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REVIEW

Chitosan-based Nanosystems as Drug Carriers

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ARTICLE INFO

Article history
Received: 20 April 2022
Revised: 27 May 2022
Accepted: 22 June 2022
Published Online: 4 July 2022

Keywords:
Chitosan
Nanochitosan
Modification
Nanoparticle synthesis
Chemical structure
Ionotropic gelation
Covalent crosslinking

ABSTRACT

The formation and application of polymeric nanomaterials is great demand in science, industry, biotechnology, and medicine due to the possibility of achieving a significant improvement in the physicochemical, mechanical, and barrier properties of polymers and using them as drug carriers and fillers, which is especially promising for biodegradable polymers such as chitosan and their derivatives. The article presents methods for creating polymer nanostructures based on polysaccharides and, in particular, chitosan. Obtaining nanostructured samples of chitosan using the approaches of chemical transformation and modification of polysaccharides is an urgent scientific problem, the solution of which makes it possible to obtain new polymer systems of great practical interest. The medical aspects of the use of polymer carriers based on chitosan for the treatment of various diseases are discussed. The unique specificity of the properties of chitosan and nanomaterials derived from it, with the properties inherent in this natural polymer, can serve as a promising future, especially in the field of medicine.

1. Introduction

The creation of polymeric nanomaterials with specific properties in the last decade has been used in various industries, innovative technologies for the production of modern drugs, since it is so far the only way to synthesize unique drugs based on biodegradable polymers, which include chitosan. Of particular interest is the establishment of the relationship between the conditions for the synthesis and formation of nanopolymer systems based on chitosan and the identification of their correlation with biological activity. The study of the possibility of forming NP nanoparticles from natural polymers (polysaccharides) - chitosan and its derivatives under the influence of chemical and physical factors, the functionalization and stabilization of polysaccharide nanoparticles, the assessment of their structure and properties by physicochemical methods is relevant for modern polymer chemistry.

Obtaining nanostructured samples of chitin, chitosan using the approaches of chemical transformation and modification of polysaccharides is an urgent scientific problem, the solution of which makes it possible to obtain

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DOI: https://doi.org/10.30564/opmr.v4i1.4644

new polymer systems of great practical interest.

One of the promising materials for creating drug delivery systems is chitosan, a deacetylated derivative of the natural polysaccharide chitin. Chitosan is practically the only polycation of natural origin, which is characterized by low immunogenicity. Chitosan is non-toxic, biocompatible, and also biodegradable, which eliminates the possibility of its accumulation in the human body and the environment. Therefore, the synthesis of mixtures based on chitosan and synthetic polymers, as well as the inclusion of drugs in these mixtures, the study of the features of their molecular and supramolecular organization and various external influence factors, is necessary to obtain polymer nanomaterials with unique properties.

Such structures have a high affinity for the cell membrane and are small in size, which makes it easy to penetrate into the cell nucleus. These studies will contribute to the development of polymer nanomaterials with fundamentally new performance indicators.

2. Characterization of Chitosan Nanoparticles

It is generally accepted that nanoparticles are referred to as submicron, colloidal particles having one size less than 100 nm. For the first time, information about polymer nanoparticles was discussed in the article [1], which led to a boom in research for the development of polymer nanocarriers for drug delivery.

Ultra-small sizes of nanoparticles allow them to purposefully deliver drugs to the desired organs [2]. Nanoparticles based on chitosan and polyethyleneimine (PEI) have been studied for the delivery of anticancer drugs.

These nanoparticles can be broadly divided into:

(1) nanospheres are spherical particles in which the drug is inside the sphere, or on the outer surface, or both options are combined;

(2) nanocapsules - consist of an inner liquid core with a solid polymer shell, drugs are located inside the core, or on the outer surface, or both (Figure 1).

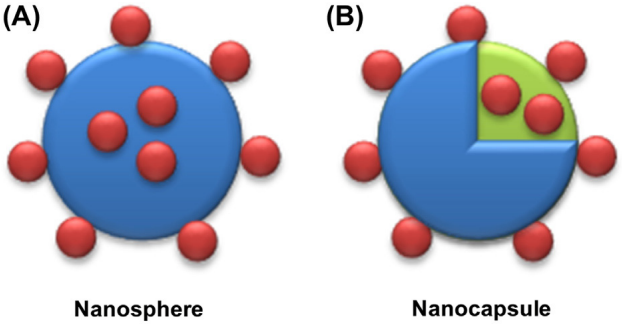


Figure 1. (A). Nanospheres, (B) Nanocapsules

Nanoparticles are of various shapes: cylinders, nanorods, nanotubes, cones, spheres, etc. [3].

Chitosan, a derivative of chitin, is a heteropolymer consisting of N-acetylglucosamines and b-(1,4)-linked glucosamine residues. Chitosan is synthesized by alkaline N-deacetylation of chitin at elevated temperature (Figure 2).

The properties of chitosan are due to its chemical structure. The formation of nanoparticles based on chitosan can serve as one of the ways to modify it. Chitosan molecules contain amino groups, which are partially protonated in slightly acidic aqueous solutions, and completely protonated at pH = 4. Thus, the chitosan molecule in solution is present in the cationic polyelectrolyte form, which opens up wide opportunities for interaction with negatively charged molecules: anions and polyanions. The choice of a simple and affordable method for the synthesis of nanoparticles from a specific chitosan derivative requires careful selection of conditions.

Modification of chitin under controlled conditions makes it possible to obtain chitosan with a degree of deacetylation (DDA) up to 98% and a molecular weight (M_W) from 5×10^4 Da to 2×10^6 Da. The physicochemical and biological properties of chitosan are directly dependent on DDA and the degree of polymerization (DP), on the basis of which the molecular weight of the polymer is determined. The study of the chemical structure shows

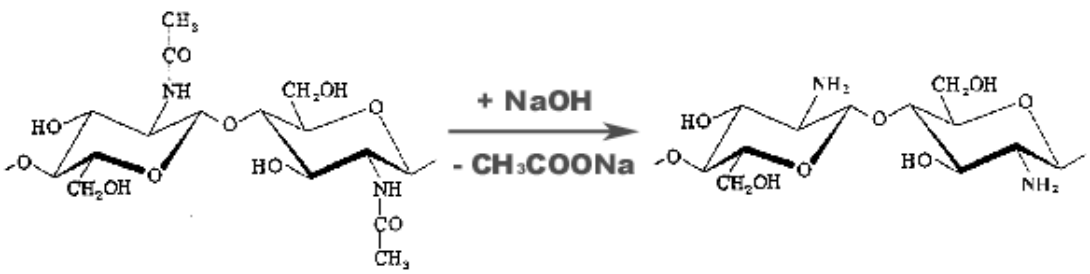


Figure 2. N-deacetylation of chitin to chitosan

that chitosan has reactive hydroxyl and amino groups. Chitosan is an amorphous polymer, unlike chitin, which has a crystalline structure. The presence of primary amino groups in chitosan with a pKa value of 6.3 makes it possible to classify it as a strong base [4].

At a pH above 6, chitosan loses its charge and solubility, since the amino groups are deprotonated at this pH. At a lower pH, the amino groups are protonated and become positively charged, providing chitosan with water solubility, which allows it to be classified as a cationic polyelectrolyte. In the pKa range between 6 and 6.5, there is a transition between solubility and insolubility. Therefore, chitosan dissolves well in a weak acid medium: acetic acid, hydrochloric acid, and does not dissolve at neutral and alkaline pH values. In addition to pH, the solubility of chitosan is highly dependent on DDA, M_W and the ionic strength of the solution. Under physiological conditions, chitosan can be easily degraded by lysozymes or chitinases, which are products of the normal flora in the human intestine or exist in the blood [5-7]. With an optimal N/P ratio (ratio of amino nitrogen in chitosan/ to DNA phosphate), chitosan can efficiently adsorb DNA to form nanoparticles with sizes that allow penetration into the cell, thus providing protection against enzymatic degradation of nuclease [8]. Due to these properties, chitosan is considered a biodegradable, biocompatible polymer and is widely studied as a matrix for drug delivery [9-15]. Since chitosan nanoparticles (CP chitosan) are currently widely used as nanocarriers for drugs, it is necessary to compare different methods for the preparation and characterization of chitosan nanoparticles.

3. Methods for Obtaining Chitosan Nanoparticles

There are several methods for obtaining chitosan nanoparticles of various sizes according to their further application:

1) Polyelectrolyte complex formation - occurs during electrostatic interaction between an anion and cation, followed by charge neutralization, NPs with a size of 50 nm~700 nm are formed;

2) Ionotropic gelation - using a cross-linking agent such as sodium tripolyphosphate (Na-TPP), the NP size can be adjusted depending on the reaction conditions;

3) Microemulsion method - nanoparticles are formed in the liquid phase of a reverse micelle using a surfactant and a crosslinking agent - nanoparticles smaller than 100 nm;

4) Covalent crosslinking - nanoparticles obtained by covalent crosslinking between chitosan and crosslinking agents, such as PEG, glutaraldehyde, etc., produce nanoparticles of various sizes;

5) Combination and incubation - This method allows you to mix the protein with chitosan, with mandatory washing in a mixture with Na-TPP, the formation of NPs with a size of 100 nm~150 nm;

6) Evaporation of the solvent - adding chitosan to an aqueous medium with the formation of an emulsion and precipitation, then evaporation is carried out, NPs with sizes of 50 nm~300 nm are obtained;

7) Co-precipitation - co-precipitation of a solution of chitosan obtained in a solution of CH_3COOH with a low pH by adding to a solution with a high pH of 8.5-9.0 (ammonium hydroxide solution) with the formation of highly monodispersed NPs up to 10 nm in size.

8) Complex coacervation - NPs are obtained by coacervation between cationic chitosan and anionic polyanions, polymers or biomacromolecules. The NP size depends on the anionic coacervate used.

3.1 Method of Polyelectrolyte Complex Formation

The formation of a polyelectrolyte complex (PEC) occurs due to the electrostatic interaction between the anion and the cation, followed by charge neutralization (Figure 3).

Due to charge neutralization, the polyelectrolyte complex self-organizes, which leads to a decrease in hydrophilicity. The formed nanocomplexes can have different sizes from 50 nm to 700 nm.

These polyelectrolyte complexes are used as carriers for proteins, peptides, drugs, and plasmid DNA.

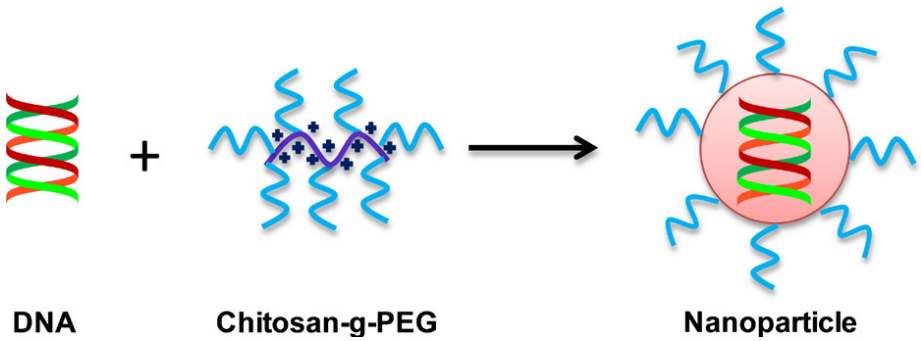


Figure 3. The method of polyelectrolyte complex formation: the formation of nanoparticles occurs during the electrostatic interaction of an anion (DNA) with a cation (chitosan) during charge neutralization.

The authors of the research [16] obtained chitosan-dextran nanoparticles by the method of polyelectrolyte complex formation with a size of 300 nm~500 nm and a zeta potential of + 40 mV~50 mV. Chitosan and dextran nanocomplexes can be used as effective delivery systems.

Another study was also carried out by the method of polyelectrolyte complex formation with the preparation of water-soluble chitosan nanocarriers for insulin [17]. The nanoparticles obtained by the method described above were about 200 nm in size.

Under simple and mild conditions, heparin and chitosan NPs were prepared by polyelectrolyte complexation [18]. The influence of pH, M_w, and concentration was studied when obtaining nanoparticles of the desired size and their yield. It has been shown that effective complex formation occurs at a lower pH and average molecular weight.

3.2 Ionotropic Gelation Method

The method of ionotropic gelation of chitosan nanoparticles was used with the help of ionic crosslinking agents [19]. Crosslinking occurs when intermolecular or cross-links are formed between polysaccharide molecules. Crosslinking sharply reduces the mobility of segments in the polymer with the formation of new interchain bonds and the formation of a three-dimensional network. At a high degree of crosslinking, the polymer matrix becomes insoluble both in water and in organic solvents [20]. Crosslinkers are broadly classified based on the interaction of crosslinkers with chitosan during crosslinking. There is physical and chemical crosslinking.

When physically crosslinked, polysaccharides form a network with counterions on the surface. A high counterion concentration requires a longer time for complete crosslinking of the polysaccharide. This method makes it possible to obtain reversible nanoparticles that are biocompatible due to the absence of harsh conditions for obtaining. Ionically crosslinked nanoparticles are typically pH sensitive, which is a prerequisite for controlled release. Ionic crosslinking occurs using sodium tripolyphosphate (Na-TPP), sulfuric acid, inorganic ions such as Fe $(CN)_6^4$, Fe $(CN)_6^3$ citrate, and calcium ions as crosslinkers [21].

An available and widely used method for obtaining chitosan nanoparticles is the ionotropic gelation of chitosan with sodium tripolyphosphate (Na-TPP), which has a triple negative charge in the physiological pH range.

During the preparation of chitosan nanoparticles using tripolyphosphate (TPP), the average size of NPs and their storage stability were controlled, and it was studied how these parameters are affected by the polymer concentration, the ratio of components, and other parameters.

Figure 4 is a diagram showing the interaction of positively charged chitosan chains with a negatively charged tripolyphosphate anion.

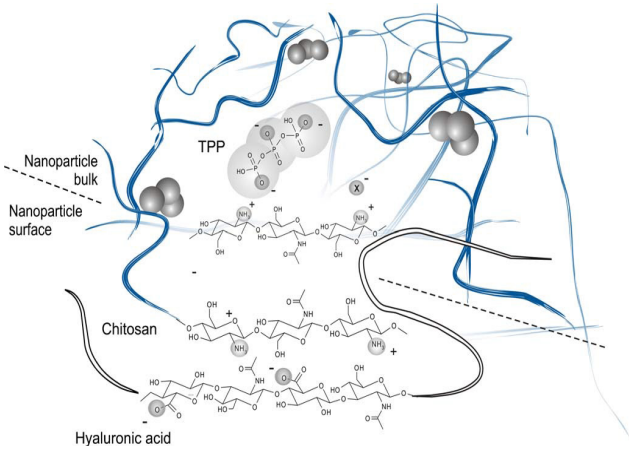


Figure 4. Graphical view of chitosan/TPF nanoparticles present in the volume of NPs connecting between positive charges of chitosan chains

Despite the fact that the processes of obtaining chitosan NPs by the method of ionotropic gelation are quite well known, the influence of various factors on the particle size requires a deeper study. Attempts were made to optimize the processing parameters of chitosan in the process of ionic gelation [22], but there are no unambiguous systematic conclusions in this area of research.

We have carried out studies on the preparation of *Bombyx mori* ChsNPs by ionotropic gelation using an ionic crosslinking agent, sodium tripolyphosphate (TPP) [23].

The concentration of the chitosan solution varied from 5mg/mL to 10 mg/mL, the concentration of the $Na_5P_3O_{10}$ solution was 1 mg • mL^{-1} at a volume ratio of chitosan-NaTPP = 1:1. Optical and AFM studies of ChsNPs samples were carried out. It was revealed that chitosan nanoparticles with sizes of 50 nm ~ 200 nm with an ordered structure of high intensity were obtained. Figure 5 shows the AFM and topography image of chitosan NPs. The resulting ChsNPs had an immunomodulatory effect on living organisms, increased the number of antibody-forming cells in the spleen, the total number of cells in the central and peripheral organs: thymus, bone marrow, lymph nodes [24], i.e. ChsNPs has a fairly pronounced immunostimulating activity. Our studies are consistent with the literature data of the authors in the work [25] in which the chitosan biopolymer stimulates cellular metabolism and, first of all, activates the functions of immune system cells.

In chemical crosslinking, crosslinkers react with polysaccharides to form either intermolecular or intramolecular covalent bonds. The covalently crosslinked polysaccharide nanoparticles allow the network structure to be permanent as irreversible chemical bonds are formed.

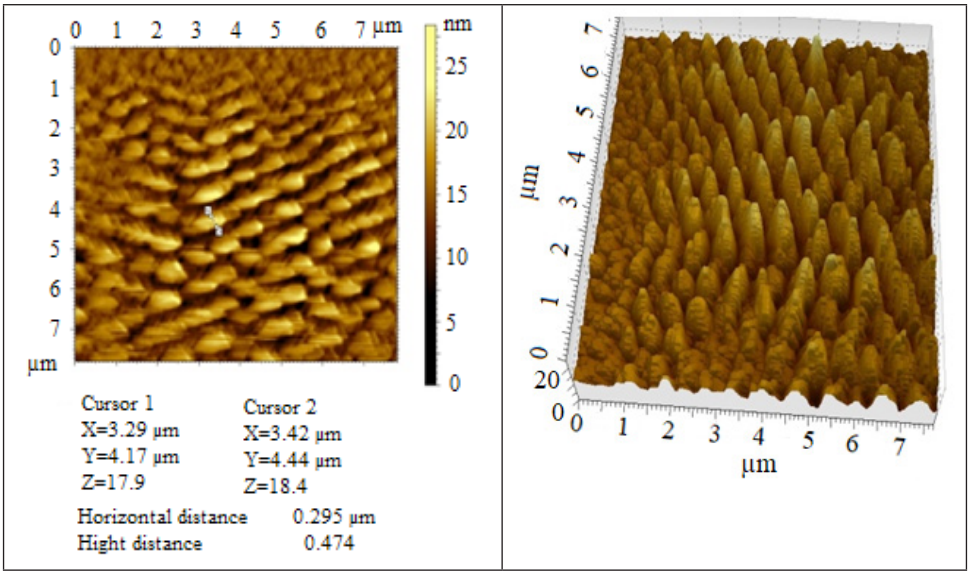


Figure 5. AFM and topography image ChsNPs, concentration of Chs solution - 0.5 mg/mL. The ratio of chitosan:TPP = 3:1, the concentration of $Na_5P_3O_{10}$ is 1 mg/mL. ChsNPs size =295 nm

Despite a sharp change in pH, the rigid network allows the absorption of water and biologically active substances without dissolving the nanoparticles. Crosslinker concentration and crosslinking time affect the degree of chemical crosslinking [20]. Chemical cross-linking is carried out using glutaraldehyde, formaldehyde, genipin, of low-toxic di- and tricarboxylic acids (succinic acid, malic acid, tartaric acid and citric acid), vanillin, epichlorohydrin, etc. [26]. Modification of chitosan can be carried out by crosslinking the molecules of this polysaccharide with biologically active compounds, in particular, glutaraldehyde. The method of cross-linking chitosan by means of glutaraldehyde (GA) is accompanied by the formation of a network supramolecular structure, which, depending on the conformational state and chain stacking, is characterized by different porosity. Of great interest is the ordered stacking of chains during crosslinking, since in this case it is possible to form the same type and evenly spaced pores in the crosslinked polymer and obtain a network material with pronounced anisotropic swelling and desorption properties.

We carried out a study in this direction for solutions of chitosan *Bombyx mori* (M = 110 kDa) in 2% CH_3COOH of various chitosan concentrations (C = 0.15%; 0.25%; 0.50%; 0.75%; 1.0%) by cross-linking with glutaric aldehyde at 25 °C, the modulus of the solution of chitosan and the cross-linking agent was: 5:1. Cross-linking of *Bombyx mori* chitosan with glutaraldehyde occurs at a concentration above 0.75 g/dL.

We have conducted studies on the reparative regeneration of skin cells in thermal injury and the possibility of correcting the identified disorders when using *Bombyx mori* chitosan-based gels cross-linked with glutaraldehyde (GA) and filled with biologically active elements (BAE). Furacilin (FC) was used as BAE, an aqueous solution of which was prepared for experiments by dissolving furacillin powder. The results of freeze drying of the swollen sample showed that cross-linked chitosan contains about 0.5% furacillin in its composition. The morphological (histological) pattern of skin cell regeneration in thermal injury was assessed. During the treatment with gels for burn injury, primary anatomical and functional changes, reactive-inflammatory phenomena and regenerative processes were observed in the affected area. Chitosan gels, especially in combination with furatsilin, significantly increased the regeneration coefficient, which, apparently, determined the earlier reduction of the burn surface (Figure 6).

The obtained data indicate that chitosan has a pronounced ability to accelerate wound healing: chitosan is capable of compacting with cell DNA, which leads to the activation of reparative processes in the nuclear apparatus of the cell and to the regeneration of unburned cells [27]. The presence of furacilin has a bactericidal effect, contributing to faster wound cleansing and earlier skin regeneration, significantly accelerate the healing rate of affected areas, increase the regeneration coefficient, contributing to an earlier reduction of the burn surface.

When tripolyphosphate (TPP) is used as an ionic crosslinking agent, two aqueous phases containing chitosan and another one consisting of polyanionic TPP are mixed to form a complex coacervate [28].

The electrostatic interaction between the cationic amino groups of chitosan and the negatively charged anions of the crosslinker promotes the formation of nanoparticles.

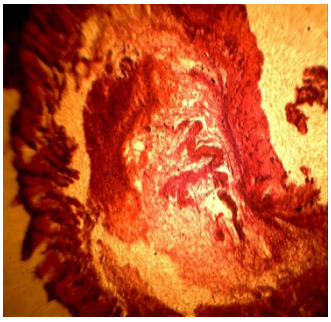
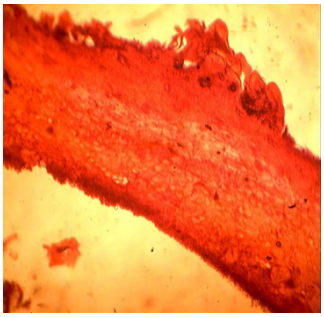
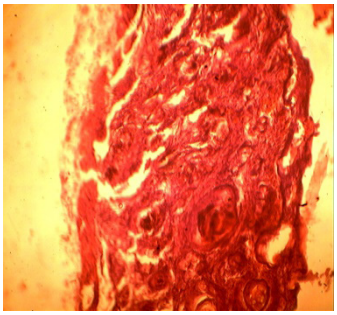
		
a	b	c
Histopreparation of skin samples of the first group with thermal burn after 3 days. Visible: necrosis and inflammation of the layers of the skin. Increase X 200	*after 7 days. Visible, multilayered flat epithelium, in which layers of the epidermis are determined. The newly formed granulation tissue, blood vessels*	*after 10 days. A newly formed granulation tissue, blood vessels are revealed, which indicates a good regeneration of the skin.*

Figure 6. Results of morphological studies

Chitosan is dissolved in acetic acid to form a polymer solution. The crosslinker is added to water to form a solution in the presence or absence of stabilizing agents such as Tween 80, polyethylene glycol. The crosslinking agent is added to the chitosan solution to form nanoparticles with stirring at room temperature. The size and surface charge of particles can be modified by changing the ratio of chitosan and stabilizer [29]. The resulting nanoparticles can be successfully used as drug carriers for biomedical applications both *in vitro* and *in vivo*.

Chitosan-TPP/vitamin C nanoparticles were obtained by ionotropic gelation between the positively charged amino groups of chitosan-TPP and vitamin C with constant stirring on a magnetic stirrer at room temperature for 1 hour to stimulate crosslinking [30]. The nanoparticles were isolated using ultracentrifugation at 10,000 rpm for 30 min and freeze-dried nanoparticles were stored for further use. ChsNP can be used as intranasal delivery for migraine therapy. Chitosan nanoparticles containing chitosan sumatrin-succinate are used to treat migraine, to improve the therapeutic effect and reduce the frequency of dosing. For the formation of nanoparticles, the method of ionic gelation was used.

For the treatment of Alzheimer's disease by ionic gelation, the authors of the investigation [31] obtained encapsulated chitosan nanoparticles for delivery to the brain through the nose. To do this, for the formation of nanoparticles, different ratios of thymoquinone were included in the chitosan solution. Other groups have also used the ionic gelation method to prepare vitamin C-containing chitosan nanoparticles *via* the gastrointestinal tract and to induce the non-specific immune system. It has been shown that chitosan nanoparticles are suitable for encapsulating vitamin C in nanoscale and maintaining the immunostimulating properties of vitamin C. Some researchers modified the ionic gelation method to obtain chitosan nanoparticles containing ampicillin trihydrate and evaluated their antimicrobial activity [32].

Staphylococcus aureus microorganisms were used to test the antimicrobial properties of such nanoparticles. They suggested that the concentration of the reagents - polymer and crosslinking agent, as well as the time of sonication are factors limiting the development of optimized nanoparticle compositions. Chitosan nanoparticles were capable of sustained delivery of ampicillin trihydrate.

Another group has also used a modified ionic gelation method to prepare dopamine-containing chitosan nanoparticles for the treatment of Parkinson's disease [33]. The formation of nanoparticles containing dopamine, a neurotransmitter, may help in the treatment of patients with Parkinson's disease.

3.3 Microemulsion Method

Reverse micelles are thermodynamically more stable liquid mixtures of water, oil and surfactant. Macroscopically, they are homogeneous and isotropic and, on a microscopic scale, are structured into water and oil microdomains separated by a film enriched with a surfactant. Surfactants are amphiphilic molecules that spontaneously form spherical or ellipsoidal aggregates (micelles) in the presence of water or an organic solvent. There are conventional micelles that exist in water at a relatively low concentration of organic solvents. Reverse micelles are

formed in a large number of organic solvents such as hydrocarbons (e.g. hexane, octane, isooctane or benzene), long chain alcohols, chloroform, diethyl ether, etc. In reverse micelles, the reaction takes place in the water core of reverse micellar droplets [34]. Here, aqueous solutions of the monomer, crosslinking agent, and other hydrophilic compounds remain in the water core (host- nanoreactor) of reverse micelles (Figure 7).

The polymerization reaction leading to the formation of nanoparticles occurs in these aqueous media through the primary growth process. Reverse micellar droplets are nanometer sized, so any polymerization reaction taking place in these droplets will result in nanometer sized polymers.

Reverse micellar drops consist of a swollen water core stabilized by a layer of surfactant molecules and dispersed in oil. The process of nucleation in such a system is continuous and can proceed during the entire polymerization reaction. The number of polymer particles formed steadily increases with time, but their size remains constant.

The authors obtained chitosan nanoparticles using the microemulsion method by involving chitosan in the water core of the reverse micellar system, followed by crosslinking with glutaraldehyde [35]. Chitosan nanoparticles were formed using a surfactant, such as AOT - sodium bis (2-ethylhexyl) sulfosuccinate. Initially, the surfactant was dissolved in n-hexane, and then chitosan and glutaraldehyde were added to the surfactant/n-hexane mixture with continuous stirring at room temperature. The free amino groups of chitosan were crosslinked with glutaraldehyde, the organic solvent was removed by evaporation at low pressure, followed by the accumulation of crosslinked chitosan nanoparticles. Excess surfactant was removed by precipitation with $CaCl_2$ and then centrifuged. The size of lyophilized nanoparticles was less than 100 nm. In addition, the size can be controlled by changing the concentration of glutaraldehyde.

The authors of the article [36] prepared chitosan-alginate nanoparticles with a core and a shell using a reversible water-in-oil microemulsion. The study concludes that these biocompatible and biodegradable nanoparticles can be used to encapsulate plasmid DNA for gene delivery via the cellular pathway of endocytosis.

In one of the studies, chitosan nanoparticles were obtained by the authors by cross-linking reverse micellar droplets with glutaraldehyde in the aqueous core [37]. An optically transparent solution was obtained by resuspension of chitosan nanoparticles in an aqueous buffer. The pH-dependent recovery of adsorbed oligonucleotides from nanoparticles *in vitro* showed that the recovery of oligonucleotides at alkaline pH is higher compared to neutral and acidic media.

3.4. Covalent Linking Method

Chitosan has the ability to form covalent bonds with various functional crosslinkers. This method for producing nanoparticles involves the formation of covalent bonds between chitosan chains with agents such as polyethylene glycol (PEG), dicarboxylic acid, glutaraldehyde, or monofunctional agents such as epichlorohydrin. Chitosan has amino groups that are protonated in an acidic aqueous solution, making it soluble in it. But it is insoluble in other solutions, which limits its use. Thus, to make it soluble in

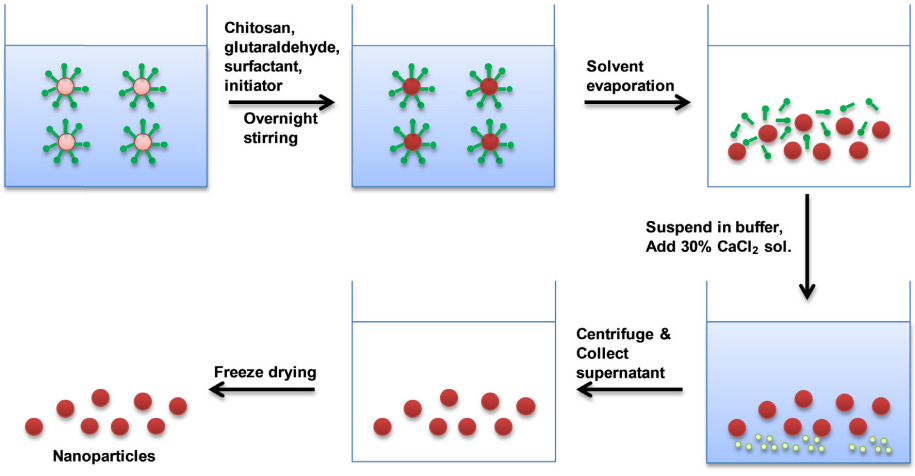


Figure 7. Microemulsion method: in reverse micelles, the reaction takes place in the water core of reverse micellar droplets.

water or other organic solvents, chitosan can be modified by the addition of PEG or other cross-linking agent.

Such attempts can be made by grafting chitosan through chemical modifications with other polymers such as PEG with different M_W [38]. PEGylation of chitosan through hydroxyl groups was first performed by Gorokhovtseva and Makushka [39]. To obtain PEGylated chitosan nanoparticles, chitosan molecules are chemically selectively modified with PEG at the C-6 position of the glucosamine units. Using phthalic anhydride, the amino groups at the C-6 position are protected. Sodium hydride (NaH) is used for catalytic esterification between chitosan and PEG. These PEGylated chitosan nanoparticles are used for gene/drug delivery.

We have obtained nanopolymers based on chitosan *Bombyx mori* and polyethylene glycol (PEG), which were synthesized by graft copolymerization to obtain water-soluble hybrid systems that combine the properties of natural and synthetic polymers [40].

The synthesis of PEG-O-ChsBm graft copolymers was carried out by a sequence of 4 stages: (1) the synthesis of polyethylene glycol monomethyl ether iodide based on the activation of PEG monomethyl ether using triphenyl phosphate to prevent hydrolysis of the product, (2) the protection of chitosan amino groups with a threefold excess of phthalic anhydride in the atmosphere N_2, (3) reaction of N-phthaloyl chitosan and polyethylene glycol monomethyl ether iodide and (4) removal of N-phthaloyl groups by hydrazine monohydrate (Figure 8).

PEG-O-ChsBm amphiphilic graft copolymers spontaneously self-assemble in an aqueous medium and generate 3D supramolecular assemblies by forming hydrogen bonds between unmodified glucosamine units of chitosan.

Various interactions contribute to the self-organization process, namely, electrostatic interactions of the amino groups of chitosan, hydrophobic interactions of $-CH_2-CH_2$ and acetyl groups, and H-bonds involving the -OH group of chitosan [41].

The solubilization effect of PEG chains on chitosan is based on a decrease in the number of hydrogen bonds between chitosan units caused by the presence of PEG chains [42].

Supramolecular 3D ensembles were characterized by various modern methods: IR and ^{1}H-NMR spectroscopy, a combination of TEM, SEM and cryo-TEM (Figure 9).

The toxicity of the nanoparticles was assessed by HeLa and THP-1 cell lines (Figure 10). The results of the experiments revealed the absence of toxicity, which may

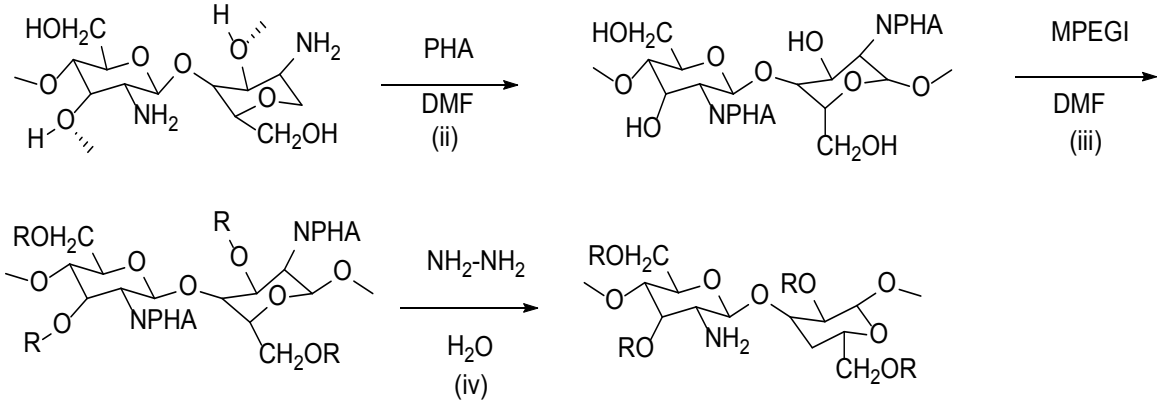


Figure 8. O-PEGylation of Bombyx Mori chitosan, where R: $CH_2CH_2(OCH_2CH_2)_mOCH_3$ and PHA: phthalic anhydride

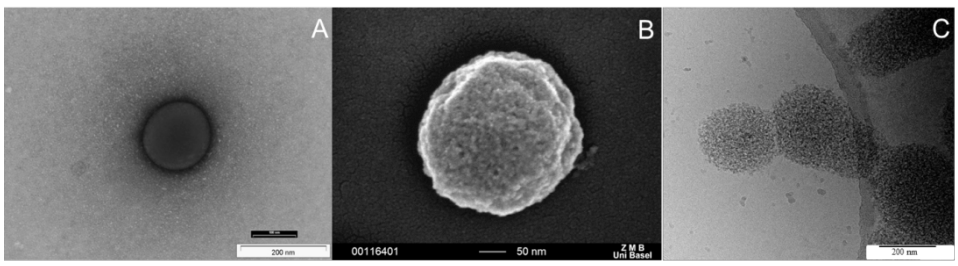


Figure 9. Nanoparticles produced by self-assembly of PEG-O-X3Bm 1.12 tested by TEM: (A) Scale: 200 nm, (B) SEM. Scale: 50 nm , and (C) cryo-TEM. Scale: 200 nm.

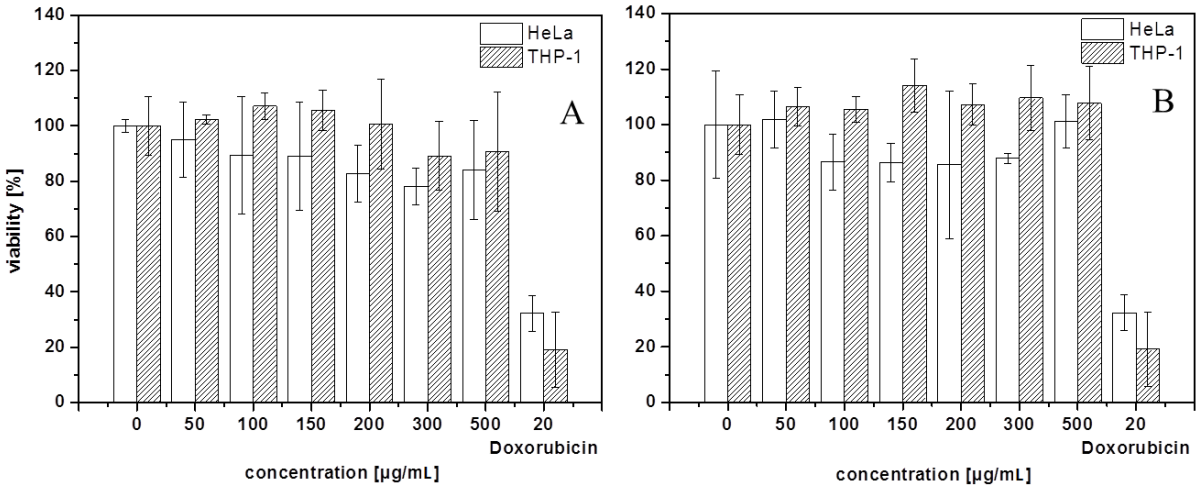


Figure 10. HeLa and THP-1 cell viability after 24 h incubation (A) PEG-O-ChsBm1.12 and (B) PEG-O-ChsBm0.70 nanoparticles.

contribute to the potential medical application of PEG-O-ChsBm nanoparticles for protein attachment.

Chitosan and polyethylene glycol based nanosystems are ideal candidates for on-demand drug delivery through direct selection of their properties that will support a wide range of biomedical applications.

3.5 Method of Incorporation and Incubation

This method is usually used to obtain chitosan nanoparticles for the delivery of protein molecules. In the incorporation method, the protein is first pre-mixed with a chitosan solution, and the pH is adjusted to 5.5 at a temperature of 20 °C [43]. Further mixing of the protein-chitosan solution with TPP leads to the spontaneous formation of chitosan-protein nanoparticles, followed by gentle stirring for 60 mins. During the process of association, protein molecules are introduced into the chitosan-protein nano-matrix, and some protein molecules are absorbed on the surface of the particle. On the contrary, in the incubation method, chitosan nanoparticles are first formed through coacervation of TPP followed by mixing with solutions containing protein at specified concentrations. These solutions are gently stirred for 60 min to ensure that the protein is adsorbed onto the nanoparticles to achieve isothermal equilibrium. In this method, the protein is involved exclusively due to adsorption on the surface of nanoparticles [44].

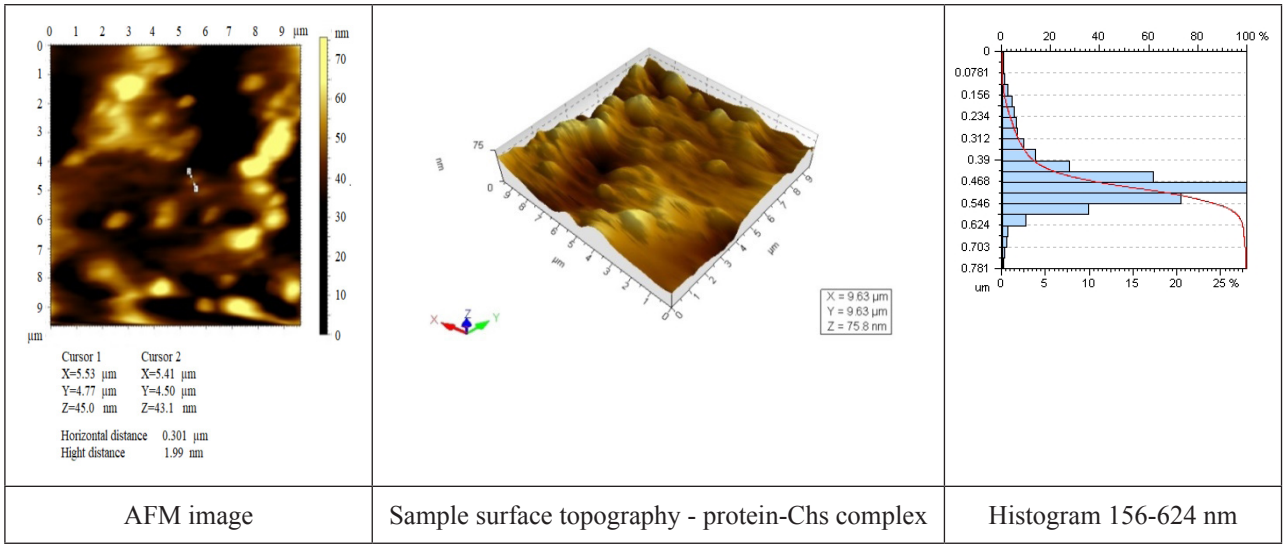
No less interesting are the data on AFM studies carried out during the preparation of the protein-chitosan complex *Bombyx mori* [45].

When analyzing the supernatant that formed after the precipitation of the complex and its separation, by AFM method nanoparticles with sizes of 100 nm~600 nm were detected. The driving forces in the formation of protein and chitosan complexes during the separation phase are electrostatics and the force of attraction between the aggregates, which become stronger when the charge density increases.

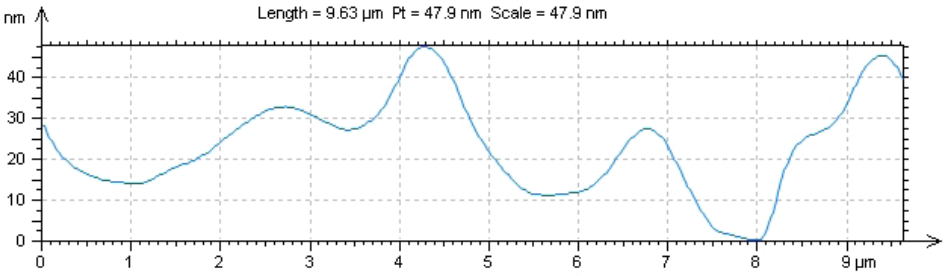
In thermodynamically compatible systems, proteins and polysaccharides have opposite charges, resulting in electrostatic discharge. When protein-polysaccharide complexes are induced electrostatically, it is assumed that stabilization must be achieved through secondary hydrogen bonds or hydrophobic interactions [46]. The results obtained by determining the particle size of the complexes in an aqueous medium revealed the formation of associates (macromolecular nanostructures) with sizes from 150 nm to 600 nm (Figure 11a). The surface cut profile (Figure 11b) showed a value of 47.9 nm. Polymer complexes in a solvent exist in the form of a globular coil, are highly mobile, and are capable of assuming various conformations. These data are consistent with the work of the authors [47], which showed that, due to the small size of the complexes, they have a high specific surface area and exhibit high physicochemical activity and sorption capacity.

3.6 Solvent Evaporation Method

The method includes the formation of an emulsion of chitosan followed by evaporation of the solvent. At the initial stage, the chitosan solution is added to the aqueous phase to form an emulsion. The evaporation of the polymer solvent then leads to the formation of nanospheres due to precipitation. Chitosan is added to ethanol, and then the pDNATris buffer is added to this solution with



a



b

Figure 11. AFM-images - - protein-Chs complex -a, b- profile

rapid pouring of ethanol while stirring on a magnetic stirrer. By applying reduced pressure, the solvent is removed from the obtained nanoparticles. The authors of investigation [48] prepared cyclosporine-A loaded with modified lipid-based nanoparticles with PEGylated chitosan using the emulsification/solvent evaporation method, where the average nanoparticle size was 89.4 nm. It was found that the efficiency of encapsulation of cyclosporine - A on nanoparticles modified with chitosan is 69.22%. In another published study, the authors of the work [49] used the modified solvent evaporation method to obtain nanoparticles in two size ranges of 126 nm~139 nm and 151 nm~181 nm. Lectin/chitosan nanoparticles were loaded with hydrochlorothiazide (HCT) and then in complex with β-cyclodextrin (HCT-β-CD). The maximum entrainment efficiency of 81.8 ± 1.7% and 91.1 ± 1.5% was obtained for nanoparticles loaded with HCT and HCT-β-CD, respectively.

3.7 Co-precipitation Method

This method involves the co-precipitation of a chitosan solution obtained in a low pH acetic acid solution by adding an ammonium hydroxide solution to a high pH of 8.5-9.0, resulting in the formation of highly dispersed chitosan nanoparticles. In a published investigation, chitosan nanoparticles grafted with lactic acid were obtained by coprecipitation with the addition of Chs with lactic acid to ammonium hydroxide to form coacervate droplets. The resulting nanoparticles were highly monodisperse with a size of ~10 nm [50]. In another study by research [51] obtained magnetic nanoparticles coated with chitosan by co-precipitation using various concentrations of chitosan. Chitosan and 6-mercaptopurine nanoparticles were obtained by this method and used as a drug delivery system [52].

To obtain nanoparticles, we used *Bombyx mori* Chs isolated from silkworm pupae, which are waste products of silk production. Synthesis of nanochitosan from *Bombyx mori* Chs was carried out by fractional coprecipitation in the presence of TWEEN-80 surface modifier. It was shown that TWEEN-80 prevents the agglomeration of nanoparticles, which makes it possible to obtain freeze-dried nanoparticles in the form of a dry powder. The particle

size of nanochitosan is 90 nm~200 nm. The sensitivity test of strains of microorganisms related to gram-positive was carried out: *Aureus, St.epidermidis, St.saprofiticus, Str.pyogens, Enteroc. faecalis*; microorganisms related to gram-negative bacteria - *Esch. Coli LP, Esch. Coli LN, Prot. vulgaris, Klebsiella, Ps. aerogenosa;* and microorganisms related to fungi - *Candida albicans, Actinomyces, Bas. Subtilis* to the action of Chs nanoparticles. It is shown that, depending on the concentration, chitosan has an antibacterial effect on almost all groups of microorganisms: *St.saprofiticus, Str.pyogens, Ent.faecalis, Esch. Coli LP, Esch. Coli LN, Prot. vulgaris, Klebsiella, Actinomyces*. In addition, nanochitosan has an immunostimulating effect on living organisms, which makes it possible to recommend preparations based on nanochitosan for raising immunity [53].

3.8 Complex Coacervation Method

The complex coacervation method was used to obtain chitosan nanoparticles by forming coacervates between cationic chitosan and anionic polyanions, polymers, or biomacromolecules. The authors of the article [54] obtained nanoparticles of chitosan-poly(acrylic acid), where it was observed that the size depends on the ratio between the two polyelectrolytes.

pH-sensitive chitosan/poly(acrylic acid) nanospheres with a diameter of 70 nm to 500 nm were prepared by emulsion polymerization of acrylic acid in the presence of chitosan.

Figure 12 shows the interaction between chitosan and polyacrylic acid, which leads to the formation of nanospheres. As shown in Figure 12, acrylic acid molecules are electrostatically bonded to chitosan, forming a micelle framework.

During polymerization, polyacrylic acid (PAA) interacts with chitosan at the interface of micelles. The contraction of the spheres through electrostatic interaction between chitosan and PAA, as well as the expansion of spheres due to the electrostatic repulsion of chitosan molecules, phase separation between the polymer and the solvent lead to the formation of polymeric nanostructures - PNS.

Chitosan/alginate nanoparticles have been obtained for the delivery of bioactive molecules such as proteins and nucleic acids [55]. The authors of the investigation [56] obtained chitosan nanoparticles using chitosans of different molecular weights and DDA, quaternized chitosans, and a trimethylated chitosan oligomer to encapsulate plasmid DNA encoding green fluorescent protein (GFP) using complex coacervation.

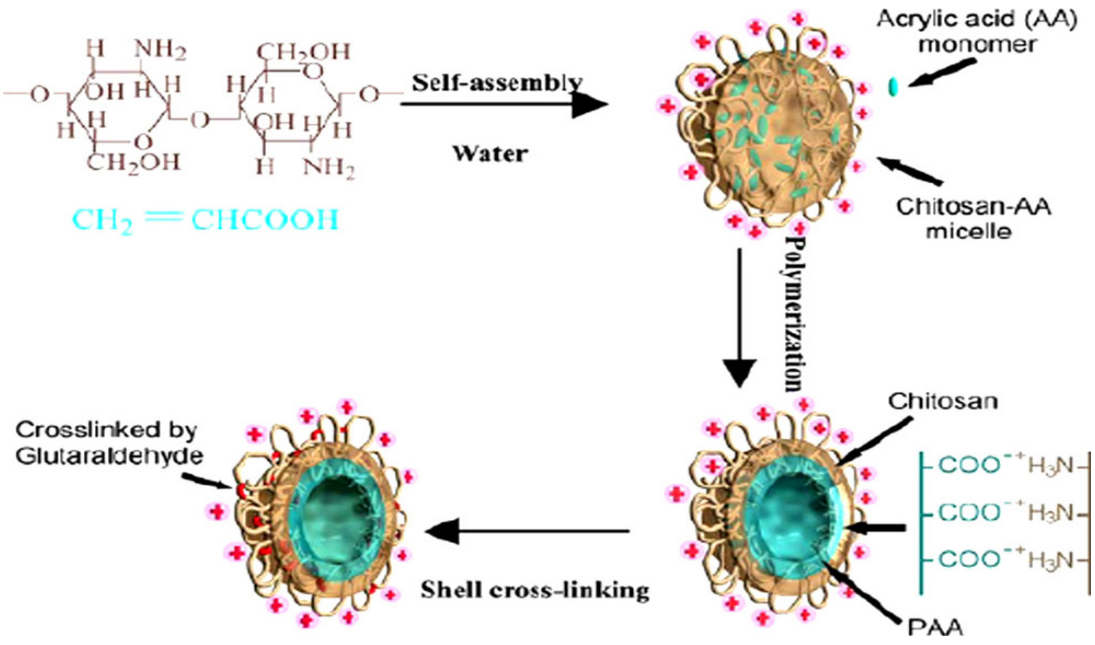


Figure 12. Scheme of the formation of chitosan/poly(acrylic acid) nanospheres

4. Conclusions

Numerous publications on the study of nanomaterials based on chitosan and its derivatives reflect the importance of this polymer. Major fundamental and technical advances have been used to increase the feasibility and improve the properties and reliability of chitosan-based nanomaterials for various applications. Chitosan and chitosan-based nanomaterials are amazing materials that have a wide range of promising applications. Chitosan can be modified with various functional groups to control hydrophobic, cationic and anionic properties. That is why chitosan is a relatively unique biopolymer. These specific properties of chitosan are the result of the presence of primary amino groups in its chain. This structure gives these polysaccharides not only very valuable physical and chemical properties, but also special interactions with cells, proteins and living organisms. Modified chitosan has an excellent ability to immobilize biomolecules, which demonstrates great potential for creating the basis of drugs.

Depending on the requirements, chitosan is functionalized to achieve targeted delivery. Thus, it is clear that chitosan nanoparticles have the potential for efficient drug/gene delivery to target organs. Therefore, the unique specificity of the properties of chitosan and nanomaterials derived from it, with the properties inherent in this natural polymer, can serve as a promising future, demonstrating emerging products on the market, especially in the field of medicine. It is reasonable to expect significant advances in the application of chitosan and nanomaterials based on it, with extensive innovative possibilities that will fundamentally change the field of nanotechnology in the near future.

Conflict of Interest

There is no conflict of interest.

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