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Design and characterization of crotamine-functionalized gold nanoparticles

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ABSTRACT

This paper describes the development of a facile and environmentally friendly strategy for supporting crotamine on gold nanoparticles (GNPs). Our approach was based on the covalent binding interaction between the cell penetrating peptide crotamine, which is a snake venom polypeptide with preference to penetrate dividing cells, and a polyethylene glycol (PEG) ligand, which is a non-toxic, water-soluble and easily obtainable commercial polymer. Crotamine was derivatized with ortho-pyridyldisulfide-polyethyleneglycol-*N*-hydroxysuccinimide (OPSS-PEG-SVA) cross-linker to produce OPSS-PEG-crotamine as the surface modifier of GNP. OPSS-PEG-SVA can serve not only as a surface modifier, but also as a stabilizing agent for GNPs. The successful PEGylation of the nanoparticles was demonstrated using different physicochemical techniques, while the grafting densities of the PEG ligands and crotamine on the surface of the nanoparticles were estimated using a combination of electron microscopy and mass spectrometry analysis. In vitro assays confirmed the internalization of these GNPs, into living HeLa cells. The results described herein suggest that our approach may serve as a simple platform for the synthesis of GNPs decorated with crotamine with well-defined morphologies and uniform dispersion, opening new roads for crotamine biomedical applications.

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1. Introduction

Crotamine is one of the main components of the venom of the South American rattlesnake, *Crotalus durissus terrificus*, corresponding to about 12–25% of the dry weight of the crude snake venom, which could potentially be influenced by the genetic background [1]. In addition to its potential as a structural model for the development of new drugs, this positively charged small polypeptide is also a promising tool for a diverse range of biotechnological and biomedical applications [2]. The most prominent amongst these potential applications in clinics is the ability of crotamine to specifically target actively proliferating cells, such as tumor cells, as previously demonstrated by us both in vitro and in vivo [3,4]. We have also demonstrated that crotamine can act on intracellular compartments, such as lysosomes and nuclei, in addition

Abbreviations: PEG, polyethylene glycol; NPs, nanoparticles; GNPs, gold nanoparticles; OPSS, ortho-pyridyldisulfide; OPSS-PEG-SVA, ortho-pyridyldisulfide-polyethyleneglycol-succinimidylvalerate; DLS, dynamic light scattering; TEM, transmission electron microscopy; SERS, surface-enhanced Raman spectroscopy.

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to forming complexes with DNA molecules without effect on its ability to be internalized selectively by dividing cells [5–7]. This demonstrates its potential as a vector for transporting (therapeutic) molecules into cancer cells [2]. However, up to now, only the transport of nucleic acid molecules has been experimentally proven [1,5,6]. Together with its well-characterized cytotoxic and antitumoral activity [5,6,8], crotamine clearly is a promising theranostic compound [4].

Nanoparticles (NPs) are small particles (mainly between 1 and 100 nanometers in size) with the ability to transport other molecules as a single unit. NPs are playing a progressively more significant role in multimodal and multifunctional molecular imaging to detect diseases, such as early stage cancer [9]. Gold nanoparticles (GNPs), in particular, present outstanding properties such as very high surface-to-volume ratio, easy functionalization of surface, high stability, size flexibility and biocompatibility [10–13]. GNPs have already shown great potential as platforms for therapeutic agents, targeting moieties and contrast agents that are to be introduced into the animal body [14–16].

With a view towards improving the potential of crotamine use as a theranostic agent, the linkage of crotamine to GNPs and the determination of the biological effects of crotamine-functionalized nanoparticles are clearly of great interest. In this report, we describe the production of GNPs with surface-immobilized crotamine. In order to avoid the denaturation of the protein caused by direct adsorption onto the NP surface [17], crotamine was first conjugated via lysine ε-amino groups to a polyethylene glycol (PEG) ligand containing an ortho-pyridyldisulfide (OPSS) group at one end and an activated carboxylate group at the other end [18]. The PEG linker may provide a more stable coating due to the strong Au-S bond [19], and also may increase the bioavailability of the GNPs [20]. The crotamine-PEG-GNPs were characterized by various physicochemical methods, and, as does the native protein, have the property of entering mammalian cells.

2. Materials and methods

2.1. Native crotamine purification from snake venom

The crude venom was extracted from *Crotalus durissus terrificus* snakes kept in the serpentarium of the Faculdade de Medicina de Ribeirão Preto (FMRP), São Paulo University – Ribeirão Preto (USP-RP; authorization of access to genetic resources No. 010426/2010 COAPG/DABS/CNPq, term of concession No. 20100104268). Crotamine was prepared and purified according to the procedure described by Hayashi et al. [1].

2.2. Synthesis of gold nanoparticles (GNPs)

The synthesis procedure used was a modified version of the well-known and frequently used method, reported previously by Turkevich et al. [21] and Frens et al. [22], and which involves the reduction of hydrogen tetrachloroaurate(III) ($HAuCl_4$, EMS catalog 16580) by sodium citrate to produce Au nanoparticles (*i.e.*, GNPs) in boiling water. Typically, 706 μL of 0.17 M sodium citrate was added to 100 mL of 3×10^{-4} M $HAuCl_4$ (1:4 mol ratio of $HAuCl_4$ to citrate) dissolved in water under reflux, and the solution was kept under strong stirring and under reflux for 20 min. This procedure yielded particles with a diameter of about 14.6 nm by number (16.8 nm by volume and 20.1 nm by intensity), with a polydispersity index (PDI) of 0.038, as determined by dynamic light scattering (DLS). The nanoparticle concentration was determined spectrophotometrically using an extinction coefficient of 1.76×10^8 M^{-1} cm^{-1} at 450 nm (based on its experimentally-determined diameter) [23].

2.3. Synthesis of gold-polyethyleneglycol-crotamine nanoparticles

Crotamine was conjugated to the GNP via a polyethyleneglycol (PEG) linker employing the procedure described by Chattopadhyay et al. [24] (Fig. 1). In this method, the ε-amino groups of lysine residues are initially reacted with the *N*-hydroxysuccinimide end of a modified PEG, ortho-pyridyldisulfide-polyethyleneglycol-succinimidylvalerate (OPSS-PEG-SVA, Laysan Bio, Inc., Arab, AL, USA). The disulfide end of the linker is subsequently reacted with the GNPs. Solid crotamine was dissolved in PBS buffer to a concentration of 0.45 mg/mL, and reacted with varying concentrations of the OPSS-PEG-SVA linker (molecular weight ~3600 Da), dissolved in 0.1 M $NaHCO_3$. The mixture was stirred for 3 h at room temperature. Unreacted OPSS-PEG-SVA was separated from the PEG-crotamine adduct by centrifugation in a Corning Spin-X UF500 concentrator, 5 kDa molecular weight cut-off (three times at 15,000*g* for 8 min at 10 °C). The concentration of adduct was determined spectrophotometrically at 280 nm, using the extinction coefficient of the protein ($\varepsilon_{280} = 1.23 \times 10^4$ M^{-1} cm^{-1}) [7]. The PEG-crotamine adduct (0.53 mL) was then mixed with 2 mL of the GNPs (500 mol PEG-crotamine/mol GNP) for 2 h at room temperature with rapid stirring. The PEG-crotamine-GNPs were finally purified by centrifugation (14,000*g*, 25 min, 4 °C) to remove the excess PEG-crotamine in solution.

2.4. Characterization of conjugated nanoparticles

The concentration of the conjugated GNPs was determined spectrophotometrically at 450 nm, as described above. DLS measurements on PEG-crotamine intermediates and PEG-crotamine-GNPs were performed in a Zetasizer Nano ZS series instrument (Malvern Instruments, Worcestershire, UK) using the software supplied by the manufacturer. MALDI mass spectrometry measurements were performed on a Microflex LT™ (BrukerDaltonics, Bremen, Germany) instrument using the software FlexControl™ version 3.4 (BrukerDaltonics). Briefly, 1.2 μL of the supernatant was spotted on each well of the steel target plate and was air dried and overlaid with 1.2 μL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid in organic solvent [50% acetonitrile and 2.5% trifluoroacetic acid]; Sigma-Aldrich, St. Louis, MO, USA) before mass spectra were generated.

2.5. Spectrophotometric titrations

Stock solutions of crotamine, azure A chloride (Sigma), and heparin (sodium salt from porcine intestinal mucosa, Sigma Grade 1-A) were prepared in 5 mM HEPES, pH 7.7. The azure A was diluted in 130 μL of this buffer to an initial A_{632} of 1.00, in the presence or absence of crotamine. Small (typically 3 μL) aliquots of the heparin stock were added, the A_{632} was read in a Cary 50 spectrophotometer, and then adjusted for the volume dilution. For calculation of $[heparin]_{res}$:[azure A], the heparin monomer molecular weight is 312 g/mol [25], and the ε_{620} for azure A is 2.2×10^4 M^{-1} cm^{-1} [26].

2.6. Transmission electron microscopy (TEM)

GNPs-PEG and GNPs-PEG-crotamine were placed on an ultrathin carbon with lacey support on 400 mesh copper grids (TedPella, Redding, CA, USA). Grids were submitted to glow discharge in an easiGlow (Pelco, Clovis, CA, USA), with the following parameters: 15 mA current; negative charge; 25 s time. Then, 3 μL of the samples were dropped onto the grid, followed by 60 s rest, blotting and air-drying. TEM analyses were carried out on a JEOL JEM-1400Plus (JEOL, Tokyo, Japan), operating at 120 kV with a lanthanum hexaboride (LaB_6) filament, using a CCD camera GatanMultiScan 794

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