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Targeted cancer theranostics using alpha-tocopheryl succinate-conjugated multifunctional dendrimer-entrapped gold nanoparticles

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ABSTRACT

Development of multifunctional theranostic nanoplatforms for targeted cancer imaging and therapy still remains a great challenge. Herein, we report the use of multifunctional dendrimer-entrapped gold nanoparticles (Au DENPs) covalently linked with α-tocopheryl succinate (α-TOS) as a platform for targeted cancer computed tomography (CT) imaging and therapy. In this study, amine-terminated poly(-amidoamine) dendrimers of generation 5 ($G5.NH_2$) conjugated with fluorescein isothiocyanate (FI), polyethylene glycol (PEG)-modified α-TOS, and PEGylated folic acid (FA) were used as templates to synthesize Au DENPs, followed by acetylation of the remaining dendrimer terminal amines. The formed multifunctional Au DENPs were characterized *via* different techniques. We show that the Au DENPs conjugated with approximately 9.8 α-TOS molecules per dendrimer and with an Au core size of 3.3 nm are water-dispersible, and stable under different pH and temperature conditions and in different aqueous media. The FA modification onto the Au DENPs enables efficient targeting of the particles to cancer cells overexpressing FA receptors (FAR), and effective targeted CT imaging of the cancer cells *in vitro* and the xenografted tumor model *in vivo*. Likewise, the covalent conjugation of α-TOS does not compromise its therapeutic activity, instead significantly improves its water solubility. Importantly, thanks to the role of FA-directed targeting, the formed multifunctional Au DENPs are able to exert the specific therapeutic efficacy of α-TOS to the FAR-overexpressing cancer cells *in vitro* and the xenografted tumor model *in vivo*. The developed multifunctional Au DENPs may hold a great promise to be used as a unique theranostic nanoplatform for targeted CT imaging and therapy of different types of cancer.

1. Introduction

Recent advances in nanoscience and nanotechnology enable the development of various platforms for cancer imaging and therapy [1,2]. Many imaging modalities such as fluorescence imaging [3,4], magnetic resonance (MR) imaging [5–7], positron-emission tomography (PET) [8], and computed tomography (CT) [9,10] have been achieved using the developed nanoparticles (NPs) with different functionalities. Similarly, a wide range of nanocarrier systems including but not limited to vesicles [11], micelles [12], nanogels [13], dendrimers [14–16], microbubbles [17,18] and carbon nanotubes [19,20] have been developed for cancer therapy applications. For efficient cancer "theranostics", it is ideal to incorporate both imaging element and cancer drug into single NPs.

As a new class of highly branched, monodispersed, and synthetic macromolecules, poly(amidoamine) (PAMAM) dendrimers have attracted a great deal of interest in the development of various imaging platforms [10,21–29] and drug delivery systems [14,15,30]. The unique structural characteristics allow one to design various dendrimer-entrapped metal NPs or dendrimer-stabilized metal NPs for CT imaging applications [10,21–28,31] by virtue of the metal NPs (e.g., Au) or for CT/MR dual mode imaging applications by

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virtue of the co-existence of Au NPs and the complexed Gd (III) ions [21,29,32]. On the other hand, the abundant amino groups on the surface of PAMAM dendrimers enable covalent conjugation of various cancer drugs for targeted cancer therapy applications [16,33–38]. Likewise, the relatively hydrophobic interior of the dendrimers allows for effective encapsulation of hydrophobic cancer drug for drug delivery applications [14–16,39]. These advantages of PAMAM dendrimers enable them to be used as an ideal multifunctional nanoplatform for both imaging and drug delivery applications. However, a thorough literature investigation reveals that there are few reports dealing with the incorporation of both imaging element and drug into a single dendrimer-based nanoplatform for cancer theranostic applications [36,40]. Not to mention that the developed nanoplatforms in the literature have not been completely investigated in terms of the *in vivo* performance of either cancer imaging or therapy.

Alpha-tocopheryl succinate (α-TOS) is known as a kind of vitamin E derivative that can induce apoptosis of various types of cancer cells, inhibit cells cycle, and disrupt the necessary autocrine signaling pathways of tumor growth [41,42]. α-TOS, composed of three domains (the functional domain, the signaling domain, and the hydrophobic domain) [43–45] can kill cancer cells, while not affecting the proliferation of most normal cells [46,47]. This is simply because it stimulates the production of reactive oxygen species *via* interaction with the coenzyme Q binding site in complex II of the mitochondrial respiratory chain and causes retardation of cell growth in malignant, but not in normal cells [48–52]. Due to its poor water solubility, the practical application of α-TOS in cancer chemotherapy has been quite limited. Therefore, development of various nanocarrier systems to improve the water solubility and bioavailability of α-TOS is necessary to maintain its anticancer efficacy [49,53,54].

Inspired by our previous successes in the development of dendrimer-based CT imaging contrast agents [10,21,27–29] and dendrimer-based drug delivery systems [14,16,39], we attempted to develop a dendrimer-based theranostic platform for targeted cancer CT imaging and therapy. In this study, amine-terminated PAMAM dendrimers of generation 5 ($G5.NH_2$) covalently conjugated with fluorescein isothiocyanate (FI), polyethylene glycol (PEG)-modified α-TOS (PEG-α-TOS), and PEGylated folic acid (PEG-FA) were used as templates to synthesize dendrimer-entrapped Au NPs (Au DENPs), followed by acetylation of the remaining dendrimer terminal amines. The formed multifunctional Au DENPs were characterized *via* different techniques. *In vitro* flow cytometry and confocal microscopy studies were used to confirm the binding specificity of the Au DENPs to cancer cells overexpressing FA receptors (FAR). The multifunctional Au DENPs were then used for CT imaging of cancer cells and the xenografted tumor model *in vivo*. Finally, the therapeutic efficacy of the Au DENPs and the performance of targeted cancer therapy were evaluated *in vitro* and *in vivo*.

2. Experimental section

2.1. Materials

Ethylenediamine core $G5.NH_2$ PAMAM dendrimers with a polydispersity index less than 1.08 were purchased from Dendritech (Midland, MI). PEG with one end of amine group and the other end of carboxyl group (NH_2-PEG-COOH) and PEG monomethyl ether with one end of carboxyl group (*m*PEG-COOH) were purchased from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). α-TOS (molecular structure shown in Fig. S1, Supporting Information) was purchased from Hubei Hengshuo Chemical Limited Corporation (Wuhan, China). FA, FI, acetic anhydride, triethylamine, sodium hydroxide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and all the other chemicals and solvents were purchased from Aldrich (St. Louis, MO) and used as received. U87MG cells (a human glioma cell line) and L929 cells (a mouse fibroblast cell line) were obtained from the Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Minimum Essential Medium (MEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18 MΩ cm. Regenerated cellulose dialysis membranes (MWCO = 14,000 and 1000) were acquired from Fisher (Pittsburgh, PA).

2.2. Synthesis of $G5.NH_2$-FI-(PEG-TOS)-(PEG-FA) dendrimers

$G5.NH_2$ dendrimers (24.71 mg, 0.00095 mmol) were dissolved into 5 mL dimethyl sulfoxide (DMSO). Then, FI with 5 molar equivalents of G5 dendrimer (1.85 mg, 0.00475 mmol) dissolved in 2 mL DMSO was dropwise added into the dendrimer solution under vigorous magnetic stirring at room temperature. The reaction was stopped after 24 h. Then, the reaction mixture was extensively dialyzed against phosphate buffered saline (PBS, 3 times, 4 L) and water (3 times, 4 L) through a 14,000 MWCO membrane for 3 days to remove the excess of reactants, followed by lyophilization to give an orange solid of $G5.NH_2$-FI dendrimers.

α-TOS with about 1.5 molar equivalents of NH_2-PEG-COOH (11.94 mg, 0.0225 mmol) dissolved in 5 mL DMSO was mixed with a DMSO solution (2 mL) containing EDC (4.31 mg, 0.0225 mmol), and the mixture was stirred for 3 h to activate the carboxylic acid group of α-TOS. Then the activated α-TOS was dropwise added to a DMSO solution (5 mL) containing NH_2-PEG-COOH (30 mg, 0.015 mmol) under vigorous magnetic stirring. The stirring process was continued for 24 h to complete the reaction. Then the reaction mixture was purified and lyophilized to get TOS-PEG-COOH according to the procedure similar to the purification of $G5.NH_2$-FI dendrimers, except that a dialysis membrane with MWCO of 1000 was used.

FA with about 1.5 molar equivalents of NH_2-PEG-COOH (11.37 mg, 0.0258 mmol) dissolved in 5 mL DMSO was mixed with a DMSO solution (2 mL) containing EDC (4.94 mg, 0.0258 mmol), and the reaction mixture was stirred for 3 h to activated the γ-carboxylic acid group of FA. Then the activated FA was dropwise added to a DMSO solution (5 mL) containing NH_2-PEG-COOH (34.36 mg, 0.0172 mmol) under vigorous magnetic stirring. The reaction and purification process was similar to that of TOS-PEG-COOH, and the product of FA-PEG-COOH was obtained.

TOS-PEG-COOH with about 34 molar equivalents of G5 dendrimer (20 mg, 0.0082 mmol) dissolved in an aqueous solution (5 mL) was activated by EDC (31.4 mg, 0.1638 mmol, in 2 mL water) under magnetic stirring for 3 h. Then the activated TOS-PEG-COOH solution was dropwise added to the $G5.NH_2$-FI dendrimers (6.71 mg, 0.00024 mmol, in 5 mL water) under vigorous magnetic stirring. The reaction and purification process were similar to that of $G5.NH_2$-FI dendrimers and $G5.NH_2$-FI-(PEG-TOS) dendrimers were obtained.

FA-PEG-COOH with about 25 equivalents of G5 dendrimer (15.57 mg, 0.00697 mmol, in 5 mL water) was activated by EDC (26.72 mg, 0.1394 mmol, in 2 mL water) under stirring for 3 h. Then the activated FA-PEG-COOH solution was dropwise added to the $G5.NH_2$-FI-(PEG-TOS) dendrimers (16.39 mg, 0.000279 mmol, in 5 mL water) under magnetic stirring. The reaction and purification process was similar to that of $G5.NH_2$-FI dendrimers and $G5.NH_2$-FI-(PEG-TOS)-(PEG-FA) dendrimers were obtained. $G5.NH_2$-FI-(PEG-TOS)-(*m*PEG) dendrimers without FA and $G5.NH_2$-FI-(PEG-FA)-(*m*PEG) without α-TOS were also synthesized for comparison under similar experimental conditions.

2.3. Synthesis of α-TOS-conjugated multifunctional Au DENPs

The procedure to synthesize α-TOS-conjugated multifunctional Au DENPs was identical to the reported method described in our previous reports [10,22,55]. The molar ratio of Au salt to $G5.NH_2$-FI-(PEG-TOS)-(PEG-FA) dendrimers was set at 200:1. Briefly, an $HAuCl_4$ solution (470.8 μL, 30 mg/mL) was added into an aqueous solution of $G5.NH_2$-FI-(PEG-TOS)-(PEG-FA) dendrimers (12.4 mg, 10 mL) under vigorous magnetic stirring. After 30 min, an icy cold $NaBH_4$ solution (3.955 mg, in 5 mL water/methanol ($v/v = 2:1$)) with 3 times molar excess to the Au salt was added to the gold salt/dendrimer mixture under stirring. Within a few seconds, the reaction mixture turned deep-red and the stirring process was continued for 2 h to complete the reaction. The formed $\{(Au^0)_{200}$-$G5.NH_2$-FI-(PEG-TOS)-(PEG-FA)$\}$ DENPs were further acetylated to neutralize the remaining dendrimer terminal amines according to the literature [22,56]. In brief, triethylamine (16.17 μL) was added to an aqueous solution of $\{(Au^0)_{200}$-$G5.NH_2$-FI-(PEG-TOS)-(PEG-FA)$\}$ DENPs under magnetic stirring. After 30 min, acetic anhydride (9.151 μL, 0.0958 mM) with 5 times molar excess of the total primary amines of G5 dendrimer was added into the Au DENP/triethylamine mixture solution under vigorous stirring and the mixture was allowed to react for 24 h. The aqueous solution of the reaction mixture was purified by dialysis against PBS (3 times, 4 L) and water (3 times, 4 L) through a membrane with MWCO of 14,000 for 3 days and lyophilized to obtain the $\{(Au^0)_{200}$-G5.NHAc-FI-(PEG-TOS)-(PEG-FA)$\}$ DENPs (for short, Au-TOS-FA). The control devices of $\{(Au^0)_{200}$-G5.NHAc-FI-(PEG-TOS)-(*m*PEG)$\}$ DENPs without FA conjugation (for short, Au-TOS) and $\{(Au^0)_{200}$-G5.NHAc-FI-(PEG-FA)-(*m*PEG)$\}$ DENPs without α-TOS (for short, Au-FA) were also synthesized under similar experimental conditions.

2.4. Characterization techniques

^1H NMR spectra were recorded using Bruker AV-400 NMR spectrometer. TOS-PEG-COOH was dissolved in D_6-DMSO and all the other samples were dissolved in