Development of chitosan-hyaluronic acid nanoparticles and study of their physico-chemical properties for targeted delivery of anticancer drugs

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Abstract. Nanoparticles from chitosan and hyaluronic acid were obtained using ionotropic gelation technology. The size of the nanoparticles was investigated using electron microscopy and dynamic light scattering. Nanoparticles were obtained of an optimal size of ~ 100 nm. A physical association method has been developed of encapsulating nanoparticles with doxorubicin, a well-known antitumor drug, and dinitrosyl iron complex (donor of nitric oxide). The surface potential of nanoparticles was determined by dynamic light scattering. It was shown that HA-DOX: CS {6: 1} nanoparticles were stable and had a potential of -45.6 meV. The localization of nanoparticles in the cancer cells has been studied by confocal and FLIM microscopy. It was found that nanoparticles with doxorubicin are located in the cell near and inside the nucleus. It was shown that the encapsulation of DNIC in the composition of nanoparticles significantly increases the stability of DNIC, prolongs the formation and increases the yield of nitric oxide. A unique nano-system has been created for the delivery of anticancer drugs into the cell.

1. Introduction
Cancer is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018. Globally, about 1 in 6 deaths is due to cancer [1]. The relevance of developing nanoparticles for targeted delivery of anticancer drugs is determined by the high frequency of oncological diseases worldwide, the low survival rate of cancer patients and the expensive treatment of cancer. Currently, the main problem in oncology is the lack of specificity of the action of many antitumor drugs.

In this work, we used chitosan (CS) as a carrier in nanoparticles. Chitosan-aminosugar, a linear polysaccharide derivative whose macromolecules consist of randomly linked β-D-glucosamine units and N-acetyl-D-glucosamine [2]. Chitosan is a highly cationic, non-toxic, biocompatible and...
biodegradable compound [3]. Due to its cationic nature, chitosan is able to form insoluble complexes with the anionic polysaccharide-hyaluronic acid (hyaluronic acid, HA). Polysaccharide-coated nanoparticles are a new trend in drug delivery systems. Following this, we used a negatively charged polymer-HA as a delivery vector. HA is a non-sulfonated glucose aminoglycan that is part of the connective, epithelial and nervous tissues [4]. HA is a biocompatible, non-toxic and easily biodegradable molecule. HA can provide protection for the delivered drug and improve the solubility of hydrophobic drugs. In addition, since HA is a polyanionic polysaccharide, it has multiple charges for interaction with polycations [5]. HA is a physiological ligand of CD44 and RHAMM (receptor for hyaluronan-mediated mobility) receptors; therefore, it specifically interacts with CD44 and RHAMM receptors on the cell membrane, ensuring the efficiency of drug delivery to the cell [6]. HA-based nanoparticles are able to recognize CD44 and RHAMM-expressing cancer cells and, thus, specifically deliver particle-bound drugs through receptor-mediated endocytosis [7]. This cell targeting strategy allows you to deliver drugs directly to the cells, rather than concentrating in the kidneys, liver and then excreted from the body by the reticulo-endothelial system [8]. In addition, cellular enzymes (hyaluronidases) cleave HA inside the cell, thereby releasing the drug directly into the cell [9]. This strategy allows for specific and selective drug delivery.

The objective of this study was to develop nanoparticles based on chitosan-HA and to encapsulate doxorubicin (DOX) and dinitrosyl iron complex (DNIC) into nanoparticles to increase the stability and effectiveness of their action.

2. Materials and Methods

2.1. Materials
Commercial grade chitosan (M.w. = 100.000-300.000) manufactured by Acros organics (New Jersey, USA) was used. HA (sodium hyaluronate, low molecular weight <0.1 MDa) was obtained from My formula (Germany), HyClone Fetal Clone III Serum was bought from GE Healthcare (USA), doxorubizin (DOX) was purchased from Ebeve Pharma (Austria).

2.2. Fabrication of nanoparticles
Nanoparticles (CS-HA, HA-DOX: CS, HA-DNIC: CS) were obtained by ion gelation. A portion of chitosan (10 mg) was dissolved in 1 ml of HCl (0.01 M, pH = 2) and stirred for 12 hours. A portion of HA (40 mg) was dissolved in 1 ml of distilled water and stirred for 12 hours. Then, the obtained solutions of chitosan and HA were mixed in the different ratio, the pH of the solution was adjusted to pH = 6.8 with phosphate buffer (2 ml, 0.1 M, pH = 6.8) and stirred on a magnetic stirrer for 36 hours. Then the solution was dried by freeze drying and analyzed by electron microscopy. Chitosan and HA were also analyzed as control samples.

2.3. Analysis of nanoparticles by electron microscopy
The analysis of nanoparticles was carried out by electron microscopy. The samples of nanoparticles were prepared by vacuum deposition of carbon on an aluminum substrate. To apply carbon coating by an arc discharge method, a VUU-4 high-vacuum deposition device was used. Then, the nanoparticles were analyzed using a Supra-25 electron microscope (Zeiss, Germany), working distance WD = 2 mm, accelerating voltage EHT = 3.56 kV, magnification Mag = 100.02 KX, Signal detector A = InLens, vacuum in the system = 1.13e-0.006 mBar, vacuum in the cathode assembly = 8.49e-010 mbar.

2.4. Synthesis of mononuclear dinitrosyl iron complex with functional sulfur-containing ligands (DNIC)
Synthesis and identification of the DNIC compound was carried out according to the procedure [10]. As the tested compound used synthetic analog of mononuclear dinitrosyl iron complex with functional sulfur-containing ligands, namely with thiourea and its derivatives [Fe(SC(NH2)2)2(NO)2]ClO4Cl
(Compound #6). DNIC secretes nitric oxide (NO) when dissolved in water solvents due to dissociation [10].

2.5. Analysis of size and zeta potential nanoparticles by dynamic light scattering (DLS)
Particle size distribution was studied using a DLS technique (Nanopartica SZ-100; HORIBA Ltd, Kyoto, Japan). The scattered light from the particles present in the sample was collected either at 90 or 173 degrees, which was automatically selected by the instrument as the optimum scattering angle based on sample concentration. The zeta potential, which is an indicator of dispersion and stability of the prepared nanoparticles, was measured, according to method [11].

2.6. Cell culture
Cell culture of HeLa cells (human cervix epitheloid carcinoma) are maintained in tissue culture flasks T-75 at 37°C in 5% CO₂ atmosphere and culture with MEM cell medium supplemented with 10 % v/v HyClone Fetal Clone III Serum and 1 % v/v Penicillin/Streptomycin solution.

2.7. Analysis cells with confocal microscopy
The cells were imaged with an inverted laser scanning confocal microscope (LSCM) LSM780 (Zeiss, Germany), illuminated by a 488 nm laser and a fluorescence detection range from 550 nm to 700 nm. Fluorescence z-stack projections were obtained and analyzed with the ZEN2010 software [12].

2.8. Analysis of cells be Fluorescent Life Imaging Microscopy (FLIM)
FLIM was performed on an upright LSM 510 microscope (Carl Zeiss, Germany) with a 1.0 NA × 40 water-dipping objective using a 650-nm short-pass dichroic and 460±25 nm emission filter. Emission events were registered by an external detector attached to a commercial time-correlated single photon counting electronics module. Scanning was performed continuously for 4 min with a pixel dwell time of 1.6 μs.

2.9. Electrochemical determination of NO concentration, isolated from DNIC
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 NO generated from the DNIC. The NO concentration in the aqueous solution was fixed for ~ 500 seconds (in 0.2 second increments). A standard aqueous solution of NaNO₂ (100 μM) was used to calibrate the sensor electrode, to which was added a mixture of aqueous solutions of KI (0.12 M, 18 mL) and H₂SO₄ (1 M, 2 mL)[13].

3. Result and Discussion
Nanoparticles (CS-HA) were obtained using the method of physical association due to ion self-assembly of hyaluronic acid molecules (negatively charged) and chitosan molecules (positively charged) in nanogels. Nanoparticle’s size was determined by electron microscopy. The obtained nanoparticles were water soluble due to the hydrophilicity of HA and chitosan. It was shown that the particles have a spherical morphology and the size is approximately equal to 100 nm as shown on Figure 1.
Figure 1. Images of nanoparticles (chitosan-HA) obtained by electron microscopy.

The nanoparticles were homogeneous in size and shape.

Thus, for the implementation of cell targeting, we have developed a technique for producing nanoparticles with a size of ~ 100 nm. This nanoparticle size is optimal for targeted delivery, since nanoparticles of this size can penetrate into the cells and they will not be removed by the reticuloendothelial system from the bloodstream [8]. Therefore, these nanoparticles will carry out cell targeting.

Encapsulation of DOX in nanoparticles (HA-DOX: CS and HA-DNIC: CS) was performed by physical association. Using different ratios of chitosan and HA, a method was developed for producing nanoparticles of various sizes with encapsulated DOX. The size of the nanoparticles was determined by dynamic light scattering. The results showed that the nanoparticles have a size of 100 ~ 400 nm, the image of nanoparticles is shown on Figure 2. The optimal nanoparticle size (100 nm) was obtained with the ratio HA: CS = 6: 1.

Figure 2. Determination of the size of nanoparticles (HA-DOX: CS) by dynamic light scattering.

The surface potential of nanoparticles was determined by dynamic light scattering. The data presented in Table 1. It was shown that HA-DOX: CS {6: 1} nanoparticles have a potential of -45.6 mV, which indicates that the nanoparticles are negatively charged and stable.

Table 1. Surface zeta potential of nanoparticles.

|   | Nanoparticles (HA: CS {4: 1}) | CS-DOX : HA {4:1} | HA-DOX : CS {6:1} stirred 10 min |
|---|-------------------------------|-------------------|---------------------------------|
| 1 | -1.74 mV                      | -2.3 mV           | -6.16 mV                        |
HA-DOX : CS {12:1} stirred 30 min -7.06 mV

HA-DOX : CS {12:1} stirred 30 мин + ultrasound -8.1 mV

After washing and ultrasound treatment

Nanoparticles (HA:CS {4:1}) -47.22 mV

HA-DOX:CS {6:1} stirred 10 min -45.6 mV

HA-DOX:CS {12:1} stirred 30 min -39.4 mV

To improve the targeted efficiency of nanoparticles, we used the concept of active targeted delivery, which consists in using bio-molecular recognition of molecules on the surface of cells, which leads to a higher degree of specificity. In this case, we decorated nanoparticles with a biologically targeted molecule. As such a molecule, we used HA, which is the main component of the extra cellular matrix in the bone marrow, connective tissues [14]. This biopolymer regulates various cellular processes, including proliferation, differentiation, motility, invasiveness, cell adhesion, and gene expression in cancer cells [15]. It is HA that increases the possibility of targeted delivery and accelerates intracellular delivery through endocytosis, since all cells express endogenous receptors for this polymer (CD44 and RHAMM receptors) [15, 16]. The undoubted advantage of our nanoparticles is that we use natural biopolymer chitosan, which is a biocompatible and biodegradable material, as a nanostructured material [5].

The penetration of nanoparticles (HA-DOX: CS) into HeLa cancer cells was studied by confocal and FLIM microscopy using DOX, which has the property of auto-fluorescence. As can be seen from Figure 3, nanoparticles (HA-DOX: CS) are localized in the cytoplasm of HeLa cells (yellow fluorescence).

![Confocal images of HeLa cells after incubation with nanoparticles (HA-DOX: CS) for 24 hours.](image)

**Figure 3.** Confocal images of HeLa cells after incubation with nanoparticles (HA-DOX: CS) for 24 hours.

The results of the analysis of the localization of nanoparticles in living cells using FLIM microscopy showed that free DOX accumulates in the nucleus of HeLa cells after 24 hours of incubation (Figure 4, bottom panel). It was found that nanoparticles (HA-DOX: CS) after incubation with HeLa cells for 24 hours were localized around and inside the cell nucleus (Figure 4, upper panel).
Both methods confirmed the passage of nanoparticles through the cytoplasmic membrane of cell and showed the localization of nanoparticles in the cytoplasm, around and inside the cell nucleus after 24 hours of incubation.

At present time, in oncology, for the treatment of cancer deceases are used drugs that are donors of nitric oxide [17]. However, the main problem of these drugs is that these drugs are often unstable, nonspecific and have a number of side effects. Therefore, the development of nanoparticles for stabilization and targeted delivery of DNICs drugs is currently relevant.

The encapsulation of DNIC6 in nanoparticles was carried out by ion gelatinization. Then we investigated the release of NO from DNIC (compound #6), in aqueous solution and in the composition of nanoparticles (Figure 5).

It was found that upon dissolution of DNIC in an aqueous solution, a sharp decrease in generation NO is observed, and after 100 seconds the level of generated NO drops to almost zero (Figure 5, curve 2). This indicated that the DNIC complex quickly decomposes in water and ceases to emit NO. Next, we analyzed the release of NO from the nanoparticle-DNIC complex (compound #6). It was shown that after adding DNIC to the nanoparticles, generation of 13 nmol NO was observed, and this level was maintained for a long time (> 500 seconds) (Figure 5, curve 3 and 4). Thus, the results showed
that the encapsulation of DNIC in the composition of nanoparticles stabilizes these compounds and also prolongs and increases the amount of NO formed. Therefore, we first encapsulated DNIC in nanoparticles and increased the efficiency of the DNIC’s action.

4. Conclusions
Thus, a universal nano-drug delivery system using nanoparticles (HA-DOX: CS) has been developed for target delivery anti-cancer drugs. The nanoparticles obtained by us are water-soluble, biocompatible and biodegradable, bio-permeable, capable of releasing the drug inside the cell due to the cleavage of HA with hyaluronidases. Such nanoparticles will undoubtedly be of interest to the pharmaceutical industry, since they are a combination of safe biomaterials, ideal for encapsulating cationic hydrophilic drugs, are able to provide intracellular drug delivery, can fulfill the necessary function of drug delivery for a long time without causing harmful effects to the human body, made simple and easy on a large scale nanotechnology, and finally are stable.

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