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# Efficient, dual-stimuli responsive cytosolic gene delivery using a RGD modified disulfide-linked polyethylenimine functionalized gold nanorod

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ABSTRACT

Controlled-release systems capable of responding to external stimuli and/or unique internal environments have received great interests in site-specific gene and/or drug delivery. In this work, a functionalized gene nanocarrier for dual-stimuli triggered cytosolic gene delivery is developed and showing high gene delivery efficacy with low cytotoxicity. The nanocarrier is prepared by conjugating gold nanorod (GNR) with multiple disulfide cross-linked short PEIs to harness the advantageous properties of GNR based near infrared (NIR) laser induced photothermal heating and intracellular stimuli-triggered degradability of disulfide cross-linked short PEIs (DSPEI). The DSPEI is further grafted with a poly(ethylene glycol) (PEG) section to afford high carrier stability in cell cultures and a terminal RGD peptide for specific targeting of cancer cells. The nanocarrier is found to effectively condense plasmid DNA to form a highly stable GNR-DSPEI-PEG-RGD/DNA complex with tumor cell-targeting ability that can be efficiently uptaken by cancer cells. Moreover, the loaded genes can be effectively released from the complex triggered by the high intracellular glutathione content and/or by photothermal effect of NIR irradiation at 808 nm. Interestingly, the GNRs-based complex can easily escape from intracellular endo-/lyso-somal compartments and release the gene load into the cytosol upon exposure to NIR irradiation, resulting in significantly improved gene transfection efficiency. Our new gene carrier exhibits high gene transfection efficiency, comparable to or even better than that of high MW PEIs, but with a much lower cytotoxicity. Additionally, neither the GNR-based carrier nor the laser treatment shows any significant evidence of cytotoxicity. This work demonstrates a promising strategy for intracellular stimuli triggered, photothermal controllable gene delivery system, which can be further applied to many other nanomedicine fields.



## 1. Introduction

Gene therapy is a promising and unique approach to treat a variety of diseases, including genetic diseases, cardiovascular diseases and cancer [1–4]. Since the nucleic acids are prone to hydrolysis in biological fluids and show low cellular uptake efficiency due to their polyanionic nature, the development of gene delivery systems with high efficiency, safety and selective targeting ability is essential for gene therapy [5,6]. Although viral vectors have shown high transfection efficiency, some shortcomings such as non-specific, immunogenic and susceptibility to enzyme degradation, have limited their clinical applications. Due to the improved safety profile and ease of preparation and manipulation, non-viral gene delivery vectors are continuing to be explored and optimized [7,8]. Significant research efforts have been focused on developing controlled-release systems that are capable of responding to external stimuli and/or the unique environments of tissues.

The exploitation of external stimuli triggered, spatially and temporally controlled drug delivery carrier has received tremendous attention in recent years. External stimulation factors include light [9–12], temperature [13], radiofrequency [14,15], magnetic field [16] and ultrasound [17,18]. Among which, light provides a great opportunity to deliver a drug at the desired area at a specific time, which is considered a key tool to amplify drug efficacy with minimum adverse effects. In particular, near-infrared (NIR) light has been particularly attractive due to the "water window" (650–900 nm) which shows minimal absorbance by skin and tissue [19], and thus providing deep tissue penetration with high spatial precision without damaging normal biological tissues.

Gold nanoparticles (GNPs) exhibit extraordinary functionality due to their unique optical and electronic properties [20]. Following excitation, the plasmons on nanoparticle surfaces can decay by either radiative damping or energetic relaxation, which create nonequilibrium "hot" electron–hole pairs [21,22]. When molecules are adsorbed on the nanoparticle surface, excited "hot" electrons can transfer to the adsorbate prior to thermalization [23,24]. Thus, photorelease strategies have sought to covalently attaching a "carrier" molecule to the nanoparticle surface through the Au – S bond. Then a "cargo" therapeutic gene

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entity loads onto the "carrier" molecule via weaker, noncovalent interactions. When this type of nanocomplex is irradiated with continuous-wave (CW) laser, the nanoparticle absorbs energy, which reduces the attraction between the "carrier" and "cargo" molecules either thermally or nonthermally, resulting in release of the therapeutic gene [25,26]. This "on demand" release strategy shows excellent promise for light-controlled delivery due to the relatively low laser power intensity and short irradiation times required to achieve the release of molecular "cargo". Notably, research showed that low CW laser intensity irradiation of cancer cells containing endosomal GNPs leads to endosome rupture without affecting the cells' viability. This underlying photochemical mechanism facilitates the GNPs' escape from the endosome into the cytosol, which is required for the therapeutic gene to be effective [25,27,28].

Gold nanorods (GNRs), which are rod-shaped GNPs, show high promising potential as light-triggered, remotely controlled molecular release trigger in response to optical excitation. The plasmon resonance of GNRs can be tuned from the visible to NIR regions that depend on the nanorod's aspect ratio [29]. The NIR laser irradiation-triggered drug and gene release is especially attractive, because GNRs can be easily made to maximally absorb in the "water window" [30]. In addition, GNRs have the advantages of efficient large-scale synthesis and can be easily decorated with multiple molecular species to simultaneously provide biological compatibility [31–33], activated drug and gene delivery [34–36] and direct cell specific targeting [37–39].

Polyethylenimine (PEI) has been regarded as the "gold" standard for gene delivery because it shows relatively high transfection efficiency from its proton sponge effect and ability to protect DNA from degradation by enzymes [40,41]. High molecular weight PEIs such as 25-kDa PEI are highly effective in gene transfection, but also induce high cytotoxicity due to the high cationic density and lack of biodegradability [42]. Low molecular weight PEIs such as 1.8-kDa PEI have a much lower cytotoxicity, but they cannot effectively condense DNA and display very poor gene transfection activity [42,43]. In order to reduce the cytotoxicity and enhance carrier unpacking into the cytosol and/or nucleus, intracellular-cleavable disulfide-linked PEIs have been designed for gene delivery [44–46]. The disulfide linkage is stable in blood circulation [47]. Once inside cells, the disulfide bonds are cleaved under the high concentration of reductive glutathione (GSH), making it favorable to unpack the PEI shell and release the infective nucleic acid [42,48]. Therefore, reducible disulfide cross-linked short PEIs (DSPEI) was synthesized as gene carrier for controlled-release systems in response to the unique environments of cells.

Integrin $\alpha_v\beta_3$, an important biomarker over-expressed on actively angiogenic endothelium and malignant glioma cell surfaces, plays a critical role in regulating tumor growth, metastasis and tumor angiogenesis [49,50]. Researches have shown that the cyclic RGD (arginine–glycine–aspartic acid) peptides (cRGD) can specifically bind with integrin $\alpha_v\beta_3$ [39,49], thus we have selected the RGD peptides as the targeting molecules for glioblastoma cell targeting and selective therapy. Here, DSPEI-PEG-RGD was developed by incorporating the RGD peptide into the terminal end of hydrophilic polyethylene glycol (PEG) spacer. The tethered RGD peptide at the end of the PEG chain may behave like a free molecule in the solution due to highly flexible and well-hydrated PEG chains so that the RGD peptide should have easy access to its receptor.

In this study, we developed a GNR-based gene delivery system. This nanosized gene delivery carrier consists of GNR covalently conjugated with RGD modified DSPEI via "round-trip" phase transfer ligand exchange. The success of the functionalized GNR-DSPEI-PEG-RGD was confirmed by UV–Vis–NIR spectrophotometer, $^1$H-NMR, transmission electron microscopy (TEM) and thermogravimetric analysis (TGA). To investigate the general utility of this delivery system, the gene was loaded onto the carrier and the tumor cell targeting ability of the GNR-based complexes was examined by dark-field microscopy and inductively coupled plasma–mass spectrometry (ICP–MS). We evaluated YOYO-1 labeled DNA release from the complex by dual-stimuli of GSH and NIR laser irradiation in solution and in living cells. FITC labeled GNR-DSPEI-PEG-RGD was further used to investigate intracellular light-triggered endosome escape. The transfection activity of GNR-DSPEI-PEG-RGD based complexes under NIR laser treatment was also evaluated in U-87 MG cells using the pGFP as reporter genes.

## 2. Materials and methods

### 2.1. Materials

Cetyltrimethylammonium bromide (CTAB), sodium borohydride ($NaBH_4$), 5-Bromosalicylic acid (5-BrSA), silver nitrate ($AgNO_3$), L-ascorbic acid (L-AA), polyethylenimine branched (PEI-25 KDa), L-glutathione (Reduced) (GSH), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-Hydroxy succinimide (NHS), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Ethidium bromide (EB), bisBenzimide H 33342 trihydrochloride (Hochest 33342), trypan blue and dimethyl sulfoxide (DMSO) were obtained from Sigma Co., Ltd. (USA). Polyethylenimine branched (PEI-1.8 KDa) was purchased from Alfa Aesar (USA). 3,3′-dithiodipropionic acid, methyl thioglycolate, BOP reagent, 1-dodecanethiol (DDT) and 11-mercaptoundecanoic acid (MUDA) were purchased from Aladdin Industrial Co., Ltd. (China). YOYO-1 and Lyso Traker Red were obtained from Invitrogen Molecular Probes (USA). C(RGDyk)-$NH_2$ (RGD) was purchased from GL Biochem (Shanghai) Ltd. (China). Maleimide polyethylene glycol *N*-Hydroxysuccinimide ester (MAL-PEG-NHS, MW 3500) was purchased from Jenkem Technology (China). Chloroauric acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$), hydrochloric acid (HCl, 36.0–38.0 wt.% in water) and nitric acid ($HNO_3$) were obtained from Sinopharm Chemical Reagent Co., Ltd. (China). The Dulbecco's Modified Eagle Medium (DMEM), penicillin–streptomycin, fetal bovine serum (FBS), 0.25% (w/v) trypsin–0.03% (w/v) EDTA solution and Phosphate buffer solution (PBS) were purchased from Gibco BRL (USA). Water was purified by distillation, deionization, and reverse osmosis (Milli-Q plus). All reagents were analytical grades and used without further purification.

Plasmid DNA encoding for green fluorescent protein (pGFP-N1) was a kind gift from Professor Tuo Jin (School of Pharmacy, Shanghai Jiao Tong University), and was transformed in *Escherichia coli* DH5R and propagated in Luria-Bertani (LB) medium at 37 °C overnight, followed by isolation and purification with a commercially available plasmid purification Qiagen Plasmid Maxi Kit (Qiagen, Valencia, CA). The concentration of plasmid solution was determined by measuring the ultraviolet (UV) absorbance at 260 nm and its optical density ratio at 260 nm to 280 nm was in the range of 1.8 to 1.9.

### 2.2. Cell culture

The U-87 MG (human glioblastoma cell) cell line was kindly donated by the School of Pharmacy, Fudan University. Cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 units/mL penicillin G sodium and 100 μg/mL streptomycin sulfate (complete DMEM medium) and maintained at 37 °C in a humidified and 5% $CO_2$ incubator. Cells grown to confluence were subcultured every other day after trypsinized with 0.25% trypsin–EDTA and diluted (1/3) in fresh growth medium.

### 2.3. Synthesis of GNRs

GNRs were synthesized using the seed-mediated growth method according to the literatures [51,52]. Briefly, a 5 mL amount of 0.5 mM $HAuCl_4$ was mixed with 5 mL of 0.2 M CTAB solution. While the solution under vigorous stirring (1200 rpm) at 30 °C, 0.6 mL of fresh ice-cold 0.01 M $NaBH_4$ was added. The solution color changed from yellow to brownish-yellow, and the stirring was stopped after 2 min. Afterwards, it was allowed to react for 2 h to form the CTAB-capped gold nanoparticles to be used as seeds for the synthesis of GNRs.



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