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ZINC-MODIFIED NANOTRANSPORTER OF DOXORUBICINE FOR MULTI-TARGETED THERAPY OF PROSTATE CANCER CELLS

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Abstract

Target therapy for oncologic diseases presents a big challenge for advance nanomedicine. In our work, we focused on multi-target approach development. Designed nanotransporter is based on polysaccharide chitosan which allows formation of nanoparticles. These nanoparticles can bind metal ions, mainly zinc (moreover, zinc stabilizes chitosan structure). The estimated zinc concentration was approximately 1 nmol/g of chitosan. In addition, chitosan nanoparticle (cage) irreversibly binds therapeutics which could be applied for targeted therapy of malignant tumours. Designed chitosan structure (LMQ, 10 g) encapsulation efficiency for doxorubicin was 50%. The pH change (tested interval 5 - 8) caused 20% release of doxorubicin from the nanocage. The nanotransporter is orientated to cancer tissue due the fact that the malignant cells highly express metallothionein (MT). The increased affinity of MT to zinc ions causes that the nanotransporter is preferentially bound to tumour regions with a high MT concentration. Our latest experimental results showed the changes in amino acid metabolism of prostate cancer signalized by increase in the amount of amino acid sarcosine. Therefore, the chitosan-based nanotransporter was modified by anti-sarcosine antibody. The functionality of designed nanotransporter was proved by ELISA with double detection of doxorubicin using fluorescence and by peroxidase activity of ABTS substrate. In another system, magnetic separation and identification of individual components of the nanotransporter were used. The sarcosine binding activity was estimated around 50%.

Keywords: Antracycline antibiotics, chitosan, prostate cancer, metallothionein, nanoparticles, nanomedicine

1. INTRODUCTION

Nowadays, polymers are greatly used for biodegradable drug carrier due to its ability to gradual release of the therapeutic compound. Among natural polymers belongs chitosan gained from chitin. Chitosan is a cationic polymer and it has three reaction centers. The primary amino group tend to quaternization and it can bind metal ions. Primary hydroxy group is often substituted by “spacers” which leads to binding of drug active groups or targeting labels. Secondary hydroxy group could be modified for improving the chitosan solubility [1]. The chitosan oligomers can be partially attributed to the ionic cross-linking using polyanions resulting in nanoparticle formation [2]. Chitosan nanoparticles have attracted more attention in drug delivery due to their stability, low toxicity and easy preparation. Deacetylated chitosan skeleton composed of glucosamine units has a high density of charged amino groups which allow strong electrostatic interactions with biomolecules [3].

Several authors described doxorubicin encapsulation to the polymeric cage formed by chitosan. Doxorubicin is widely used anthracycline antibiotic for the cancer treatment. Although, doxorubicin shows serious side

effects such as cardiotoxicity [4]. The development of new chitosan-based nanotransporters is aimed at reducing the cardiotoxicity of anthracycline antibiotics. Raja et al. published the pH-sensitive, self-assembled nanoparticles formed by N-acetyl histidine and arginine-grafted chitosan for doxorubicine delivery. Prepared nanocarriers shows good results in resistant human breast tumor cell line (MCF-7) efficiently in a dose-and time-dependent pattern [5]. Collagen peptide functionalized chitosan nanoparticles as a smart drug delivery carrier in advanced cancer therapy was presented by Anandhakumar et al. Observed nanoparticles showed stability under physiological conditions, high encapsulation efficiency towards doxorubicin hydrochloride as well as pH controlled release. Moreover, the excellent anti-proliferative characteristics against HeLa cells with favorable biocompatibility against normal cells have been proven [6]. Further modifications are described across the literature. We would like to highlight the myristate [7], Dextran-sulfate [8], folate [9], PEGylated liposome [10], glycyrrhetinic acid-modified chitosan-cystamine-poly(epsilon-caprolactone) copolymer [11], and stearic acid [12]. Summarily, the chitosan structure allows various modifications and functionalizations aimed to be a nanocarrier useful with unique properties beneficial in the cancer treatment.

In our previous study we described DOX enclosed in chitosan nanoparticles functionalized by Zn^{2+} ions [13]. We observed the affinity of nanotransporter to metallohionein, which is overexpressed in the tumor tissue and plays an important role in breast cancer. In the further work, we want to focus on multi-targeted therapy provided by functionalized chitosan nanoparticles (CS NPs) by anti-sarcosine antibodies. For CS NPs stabilization we investigated the influence of zinc ions (**Figure 1**).

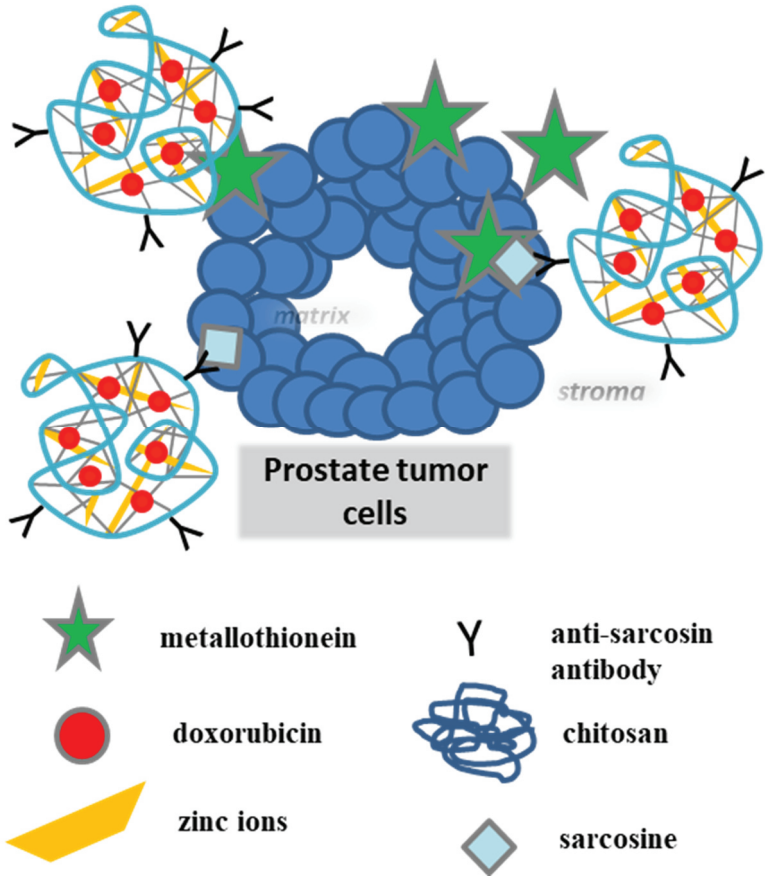

Figure 1 The structure of chitosan-based nanotransporter in multi-target therapy in the prostate cancer treatment. Prostate tumor cells highly express amino acid sarcosine. Via anti-sarcosine antibodies is CS NPs nanotransporter targeted to the tumor cells. Nanocarrier decorated by zinc ions facilitate enhance retention permeability effect of CS NPs in the blood stream

2. MATERIAL AND METHODS

Chemicals

Low molecular weight chitosan, sodium tripolyphosphate penta basic (TPP), DMSO, sodium acetate, acetic acid, doxorubicin HCl, zinc chloride and other chemicals unless noted otherwise were purchased from Sigma-Aldrich (USA). Aqueous solutions analysis were prepared using Milli Q water from Millipore Systems (USA) with conductivity less than 0.2 mS/cm.

Preparation of chitosan nanoparticles

In the case of Zn inner type of CS NPs, 12.5 mg of low MW chitosan was dissolved in 5 mL of 1 % acetic acid. Doxorubicin (30 μM) and Zinc (0.5 m/mL) was added to the chitosan solution under stirring (3 hours, 24 °C). After chitosan dissolution the sodium tripolyphosphate (0.25 % w/v) was added dropwise and incubated in rotator Multi-Rotator RS-60 BIOSAN. Zn outer type NPs was prepared in the same way, however the Zinc (0.5 mg/mL) was added after TPP gelatation. The mixture was stirred 3 hours, 24 °C.

Ninyhdrin assay

The reaction solutions were then processed as described in articles [14, 15]. The ninhydrin reagent was freshly prepared on the day of the assay by adding 25 ml of 4 M acetate buffer (pH 5.2) to 2 g ninhydrin and 0.3 g hydrindantin in 75 ml DMSO. For the assay, 75 μL of reagent was added to 100 μL of the sample in microtube. The microtubes were immediately capped, briefly shaken by hand and heated thermoblock for 30 min to allow the reaction to proceed. After cooling, 15 ml of a 50:50 ethanol:water mixture was added to each sample. The color intensity of the complex was spectrophotometrically evaluated as a measurement of depolymerized chitosan activity. Accurately weighed depolymerized chitosan was dissolved in 1% w/v acetic acid. A blank solution was also prepared in an identical manner, wherein 1% w/v acetic acid was used instead of a chitosan solution to prepare the reaction mixture. The effect of the solvent system was nullified by calibrating the instrument to 100% transmittance of the blank solution.

Dot blot assay

On the nitrocelulose membrane 0.45 μm pore diameter (SERVA, Heidelberg, Germany) was pippeted 1-2 μl of Rabbit AntiChicken antibody. After drying, the membrane was blocked with 1% BSA in PBS buffer. After 30 min incubation in the rotator the membrane was soaked in the CSNPs (AntiSar/CS NPS/Dox-AuMNPs) solution for 1 hour. The incubation was carried out in the 50 ml tube on rotator (15 rpm). The membrane was 3x washed using PBS-T. Further, the membrane was soaked in AuNPs labeled antibody or sarcosine for 1 hour. Each sample was washed from unbounded parts by PBS-T buffer. After dying the membrane, the samples were prepared for evaluation.

3. RESULTS

Characterization of CS NPs

For basic characterization of the formed nanoparticles spectrophotometric analysis based on the reaction of CS with ninhydrin solution have been used which enabled to react with primary and secondary amino groups. The chromophore is created by amine condensation with a molecule of ninhydrin to give Ruhemann purple. Firstly, the effects of several factors including the reaction temperature, reaction time and the ninhydrin concentration on the chitosan-ninhydrin reaction were investigated to optimize the specificity and sensitivity. **Figure 2 A** shows the dependence of absorbance on reaction temperature 70 - 100 °C, where the increase of chromophore absorbance in correlation with higher temperature in absorbance maxima is obvious. The 100 °C of reaction temperature was used for testing the reaction time (0 - 60 min). **Figure 2B** shows increasing trend of signal in correlation with the increasing ninhydrin amount up to the concentration of 32 μg per reaction.

Higher concentration caused slightly decrease in the signal. In the graph (**Figure 2C**) is clearly shown that the reaction time up to 40 min lead to the highest absorbance which were also stable after 50 and 60 min. The dependence of ninhydrin amount entering the reaction with CS was investigated. The dependence of absorbance on CS concentration (0-0.5 mg/mL) is shown in **Figure 2D**. Calculated limit of detection was 1.7 µg/mL.

We used ninhydrin assay to monitor of chitosan properties in the presence of TPP and zinc. As we described in the introduction, TPP as well as zinc ions stabilize chitosan crosslinked structure and provide nanoparticle formation. We studied the dependence of various CS concentrations 0.06 - 2.00 mg/mL in the presence of 2 mg/mL TPP. The low CS concentration in the presence of TPP showed similar signal intensity as CS alone. Up to the CS concentration of 0.5 mg/mL it was evident that the signal intensity of TPP was lower than CS alone. The CS NPs were well formed in the concentration of 2 mg/mL of CS and the higher TPP concentration caused its stabilization. The apparent signal intensity of CS NPs decreases in the dependence of higher TPP concentration that results in more crosslinked structure.

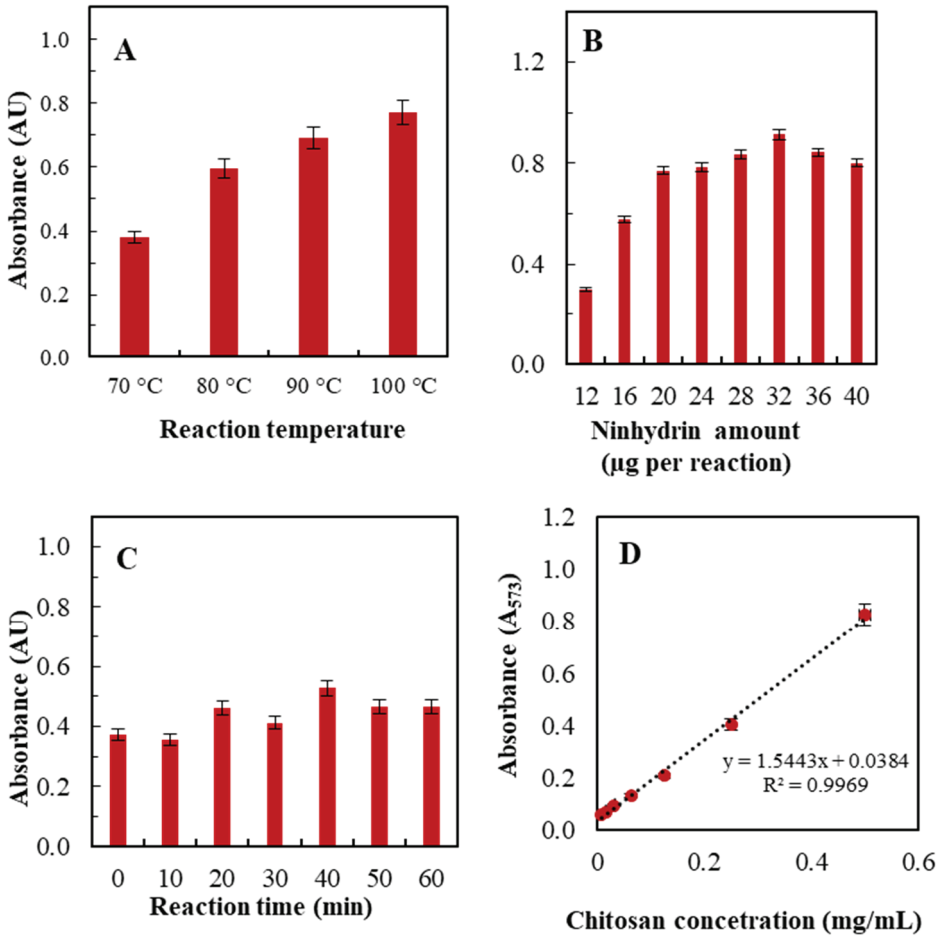

Figure 2 Optimization the ninhydrin assay: **A**) reaction temperature (70-100 °C), **B**) ninhydrin amount per reaction (12-40 µg). **C**) reaction time (0-60 min). **D)** Dependence of CS concentration (0-0.6 mg/mL) on absorbance

Doxorubicin release in pH range (5-8)

In our experiment, we studied the stability of CS NPs and doxorubicin release in the intervals from several hours to several days. For 28 hours CS NPs were subjected to doxorubicin (DOX) fluorescence sensing and the cumulative release of DOX amount were calculated in selected environmental conditions: blood, phosphate

buffer (1 mM) pH 5, 7 and 8. Results clearly showed the rapid DOX release which began immediately and ended after 120 min. Further DOX release was not so intensive and the NPs were stable. Apparent differences between each environment showed the highest stability of CS NPs at pH 5. The strongest cumulative release of DOX amount occured in the blood environment. Not surprisingly, blood is complex matrix and the presence of enzymes, proteins and specific components caused the rapid DOX release from CS nanostructure.

AntiSar antibody - CS NPs conjugation

Our goal was to design a nanocarrier which can target into the cancer tissue with highly expressed levels of sarcosine, such as prostate cancer. We utilized non-specific conjugation of chicken AntiSar antibody to CS NPs surface. This interaction is mediated by electrostatic and hydrophobic interactions between positively charged CS and proteins. For the verification of this interaction, we employed the dot blot analysis as a rapid and precise immunochemical method. We utilized the ability of AuNPs aggregation which was visible as a purple dot on the membrane. After initiation of primary antibodies, the membrane surface was blocked by bovine serum albumin (BSA) to avoid false positive results. The dot blot analysis showed positive signal in both cases; control sample (labeled AntiSar antibodies) and the CS-AntiSar NPs. From this result, we assume that the proposed system is applicable and we can proceed with chitosan testing itself. The chicken AntiSar antibodies were attached on the surface of CS NPs. The formation of this complex was confirmed by the interaction with AuNPs labeled sarcosine (AuSar). The stronger interaction was occurred when primary rabbit AntiChicken antibodies was immobilized on the membrane. We continued with the sandwich method and the CS NPs-AntiSar labelled with rabbit AntiChicken-AuNPs showed the strong positive reaction. This effect is due to a stronger binding of the first antibody to the second antibody.

4. CONCLUSION

A detailed interaction study of chitosan nanotransporter, zinc ions and TPP has been performed. The Zn^{2+} concentration shows the strong effect on crosslinking and coiling of CS NPs with the result of better nanoparticle stabilization. The entrapment efficiency for doxorubicin of designed CS NPs was 20%. The highest drug release from the CS nanocarrier appeared after 120 min at the pH 5, 6, 8 and in blood environment. Functionality and ability of the AntiSar/CS NPs/DOX binding to the sarcosine molecule was proved by dot blot analysis.

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