chemicals (Czech Republic).

5.2 Fabrication of PENs

Polysorbate 80 (0.5%) was dissolved in CS solution (1mg/ml in acetic acid 1%, pH 5) and WPI solution (1 mg/ml in dH_2O, pHs 4-7) under stirring for 1 hour. After that, different ratios of WPI solution were mixed with CS solution under stirring for 2 h (CS /WPI: 1/1, 1/2, 1/4, 1/6, 1/8, and 1/10). Then, TPP (1 mg/ml, pH 8) was added to the solution at different w/w ratios of 0.05, 0.075, 0.1, 0.125, 0.2, 0.4 and 0.6 under stirring at 1000 rpm for 30 min. Finally, the optimized formulation was characterized by DLS, SEM, FT-IR, and TGA.

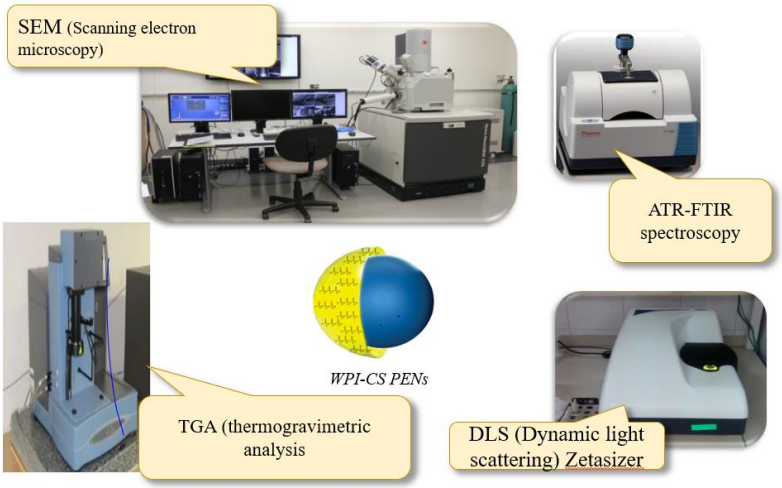

Figure 4 WPI-CS PENs characterization.

5.3 Characterization of NPs

The average diameter, polydispersity index (PDI), and ζ-potential of PENs were determined by DLS (Nano ZS, Malvern, UK). The surface morphology of the PENs was examined using a Nova450 NanoSEM, FEI (Brno, Czech Republic) at an elevated voltage of 10 kV. The dried samples on a sheet of aluminum foil were attached to the SEM specimen stub using a double-sided carbon adhesive disc (Taab, Berkshire, UK) and then coated by gold/palladium sputter (SC7620 Mini Sputter Coater, Quorum Technologies, Laughton, UK, 10mA for 45 s). The Nicolet iS5 FTIR spectrometer analyzed the specific functional groups of the raw materials and nanostructure with iD5 ATR accessory Ge crystal at a resolution of 4 cm^{-1} and 64 scans in the wavelength region of 4000–600 cm^{-1}. Finally, the thermogravimetric analysis was carried out using a TA Instruments Q500 Thermogravimetric Analyzer (Wilmington, USA) at a heating rate of 10 °C min^{-1} in a nitrogen atmosphere between 25 and 600 °C. The Universal Assessment 2000 system was then utilized to calculate the weight loss percentage of the components.

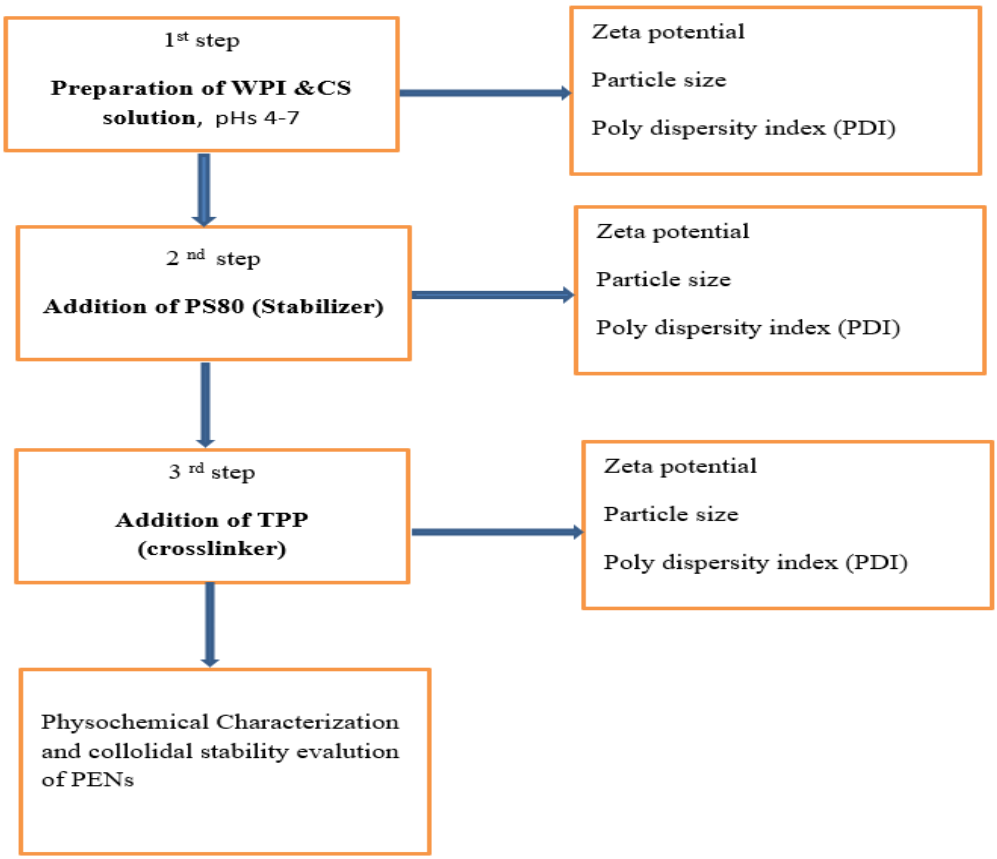

Figure 5 Schematic representation of the research outline

5.3.1 Effect of PH and concentration on hydrodynamic size

Considering the crucial role of NP size on therapeutic efficacy (i.e., long circulation, biodistribution, and clearance), the size distribution of NPs was first explored at different pHs and polymer concentrations. pH is one of the primary characteristics that impact the electrostatic interactions among the ionized functional groups and, subsequently, the particle size and ζ-potential [79]. Therefore, the impact of pH on the size distribution of WPI was first assessed. According to Fig. 6a, there is no significant difference among the size distribution of WPI at pH values of 5 (359.83±7.67 nm and 0.59± 0.09), 6 (338.07±17.35 nm and 0.59± 0.10) and 7 (341.30±17.78 nm and 0.57± 0.09) as opposed to pH 4 (383.23±14.08 nm and 0.58± 0.15). WPI undergoes shifting in its ionized and unionized forms and experiences increasing repulsive electrostatic forces when above or below its isoelectric point (pI) of 4.9, which plays an essential role in unfolding and reducing self-aggregation [80]. After that, the average size of the particles was evaluated in the presence of PS 80 at different pH values, which displays significant differences at pH 4 to pH values of 5, 6, and 7. The smallest size and relatively narrow distribution of WPI (268.28±51.68 nm and 0.76± 0.20) were observed at the pH of 7 (Fig. 6b). The action of co-solvents is extremely pH and concentration-dependent [80], which confirmed that PS 80 decreased WPI size considerably at pH 7 and concentration of 0.5 %. As shown in Fig. 6c, in the presence of CS with pH values of 5.0, significantly small particles were obtained at the CS: WPI w/w ratio of 1:4 (302.73±6.82, 0.61±0.11) [38]. However, these findings confirmed that, aside from pH, the biopolymer ratio significantly impacted the average size of the PENs [38]. Finally, the PENs were synthesized in the presence of TPP at a concentration of 0.075 and pH 8, which affected the hydrodynamic size considerably (Fig. 6d). The smallest size of the particles was about 248.57±5.00 with a size distribution of 0.41±0.02 At pH 8, TPP is dissociated into OH^- and TPP ions ($HP_3O_{10}^{4-}$ and $P_3O_{10}^{5-}$), which interact electrostatically with positive binding sites of CS and overcome repulsive forces among positive amino groups of CS chains [81].

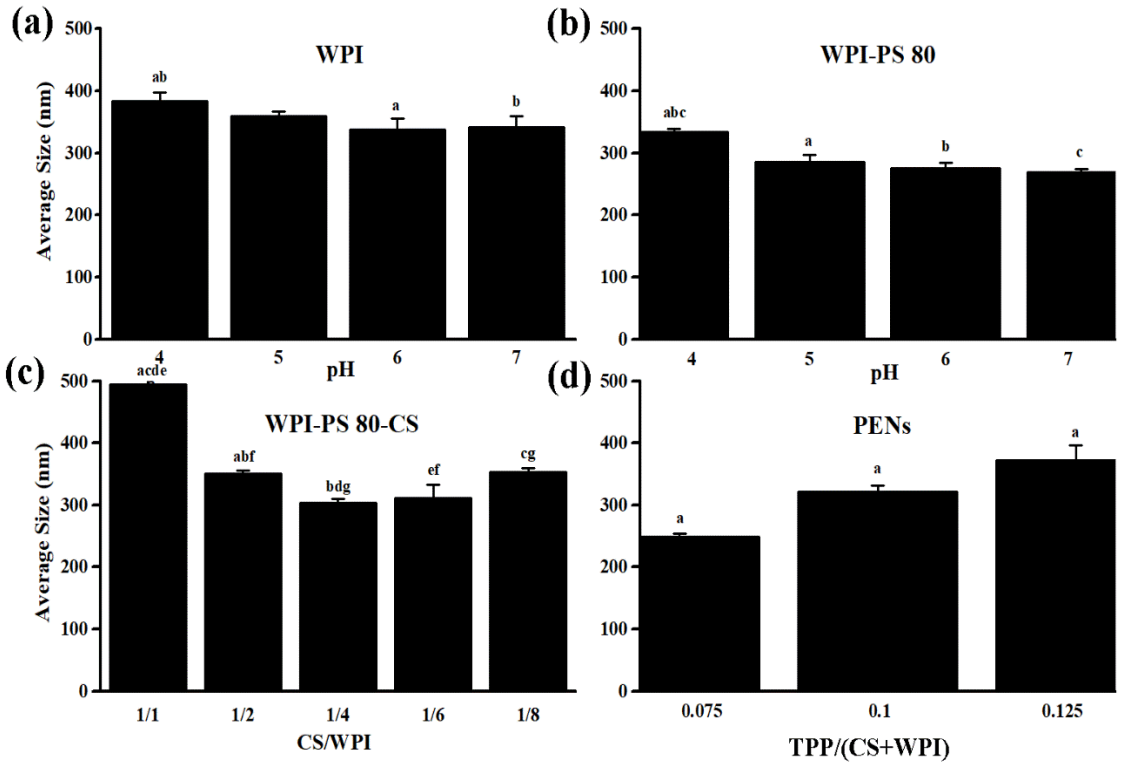

Figure 6 The relationship between z-average size to pH of WPI (a), PS80 at different pH (b), CS concentration (c), and TPP concentration (d). n= 3, mean ± standard deviation, the same letters indicate significant differences between the means of size (p value <0.05), and the values marked with the different letters are not statistically. different.

Another crucial factor that significantly affects the physicochemical properties of the PENs is the ζ-potential. The ζ-potential of WPI at different PH values of 4 to 7 was around -19.57 ± 0.70, -22.50± 0.92, -23.57± 1.17, -26.83± 1.30, respectively. However, the pH increased in the presence of PS 80 to -23.83±0.058, confirming the stabilizer's effect on the surface charge of the PENs. After the addition of CS to the core structure, the ζ-potential value increased to 22.97±1.08. Therefore, the complexation happened due to the electrostatic interactions between the free primary amino groups of CS and the anionic groups of the WPI [38]. Moreover, the smallest PENs with the most uniform distribution showed a ζ-potential of 10.80 ±0.43 mV due to the presence of TPP ions, which interacted with the CS backbone and decreased the number of free amino groups.

5.3.2 Morphology of PENs

SEM micrographs of PENs are shown in Fig. 7. These images showed spherical particles with an average size of 329.10±65.24 nm (WPI), 399.91±55.56 nm (WPI/CS), and 256.26±34.52 nm (PENs) in consistent with DLS data. WPI particles identified by SEM analysis were smooth, spherical, and more apparent in shape (Fig. 7a). In contrast, the addition of CS changed the particles morphology to the particles with vacuoles of various sizes scattered across it (Fig. 7b), as confirmed by Huang et al. [82]. Finally, in the presence of TPP, the PENs showed more compact structures with rough edges (Fig. 7c). These average size differences are due to the increased electrostatic interactions among opposing charges of two macromolecules and TPP with CS.

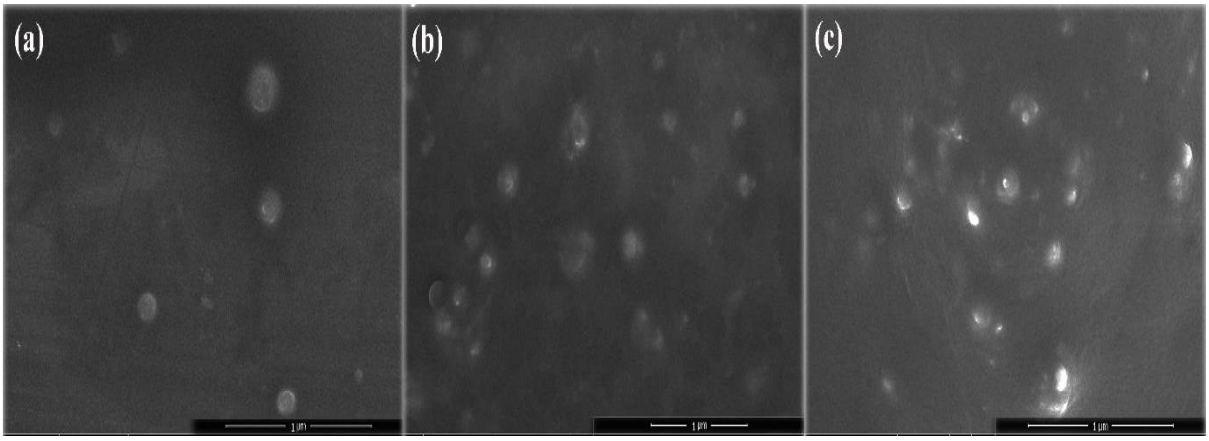

Figure 7 SEM images of WPI (a), WPI-CS (b) and PENs (c) in a dried state.

5.3.3 Infrared spectrophotometry analysis of PENs

To assess the occurrence of intermolecular interactions in WPI-CS complexation, FTIR-ATR analysis was conducted at a wavenumber of 400–4000 cm^{-1} (Fig. 8). The representative peaks of WPI backbone are as follows: the stretching of C =O at 1644 cm^{-1} (amide I), the bending of N-H (amide II) at 1542 cm^{-1}, and the N-H bending and C-N stretching vibrations (amide III) at 1400 cm^{-1} [38]. The IR spectrum of CS showed the peak of amid I at 1660, amid II (NH_3^+ groups) at 1578, an amino functional group at 3446 cm^{-1}, extending the vibration of the C-H bond at 2927 cm^{-1}, the vibration bands of -OH and -CH groups at 1417 cm^{-1}, the symmetrical stretching of C-O-C at 1152 cm^{-1}, vibrational stretching of C-O

at1043 cm^{-1} and pyranose ring at 896 cm^{-1} [83]. Finally, the spectra changes in NH_3^+ groups of CS (1578 cm^{-1}), the C =O stretching (1644 cm^{-1}) and the N-H bending (1542 cm^{-1}) of WPI, P=O band of TPP (1120 cm^{-1}) and the appearance of two strong stretching bands at 1583 and 1564 cm^{-1} in the COO^- antisymmetric region of PENs confirmed electrostatic interaction between the amine groups of CS ($-NH_3^+$) and the carboxyl groups of WPI (COO^-). In addition, the spectra of the PENs showed a broader band at around 3000–3600 cm^{-1} compared to the spectra of WPI and CS, which demonstrated hydrogen bonding [38]. The C=O stretching vibrations at 1600-1700 cm^{-1} also declared the intensity of hydrogen bonds and interactions across amide units on the protein structure [84]. These findings suggest the presence of hydrogen bonding in addition to electrostatic interactions in forming PENs.

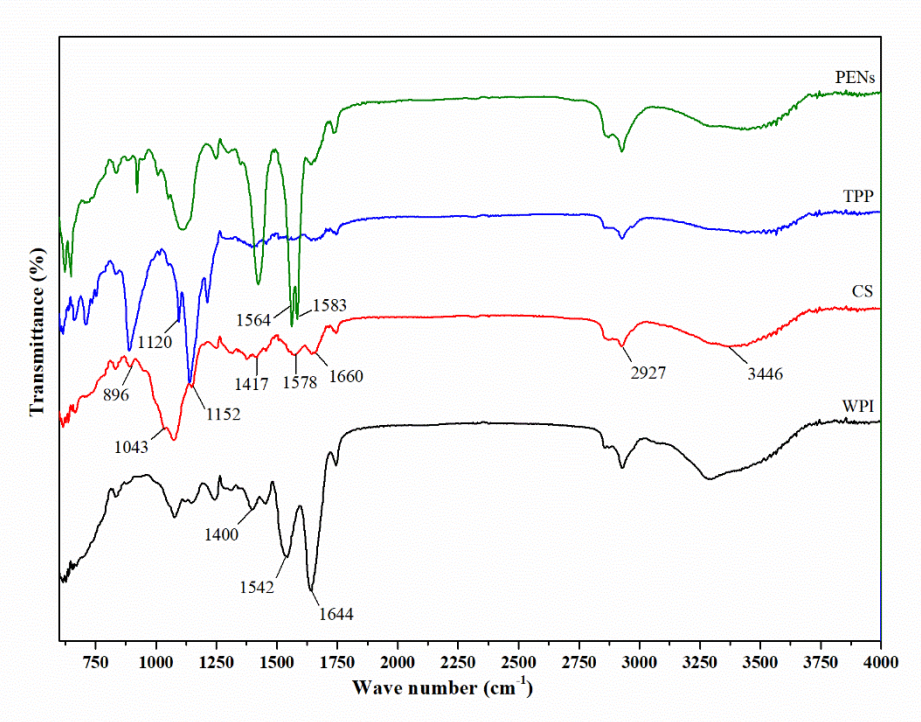

Figure 8 FTIR spectra of WPI, CS, TPP and PENs.

5.3.4 Thermogravimetric analysis

The thermal stability of the pure materials and PENs was evaluated by determining their total percentage weight loss at 25°C to 600 °C (Table 1). As shown in Fig. 9, the regular diminishment of the weights below ∼100 °C was mainly due to free and bonded CO_2, H_2O, and other gases. CS lost about 60% of its mass from 293.34 °C to 594.88 °C due to depolymerization and loss of amino and CH_2OH moieties [85]. The two other thermal mass loss events of WPI happened at Tmax of 164.36 °C and 303.20 °C. Tmax of 164.36 °C can be attributed to the depolymerization of WPI by breaking the peptide linkages, resulting in a weight loss of 4.86%. The last weight loss of 65.81% happened between 210.78 °C to 591.65 °C due to protein decomposition [86]. TGA curves of PENs showed four primary thermal degradation and weight loss zones. Firstly, weight loss behavior of approximately 5.00 % at temperatures ranging from 25 to 122.45°C was related to the diminishment of freezing-bound water. A further increase to 265.72°C resulted in weight losses of 5.52 % attributed to covalent peptide bonds, and the third stage at 265.72–394.84 °C with the highest degradation rate of 53.73 % is correlated to the degradation of CS, WPI. The final stage, with weight loss of 15.15%, was 394.84 to 471.35 °C associated with the complete degradation of organic compounds. Moreover, the PENs had a higher Tmax than their components, which can be attributed to the higher water trapped in the nanostructure.

Table 1 Total percentage weight loss in pure components and PENs

Compounds	Total loss of mass (%)
CS	65.87
TPP	1.456
WPI	77.48
PENs	79.41

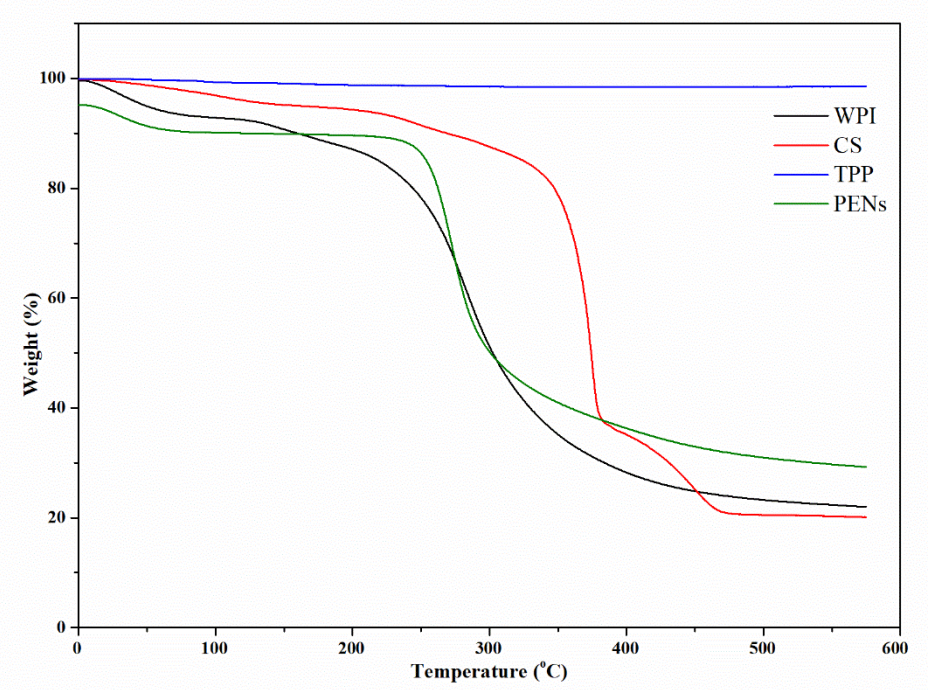

Figure 9 Thermogravimetric analysis of WPI, CS, TPP and PENs.

5.3.5 Colloidal stability analysis

Due to the low conformational stability of proteins, their complexation by polysaccharides through electrostatic interactions can lead to new rheological behaviours with high stability [87]. Herein, anionic WPI was coated by a cationic low MW CS in the presence of TPP, and then the stability was evaluated by DLS upon storage at 4 °C for one month. According to Table 2, the sample presented negligible increases in size (261.87±9.16) with no aggregates confirmed by a PDI value of 0.49±0.04 for three weeks. It can be deduced that the stabilization of the nanosystem occurred through electrostatic interactions or hydrogen bonds among the biopolymers by adjusting pH and ratio. Nonetheless, PENs started to swell and aggregate in the fourth week, confirmed by a significant increase in z-average size (345.03±44.64) and PDI value of 0.80±0.21 (Table 2). It can be concluded that a water inflow to the PENs happened, which increased the size significantly [88].

Table 2 Comparison of PENs' z-average size and PDI by DLS upon storage at 4 ℃ for one month.

Day	*Z-Average Size*	*P-value*
1	248.57 ± 5.00^{a}	0.41 ± 0.02^{e}
8	253.10 ± 6.21^{b}	0.44 ± 0.06^{f}
15	261.20 ± 8.35^{c}	0.46 ± 0.05^{g}
22	261.87 ± 9.16^{d}	0.49 ± 0.04^{h}
29	345.03 ± 44.64^{abcd}	0.80 ± 0.21^{efgh}

n = 3, Mean ± Standard Deviation, p value<0.05

5.3.6 Drug loading and *in vitro* release assays of PENs

In order to fabricate DOX-loaded PENs, DOX at the concentration of 0.04 mg/mL was first added to the WPI solution under stirring for 30 min, and then CS and TPP were added, as mentioned before. Drug loading and *in vitro* release of DOX-loaded PENs were assessed by a dialysis method and in the presence of phosphate buffer solution (PBS 10mM, PH=7.4). For evaluation of encapsulation efficiency (EE) and loading capacity (LC), a dialysis tube containing DOX loaded-PENs' solution (~ 11 mL) was placed into 100 mL PBS, and then the entire system was kept in an orbital incubator (Stuart SI500, UK) at 37±0.5°C, 40 rpm for 1 h. EE (%) and LC (%) were calculated according to Equations (5.1) and (5.2), respectively [79], where Total DOX was the quantity of primary DOX and Free DOX was measured by UV-vis spectrophotometry (CARY 300 Conc, USA) at 480 nm versus a calibration curve (R^2= 0.998, n=3).

$$\mathrm{LC}(\%) = \frac{\text{Total DOX} - \text{Free DOX}}{\text{Nanoparticle Weights}} \times 100 \qquad \text{Equation (5.1)}$$

$$\mathrm{EE}(\%) = \frac{\text{Total} - \text{Free}}{\text{Total DOX}} \times 100 \qquad \text{Equation (5.2)}$$

After that, the medium was changed to 50 mL fresh PBS, and the system was kept in the orbital incubator at 37±0.5°C, 40 rpm for 72 h to assess the *in vitro* release

rate. 3 mL of the medium was taken at regular intervals, and the same volume of the fresh medium was introduced to the system. UV-vis spectrophotometry was utilized to measure the concentration of DOX in the medium compared to a calibration curve at 480 nm for *in vitro* drug release testing of all formulations.

In this stage, the impact of polyelectrolyte structure on loading capacity and release behaviour of DOX-loaded PENs were examined. The results showed the EE (%) and LC (%) of around 92.79±0.69 and 4.12±0.03, respectively. Due to the strong electrostatic interactions between positively charged DOX and negatively charged WPI, the nanostructure displayed high amounts of EE. Nonetheless, the low amount of LC (%) can be explained by the strong dependency of LC on the weight ratio of NPs, in consistence with Equation (5.1).

The in vitro release profile in Fig. 10 demonstrated a two-step biphasic process with an initial burst release for 4 h and a subsequent steady release for 72 h. During the initial burst release, adsorbed or trapped DOX molecules on the polymer surface coatings are released into the media. The subsequent slower release can be mainly attributed to the opposite charges of the functional groups between DOX-WPI, CS-WPI, and TPP-CS. According to Mattu et al., the interaction of fully protonated amino-binding sites of the shell (CS) with TPP dissociated into OH^- , $HP_3O_{10}^{4-}$ and $P_3O_{10}^{5-}$ resulted in a more compact structure and lower release rate [89]. Therefore, in addition to the electrostatic interaction of WPI-DOX, the introduction of CS and TPP affects the release rate by improving the inter/intramolecular forces.

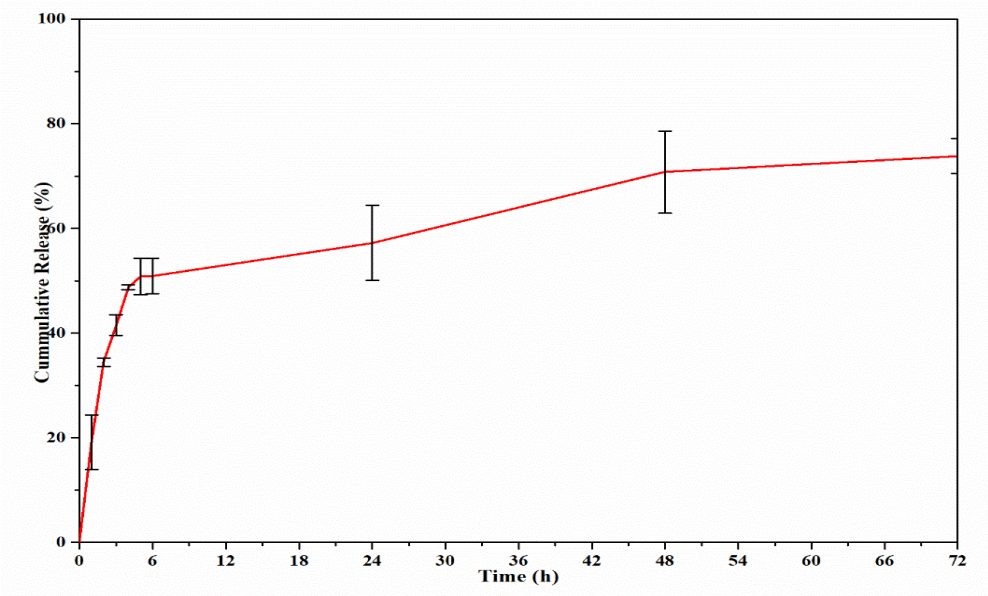

Figure 10 In vitro release profiles of DOX loaded PENs at physiologic pH of release media (7.4).

6. Conclusion

Novel PENs were synthesized through polyelectrolyte complexation and ionic gelation techniques utilizing a WPI-based core, a CS-based shell, and an ionic cross-linked polyanion, TPP. The results of Z-average size and size distribution PDI obtained through DLS and zeta-potential presented a negligible increase in size and aggregates during three weeks. The vacuolated spherical core/shell nanostructures showed high colloidal stability at the predetermined time intervals for three weeks. The particle complexation and thermal stability were confirmed by ATR-FTIR and TGA, respectively. The PENs also demonstrated high encapsulation efficiency and sustained release rate of DOX at physiological pH, governed significantly by a high amount of hydrogen bonds and electrostatic interactions between WPI and DOX. These results offer a promising drug delivery system with desirable stability and prolonged systemic circulation that should be developed. The future challenge lies in the investigation of DOX-loaded PENs in-vitro and in-vivo studies.

6.1 Future plan

Further from the doctoral research goals, which have been successfully accomplished, the upcoming immediate concern is going to focus on the subsequent development:

a) Leveraging biopolymers for designing bio nanocomposite with high Enhanced permeability and retention (EPR) effect
Building upon the results of our research, the next steps aim to optimize and enhance the EPR effect of WPI-CS PENS for improved cancer therapy. By harnessing the unique properties of biopolymers of WPI and CS, we can design advanced drug delivery systems that selectively target tumour tissues. Here are some future research directions based on our findings:

- In Vitro and In Vivo Studies
- Size of Charge Optimization
- Surface Modification for Active Targeting
- Targeted Drug Delivery
- Incorporation of Imaging Agents
- Combination Therapies for Synergistic Effects
- Long –term Stability and Biocompatibility
- Regulatory Compliance and Clinical Trials
- Scalability and Industrial Production

b) Stimuli-responsive WPI-CS PENs carrier for cancer therapy
c) Synthesis of magnetic PENs for hyperthermia-mediated drug delivery
d) CS and WPI bioinks for 3D -printing application with focus on delivery system.

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List of symbols, units, abbreviations and acronyms

WP	Whey Proteins
WPI	Whey Protein Isolated
WPC	Whey protein concentrate
PENS	Poly Electrolyte Nanoparticles
CS	Chitosan
TPP	Tripolyphosphate
β-lg	β-lactoglobulin
DDS	Drug Delivery System
PNPs	Polymeric nanoparticles
PECs	Poly Electrolyte complexes
μm	Micrometre
NPs	Nanoparticles
DOX	Doxorubicin
pH	The decimal logarithm of the reciprocal of the hydrogen ion activity
^{0} C	Degrees centigrade
pKa	pKa is the negative value of the logarithm of Ka. Indication of Acidity pKa indicates whether an acid is a strong acid or a weak acid.
NDS	Nano delivery system
SEM	Scanning electron microscope
TGA	Thermogravimetric analysis
DLS	Dynamic Light Scattering
FTIR	Fourier transform infrared spectroscopy
cm	Centimeter
mg/ml	Milli gram per milli litr
Tmax	Temperature maximum

O/W	oil-in-water
PDI	Polydispersity index
nm	nano meter
PS80	Polysorbate 80
pI	Isoelectric point
ζ-potential	Zeta potential
kDa	Kilo Dalton
DA	Degree of acetylation
DD	Degree of Deacetyltion
EE	Encapsulation efficiency
LC	Loading capacity

Curriculum Vitae

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- IGA/CPS/2022/003- Member of the research team Advanced use of residual materials from agriculture production
- IGA/CPS/2023/003 - Synthesis of magnetic nanomaterials for hyperthermia-mediated drug delivery.
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- Internal competition mobility scholarship of Tomas Bata University in Zlin for international collaboration, University of Waterloo, Canada, February – May 2024.
- Development of 3D printable inks for wound dressing applications. Mitacs Globalink Research Award - for research in Canada.

List of publications

1. Drohsler, P., Yasir, M., Fabian, D. R. C., Cisar, J., Yadollahi, Z., & Sedlarik, V. (2022). Comparative degradation study of a biodegradable composite based on polylactide with halloysite nanotubes and a polyacrylic acid copolymer. *Materials Today Communications*, *33*, 104400. https://doi.org/10.1016/j.mtcomm.2022.104400Get rights and content
2. Yadollahi, Z., Motiei, M., Kazantseva, N., Císař, J., & Sáha, P. (2023). Whey Protein Isolate-Chitosan PolyElectrolyte Nanoparticles as a Drug Delivery System. *Molecules*, *28*(4), 1724. https://doi.org/10.3390/molecules28041724

Conferences attended and outgoing activities

1. Yadollahi, Z., Motiei, M., Kazantseva, Sáha, P. (2023). Whey Protein Isolate-Chitosan core shell as a Drug Delivery System. *38 th International Conference of the Polymer Processing Society (PPS-38) St. Gallen, Switzerland May 22-26,2023*

2. Internal competition mobility scholarship of Tomas Bata university for international collaboration, waterloo university, Canada, Febrauray-May2024.

3. Development of 3D printable inks for wound dressing applications. Mitacs Globalink Research Award - for research in Canada.

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Biopolymer-Based nanocomposite as Drug Delivery System

Nanokompozit na bázi biopolymeru jako systém dodávání léčiv

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