

Design of nanogels for targeted delivery of dinitrosyl iron complexes (nitric oxide donors)

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Abstract: The main problem in oncology is the lack of specificity of action of many antitumor drugs, which creates a need for the development of new technologies providing targeted delivery of drugs to cancer cells. Nanogels from chitosan and hyaluronic acid were obtained using cross-linking reagents. To obtain nanogels, various ratios of chitosan and hyaluronic acid (1:1.25, 1:2.5, 1:5, 2:1, 5:1) were used. As a result, nanogels of various sizes and morphologies were obtained. Electron microscopy showed that nanogels are polydisperse and have a size of 100–400 nm. Dynamic light scattering method established that the obtained nanogels are positively charged and have a surface potential of (+) 5.3 ± 0.31. Stability of nanogels in water, fetal bovine serum, and saline, as well as that of nanogels-dinitrosyl iron complex was studied. Results of dynamic light scattering showed that nanogels retain high stability and are resistant to aggregation in water and in physiological solutions. Cytotoxicity of these nanogels was studied using the fluorescence method, which showed that nanogels in the growth medium at 20, 40, and 60 % are compatible with cells, with cell viability maintained at levels ranging from 100 to 78 %. The nanogels-dinitrosyl iron complex has been proved to suppress the viability of prostate cancer cells 1.5 times. These results underscore the potential of nanogels in improving cancer treatment through enhanced targeted delivery and inhibition of cell viability of cancer cells.

Keywords: chitosan, electron microscopy, hyaluronic acid, nanogels

Introduction

Biotechnology, nanomedicine in particular, is developing rapidly worldwide. Most promising areas of nanomedicine application are the treatment of cancer and cardiovascular diseases (Abdullah et al., 2025). Introduction of nanotechnology into medicine enables eliminating costly and traumatic surgical treatments. One of the most rapidly developing areas of nanomedicine is the development of technologies for targeted delivery of drugs and diagnostic substances in nanocapsules/nanoparticles or vectors (Fortune et al., 2025) to ensure more effective drug action without harming healthy cells and the reduction of the dosage. The development of nanoparticles for targeted drug delivery is urgent given the high incidence of cardiovascular diseases and cancer worldwide, the low survival rates of patients, and the high cost of treatment (Kidenko et al., 2023; Yao et al., 2020). Furthermore, many drugs are toxic, exhibit side effects, have poor solubility, cellular permeability, and poor biocompatibility.

Nanogels are composed of chemically or physically cross-linked polymers and represent a three-dimensional network structure (Soni et al., 2016). They have unique properties, including high

water absorption, stability, drug encapsulation ability, and sensitivity to various stimuli, which makes them important candidates for biomedical applications, including drug delivery (Ferozekhan et al., 2024). The use of nanogels as drug carriers ensures prolonged action of drugs, which reduces its side effects and increases the effectiveness of treatment, improving the quality of life of patients (Ferozekhan et al., 2024). Currently, most nanogels available on the global market are based on synthetic polymers. However, research is underway to utilize natural polymers such as chitosan and hyaluronic acid, as these polysaccharides are biodegradable, biocompatible, and possess antibacterial and anti-inflammatory properties (Wu et al., 2020; Cadete et al., 2019). The microstructure of nanogels resembles the intercellular matrix of many tissues and can mimic its physicochemical properties. Thus, they represent an ideal cellular microenvironment for cell proliferation and differentiation (Kurian et al., 2021).

The development of multilayer nanogels consisting of a biopolymer carrier of chitosan (CHI), and a delivery vector of hyaluronic acid (HA) and encapsulated drugs has been studied (Della Sala et al., 2024). The design and synthesis of nanogels is based on a simple and efficient method of

chemical cross-linking using cross-linking agents (Blagojevic and Kamaly, 2025), where two types of polymers containing amino groups (chitosan) and aldehyde groups (hyaluronic acid) are covalently linked by cross-linking reagents such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Mauri et al., 2021; Sutradhar et al., 2025). Chitosan is an amino sugar, a derivative of a linear polysaccharide which macromolecules consist of randomly linked β -D-glucosamine units and N-acetyl-D-glucosamine (Aranaz et al., 2021) and it is often used as a carrier in nanogels. One of the sources of chitosan is the shells of crustaceans and insects (Pellis et al., 2022). Chitosan has many properties that make it suitable for various fields, particularly in biomedicine. It is a highly cationic, non-toxic, biocompatible, and biodegradable compound (Jiménez-Gómez et al., 2020). Due to its cationic nature, chitosan forms insoluble complexes with the anionic polysaccharide hyaluronic acid (Maiz-Fernández et al., 2021) which allows its use as a carrier in nanogels/nanoparticles (Fard et al., 2025; Arpa and Akbuğa, 2025).

Chitosan-based nanogels perform their necessary function in the human body for a long time, which is followed by their decomposition and elimination without causing harm. Polysaccharide-coated nanogels are a new trend in drug delivery systems (Wang et al., 2023). Active targeted delivery of drugs associated with hyaluronic acid (HA) is a promising approach in biomedicine since HA is a biocompatible, non-toxic and easily biodegradable substance (Tripodo et al., 2015). Thus, nanoparticles/nanogels utilize a negatively charged polymer, HA, as a delivery vector. HA is a non-sulfated glycosaminoglycan composed of alternating units of D-glucuronic acid and N-acetyl-D-glucosamine (Li et al., 2020). It is one of the main components of extracellular matrix found in many biological fluids (saliva, synovial fluid, etc.), and it is a part of connective, epithelial and nervous tissues (Neumann et al., 2021). HA has negative charge ensuring rapid passage of nanoparticles through the cell membrane, which has negative charge (Kampen et al., 2024). In addition, cellular enzymes (hyaluronidases) are able to break down HA inside a cell releasing the drug directly into the cell (Kaul et al., 2021). Furthermore, since HA is a polyanionic polysaccharide, it has multiple charges for interaction with polycations. HA is a physiological ligand for CD44 and RHAMM (receptor for hyaluronan-mediated motility) receptors, so it specifically interacts with these receptors on the cell membrane surface ensuring targeted drug delivery to the cell (Entwistle et al., 1996; Carvalho et al., 2023; Esguerra et al., 2015). This cell-targeting strategy allows for the delivery of drugs directly to

cells, rather than concentrating them in the kidneys and liver, and their elimination from the body by the reticuloendothelial system. This strategy enables specific cell-targeted drug delivery.

Nitric oxide (NO) is a biologically active molecule used to treat cardiovascular, oncologic, and inflammatory diseases (Lei et al., 2013; Mysliwiec et al., 2025). New dinitrosyl iron complexes were previously synthesized with sulfur-containing ligands of the thiourea series (DNICs, nitric oxide donors) (Shmatko et al., 2017). However, since DNICs are not entirely stable, we developed nanoparticles to encapsulate DNICs using the physical association method (Gizatullin et al., 2018). These nanoparticles increased the DNICs stability when dissolved in aqueous solutions and prolonged the release of NO (Gizatullin et al., 2018; Akentieva et al., 2020). However, it was necessary to create more stable nanogels using covalent chemical cross-linking to encapsulate DNICs.

The aim of this work is to create nanogels from chitosan and hyaluronic acid using cross-linking reagents and to study their physicochemical properties: morphology, size, surface charge, stability and cytotoxicity.

Material and Methods

Chemicals

Chitosan (CHI, MW = 100,000–300,000) was obtained from Acros Organics (New Jersey, USA). Sodium hyaluronate (HA, low molecular weight <0.1 MDa) was obtained from My Formula (Germany). EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, $C_8H_{17}N_3$) and NHS (N-hydroxysuccinimide, $C_4H_5NO_3$) were purchased from ProteoChem (USA). Fetal bovine serum (10 %) was purchased from Cell Technologies (Canada).

Fabrication of nanogels

Nanogels (CHI/HA) were obtained by chemical modification (cross-linking).

To covalently link hyaluronic acid (HA) and chitosan (CHI), cross-linking reagents such as EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and NHS (N-hydroxysuccinimide) were used. A sample of CHI (5 mg/ml) was dissolved in 0.01 M HCl, pH = 2, stirred and heated, in a water bath for 12 hours. A sample of HA (5 mg/ml) was dissolved in 1 ml of distilled water (pH 5.5) and stirred for 12 hours. The resulting CHI and HA solutions were then mixed in the ratio of CHI:HA = 1:1.25, 1:2.5, 1:5, 2:1, 5:1. Solutions of EDC (15 mg/ml in distilled water, pH 5.5) and NHS (30 mg/ml in distilled water, pH 5.5) were prepared in the 1:1 ratio. The mixture of EDC and NHS reagents was then

added dropwise until opalescence occurred. The solution was then stirred with a magnetic stirrer at room temperature for 24 h. A part of the solution was then freeze-dried, and the nanoparticles were analyzed by electron microscopy. Another portion of the nanoparticle solution was analyzed by dynamic light scattering. Chitosan and HA were also analyzed as control samples.

Morphological analysis of nanogels by electron microscopy

Prepared nanogels were analyzed using electron microscopy using a Zeiss Supra 25 (Germany) scanning electron field-emission microscope with a Schottky cathode and an in-lens detector to collect secondary electrons from the sample. Carbon was sputtered onto the sample surface. The conductive carbon layer was thin (a few nanometers) and contributed minimally to the sample signal, as it is also a light element. Nanogel samples were prepared using vacuum deposition of carbon on aluminum substrate. A VUP-4 high-vacuum sputtering device was used to apply the carbon coating using an arc discharge method and the nanogels were then analyzed using a Supra-25 electron microscope, working distance WD = 2 mm, accelerating voltage EHT = 3.56 kV, detector Signal A = InLens.

Stability of nanogels obtained by cross-linking

Nanogels stability was determined using dynamic light scattering. The following samples were prepared: nanogels were dissolved in 0.9 % NaCl (2 ml, human saline), and 10 % fetal bovine serum (2 ml), or distilled water (2 ml). Nanogel-containing samples were then incubated for 24 hours at 37 °C in a CO₂ incubator. Additionally, a DNIC#4 solution (concentrations of 4.87×10^{-3} M, 4.87×10^{-4} M and 4.87×10^{-2}) was added to the nanogels in water, and the samples were incubated for 24 hours at 37 °C in a CO₂ incubator. The surface potential and size of nanogels were then measured using dynamic light scattering.

Physico-chemical analysis: electrophoretic mobility

ξ -potential measurements of HA, CHI, and nanogels CHI/HA were performed at 23 °C by a Malvern Zetasizer Nano-ZS (ZEN3600) instrument (England) using a rectangular quartz 1 cm cell. For each sample, the ξ -potential was determined at least ten times and the average value is reported.

Analysis of nanogel size by dynamic light scattering

Determination of the intensity size distribution functions of HA, CHI, and nanogels CHI/HA was performed by Malvern Zetasizer Nano-ZS

(ZEN3600) instrument (England) using a rectangular quartz 1 cm cell. For each sample, the measurement was repeated seven times. Mixed complex solutions were used without filtration. The detected scattering light intensity was processed by Malvern Zetasizer Nano software.

Synthesis of dinitrosyl iron complexes (DNICs)

Synthesis and identification of DNICs compounds was carried out according to literature (Shmatko et al., 2017; Wu et al., 2020). As the tested compounds, synthetic analogs of mononuclear dinitrosyl iron complex with functional sulfur-containing ligands, namely thiourea and its derivatives, $[\text{Fe}(\text{SC}(\text{NH}_2)(\text{NHC}_2\text{H}_5))_2(\text{NO})_2]\text{Cl}$ $[\text{Fe}(\text{SC}(\text{NH}_2)(\text{NHC}_2\text{H}_5))\text{Cl}(\text{NO})_2]$ (DNIC#4), $[\text{Fe}(\text{SC}(\text{NHCH}_3)_2)_2(\text{NO})_2]\text{BF}_4$ (DNIC#7) were used. DNICs secrete nitric oxide (NO) when dissolved in water solvents due to dissociation (Shmatko et al., 2017; Sanina et al., 2015).

Cell lines

Renal epithelial cells of African green monkey (line *Vero*) were purchased from the Russian Collection of Vertebrate Cell Cultures (BioloT, St. Petersburg, Russia).

Adherent human prostate adenocarcinoma cells (PC-3 line) were obtained from the Institute of Experimental Oncology and Carcinogenesis (Blokhin National Research Center of Oncology, Moscow, Russia).

Cell culture

Renal epithelial cells of African green monkey (line *Vero*) and prostate adenocarcinoma cells (PC-3 line) were cultured in DMEM medium containing HEPES (10 mM, pH 7.2) supplemented with 10 % fetal bovine serum (FBS), glutamine (0.15 %), and gentamicin (50 mg/mL). The cell culture was incubated in an atmosphere of 5 % CO₂ at 37 °C and 95 % humidity.

Cytotoxicity of nanogels

To analyze the cytotoxic effect of nanogels (CHI/HA), the AlamarBlue® Cell Viability Assay (ThermoFisher Scientific, USA) was used. This method allows determining the activity of mitochondrial NADH dehydrogenases, which break down NADH into NAD and H⁺, and the resulting proton reduces the dye resazurin to fluorescent resofurin (Schreer et al., 2005). To assess the cytotoxicity of nanogels, renal epithelial cells (*Vero* line) were seeded in 96-well plates in the growth medium (DMEM) at the seeding density of 10,000 cells/well (4 wells for each sample) and incubated at 37 °C and 5 % CO₂ for 24 hours. After 24 hours of incubation, the growth

medium was removed and replaced with gradually increasing amounts of nanogels suspension (20, 40, 60, 80, and 100 μ l in DMEM medium). The cells were incubated with nanogels for 24 hours at 37 °C in a CO₂ incubator. DMEM medium without the addition of nanoparticles served as a control; an equal amount of phosphate-buffered saline (PBS, 100 mM, pH 7.2) was added to the control wells. After 24 hours, the nanogels were removed, cells were washed with growth medium, and 100 μ l of DMEM/well were added. AlamarBlue® reagent was then added to the cells (10 μ l/well) and the cells were then incubated at 37 °C and 5 % CO₂ for 1 hour. After 1 hour, fluorescence intensity was measured at E_{ex}/E_{em} = 540/590 nm using a Varian Cary Eclipse spectrofluorimeter (Agilent Technologies, USA). The percentage of cell viability was then calculated relative to untreated cells (control). The percentage of cytotoxicity can be calculated using the following equation: Cytotoxicity, % = (Absorbance of cells treated with nanogels/Absorbance of control cells) $\times$ 100.

Encapsulation of DNICs into nanoparticles by physical association

Dinitrosyl iron complexes DNIC#4 and DNIC#7 were encapsulated into nanogels (CHI/HA) using the physical association method as described previously (Akentieva et al., 2020). DNIC#4 (concentrations of 4.87×10^{-2} M, 4.87×10^{-3} M and 4.87×10^{-4} M) and DNIC#7 (concentration of 4×10^{-4} M) solutions were added during mixing of polymer solutions, chitosan, and hyaluronic acid.

Effect of nanogels+DNIC#7 on prostate cancer cells viability

The effect of the nanogels+DNIC#7 complex on cell viability was analyzed using a fluorescence assay (Alamar Blue® Cell Viability Assay, Thermo-Fisher Scientific, USA) (Schreer et al., 2005). Human prostate adenocarcinoma cells (line PC-3) were seeded in 96-well plates (7,000 cells/well) and incubated in an atmosphere of 5 % CO₂ at 37 °C and 95 % humidity for 24 hours. The following day, nanogels+DNIC#7 (4×10^{-4} M) were added to the cells. Cells without the addition of nanogels+DNIC#7 were used as a positive control. An equal amount of phosphate-buffered saline (PBS, 100 mM, pH 7.2) was added to the control wells. Cells were incubated with nanogels+DNIC#7 for 20 min. Alamar Blue (10 μ l) was then added directly to the cells in the growth medium in each well, and the samples were incubated at 37 °C for 1 hour. Fluorescence was measured using a spectrofluorimeter (Varian, Cary Eclipse, USA) at E_{ex}/E_{em} = 570/585 nm.

Statistical analysis

All data presented are the result of triplicate experiments. Statistical analysis of all obtained data was performed using the GraphPad Prism One-Way ANOVA software. Differences were considered significant at $p < 0.05$.

Results and Discussion

Nanogel production

Chitosan-hyaluronic acid (CHI/HA) nanogels were obtained using the cross-linking method. To covalently link hyaluronic acid (HA) and chitosan (CHI), cross-linking reagents such as EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, C₈H₁₇N₃) and NHS (N-hydroxysuccinimide, C₄H₅NO₃) were used. EDC is an organic compound, a water-soluble carbodiimide, typically in the form of its hydrochloride salt, which is used to activate the carboxyl group for subsequent binding of a primary amine to form an amide bond. It is used in peptide synthesis, for cross-linking proteins with nucleic acids, and for the preparation of immunoconjugates (Mitchell et al., 2019). Here, this reagent was used in combination with N-hydroxysuccinimide (NHS, C₄H₅NO₃) to increase coupling efficiency and stabilize the amine-reactive product. NHS is a cyclic imide with a hydroxyl group (—OH) attached to the nitrogen atom (Montalbetti and Falque, 2005). To obtain nanogels, different ratios of chitosan and hyaluronic acid (CHI:HA = 1:1.25, 1:2.5, 1:5, 2:1, 5:1) were applied and nanogels of different sizes and morphology were obtained.

Morphology and size of nanogels

Morphology and size of nanogels were examined by electron microscopy. Figure 1 shows CHI/HA images of nanogels (ratio CHI:HA = 1:1.25) obtained by electron microscopy.

As it can be seen from the presented data, these nanogels are characterized by low asymmetry, and square or rectangular shape, they are weakly linked to each other, monodisperse, and have almost the same size (average size is 100 nm).

Nanogels obtained at the CHI:HA ratio of 1:2.5 show completely different morphologies and sizes (Fig. 2). They are larger (ranging in size from 200–400 nm), polydisperse, asymmetrical and prone to association with each other. They are basically rectangular in shape with projections along the edges.

Electron microscopy has proved that chitosan has a lamellar structure, and the structure of hyaluronic acid consists of tightly packed long filaments (Gizatullin et al., 2018). Figure 3 shows images of nanogels at the CHI:HA ratio of 1:5, where threads of

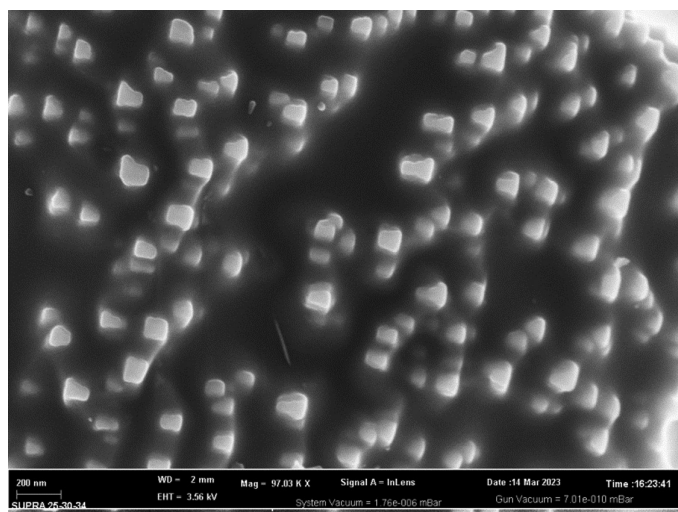


Fig. 1. Electron microscopy images of nanogels (ratio CHI:HA = 1:1.25). Nanogel size is 100 nm. Supra-25, working distance WD = 2 mm, accelerating voltage EHT = 3.56 kV, magnification Mag = 97.03. KX, Signal A detector = InLens, system vacuum = 1.76e-0.006 mBar, cathode unit vacuum = 7.01e-010 mBar.

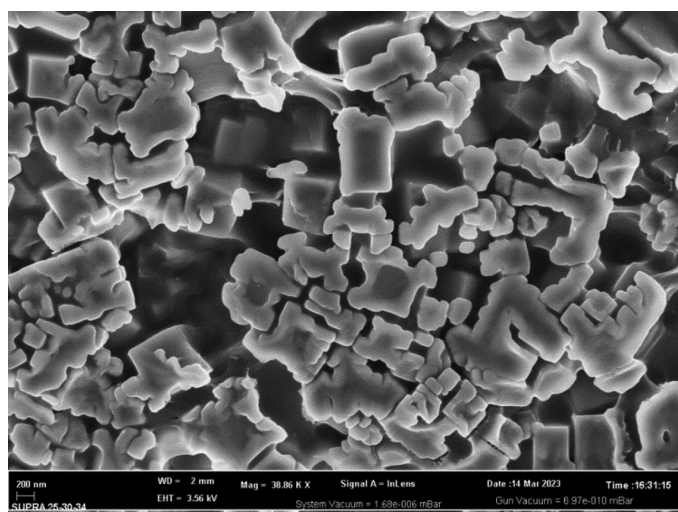


Fig. 2. Electron microscopy images of nanogels (ratio CHI:HA = 1:2.5) in the size range from 200–400 nm. Supra-25, working distance WD = 2 mm, accelerating voltage EHT = 3.56 kV, magnification Mag = 38.86 KX, Signal A detector = InLens, system vacuum = 1.68e-0.006 mBar, cathode unit vacuum = 6.97e-010 mBar.

hyaluronic acid have chitosan molecules arranged in orderly chains, which have the shape of squares and rectangles (Fig. 3a). These structures have indefinite shape and are large in size, approximately 600–800 nm (Fig. 3a).

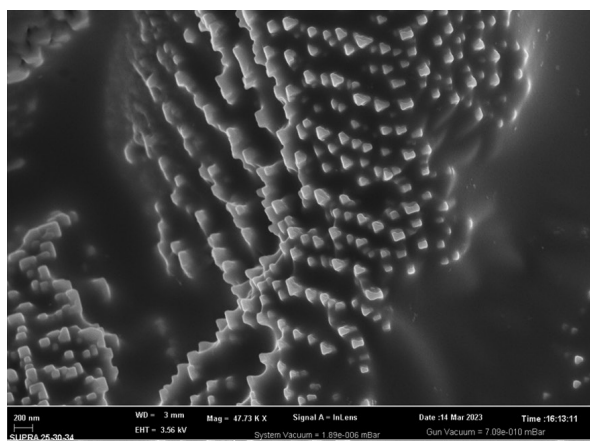
A large excess of hyaluronic acid creates a kind of scaffold on which chitosan molecules are arranged in chains. High magnification (Fig. 3b) revealed tightly associated rectangular chitosan molecules located on the surface of hyaluronic acid.

Figure 4 shows nanogels where chitosan is in excess relative to hyaluronic acid (ratio CHI:HA = 2:1). In this case, a gel-like, shapeless structure of hyaluronic acid is observed with firmly packed spherical

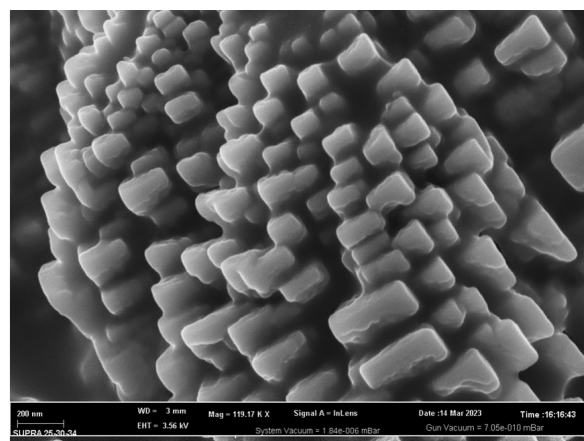
clusters of chitosan molecules (Fig. 4). The size of these gels cannot be determined; they are shapeless structures of uncertain size.

Figure 5 shows nanogels obtained at the CHI:HA ratio of 5:1. In this case, a shapeless substrate of hyaluronic acid with chitosan molecules of various shapes: square, round, rectangular, on its surface. To conclude, nanogels of different sizes and shapes are obtained during chemical cross-linking at different ratios of chitosan and hyaluronic acid.

To analyze the size and surface charge of nanogels, the nanogel sample with the ratio CHI:HA (1:1.25) was used. The size and surface charge of nanogels were determined by dynamic light scattering.



a)



b)

Fig. 3. Electron microscopy images of nanogels (ratio CHI:HA = 1:5):

a) magnification Mag = 47.73 KX; b) magnification Mag = 119.7 KX.

Supra-25, working distance WD = 3 mm, accelerating voltage EHT = 3.56 kV, Signal A detector = InLens, system vacuum = 1.89e-0.006 mBar, cathode unit vacuum = 7.09e-010 mBar.

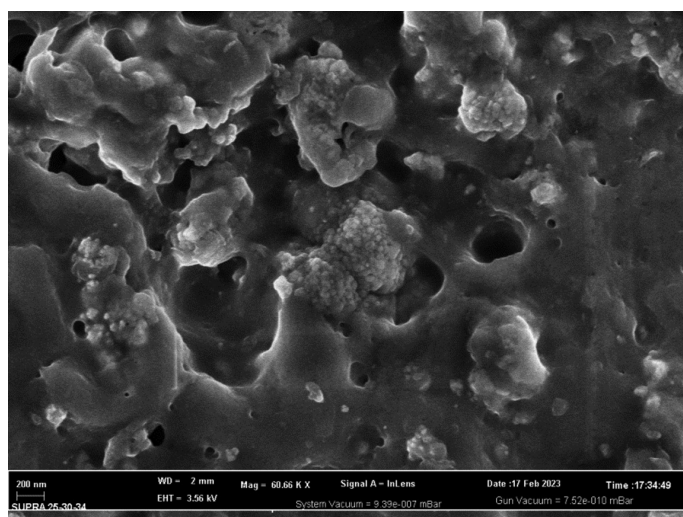


Fig. 4. Electron microscopy images of nanogels (ratio CHI:HA = 2:1). Supra-25, working distance

WD = 2 mm, accelerating voltage EHT = 3.56 kV, magnification Mag = 60.66 KX,

Signal A detector = InLens, system vacuum = 9.39e-0.007 mBar,

cathode unit vacuum = 7.52e-010 mBar.

Fig. 6 shows the intensity size distribution functions for HA and CHI solutions and for a complex CHI/HA mixture. The scattering correlation functions indicate that the data is good enough to calculate the mean size of the particles. As it can be seen in Fig. 6, HA is present in solution mainly as individual macromolecules with the average size of 12 nm, whereas CHI is present in aggregated form with the average size of 190 nm.

Since the main peak of HA with an average size of 12 nm is far enough from the main CHI peak (Fig. 6), possible changes of each peak can be discerned after complexation. Fig. 6 shows that in the presence of CHI in the HA solution, a new rela-

tively narrow peak appears (instead of the CHI and HA peaks) corresponding to an average radius of 340 nm. This peak corresponds to the formation of CHI/HA nanogels. Zeta potential measurements showed that nanogels are positively charged with $\xi = +5,3 \pm 0,31$ mV.

The scattering correlation functions indicate that the data is good enough to calculate the mean size of particles (Fig. 7.)

Effect of solvents on the average sizes of CHI/HA nanogels

The influence of a number of solvents, namely water, serum, saline, and DNIC#4 on the distribu-

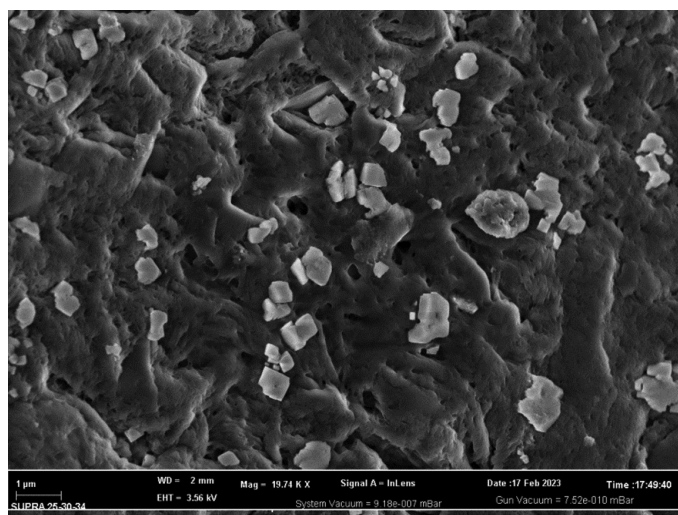


Fig. 5. Electron microscopy images of nanogels (ratio CHI:HA = 5:1): Supra-25, working distance WD = 2 mm, accelerating voltage EHT = 3.56 kV, magnification Mag = 19.74 KX, Signal A detector = InLens, system vacuum = 9.18e-0.007 mBar, cathode node vacuum = 7.52e-010 mBar.

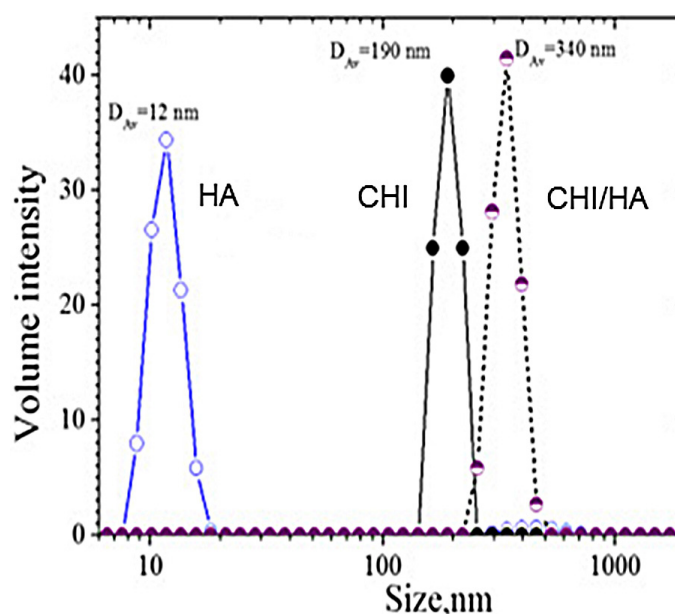


Fig. 6. Scattering intensity-size distribution functions for solutions of HA, CHI, and nanogels CHI/H at CHI:HA ratio of (1:1.25).

tion of CHI/HA nanogels was studied. We have previously shown that DNIC#4 is a cytoprotective compound (Akentieva et al., 2019).

The corresponding data is presented in Fig. 8.

Results show that the suspension of nanogels CHI/HA in a physiological solution (sample N1), in addition to the main peak, contains large, rapidly sedimenting particles with sizes above 10 μm , the surface charge is negative, $\zeta = -18.8$ mV.

Nanogels CHI/HA in fetal bovine serum (sample N2) showed a major peak at 8.7 nm (95 %) and negative surface charge, $\zeta = -7.52$ mV. Consequently, polydisperse nanogels were obtained with

the main size of 6–8 nanometers, as well as from 10 to 340 nm.

Nanogels CHI/HA in water (sample N3) showed a major peak at 545 nm (100 %) and a negative surface charge of $\zeta = -26.5$ mV.

Nanogels CHI/HA+DNIC#4 (sample N4, concentration of DNIC#4 = 4.87×10^{-3} M) showed a main peak at 2041 nm (100 %) and negative surface potential, $\zeta = -0.047$ mV. Large nanoparticles were obtained; 1–2 microns in size (by volume and intensity), the potential was almost equal to 0.0.

Nanogels CHI/HA+DNIC#4 (sample N5, concentration of DNIC#4 = 4.87×10^{-4} M) showed a main

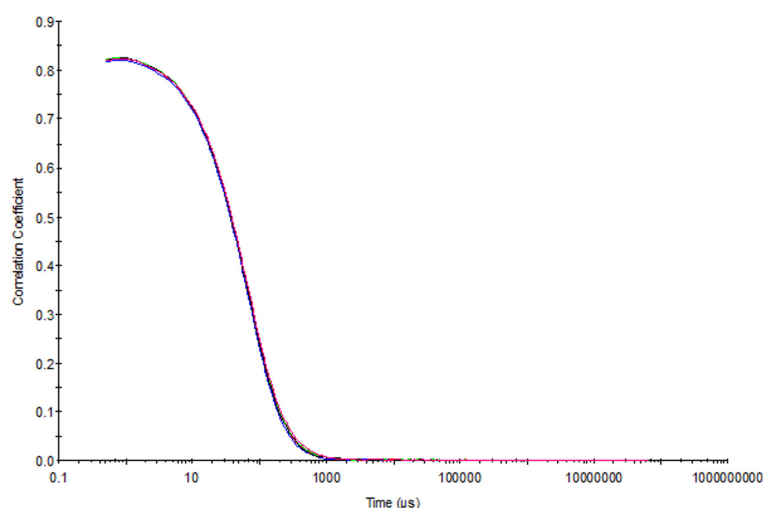


Fig. 7. Correlogram of the CHI/HA system.

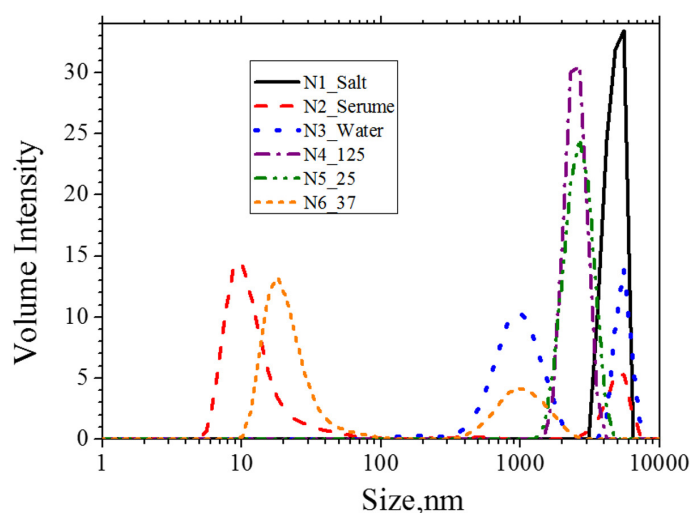


Fig. 8. Stability of nanogels (CHI/HA) in physiological solution (N1, 0.9 % NaCl solution), in fetal bovine serum (N2, 10 % serum), in water (N3), N4 (nanogels+DNIC#4, concentration of 4.87×10^{-3} M), and in N5 (nanogels+DNIC#4, concentration of 4.87×10^{-4} M).

narrow peak, the size of nanogels is 2.5 μm (by volume and by intensity) (100 %) and $\zeta = -3.91$ mV. Nanogels CHI/HA+DNIC#4 (sample N6, concentration of DNIC#4 = 4.87×10^{-2} M) showed polydispersity too high for reliable average size measurements.

Samples N1, N4, and N5 demonstrated 100 % reproducibility and moderate polydispersity (PDI). Their measurement data are identical for both the volume-size distribution function and the scattering intensity distribution function. For samples N2 and N3, the measurement results are quite satisfactory and are well defined by the volume-size distribution function.

Nanogel aggregation was observed in saline, which indicates that the system lacks colloidal stability. High concentrations of salt (0.9 % NaCl solution) in the solution shield the electrostatic charges of na-

noparticles, reducing thus the electrical repulsion of the double layer, which allows particles to easily overcome the energy barrier and stick together.

The addition of DNIC#4 (concentration of 4.87×10^{-3} M and 4.87×10^{-4} M) to the nanogels also leads to a significant reduction in surface potential ($\zeta = -0.047$ mV; $\zeta = -3.91$ mV), indicating high risk of particle aggregation, flocculation, and colloidal instability as the system lacks the electrostatic repulsion necessary to keep particles apart. The addition of DNIC#4 (concentration of 4.87×10^{-2} M) resulted in significant aggregation and made it impossible to perform measurements. It is known that dinitrosyl iron complexes are very dynamic in nature. In aqueous or biological fluids, DNICs tend to undergo rapid degradation, hydrolysis or denitrosylation. This changes the surface chemistry of the nanoparticles, destroying their protective

coatings. Therefore, the addition of dinitrosyl iron complex (DNIC#4) causes rapid aggregation, and nanoscale assemblies to form micrometer-sized particles.

Thus, the results show that nanogels CHI/HA maintain good stability just in water and in fetal bovine serum.

It is known that the polydispersity index (PDI) is a critical quality indicator used to determine the width of the particle size distribution in a nanoparticle system. High PDI (>0.5) can result in drug delivery resulting in inconsistent drug release rates, poor cellular uptake, and compromised long-term stability. Results of the DLS study of nanogels show that the obtained nanogels are polydisperse and have polydispersity index values ranging from 0.2 to

0.4. Such polydispersity index values are generally considered the optimal threshold for therapeutic and pharmaceutical nanoparticles.

Therefore, our obtained nanogels meet the “critical quality attributes” (CQA) of nanoparticles and can be used for therapeutic purposes.

Cytotoxicity of nanogels

Cytotoxicity of CHI/HA nanogels was evaluated at different nanogel concentrations 24 hours after incubation with *Vero* cells. The results showed that CHI/HA nanogels at 20, 40, and 60 % concentration in the growth medium were compatible with *Vero* cells, with cell viability maintained at levels ranging from 100 to 78 %. However, at nanogel concentration of 80 or 100 % in the growth me-

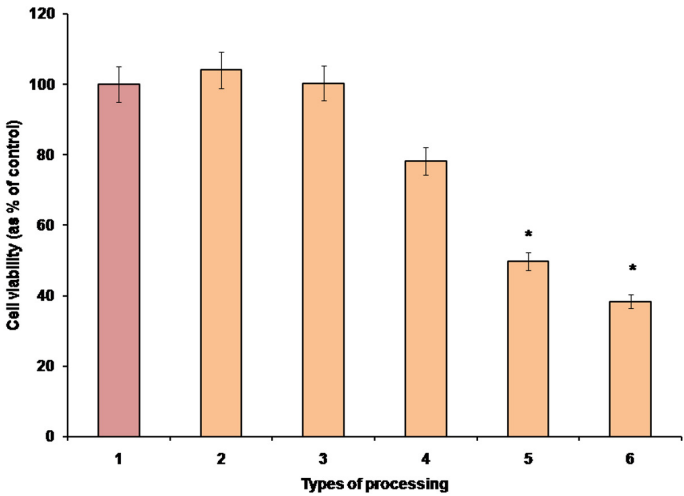


Fig. 9. Cytotoxicity of CHI/HA nanogels. *Vero* viability in the presence of various nanogel concentrations: 1 – cells (control), 2 – 20 % of nanogels, 3 – 40 % of nanogels, 4 – 60 % of nanogels, 5 – 80 % of nanogels, 6 – 100 % of nanogels.

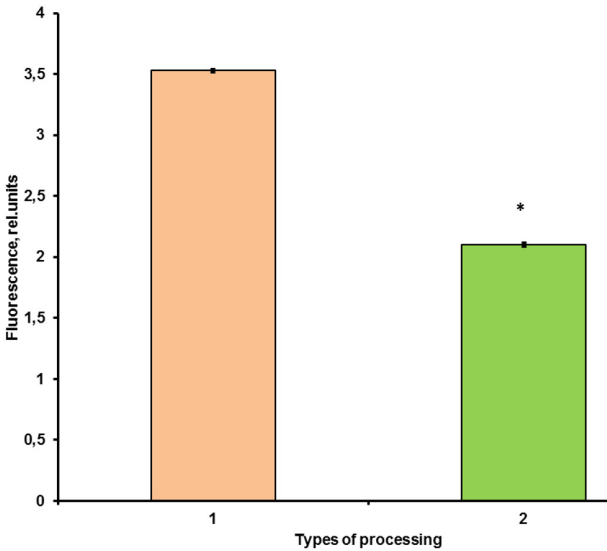


Fig. 10. Effect of complex (CHI/HA nanogel+DNIC#7) on prostate cancer cells viability: 1 – PC-3 cells, 2 – PC-3 cells + (CHI/HA nanogel+DNIC#7). $M \pm m$, $n = 3$, $*p < 0.05$.

dium, cell viability decreased to 49.8 and 38.3 %, respectively (Fig. 9).

The results of this experiment show that the cytotoxic effect of the nanogels is higher at higher nanogel concentrations (80 or 100 % in the growth medium). This may be due to chitosan which interacts with cellular components and thereby kills cells (Dalal et al., 2025). It is known from literature that high concentrations of chitosan can induce oxidative stress and membrane destruction (Naghib et al., 2024).

Thus, the results show that CHI/HA nanogels are nontoxic, compatible with *Vero* cells at <80 % concentration in the growth medium.

Effect of complex (CHI/HA nanogel+DNIC#7) on prostate cancer cells viability

DNIC#7 has been proved as a cytotoxic compound suppressing the viability of multiple myeloma cells by 1.8 times (Akentieva et al., 2019).

In this study, the effect of DNIC#7 in the composition of nanogels (CHI/HA) on the viability of prostate cancer cells (human prostate adenocarcinoma, PC-3 cell line) was studied using the AlamarBlue® Cell Viability Assay. The results of the study showed that complex (CHI/HA nanogel+DNIC#7) (concentration of 4×10^{-4} M) suppresses the viability of prostate cancer cells by 1.5 times (Fig. 10).

Therefore, the results show that complex (CHI/HA nanogel+DNIC#7) exhibits cytotoxic properties and has potential for the treatment of human prostate cancer.

Conclusion

New dinitrosyl iron complexes with sulfur-containing ligands of the thiourea series have been synthesized previously. However, the resulting DNICs were not entirely stable and easily destroyed when dissolved in aqueous solutions and thus, they could not generate NO for a long time. Therefore, it was necessary to develop nanogels and encapsulate DNICs in them to increase the stability and efficiency of these compounds.

In this study, chitosan, a natural polysaccharide, and hyaluronic acid, a non-sulfated glycosaminoglycan, were chosen to create nanogels. These compounds are biocompatible and easily biodegradable. Hyaluronic acid is used extensively as a delivery vector because it specifically binds to CD44, RHAMM receptors on the surface of body cells. Consequently, the obtained nanogels can interact specifically with the cells of the body.

Using a chemical modification method, stable nanogels were created. Results of the study show that at different ratios of chitosan and hyaluronic acid,

nanogels of different sizes, shapes and charges are obtained. It was found that at the ratio of CHI:HA = 1:1.25, nanogels of optimal size and surface charge are formed. Nanogels were found to be stable in water and physiological solutions. Complex (CHI/HA nanogels+DNIC#4) (cytoprotector) has been proved non-toxic and biocompatible with renal epithelial cells. At the same time, complex (CHI/HA nanogels+DNIC#7) (cytostatic) demonstrated anticancer activity, which suggests its use in the treatment of human prostate cancer. Therefore, depending on the properties of DNICs, the resulting nanogels can be used in various biomedical applications. Further studies to analyze cell death (apoptosis, necrosis, lactate dehydrogenase assay, trypan blue exclusion) due to (CHI/HA nanogels+DNIC#7) are planned.

In this work, nanogels for stabilization and targeted delivery of DNICs into body cells have been prepared. However, the results presented were obtained *in vitro*. Therefore, further studies in animal models (*in vivo*) are needed to evaluate their biocompatibility, specificity of action and therapeutic potential. At the same time, it should be noted that the results of the study are of practical importance since a technique for stabilization and targeted delivery of DNICs into body cells using nanogels has been developed, which may be in demand in biotechnology, nanomedicine, and pharmaceutical industry. The results obtained can be further used in the pharmaceutical industry to create innovative drugs for the treatment of socially significant diseases.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Compliance with ethical standards

This work does not contain any human or animal studies.

Author Contributions

Natalia Akentieva: conceptualization; data curation; methodology; writing – original draft.

Natalia Sanina: synthesis DNIC, data curation; methodology; writing – review.

Yurii Antonov: data curation; methodology; DLS; writing – review & editing.

Nadezhda Dremova: visualization; methodology; electron microscopy; data curation.

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