Gold-Mediated Modification of Apoferritin Surface with Targeting Antibodies

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Abstract—To ensure targeting of apoferritin nanocarrier with encapsulated doxorubicin drug, we used a peptide linker based on a protein G with N-terminus affinity towards Fc region of antibodies. To connect the peptide to the surface of apoferritin, the C-terminus of peptide was made of cysteine with affinity to gold. The surface of apoferritin with encapsulated doxorubicin (APODOX) was coated either with gold nanoparticles (APODOX-Nano) or gold(III) chloride hydrate reduced with sodium borohydride (APODOX-HAu). The reduction with sodium borohydride caused a loss of doxorubicin fluorescent properties and probably accompanied with the loss of its biological activity. Fluorescent properties of APODOX-Nano were similar to the unmodified APODOX; therefore it was more suited for the intended use. To evaluate the specificity of apoferritin modified with antibodies, ELISA-like method was used with the surface of microtitration plate wells coated by the antigen (goat anti-human IgG antibodies). To these wells, the nanocarrier was applied. APODOX without the modification showed 5× lower affinity to the antigen than APODOX-Nano modified gold and targeting antibodies (human IgG antibodies).

Keywords—Antibody targeting, apoferritin, doxorubicin, nanocarrier.

I. INTRODUCTION

In the past, the development of new and improved drugs was solely based on their efficiency against the disease with almost no regard to the negative side effects to the rest of patient’s body. Nowadays, the attention is paid towards the elimination of these negative side effects as much as possible [1]. Nanocarriers can very conveniently be applied to just this outcome. They can be made of various materials, including metals, carbon or organic materials such as proteins [2].

The suitable nanocarrier needs to be small to utilize the Enhanced Permeability and Retention (EPR) effect (i.e. ≤100 nm), but large enough so it is not removed from body by renal clearance [3]. It should also be produced easily, encapsulate large quantities of drug and have reliable mechanism of drug release [4]. The size and shape of nanocarrier particles should be uniform and it should enable the surface modification with targeting moieties [5].

Inorganic nanocarriers are usually easier to produce; however, they are often immunogenic or even toxic to human cells. Therefore, nanomedicine research often focuses on natural nanocarriers that can be found in human body [6].

One of these natural nanocarriers is the protein ferritin, or rather its hollow shell apoferritin [7]. Apoferritin seems to be a very promising structure for use as a nanocarrier. The intracellular ferritin protein can be naturally found in most organisms. The role of ferritin proteins is to store and transport ferrous ions which can be toxic in higher doses [8]. The apoferritin protein shell is a hollow cage without ferrous ions that can be prepared from ferritin by reduction with thioglycolic acid or dithionite [7].

The structure of apoferritin is composed of 24 protein subunits [9], creating a roughly spherical (icosahedral) cage with 12 nm in diameter. The inner cavity has a diameter of 8 nm [10]. The drug encapsulation process is based on the response of apoferritin structure to the pH changes of surrounding solution. In low pH, apoferritin is disassembled into individual subunits and its structure is “opened”. It can then be mixed with any desired cytotoxic drug and after adjustment of pH back to neutral the subunits are reconnected again and the drug is encapsulated within the apoferritin particles [11]. Excess drug molecules can be removed by dialysis or filtration.

II. MATERIALS AND METHODS

A. Chemicals

All chemicals of ACS purity were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise. Doxorubicin hydrochloride was obtained in HPLC purity from Sigma-Aldrich (St. Louis, MO, USA).

The composition of buffer for 6% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was as follows: 6 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 40 mM imidazole pH 7.4. The proteins separated on gel were visualized using rapid coomassie blue staining according to [12].

B. Encapsulation of Doxorubicin in Apoferritin

200 µl of 1 mg/ml doxorubicin (DOX) was added to 20 µl of 50 mg/ml horse spleen apoferritin (APO) and 100 µl of water. 2.5 µl of 1 M hydrochloric acid was added to decrease the solution pH and disassociate the APO. The solution was mixed for 15 minutes. 2.5 µl of 1 M sodium hydroxide was added to increase the pH and encapsulate the DOX inside APO (creating APODOX). The mixture was kept at 20°C for 15 minutes and then rinsed twice with water using Amicon®...
Ulta - 0.5 ml 3K (Merck Millipore, Billerica, MA, USA). Absorbance and emission spectra of APODOX were subsequently measured using Tecan Infinite 200 PRO (Tecan, Männendorf, Switzerland).

C. Modification of APODOX Surface with Gold

The surface of APODOX was modified with either gold nanoparticles or gold (III) chloride hydrate. To APODOX, 25 µl of 1 mM solution of AuNP or 200 µl of 1 mM HAuCl4 (followed with 3 mg of NaBH4 and observed hydrogen evolution) was added and the mixture was shaken on Orbital Shaker (Biosan, Riga, Latvia) at 20°C for 12 h. The resulting product was rinsed six times with water on Amicon® Ultra - 0.5 ml 3K (Merck Millipore, Billerica, MA, USA). Absorbance and emission spectra of resulting products were measured.

D. Gel Electrophoresis and Gold Determination

APODOX modified with gold nanoparticles and gold(III) chloride hydrate were run on 6% non-denaturing PAGE using a continuous buffer system. Non-denaturing PAGE gels were prepared and run using 60 mM HEPES with 40 mM imidazole pH 7.4 buffer systems as described by [13]. The samples were loaded with a loading buffer (5× buffer; 50% glycerol) and gels were run with the appropriate buffer at 10 mA for 2 h. In order to avoid overheating the gels, the electrophoretic apparatus was kept at 6°C. The DOX was visualized at 312 nm and the gels were stained with coomassie blue.

To determine gold in the bands of apoferritin, the bands were cut out, mineralized; filled up to 10 ml by ultrapure water and analyzed by the means of quadrupole inductively coupled plasma mass spectrometer (ICP-MS) Agilent 7500 CE (Agilent, Japan) which is equipped with a collision-reaction cell (CRC) for suppressing polyatomic interferences. Optimization of ICP-MS parameters was performed with respect to the maximum S/N ration of signal of 197Au isotope. The applied amount of gold in the form of gold(III) chloride hydrate was 10 times higher than in the case of gold nanoparticles. However, after removal of the excess unbound gold, the peptide linker was used, based on a protein G with N-terminus affinity towards Fc region of antibodies [15]. To connect the peptide to the surface of apoferritin, the C-terminus of peptide was made of cysteine with affinity to gold [16]. The surface of apoferritin with encapsulated doxorubicin (APODOX) was coated either with gold nanoparticles (APODOX-Nano) or gold(III) chloride hydrate reduced with sodium borohydride (APODOX-HAu). To this gold, the peptide linker was attached by its cysteine end and on the other end of peptide linker, the human IgG antibody was attached. The schematic visualization of created nanocarrier is shown in Fig. 1.

The applied amount of gold in the form of gold(III) chloride hydrate was 10 times higher than in the case of gold nanoparticles. However, after removal of the excess unbound ions by electrophoretic separation (Fig. 2 (a)), the concentration of gold on the surface of apoferritin was only 6 times higher for APODOX-HAu in comparison with APODOX-Nano (Fig. 2 (b)). Moreover, the influence of gold on the spectrophotometric properties of the nanocarrier was studied by measurement of absorbance and fluorescence spectra of nanocarriers with and without the gold modification (Fig. 3). The reduction with sodium borohydride caused a loss of typical doxorubicin absorption maxima at 480 nm (Fig. 3 (a), green line) as well as its fluorescent properties (excitation maximum at 480 nm with emission maximum at 600 nm, Fig. 3 (b), green line) and probably also its biological activity. Absorbance (Fig. 3 (a), blue line) and fluorescent properties of APODOX-Nano (Fig. 3 (b), blue line) were similar to the
unmodified APODOX; therefore it was chosen as more suited for the intended use.

Fig. 1 Schematic illustration of the created nanocarrier. The anthracycline drug doxorubicin was encapsulated in apoferritin protein cage. The surface of apoferritin was coated with gold. To this gold, a peptide linker was attached, with cysteine on C-terminus and N-terminus derived from Protein G with affinity to Fc region of various antibodies.

Fig. 2 The determination of gold in apoferritin after the removal of excess gold by electrophoretic separation: (a) The apoferritin bands on non-denaturing 6% HEPES-Imidazole PAGE gel after staining with coomassie blue. (b) The measured concentration of gold in cut and mineralized apoferritin bands. The measurement was performed using the ICP-MS with RF power 1500 W; plasma gas (Ar) 15 l/min; sheath gas (Ar) 0.84 l/min; carrier gas (Ar) 0.21 l/min and He in CRC 2.5 ml/min.

To evaluate the specificity of apoferritin modified with antibodies, we used ELISA-like method with the surface of microtiteration plate wells coated by the antigen (goat anti-human IgG antibodies). APODOX without gold modification and APODOX modified with gold nanoparticles were both incubated with the linker peptide and targeting human IgG antibody and applied to antigen coated wells. The fluorescence of bound nanocarrier at the excitation of 480 nm and emission of 600 nm was measured after incubation and rinsing of unbound nanocarrier molecules.
and targeting antibodies and APODOX-Nano modified with targeting antibodies (human IgG antibodies). The amount of unmodified APODOX on antigen after incubation and subsequent rinsing with water was 5 times lower than in the case of APODOX-Nano modified with targeting antibodies. It can therefore be concluded that the demonstrated procedure allows us to create nanocarrier with enhanced targeting properties, suitable for nanomedicine.

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REFERENCES


