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Redox- and pH-responsive fluorescent carbon nanoparticles-MnO_2-based FRET system for tumor-targeted drug delivery *in vivo* and *in vitro*

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ABSTRACT

The carbonized boronic acid-conjugated fluorescent carbon nanoparticles (FNPs) were crosslinked with carbonized dopamine-conjugated hyaluronic acid (HA) *via* the diol boronic acid-linked catechol groups [C-FNP] as extracellular targeting ligands and pH responsiveness. Into that C-FNP, the redox-responsive MnO_2 nanosheets were loaded to demonstrate quenching effect, which recovered after glutathione (GSH) treatment. The MnO_2-loaded C-FNP showed excellent stability and tumor-targeting ability guided to the localized tumor using "on–off" fluorescent switch. Furthermore, paclitaxel (PTX) inside the MnO_2-loaded C-FNP performed *in vitro* and *in vivo* bioimaging and chemotherapeutic activities analyzed towards cancer and non-cancerous cells.

Introduction

Controlled drug-delivery system (DDS) for targeting specific cancer sites is becoming an attractive concept compared to conventional chemotherapeutics, siRNA technology, and gene therapy [1,2]. Recently, stimuli-responsive materials have been developed, which have immense potential in achieving effective and controlled release of drugs by sensing the cellular environment [3]. Mostly, the intracellular pH of normal cells is ~7.2–7.4 and that of endosomes is ~5; however, the extracellular pH of tumor cells is ~6.5–6.9 [4,5]. Additionally, the cells have an important non-protein thiol, namely glutathione (GSH), which plays an important role in regulating cellular processes such as cell differentiation, proliferation, and apoptosis [6,7]. In tumor cells, the GSH concentration is ~2–10 mM, which is at least four times higher than that in the normal cells [8]. Considering the above facts, a smart material for DDS can be developed in the form of a biological-stimuli responsive system.

Chemotherapy suffers from several limitations, such as lack of specificity in drug delivery and adverse effects on noncancerous tissues [9]. To enable drug delivery to the appropriate intracellular compartment, promising techniques that result in intracellular accumulation (and not merely cellular uptake) and also minimize the unwanted side effects should be considered. Targeted imaging using DDS might prove to be a reliable technique to overcome this issue, using extracellularly active ligands and fluorescence for tracking whether the drug is delivered at the target site or not. So far, several chemical conjugations have been used to synthesize multi-stimuli-responsive DDS; however, these processes were complex and not effective for DDS material preparation. It is well known that the fluorescent carbon nanoparticles (FNPs) have attracted considerable attention in recent years owing to their unique properties, including low cost, high quantum yield, water solubility, low toxicity, good photostability, and excellent biocompatibility [10]. Previously, our group successfully fabricated FNPs derived from biocompatible hyaluronic acid (HA) for targeted bioimaging applications [10,11]. The results indicated the possibility of designing a combination of stimuli-responsive materials and targeted FNPs, as cooperative tools for efficient cellular drug uptake using a single platform.

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Various studies related to the quenching effect induced by nanomaterials synthesized using graphene oxide, tungsten oxide, gold, and organic dyes have been performed for developing sensors for on-field applications; however, this approach was limited in applicability since molecular dynamics, such as protein conformational changes, protein–protein interactions, and intracellular imaging could not be monitored [12–14]. Recently, many researchers have developed reagents for switching the fluorescence of probes using manganese oxide (MnO_2) as a quencher, taking into consideration the effects of cellular environment on quenching [15]. Since MnO_2 has a superior light absorption property, it allows the occurrence of Förster resonance energy transfer (FRET), resulting in spectral overlap between the fluorescence emission spectrum of the donor and the absorbance spectrum of the acceptor [16]. In addition, MnO_2 nanosheets possess strong oxidation ability and can oxidize many organic compounds. Therefore, they might be GSH responsive [17].

Inspired by our previous work, a pH-responsive nanocarrier was required to improve the release of a drug from endosomal vesicles into the intracellular spaces [18,19]. This could be achieved by using a pH-labile linker, such as a boronic acid (BA)–diol complex or a hydrazine bond [18]. Moreover, poor aqueous solubility and serious side effects associated of commercial drug like paclitaxel (PTX) developed demanding of alternative PTX formulations. To date, the integrated PTX with super soluble nanoparticles containing pH/redox triggered release moieties can be potential choices to eliminate undesired side effects. In our recent study, we synthesized FNPs using hyaluronic conjugated polydopamine [FNP(HA-D)] *via* carbonization using an acid catalyst. Based on this study, we designed redox and pH responsive crosslinked FNP based FRET system between carbonized HA-D [FNP(HA-D)] and carbonized 4-chlorophenylboronic acid-conjugated polyethylene glycol-g–poly(dimethylamino) ethyl methacrylate [FNP(B-PgD)] using MnO_2 and by employing a diol-complex bind that generated pH-responsive site, inclusion complexed with PTX. The introduction of MnO_2 in C-FNP addressed the issue of quenching of fluorescence and resulted in enhanced sensitivity, responsiveness to GSH concentration, and recovering the fluorescence of C-FNP for biosensor potential, whereas the crosslinking site regulated the pH-direction for delivering PTX to the tumor tissues. Moreover, the remaining HA moieties regulated the C-FNP for stimuli-responsive targeted drug delivery and intracellular localization, in order to increase the efficacy of released PTX. We investigated the *in vivo* antitumor efficacy of C-FNP on MDA-MB-231 tumor-bearing mice, guided by the targeting behavior of HA and using fluorescence "on–off" bioimaging. The presence of high level of GSH in the mice model, guided the "turn-on" of the quenching behavior in our bioimaging system. The combined nanoplatform offered a smart material with tumor microenvironment-triggered multi-responsive effects to enhance drug efficacy against the devastating malignant tumors.

Materials and methods

Materials

Polyethylene glycol (PEG, Mn 3500), 2-(dimethylamino)ethyl methacrylate (DMA), 4-chlorophenylboronic acid, hyaluronic acid (HA; MW 700,000), dopamine hydrochloride, $KMnO_4$ solution, 2-(N-morpholino)ethanesulfonic acid (MES) buffer, paclitaxel (PTX), glutathione (GSH), *N*-ethylmaleimide (NEM), thioctic acid (TA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich, Korea. Penicillin–streptomycin, fetal bovine serum (FBS), trypsin (0.25% w/v), ethylene diamine tetra acetic acid (EDTA, 0.03% w/v), and RPMI 1640 medium were purchased from Gibco BRL (Carlsbad, CA, USA). 4-chlorophenylboronic acid-conjugated polyethylene glycol-g– poly(dimethyl amino)ethyl methacrylate (PgD, Mn 11,300 g/mol) and dopamine-conjugated hyaluronic acid (HA-D) were synthesized as described previously [18,20].

^{1}H NMR spectra were recorded using the Bruker Avance 400 MHz spectrometer with deuterated dimethyl sulfoxide (DMSO-d6) as the solvents. The UV–vis spectra were recorded using the Optizen 2020UV spectrometer (Mecasys Co.) and particle size was measured using dynamic light scattering (DLS; Zetasizer Nano, Malvern, Germany). Photoluminescence (PL) spectra were obtained using the L550B luminescence spectrometer (Perkin Elmer). The FilterMax F3 multi-mode microplate reader (Molecular Devices, LLC.) was used for the MTT assay. Transmission electron microscopy (TEM; JEM-2100F, JEOL) was performed using an 80–200 kV electron gun. Propidium iodide (PI), calcein acetoxymethyl ester (calcein-AM), LysoTracker Green DND-26, and MitoTracker Red CMXRos were purchased from Molecular Probe, Life Technologies (Invitrogen). Confocal laser scanning microscopic images of the samples were recorded using the LSM510 confocal microscope (Carl Zeiss, Germany).

Synthesis of carbonized 4-chlorophenylboronic acid-conjugated polyethylene glycol-g–poly(dimethylamino)ethyl methacrylate [FNP (B-PgD)]

B-PgD was synthesized as described previously [20]. To synthesize FNP(B-PgD), we used the acid treatment method adopted from a procedure described previously [10]. Briefly, pristine B-PgD (1 g) was dissolved in water (5 mL). Next, H_2SO_4 was added and the solution was incubated for 1 min at room temperature, followed by neutralization with water (185 mL). Finally, the mixture was purified by dialysis (molecular weight cut-off, MWCO 1000 Da) against water and freeze dried to obtain the final product [FNP(B-PgD)].

Synthesis of carbonized dopamine-conjugated hyaluronic acid [FNP (HA-D)]

HA-D was obtained using the EDC/NHS reaction as described previously [18]. Next, to 5 mL solution of HA-D (1 g), H_2SO_4 was added and stirred for 1 min at room temperature, followed by neutralization with water (185 mL). Finally, the solution was purified by dialysis (MWCO, 1000 Da) against water and freeze dried to obtain the final product [FNP(HA-D)].

Synthesis of C-FNP by crosslinking FNP(B-PgD) and FNP(HA-D)

FNP(B-PgD) (1 g) in 9 mL and FNP(HA-D) (0.1 g) in 1 mL of TBS buffer (pH 12) were mixed. The solution was allowed to react overnight at room temperature to obtain the C-FNP. Next, the solution was dialyzed (MWCO, 1000 Da) for 24 h and freeze dried.

Loading MnO_2 in C-FNPs (MnO_2-loaded C-FNP)

The C-FNP (10 mg/mL in water) were dissolved in MES buffer (10 mL, 0.1 M, pH 6). $KMnO_4$ (5 mM solution) was added to this mixture. The solution was sonication for 30 min. After the solution turned brown, it was subjected to centrifugation (4000 rpm) for 15 min and washed three times. The final product was freeze dried.

Loading PTX into MnO_2-loaded C-FNP (MnO_2/PTX-loaded C-FNP)

PTX was loaded into the MnO_2-loaded C-FNP using the widely adopted inclusion complexation method. Briefly, MnO_2-loaded C-FNP (100 mg) and PTX (5 mg) were dissolved in ethanol (20% v/v) to

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