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RESEARCH PAPER

Fabrication of chitosan-hyaluronic acid nanoparticles and encapsulation into nanoparticles of dinitrosyl iron complexes as potential cardiological drugs

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ABSTRACT

Objective(s): Currently, the development of nanoparticles for the stabilization and targeted delivery of cardiac drugs has gained significance. The present study aimed to develop nontoxic nanoparticles based on chitosan-hyaluronic acid (HA), encapsulate dinitrosyl iron complexes (DNICs, donors NO) into the nanoparticles to increase the stability and effectiveness of their action, and assess the effect of the nanoparticle-DNIC complex on the cell viability of cardiomyocytes.

Materials and Methods: Nanoparticles were obtained from chitosan-HA using the ionotropic gelation technology, and the morphology and size of the nanoparticles were determined using electron microscopy. The DNICs were built into the nanoparticles using the physical association method, and the stability of the nanoparticle-DNIC complexes and NO release was investigated using the electrochemical method.

Results: Analysis by the electron microscopy showed that the nanoparticles were homogeneous in terms of shape and had an optimal size of ~100 nanometers. In addition, the incorporation of the DNICs into the composition of the nanoparticles significantly increased the stability of the DNICs, while also prolonging the generation of NO and enhancing the yield of nitrogen monoxide. Fluorescence analysis indicated that the chitosan-HA nanoparticles increased the cell viability of rat cardiomyocytes.

Conclusion: The nanoparticles were fabricated from chitosan and HA. The encapsulation of the DNICs into the composition of the nanoparticles could stabilize these compounds, while prolonging and increasing the generated nitric oxide. The nanoparticle-DNICs were water-soluble, biocompatible, biodegradable, and nontoxic, which could be used as potential cardiac drugs for the treatment of cardiovascular diseases.

Keywords: *Chitosan, Dinitrosyl Iron Complexes, Hyaluronic Acid, Nanoparticles, NO Donors*

How to cite this article

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INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of death worldwide. The World Health Organization (WHO) estimated that in 2016, 17.5 million died due to CVDs, among which 7.4 million died due to coronary heart disease and 6.7 million died due to stroke [1]. Currently, the cardiovascular drugs for the treatment of coronary artery disease are widely used as donors of nitric oxide (NO) [2-4]. The most common drugs in this regard are nitroglycerin, nitrosorbide, nitroprusside, and nitrite, which release NO into cells and body tissues. However, these drugs are often unstable, nonspecific, and toxic, leading to various side-effects [5].

Presently, the development of nanoparticles for the stabilization and targeted delivery of cardiac drugs has gained significance. The new analogues of dinitrosyl iron complexes (DNICs) with sulfur-containing ligands of thiourea have been synthesized previously [6]. However, these DNICs are not completely stable and are easily

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destroyed when dissolved in aqueous solutions; furthermore, they are not able to generate NO continuously. Therefore, the development of nanoparticles for the stabilization of DNICs is of utmost importance. According to the literature, numerous carriers are known for targeting drug delivery. In the current research, we used chitosan as a carrier of DNICs in nanoparticles. Chitosan is an amino sugar and a derivative of a linear polysaccharide, the macromolecules of which consist of randomly bound β-D-glucosamine units and N-acetyl-D-glucosamine [7]. Chitosan is a highly cationic, nontoxic, biocompatible, and biodegradable compound [8], and shells of crustaceans are one of its major sources [7].

Chitosan has numerous properties, which render it proper for use in different fields, especially in biomedicine and the pharmaceutical industry. Chitosan has unique adhesive properties and is widely employed as a carrier for drug delivery, increasing drug transport through the intestinal epithelium [9, 10]. Moreover, chitosan is known to interact electrostatically with negatively charged macromolecules, such as plasmid DNA and anionic proteins (e.g., insulin), thereby forming polyelectrolyte complexes [11, 12]. Owing to its cationic nature, chitosan is able to form insoluble complexes with an anionic polysaccharide-hyaluronic acid (HA), which render it a viable choice for use as a carrier in nanoparticles [13, 14].

Chitosan-based nanoparticles are able to perform the required function for long periods in the human body, decompose, and be excreted from the body without causing harm. Currently, the surface properties of nanoparticles are considered to be the key factors in the modulation of their biodistribution parameters [15]. Nanoparticles with more hydrophobic surfaces are preferably bound in the liver, spleen, and lungs [16]. Plasma proteins play a pivotal role in the recognition of particles by the macrophages of the phagocytic system and their rapid removal from the bloodstream. Comparatively, hydrophilic polymers could generate a cloud of chains on the surface of particles, repelling the plasma proteins. As such, the surfaces of nanoparticles with hydrophilic modification have reduced opsonization, and the nanoparticles coated with polysaccharides are considered to be a new trend in drug delivery systems. In the present study, we used a negatively charged polymer (i.e., HA) as the delivery vector. This polysaccharide was selected because it is able to prolong the circulation time in the plasma of liposomes [17, 18], polymeric nanoparticles [19], nanoparticle-like clusters [20], and solid lipid nanoparticles [21]. HA is a non-sulfur glucose-aminoglycan, which is part of the connective, epithelial, and nerve tissues [22]. HA is also a major component of the extracellular matrix, which is found in many biological fluids (e.g., saliva, synovial fluid) [23]. The active targeting of the drugs associated with HA is a promising approach in biomedicine since HA is a biocompatible, nontoxic, and easily biodegradable molecule [24]. HA could protect the delivered drug and improve the solubility of hydrophobic drugs. Furthermore, it could be employed as a carrier and delivery vector for the composition of nanoparticles since HA molecules are capable of ion self-assembly in nano-gels [25]. HA could also physically interact with nanoparticles through non-covalent attraction forces such as hydrophobic/hydrogen bonds or ionic interactions [25]. Since HA is a polyanionic polysaccharide, it has multiple charges for interaction with polycations [25].

HA is the physiological ligand of CD_{44}- and receptor for hyaluronan-mediated motility (RHAMM), which specifically interacts with CD_{44} [26] and RHAMM on the cell membrane, ensuring the effectiveness of drug delivery to the cells [27]. HA-based nanoparticles are able to recognize CD_{44}- and RHAMM-expressing cells, thereby specifically delivering particle-bound drugs through receptor-mediated endocytosis [28]. This strategy of cellular targeting allows direct drug delivery into the cells rather than concentrating on the kidneys and liver and excretion from the body through the reticuloendothelial system [29]. In addition, cellular enzymes (e.g., hyaluronidases) cleave HA within the cells, thereby releasing the drug directly into the cells [30]. Such a strategy allows for specific and selective drug delivery.

The present study aimed to develop nontoxic nanoparticles based on chitosan-HA and encapsulate them into DNIC nanoparticles in order to improve the stability and effectiveness of their action. Moreover, we investigated the effects of the nanoparticle-DNIC complex on the viability of cardiomyocytes.

MATERIALS AND METHODS

Experimental materials

In this study, chitosan (molecular weight=100,000-300.000) was purchased from

Acros Organics (New Jersey, USA), HA (sodium hyaluronate, low molecular weight<0.1 MDa) was purchased from My Organic Formula (Madison, USA). The other materials were supplied by Merck (Darmstadt, Germany), including sodium dihydrogen phosphate (Na_2HPO_4), potassium iodide, and hydrochloric acid. All the reagents and chemicals were of the analytical grade and used as received without purification.

Dulbecco's modified Eagle's medium (DMEM) was used as the growth medium, which contained low glucose (1 g/l), L-glutamine, HEPES (25 mM), sodium pyruvate, fetal bovine serum (ultra-low endotoxin), trypsin with 0.25% EDTA and 0.02% HBSS, gentamicin (10 mg/ml) and was purchased from Biowest (Nuaillé, France).

Rat cardiomyocytes (H9c2 (2-1) cell line; ATCC®CRL-1446™) were purchased from ATCC (Merck, Manassas, USA). The Alamar Blue® cell viability assay was obtained from Thermo Fisher Scientific (Kansas City, USA). Deionized water was used throughout the experiments, and plastic dishes were also utilized (petri dishes and disposable pipettes), which were provided by the BD Falcon Company (Franklin Lakes, USA).

Preparation of the chitosan-HA nanoparticles by ion gelation

The chitosan-HA nanoparticles were obtained by ion gelation [31]. A sample of chitosan (10 mg) was dissolved in one milliliter of HCl (0.01 M; pH: 2) and stirred for 12 hours. A portion of HA (40 mg) was dissolved in one milliliter of distilled water and stirred for 12 hours. The resulting solutions of chitosan and HA were mixed at the ratio of 1:4, the pH of the solution was set at 6.8 with phosphate buffer (2 ml; 0.1 M; pH: 6.8), and the solution was stirred using a magnetic stirrer for 36 hours. Afterwards, the solution was dried via freeze-drying and analyzed using electron microscopy. In the control samples, chitosan and HA were analyzed as well.

Analysis of the chitosan-HA nanoparticles and control chitosan and HA using electron microscopy

The analysis of the obtained nanoparticles and control samples of chitosan and HA was carried out via scanning electron microscopy [32]. The samples were assessed using a new generation Zebra scanning Supra 25 electron emission field emission microscope and a Schottky cathode, and an in-lens detector was applied to collect the secondary electrons from the object of the investigation. Data on these samples were read based on the number of the secondary electrons emitted by the object of the investigation during the interaction with the primary electron beam. Moreover, a synchronous scan was used along with the television scanning of the electronic probe over the sample, which allowed obtaining an image of an object reflecting the topography and composition as the number of the secondary electrons depends on these factors.

The main challenges in the experiments were associated with the non-conductive composition of the samples, which led to operation at low current probes with a sufficiently large signal-to-noise ratio. Sputtering carbon on the surface of the samples was the practical solution to this problem without the loss of the data of the object. The conductive layer of carbon is thin (nanometer units), making a minimal contribution to the signal from the sample since it is also an easy element.

The investigated nanoparticle samples were prepared via vacuum deposition with carbon on an aluminum substrate. A high-vacuum device was used for the deposition of VUP-4 in order to deposit the carbon coating using the arc-discharge method. At the next stage, the nanoparticles were analyzed using the Supra 25 electron microscope at the working distance of WD=2 mm, accelerating voltage of EHT=3.56 kV, increased Mag=100.02 KX, signal detector of A=in-lens, vacuum in the system=1.13e-0.006 mBar, and vacuum in the cathode node=8.49e-010 mBar.

Synthesis of the mononuclear dinitrosyl iron complexes (DNICs) with functional sulfur-containing ligands

As the test compounds, we used synthetic analogues of the mononuclear DNICs with functional sulfur-containing ligands, including thiourea and its derivatives, as follows:
$[Fe(SC(NH_2)_2)_2(NO)_2]_2[Fe_2(S_2O_3)_2NO_4]$(DNIC#3),
$[Fe(SC(NH_2)_2)_2(NO)_2]ClO_4Cl$ (DNIC#6).

The synthesis and identification of DNIC#3 and #6 was performed in accordance with the procedure [33], and the DNICs released NO upon dissolution in the proton solvents due to dissociation [33].

Electrochemical determination of the no concentration isolated from the DNICs

The NO release from the DNICs was determined

using the amperometric method [34, 35]. To do so, an amperometric sensor electrode of the amiNO-700 system inNO nitric oxide measuring system (Innovative Instruments Inc., USA) was used to measure the concentration of the NO generated from the DNICs. The NO concentration in the aqueous solution was set at ~500 seconds (0.2-second increments). A standard aqueous solution of $NaNO_2$ (100 µM) was used to calibrate the sensor electrode, which was combined with a mixture of the aqueous solutions of KI (0.12 M; 18 ml) and H_2SO_4 (1 M; 2 ml). For the analysis of the NO released from the DNICs, the DNIC samples were prepared and dissolved in water to the final concentration of 4×10^{-4} M. Afterwards, the solutions were stirred magnetically at room temperature for one and 10 minutes. At the next stage, the aliquots of the DNIC solution (0.5 ml) were added to a measuring electrochemical cell, and 49.5 milliliters of the buffer (Buffers, Hydrion Envelope Sigma-Aldrich, USA; pH: 7.0) was added to the well, in which the thermosensor and electrode were immersed. The NO emission was recorded in the system at 500 seconds (~8.3 minutes) at the temperature of 25^0C.

Analysis of NO release from the DNIC-nanoparticle complexes (chitosan-HA)

The release of NO from the DNIC-nanoparticle complex was determined using the amperometric method [35]. To analyze the NO donor activity of the DNIC-nanoparticle complexes, a solution of DNICs with the nanoparticles was prepared (DNIC concentration=$4x10^{-4}$ M). To this end, one milliliter of the nanoparticle solution was stirred for five, 10, 20, 60 or 100 minutes. Following that, nine milliliters of water was added to the solution and mixed, and 0.5 milliliter of the aliquots of the complex solution was transferred to a measuring electrochemical cell containing 49.5 milliliters of the buffer (pH: 7). The NO release was recorded for 500 seconds at the temperature of 25^0C.

Culturing of the rat cardiomyocytes

Adhesive rat cardiomyocytes (H9c2 [2-1]; ATCC® CRL-1446™) were isolated from cardiac myocardial tissue, and the cells were cultured in DMEM also containing the 10% (v/v) solution of embryonic bovine serum and HEPES (10 mM) at the pH of 7.2. The cells were incubated in a humidified atmosphere (5% CO_2) at the temperature of 37^0C. After reaching 90% of cell density, the cells were treated with a solution of 0.25% Trypsin-EDTA for detachment from the surface and neutralized using the growth medium. Afterwards, the cells were placed inside plates and used in an experiment to determine the effect of the nanoparticle-DNIC complex on the viability of the cardiomyocytes.

Evaluation of the viability of the cardiomyocytes using the fluorescent method (AlamarBlue)

To analyze the effect of the nanoparticle-DNIC complex on the viability of the cardiomyocytes, a fluorescent method (AlamarBlue® cell viability assay) was used, the active ingredient of which was resazurin [36]. Resazurin is a nontoxic, cell-permeable dye with a blue color and weak fluorescence. The method is used to measure the activity of mitochondrial NADH dehydrogenases, which cleave NADH into NAD and H^+, as a result of which the proton ion reduces resazurin to fluorescent resofurin. The reduced resorufin has a pink color and is strongly fluorescent. The rat cardiomyocytes (H9c2) were placed in 96-well plates (4,000 cells per well) in 290 microliters of the growth medium, and the cells were grown for 24 hours in an incubator at the temperature of 37^0C with 5% CO_2 and 95% humidity. Following that, nanoparticles-DNIC#3 (3.4×10^{-3} M) was added to each well, and an equal amount of the growth medium was added to the positive control and incubated for 20 minutes at the temperature of 37^0C. A minimum of four wells were used for each dose and control. The AlamarBlue reagent (20 µl) was directly added to each well, and the fluorescence intensity was measured for 30 hours at E_{ex}/E_{em}=570/590 nanometers using a Varian spectrophotometer (Cary Eclipse, USA). Cell viability under the effect of the nanoparticle-DNIC#3 complex was evaluated by comparing the fluorescence of the experimental wells with the fluorescence of the positive control, and the obtained data were expressed as the mean values of the three repeated experiments.

RESULTS AND DISCUSSION

Fabrication and characterization of the nanoparticles

In the present study, the chitosan-HA nanoparticles were obtained using the physical association method through the ion self-assembly of the HA molecules (negatively charged) and chitosan molecules (positively charged) in

nano-gels. The obtained nanoparticles were water-soluble due to the hydrophilicity of HA and chitosan. The morphology and size of the nanoparticles were evaluated via electron microscopy. According to the findings, the particles had spherical morphology and size of approximately 100 nanometers (Fig 1).

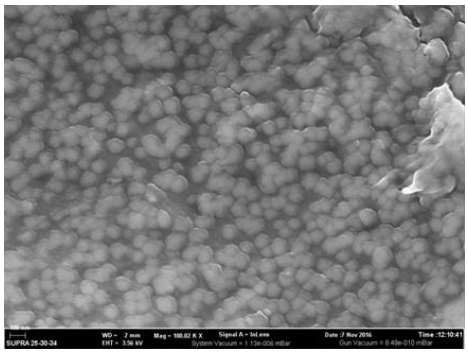

Fig 1. Chitosan-HA Nanoparticles Obtained by Electron Microscopy

In addition, the nanoparticles were homogeneous in terms of the size and shape. According to the current research, the morphology of the obtained chitosan-HA nanoparticles completely differed from the shape of the control samples, as well as the chitosan and HA molecules (Fig 2 and 3).

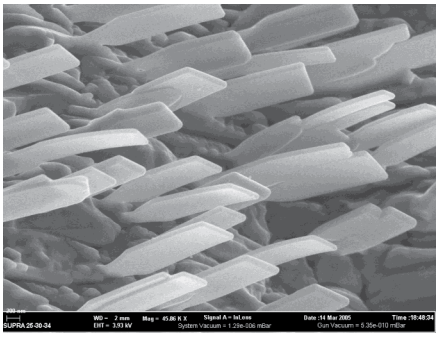

Fig 2. Chitosan Images Obtained by Electron Microscopy

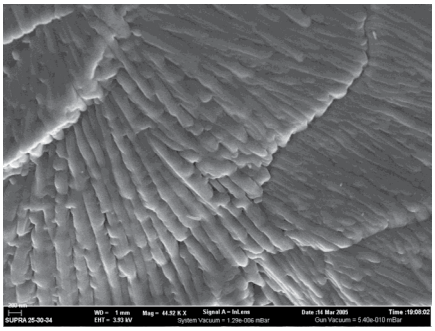

Fig 3. Images of HA Obtained by Electron Microscopy

As is depicted in Fig 2, the molecules of chitosan had a plate form It is notable that the HA molecules had an elongated shape in the form of tightly packed chains (Fig 3).

Encapsulation of the DNICs in the nanoparticles

To increase the stability of the DNICs, the DNICs were encapsulated in the nanoparticles using the physical association method. Since HA is a polyanionic polysaccharide, it has multiple charges to interact with polycations. DNICs are cationic compounds, which cause HA to physically bind to DNICs through non-covalent attractive forces, especially due to hydrophobic and hydrogen bonds or ionic interactions. At the next stage, we investigated the release of NO from the nanoparticle-DNIC#3 complex using the electrochemical method, as well as the release of NO from DNIC#3 alone in the aqueous solution and nanoparticle composition. Inirially, the NO generation from the DNIC#3 was analyzed (Fig 4; curves 1 & 2).

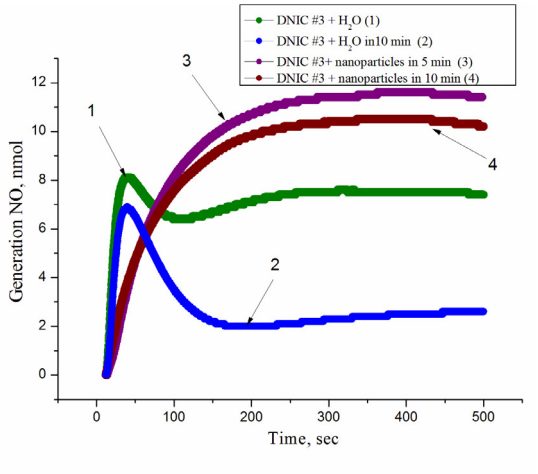

Fig 4. Generation of NO from DNIC#3 and Nanoparticle-DNIC#3 Complexes

As can be seen in Fig 4 (curve 1), when DNIC#3 dissolved in water, NO (8 nmol) was released for 50 seconds, and after 100 seconds, the NO level dropped to 6 nmol, remaining constant for 500 seceonds. Moreover, the dissolution of DNIC#3 in the aqueous solution during 10 minutes indicated that the NO level decreased to 7 nmol and dropped sharply to 2 nmol after 150 secconds (Fig. 4; curve 2), demonstrating that DNIC#3 was unstable in the aqueous solution, and the generation of NO practically ceased after 10 minutes.

At the next stage, DNIC#3 was added to the nanoparticles and incubated for five and 10

minutes. The obtained results idnicated that the preliminary incubation of DNIC#3 with the nanoparticles for five and 10 minutes caused the generated NO to gradually increase for 200 seconds, reaching the maximum value (10-11 nmol) and remaining constant for 500 seconds (Fig 4; curves 3 & 4). Therefore, it could be inferred that the encapsulation of DNIC#3 in the nanoparticles significantly increased its stability, while also prolonging and increasing the NO release.

In the present study, the release of NO from DNIC#6 in the aqueous solution and nanoparticle composition was also investigated (Fig 5). According to the findings, when DNIC#6 alone dissolved in the aqueous solution, 16 nmol of NO was observed after 25 seconds (Fig 5; curve 1). In addition, the incubation of DNIC#6 for 10 minutes in water resulted in a significant reduction in the NO release at four time intervals (from 16 to 4 nmol), and after 100 seconds, the level of the generated NO practically dropped to zero (Fig 5; curve 2), indicating that DNIC#6 alone could rapidly decomposes in water and cease to release NO.

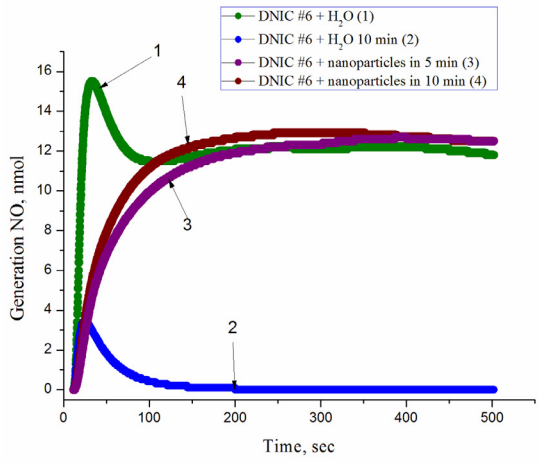

Fig 5. Generation of NO from DNIC#6 and Nanoparticle-DNIC#6 Complexes

We also analyzed the release of NO from the nanoparticle-DNIC#6 complex. According to the findings, after the addition of DNIC#6 to the nanoparticles and incubation for 5-10 minutes, the release of NO (13 nmol) was observed, and the level persisted for a long time (>500 seconds) (Fig 5; curves 3 & 4). In addition, even the pre-incubation of DNIC#6 with the nanoparticles for 20-40 minutes was associated with the significant release of NO (10 nmol and 8 nmol, respectively; data not shown). Therefore, it could be concluded that the encapsulation of DNICs in the composition of the nanoparticles stabilized these compounds, while also prolonging and increasing the amount of the generated NO.

Effect of the nanoparticle-DNIC complex on cell viability

The effect of the nanoparticle-DNIC#3 complex on the viability of rat cardiomyocytes was investigated *in vitro*. According to the obtained results, DNIC#3 alone could stimulate the viability of the cardiomyocytes, with the peak of maximum cell activity observed after three hours (Fig 6). On the other hand, Fig 6 depicts that after three hours, the fluorescence value decreased from 19 units to three units, indicating the reduced viability of the cardiomyocytes.

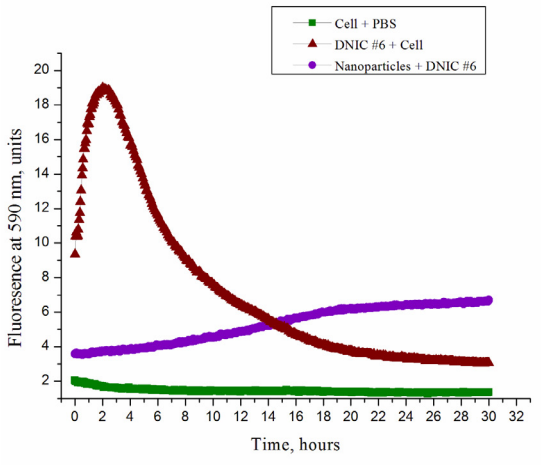

Fig 6. Effect of Nanoparticle-DNIC#3 Complex Viability of Rat Cardiomyocytes

Following that, the cardiomyocytes were incubated with the nanoparticle-DNIC#3 complex, and the obtained results showed that the pre-incubation of the nanoparticle-DNIC#3 complex with the cells led to the gradual increase in the fluorescence from four units to seven units, with the effect persisting for 30 hours. Therefore, it could be concluded that DNIC#3 in the nanoparticle composition most effectively increased cell viability. The chitosan-HA nanoparticles were nontoxic and had cytoprotective effects on the rat cardiomyocytes.

Currently, several approaches are used for the development of nanoparticles for drug delivery. Numerous studies have also been focused on the development of inorganic and organic

nanoparticles. Some inorganic nanoparticles include gold nanoparticles [37], magnetic iron oxide [38], hydroxyapatite [39], zinc oxide [40], quantum dots [41], and carbon nanotubes [42]. Some examples of nanoparticles based on organic materials are polymeric nanoparticles [43], nanogels [44], liposomes [45], dendrimers [46], and nanoparticle peptides [47].

The most important function of NO its role in vasodilation as NO has a therapeutic potential for the treatment of acute pulmonary hypertension, coronary artery disease, ischemia, and other CVDs. NO is currently delivered using gaseous NO or a precursor of NO, such as N-diazeniumdiolate, S-nitrosothiol, DNICs, organic nitrate, and nitroprusside [48]. Gaseous NO has some notable limitations since its delivery requires the use of gas cylinders under pressure, which is costly and challenging. On the other hand, NO precursor molecules have several advantages; for instance, their half-life is from a few minutes to hours depending on pH, temperature, and molecular structure. A principal approach to changing the biodistribution and pharmacokinetic properties of NO donors is their encapsulation in the nanoparticle compositions. The ability to ensure the stable delivery of NO from long-circulating nanoparticles is particularly important for the treatment of CVDs. Currently, numerous nanoparticles are utilized for the exogenous delivery of NO [49], which could be classified as the silica nanoparticles derived from sol-gel processes, surface functionalized metal/metal oxide nanoparticles, polymer-coated metal nanoparticles, dendrimers, micelles, and core-crosslinked star polymers [50].

Colloidal silica particles were first developed using the N-diazeniumdiolate group as a method for the production of NO-releasing particles [51]. Although the particle size was within the range of 0.2-0.3 μm and the resulting particles were not precisely nanoparticles, the mentioned study could be the milestone for further investigations regarding nanoparticles. These specific particles were applied in thromboresistant tubing, which decreased the number of blood clots and lowered the degree of platelet activation in the extracorporeal blood circulatory system of rabbits [51].

In addition to the studies focused on silica nanoparticles, the other experiments in this regard have employed gold nanoparticles as the potential hosts for the release of NO. Gold nanoparticles were developed by simply modifying the surface of gold using 11-bromo-1-undecanethiol, which released NO [52], and the gold nanoparticles had a small size (2 nm). Later, dendrimers were used as the basis for NO release through functionalizing polypropyleneimine dendrimers with N-diazeniumdiolate moieties when exposed to gaseous NO at the pressure of 5 atm [53]. However, it has been demonstrated that the primary amine derivative N-diazeniumdiolate released nitroxyl rather than NO under biologically significant conditions (pH: 7.4). Only at lower pH (approximately 3), a significant amount of NO was released. Afterwards, NO-releasing micelles were prepared, in which S-nitrosoglutathione was conjugated in the hydrophobic domain of an oligo copolymer methacrylate (ethylene glycol methyl ether) and the functional monomer 2-vinyl-4, 4-dimethyl-5-oxazolone [54]. It is also notable that the size of the obtained micelles was 40 nanometers. According to the findings, the NO-releasing micelles in combination with cisplatin were active against neuroblastoma with no impact on non-cancerous fibroblast cells.

Recently, core-crosslinked star polymers incorporated with N-diazeniumdiolate have been developed and used for NO release [55]. Core-crosslinked star polymers have advantages such as stability over micelles and easier synthesis than dendrimers. The cardiovascular applications of NO-releasing nanoparticles are based on their role in vasodilation. NO-releasing nanoparticles were first used for the treatment of the cardiovascular system and assessment of the effect of infusion of NO-releasing sol-gel nanoparticles on the jugular vein of Syrian hamsters [56]. According to the findings, significant changes occurred in the parameters of the gas composition of the blood in the hamsters treated with NO-releasing nanoparticles. Furthermore, methemoglobin increased compared to baseline after two hours, and the plasma nitrates and nitrites also increased. The arterial PO_2 was also observed to increase simultaneous with the reduction of the arterial pH. As was expected, the NO-releasing nanoparticles decreased the mean arterial blood pressure, which reached the minimum value after 90 minutes and returned to baseline after three hours. Fluorescence studies have also been focused on the estimation of the circulation time of NO-releasing nanoparticles, reporting that the particles circulated even six hours after

the infusion, while the circulatory system was completely cleared after 24 hours. Moreover, the nanoparticles were reported to cause microvascular vasodilation and increase the blood flow, while the control particles had no effects on the arteriole diameter and blood flow. Regarding the direct impact of exposure on blood cells, the NO-releasing nanoparticles did not increase the proportion of immobilized leukocytes. Therefore, it could be concluded that the NO-releasing nanoparticles could be applied in the treatment of hypertensive disorders. In addition, they could reverse the induced vasoconstriction [57]. According to the results of the mentioned study, the infusion of the NO-releasing nanoparticles effectively lowered the mean arterial pressure in the hamsters. The functional density of the capillaries also improved through the infusion of the NO-releasing nanoparticles. In general, these findings indicated that NO-releasing nanoparticles could be effective in the induction of vasodilation, as well as the treatment of CVDs.

The potential application a cation of nanoparticles with the release of NO obtained using sol-gel has also been investigated to maintain the microvascular function during hemorrhagic shock [58]. According to the findings, the infusion of NO-releasing nanoparticles was effective in supporting the cardiac rhythm, while also causing changes in the chemistry of blood gases, with the methemoglobin increasing in particular. Therefore, it could be inferred that NO-releasing nanoparticles are effective in alleviating the effects of hemorrhagic shock and could be used to support the patients until an appropriate volume is returned to the cardiovascular system, confirming the key role of NO in maintaining the function of the cardiovascular system.

Ample evidence suggests that after myocardial infarction, the immediate reperfusion of the ischemic myocardium reduces the degree of the infarction and preserves the mechanical cardiac function. Maintaining appropriate low concentrations of NO during repeated perfusion could be critical to reducing the risk of the ischemia/reperfusion of the heart. As such, the use of S-nitroso-N-acetyl-D, L-penicillamine functional dendrimers to prevent ischemic damage has been investigated in the isolated perfused rat heart [59]. According to the obtained results, the conjugation of the low-molecular-weight NO donors for the scaffolds of nanoparticles (dendrimers) could increase the effectiveness of the drug, and the nanoparticles may be useful in the reduction of ischemia reperfusion injury following myocardial infarction.

Several platforms could be used for the exogenous delivery of NO, each of which has certain advantages and disadvantages. Dendrimers are highly monodisperse, costly, and time-consuming to produce. The particles that include metallic or inorganic nanoparticles are highly stable and could exhibit significant toxicity when administrated via certain routes (e.g., the lungs). The use of polymer platforms is particularly appealing owing to the low cost of production, while they may not be utterly biocompatible. Therefore, recent studies have indicated that all types of nanoparticles are associated with limitations in interaction with cells. This is cause nanoparticles (>100 nm) cannot cross the pores of the blood vessels and enter the cells, large nanoparticles are absorbed by macrophages in the bloodstream and, and nanoparticles are often internalized cells in endocytosis, which is a slow process, requiring high concentrations of nanoparticles in the extracellular space. In addition, nanoparticles are often inadequate due to the lack of specificity of the drugs they deliver. A prerequisite for the effective use of nanoparticles in medicine is their nontoxicity, biocompatibility, and biodegradability. Therefore, the nanoparticles that are currently available have some limitations, such as the lack cell and molecular targeting and specificity, as well as high toxicity to the body.

In the present study, we aimed to develop nanoparticles to carry out cellular and molecular targeting, which had to be nontoxic, biocompatible, and biodegradable. As such, a technique was developed for obtaining nanoparticles with the size of ~100 nanometers for cellular targeting. The determined nanoparticle size is optimal for target delivery since these nanoparticles could penetrate the cells and are not excreted by the reticuloendothelial system via the bloodstream [25], which renders them viable options for cellular targeting.

To improve the targeted efficiency of the nanoparticles, we used the concept of active targeted delivery, which involves the biomolecular recognition of the molecules on the surface of cells for higher specificity. In this case, we proposed to decorate the nanoparticles with a biologically targeted molecule such as HA, which is the main component of the extracellular matrix in the

bone marrow and connective tissues [60]. This biopolymer regulates various cellular processes, including proliferation, differentiation, mobility, invasion, cell adhesion, and gene expression [60]. It is also known that HA increases the possibility of targeted delivery and accelerates intracellular delivery through endocytosis since all cells express endogenous receptors for this polymer (CD_{44} and RHAMM) [61, 62]. To date, the most common types of drug delivery systems based on HA have only involved the use of the methods of HA chemical association with a carrier *via* covalent bonds. Such examples of these nanocarriers are drug-HA complexes, HA covalently-modified liposomes, histidine, superparamagnetic iron oxide nanoparticles, nano-like clusters, peptide carriers linked via nucleotides, proteins, and peptides to drugs [20, 21, 63-76].

In the current research, the chitosan-HA nanoparticles were obtained by ion gelation. Unlike most nanoparticles with HA, our approach did not involve the formation of chemical covalent bonds and was based on the simple ionic interaction between HA as a negatively charged molecule and chitosan as a positively charged molecule.

For the first time, we encapsulated DNICs into nanoparticles, which were the donors of NO and effective inhibitors of myeloperoxidase [33, 77]. These compounds could specifically act on the molecular target within the cell and myeloperoxidase enzyme, which is a biomarker of CVDs [77]. As such, our nanoparticles were able to carry out molecular targeting, which increases the selectivity of the action of nanoparticles. In the present study, we encapsulated DNICs into the nanoparticles, which increased their stability, as well as the time and amount of the released NO, thereby improving the efficiency of these compounds. The nanoparticles were nontoxic and increased the viability of the cardiomyocytes. The undoubted advantage of our nanoparticles was that as a nanostructured material, we used a natural biopolymer (chitosan), which is biocompatible and biodegradable.

CONCLUSION

According to the results, the obtained nanoparticles were water-soluble (HA hydrophility), biocompatible and biodegradable (chitosan), bioprotective (negative charge of HA), and capable of releasing the drug inside the cell due to the cleavage of HA with hyaluronidases. The chitosan-HA nanoparticles were first obtained with the optimal size by ion gelation, and the DNICs were encapsulated into the nanoparticle composition, increasing the stability of these compounds. According to the findings, the encapsulation of DNICs into the nanoparticle composition prolonged and increased the generation of NO. It was also observed that the nanoparticle-DNIC complex was nontoxic and enhanced the viability of the cardiomyocytes. Therefore, the chitosan-HA nanoparticles could be used as potential cardiac drugs for the treatment of CVDs.

Such nanoparticles will undoubtedly be of great interest to the pharmaceutical industry as they are a combination of safe biomaterials, ideal for encapsulating cationic hydrophilic preparations, and capable of providing intracellular drug delivery, while they could also perform the required function to deliver medications with no side-effects in humans. Furthermore, they could be produced simply and easily on a large scale using nanotechnology and have high stability. However, it is recommended that further investigations be focused on the use of these nanoparticles in animal models.

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