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PEGylated polyethylenimine-entrapped gold nanoparticles modified with folic acid for targeted tumor CT imaging

Benqing Zhou[a,1], Jia Yang[b,1], Chen Peng[c], Jianzhi Zhu[a], Yueqin Tang[d], Xiaoyue Zhu[a], Mingwu Shen[a], Guixiang Zhang[b,**], Xiangyang Shi[a,c,e,*]

[a] College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, People's Republic of China
[b] Department of Radiology, Shanghai General Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200080, People's Republic of China
[c] Department of Radiology, Shanghai Tenth People's Hospital, School of Medicine, Tongji University, Shanghai 200072, People's Republic of China
[d] Experiment Center, Shanghai General Hospital, School of Medicine, Shanghai Jiaotong University, Shanghai 200080, People's Republic of China
[e] CQM—Centro de Química da Madeira, Universidade da Madeira, Campus da Penteada, 9000-390 Funchal, Portugal

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ABSTRACT

Development of various cost-effective contrast agents for targeted tumor computed tomography (CT) imaging still remains a great challenge. Herein, we present a facile approach to forming folic acid (FA)-targeted multifunctional gold nanoparticles (AuNPs) using cost-effective branched polyethylenimine (PEI) modified with polyethylene glycol (PEG) as a template for tumor CT imaging applications. In this work, PEI sequentially modified with PEG monomethyl ether, FA-linked PEG, and fluorescein isothiocyanate was used as a template to synthesize AuNPs, followed by transformation of the remaining PEI surface amines to acetamides. The formed FA-targeted PEI-entrapped AuNPs (FA-Au PENPs) were fully characterized. We show that the formed FA-Au PENPs with an Au core size of 2.1 nm are water soluble, colloidally stable, and non-cytotoxic in a given concentration range. Flow cytometry and confocal microscopy data reveal that the FA-Au PENPs are able to target cancer cells overexpressing FA receptors (FAR). Importantly, the developed FA-Au PENPs can be used as a nanoprobe for targeted CT imaging of FAR-expressing cancer cells *in vitro* and the xenografted tumor model *in vivo*. With the demonstrated biocompatibility by organ biodistribution and histological studies, the designed FA-Au PENPs may hold great promise to be used as a nanoprobe for CT imaging of different FAR-overexpressing tumors.

1. Introduction

Computed tomography (CT) has been considered as one of the most commonly utilized imaging techniques for disease diagnostics owing to its deep tissue penetration, better spatial and density resolution than other imaging modalities, and cost effectiveness [1–4]. High-quality CT imaging usually requires the use of contrast agents, however, the conventionally used iodinated small molecular CT contrast agents (e.g., Omnipaque) can be rapidly cleared by kidney [5–7], resulting in short imaging time. In addition, the iodinated small molecular CT contrast agents also suffer problems of renal toxicity and nonspecificity, quite limiting their applications in tumor CT imaging [7,8]. Recent progresses in nanotechnology have shown that various nanoparticulate CT contrast agents have been developed. Most of the developed nanoparticles (NPs) have enhanced X-ray attenuation property, low toxicity, and prolonged blood circulation time, enormously overcoming the drawbacks of iodinated small molecular CT contrast agents [9–11].

Among the currently available nanoparticulate CT contrast agents such as Au-, Pt-, Ta-, Yb-, and Bi-based inorganic NPs [12–16], gold NPs (AuNPs) have received immense interest owing to their better X-ray attenuation property than that of iodinated CT contrast agents, tunable surface chemical modifications, easy control of the size, and biocompatibility after surface functionalization [17–20]. For example, AuNPs modified with polyethylene glycol (PEG) with an average size of 30 nm possess long blood circulation time and good biocompatibility, and are able to be accumulated in phagocytic cells of the liver and spleen for CT imaging of hepatocelluar carcinoma [17,20]. Biocompatible AuNPs stabilized with gum-arabic (GA) matrix can be prepared and used as a CT

* Corresponding author at: College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, People's Republic of China. Fax: +86 21 67792306 804.
** Corresponding author.
E-mail addresses: guixinagzhang@sina.com (G. Zhang), xshi@dhu.edu.cn (X. Shi).
[1] Authors contributed equally to this work.

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contrast agent [21]. Recently, dendrimers have been shown to be used as templates or stabilizers to create dendrimer-entrapped or dendrimer-stabilized AuNPs for CT imaging applications, in particular tumor CT imaging [11,22–24].

For improved tumor CT imaging performance, it is desirable to modify AuNPs with targeting ligands to significantly improve the imaging specificity. AuNPs conjugated with peptides [25], antibody [9,18], or folic acid [11,26] can be used for targeted tumor CT imaging. In order to improve the cytocompatibility and colloidal stability of the NPs for applications in living organism, the surface of the NPs is often modified with polyethylene glycol (PEG) [17,27,28]. Meanwhile, PEGylation is able to effectively prevent the NPs from rapid uptake by scavenger cells, leading to prolonged circulation times, which is beneficial for tumor CT imaging. Unfortunately, the development of such NP-based CT contrast agents with targeting specificity, good biocompatibility, significant CT contrast performance and cost effectiveness still remain an enormous challenge.

Branched polyethylenimine (PEI) with abundant surface primary amines has been used as a multifunctional nanocarrier for drug/gene delivery [29,30] and for NP stabilization [31–34]. In our previous work, we have shown that branched PEI modified with PEG can be used as a template for the synthesis of AuNPs, which can be subsequently used as a high-performance contrast agent for blood pool and tumor CT imaging [35]. The major advantages of the use of PEGylated PEI as a template is that (1) PEGylation modification of PEI surface amines enables enhanced entrapment of AuNPs within its interior, similar to the case of dendrimers [36]; (2) compared to highly expensive commercial high-generation dendrimers, the use of branched PEI is more cost-effective. Likewise, the molecular structure of dendrimers is significantly different from that of branched PEI. However, until now PEGylated PEI-entrapped AuNPs (Au PENPs) modified with targeting ligand have not been reported for targeted tumor CT imaging applications. With the PEI amine-mediated conjugation chemistry similar to aminated dendrimers, it is expected that PEGylated Au PENPs with targeting specificity can be readily prepared and used for targeted tumor CT imaging.

To test our hypothesis, in this present study, branched PEI sequentially modified with PEG monomethyl ether with one end of carboxyl group (*m*PEG-COOH), FA-linked PEG with the other end of carboxyl group (PEG-FA-COOH), and fluorescein isothiocyanate (FI) was used as a template to prepare AuNPs, followed by acetylation of the remaining PEI surface amines (Scheme 1). The formed multifunctional FA-targeted Au PENPs (FA-Au PENPs) were characterized using different techniques. The cytocompatibility of the NPs were evaluated by quantitative cell viability assay and qualitative cell morphology observation. The targeting specificity of the FA-Au PENPs to cancer cells overexpressing FA receptors (FAR) was investigated by flow cytometry and confocal microscopy. The potential to use the developed FA-Au PENPs as a nanoprobe for targeted CT imaging of FAR-overexpressing cancer cells *in vitro* and the xenografted tumor model *in vivo* was investigated in detail. Finally the FA-Au PENPs were subjected to *in vivo* biodistribution and organ hematoxylin and eosin (H&E) staining studies. To our knowledge, this is the first report related to the synthesis of PEGylated Au PENPs with targeting specificity for tumor CT imaging applications.

2. Result and discussion

2.1. Synthesis and characterization of the FA-Au PENPs

Different from the synthesis of PEGylated Au PENPs reported in our previous work [35], in this study branched PEI was sequentially modified with *m*PEG-COOH, PEG-FA-COOH, and FI. The formed multifunctional PEGylated PEI was used as a template for the synthesis of AuNPs, followed by full acetylation of the remaining PEI surface amines (Scheme 1). The formed FA-Au PENPs were characterized *via* different techniques.

The number of PEG, FA, and FI moieties conjugated onto each PEI can be estimated by comparing the difference among the ^{1}H NMR integration areas of FA-PEG-COOH, PEI-*m*PEG, and FI with PEI -CH_2- protons (Fig. S1, Supporting information). The average number of FA moieties conjugated onto each PEG was estimated to be 0.7 (Fig. S1a, Supporting information), and the numbers of PEG, FA and FI moieties linked to each PEI were estimated to be 24, 5.5, and 6.3, respectively (Fig. S1b and S1c, Supporting information). Mass spectrum of the formed FA-PEG-COOH conjugate shows that the Mw of the FA-PEG-COOH conjugate is 2322.7 (Fig. S2, Supporting information). By comparison the average Mw of the NH_2-PEG-COOH (Mw = 2000), the number of FA moieties linked to each PEG was calculated to be 0.73, which is consistent with the ^{1}H NMR data. Similarly, based on the Mw of PEI-*m*PEG (Mw = 76158.3) and PEI (Mw = 25000) (Fig. S3, Supporting information), the number of *m*PEG moieties linked to each PEI was calculated to be 25.6, quite similar to that caulated based on ^{1}H NMR inegration. ^{1}H NMR was also used to confirm the successful PEI amine acetylation (Fig. S1d, Supporting information). The FA-Au PENPs after acetylation display two new peaks, one is the acetyl protons linked to the secondary PEI amides (1.90 ppm), and the other is the acetyl protons linked to the PEI tertiary amides (2.05 ppm), in agreement with the literature [35,37].

UV–vis spectroscopy was used to follow the PEI surface modification and AuNPs synthesis (Fig. 1a). By comparison of the FA-PEG-COOH and the PEI-FI-(PEG-FA), the FA-Au PENPs display the peaks at 290 nm (which is attributed to the attached FA moieties) and 500 nm (which is associated to overlapped absorption peaks of FI and surface plasmon resonance (SPR) peak of AuNPs). This indicates the successful formation of FA-Au PENPs. In addition, the acetylation reaction does not seem to significantly affect the optical property of the FA-Au PENPs.

TEM was used to characterize the size and morphology of the formed FA-Au PENPs (Fig. 2). It is clear that the Au core NPs possesses a spherical shape and are quite uniform with a mean diameter of 2.1 ± 0.4 nm (Fig. 2a and 2b). High resolution TEM shows the clear lattice structure, confirming the crystalline nature of the Au core NPs (Fig. 2c). The crystalline structure of the Au core NPs was further confirmed by selected area electron diffraction (SAED) pattern, where the (111), (200), (220), and (311) rings are typical for the face-centered-cubic (fcc) Au crystal structure (Fig. 3d). The hydrodynamic size of the FA-Au PENPs was measured to be 202.4 nm *via* DLS (Fig. S4, Supporting information). The larger size of the particles measured by DLS than that measured by TEM could be due to the fact that DLS measures the clustered Au PENPs that may consist of many single AuNPs in aqueous solution, while TEM only measures single Au core NPs.

Zeta potential measurements were also employed to confirm the successful acetylation reaction (Table S1, Supporting information). We show that the surface potential of the $\{(Au^0)_{200}$-PEI.NH_2-FI-*m*PEG-(PEG-FA)$\}$ PENPs (14.4 ± 2.4 mV) decreases to 6.1 ± 0.1 mV after acetylation. Similarly, the surface potential of the $\{(Au^0)_{200}$-PEI-*m*PEG$\}$ PENPs without FA and FI modification (29.77 ± 8.59 mV) decreases to 8.6 ± 1.8 mV after acetylation. Due to the nature of the incomplete acetylation reaction, the particles after acetylation still display a slight positive surface potential, in agreement with our previous work [38].

The stability of the FA-Au PENPs under different conditions was analyzed by UV–vis spectroscopy (Fig. S5, Supporting information). The results show that the FA-Au PENPs have good stability at different pHs and temperatures, similar to PEGylated Au PENPs [35]. Furthermore, the FA-Au PENPs dispersed in water, PBS, and cell culture medium are quite colloidally stable and no precipitation