One-Pot Synthesis of Diphenylalanine-Based Hybrid Nanospheres for Controllable pH- and GSH-Responsive Delivery of Drugs

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Supporting Information

ABSTRACT: Nano drug delivery systems have attracted much research and clinical interest but they remain challenging with respect to developing controllable, nontoxic, biocompatible and biodegradable nanocarriers. Herein, we report the first pH- and glutathione (GSH)-responsive diphenylalanine (FF)-based hybrid spheres using natural alginate dialdehyde (ADA) as the cross-linker to induce self-assembly of FF and an in situ reducer of Au\(^{3+}\) ions into Au nanoparticles (Au NPs). Not only is the synthesis simple and high-yielding, but also these biocompatible hybrid spheres can encapsulate more than 95% of hydrophobic drug (camptothecin, CPT). Moreover, the CPT-loaded carriers are stable under normal physiological environments, and have excellent pH- and GSH-responsive release at pH 5.0 with 10 mM GSH, which is similar to the tumor microenvironment. Also, these nanocarriers can be taken up by cancer cells and have greater cytotoxicity than free drugs. These attributes make nanospheres very promising for drug loading and delivery, and the method may be used for synthesis of other natural nanospheres as delivery systems.

1. INTRODUCTION

Nano drug delivery systems are very attractive for their unique properties as cancer therapies, offering better efficacy against resistant tumors and fewer side effects compared to conventional free drugs. Various materials, including inorganic nanoparticles, liposomes, hydrogels, and polymer nanoparticles made of synthetic or natural polymers, have been exploited or designed as anticancer drug carriers.\(^1\)–\(^6\) Especially, the supramolecular structures from self-assembly of natural and biocompatible diphenylalanine (FF), namely the core recognition motif of Alzheimer’s β-amyloid, have been studied for their unique physicochemical properties since the discovery of the first FF nanotubes by Reches and Gazit in 2003.\(^7\)–\(^12\)

To date, most products derived from supramolecular self-assembly of FF through π−π stacking interactions and hydrogen bonding have a tube-like morphology, with diameters ranging from several nanometers to tens of micrometers.\(^13\)–\(^15\) Such micro/nanotubes have high length homogeneity, and more importantly, high stability in severe thermal and enzymatic conditions.\(^16\)–\(^18\) For example, Silva and co-workers synthesized tubes with diameters of approximately 0.7–10 \(\mu\)m through spontaneous self-assembly of FF by diluting the stock solution of FF in 1,1,1,3,3,3-hexafluoro-2-propanol.\(^19\) The obtained tubes were evaluated as potential vehicles for rhodamine B delivery, and low toxicity was observed. However, large dimensions of the FF-tubes may induce membrane damage, reducing carrier efficacy for parenteral application.\(^20\)

Zhang and co-workers recently synthesized FF-based nanospheres with a diameter of \(~\)450 nm, using glutaraldehyde to induce self-assembly of cationic FF into nanospheres.\(^21\) The nanospheres had high encapsulation ability for doxorubicin and the drugs could be triggered to release with tyrosin under physiological conditions. Meanwhile, the doxorubicin-loaded nanocarriers could be taken up by HeLa cells through endocytosis and greater killing capacity was observed. However, toxic glutaraldehyde may impede practical applications and neither pH nor glutathione (GSH) response has been studied to currently reported FF assemblies. Obviously, an ideal drug delivery system should not only efficiently encapsulate drugs and shield it from degradation under normal physiological environment, but also cause rapid release in the tumor microenvironment which has specific pH and GSH concentration.

In fact, pH- or GSH-stimulus-responsive nanocarriers have been prepared for controllable drug release at tumor locations because of the aberrant tumor intracellular environment compared with normal tissues, such as acidic pH levels of endosome and lysosome (5.0–5.5), and high levels of GSH concentration (2–10 mM).\(^22\)–\(^27\) However, most GSH-responsive materials are based on the reduction of disulfide linkages by GSH, for which disulfides are prone to cleavage in all redox compartments, from the blood vessel lumen to the intracellular environment, consequently leading to undesirable drug leakage during delivery. Moreover, disulfide exchange

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reactions usually do not occur efficiently in low pH in endolysosomal organelles due to the protonation of thiolate required for the reaction.28–30 Therefore, it is highly desirable to synthesize intelligent FF-based nanocarriers with controllable environment responses.

In this study, we report the synthesis of the first pH- and GSH-stimulus-responsive FF-based hybrid nanospheres obtained using natural alginate dialdehyde (ADA) as both a cross-linker to induce self-assembly of FF and an in situ reducer of Au+ ions into Au nanoparticles (Au NPs), thus forming FF–ADA–Au hybrid nanospheres (FHNS). Here, the trick of introducing Au NPs into the system not only gives the hybrid nanospheres with structure rigidity, but also endows the spheres with GSH-responsive properties through the ligand exchange reaction of GSH with ADA molecules on Au NP surfaces. Consequently, this assembly can be degraded into nanoparticles (mean size 15 nm) at tumor microenvironment (at pH 5.0 with 10 mM GSH) through the breakage of Schiff base covalent bonds and the ligand exchange reaction of GSH thiols with ADA molecules on Au NP surfaces, thus presenting excellent pH- and GSH-responsive release of drugs. Accordingly, not only is the synthesis process very simple and high-yielding, but also the obtained hybrid spheres are natural and biocompatible. Moreover, the loaded drug is stable under normal physiological condition and quickly released in the tumor microenvironment.

2. EXPERIMENTAL SECTION

Materials. All chemicals were analytical grade and used as received. Diphenylalanine peptide (FF, 98%), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), sodium alginate, chlorauric acid (HAuCl4) and camptothecin (CPT, ≥98%) were purchased from Aladdin Chemical Reagent Co. Ltd. Sodium periodate (NaIO4) was obtained from Sinopharm Chemical Reagent Co., Ltd. Deionized water (17 MΩ cm) was used for all experiments.

Synthesis of FF–ADA–Au Hybrid Nanospheres (FHNS). ADA was obtained by typical periodate oxidation of sodium alginate.31 FHNS were synthesized using a one-pot method as follows: The ADA aqueous solution (4 mL, 10 mg/mL) was mixed with HAuCl4 (0.2 mL, 50 mM), and then mixed with lyophilized FF (15 mg) in 0.5 mL 1,1,1,3,3,3-hexafluoro-2-propanol, followed by incubation at 50 °C in the dark for 0.5 h. The solution changed from light yellow to dark purple, indicating Au+ ions were reduced to Au NPs and that FF was dialyzed against deionized water (200 mL) for 3 h to eliminate cytotoxic 1,1,1,3,3,3-hexafluoro-2-propanol and residual CPT. Dialysate was collected and unloaded-CPT was measured using a fluorescent spectrum (λex 364 nm; λem 440 nm; y = –0.00297 + 0.00870x; R² = 0.999936, analytical curve obtained during measurements).

Drug Loading of FHNS and Release in Vitro. The incorporation of CPT into FHNS was carried out as follows: CPT (5, 10, 15, 20 and 25 mg) was added to a suspension of FHNS (4.7 mL, 12.8 mg/mL) before dialysis purification, and then incubated at room temperature for 24 h. The CPT-loaded FHNS was dialyzed against deionized water (200 mL) for 3 h to eliminate cytotoxic 1,1,1,3,3,3-hexafluoro-2-propanol and residual CPT. Dialysate was collected and unloaded-CPT was measured using a fluorescent spectrum (λex 364 nm; λem 440 nm; y = –0.00297 + 0.00870x; R² = 0.999936, analytical curve obtained during measurements).

pH- and GSH-responsive release behavior of CPT-loaded FHNS was measured in PBS (pH 7.4 and 5.0) with or without 10 mM GSH. First, 2 mL of (10 mg/mL) CPT-loaded FHNS suspension was dialyzed against 200 mL of PBS in a water bath at 37 °C. Then, an aliquot of dialysate (1 mL) was withdrawn and 1 mL of blank solution was added into the dialysate to keep a constant volume. Released CPT was measured with the above-mentioned fluorescent spectrum.

Characterization. Product morphology was observed using transmission electron microscopy (TEM, Tecnai G2 20 TWIN) at an accelerating voltage of 200 kV and field-emission scanning microscopy (FESEM, Ultra 55) instrument at an accelerating voltage of 3.0 kV. The chemical composition and elemental distribution were characterized using high-resolution TEM (HRTEM, Philips XL30) and energy-dispersive X-ray spectroscopy (EDS) at an acceleration voltage of 200 kV. Fluorescent images were observed under a confocal laser scanning microscope (CLSM, Nikon, Japan). Dynamic light scattering (DLS) measurements were carried out in aqueous solutions to produce particle sizes and size distribution using Nano-ZS90 (Malvern).

FTIR spectra were scanned on a Nicolet Nexus 470 FTIR spectrometer with powder-pressed KBr pellets. Fluorescent emission spectra (FL) of samples in water were recorded using a PTI QM40 Spectrometer. X-ray photoelectron spectroscopy (XPS) spectra were performed with a VG ESCA-LAB220i-XL. Mass spectrometry was used to collect matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS, AB SCIEX 5800) in linear mode (c-cyano-4-hydroxycinnamic acid was used as matrix for sample ionization).

Cytotoxicity Assay. Human lung adenocarcinoma epithelial A549 cells were used to assess cytotoxicity of FHNS with a standard 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cells were split into 96-well plates using a standard tyrosinase-based technique. After 24 h, FHNS (0, 7, 20, 60, 200 μg/mL) was added wells and cocultured for 24 h. MTT (20 μL, 5 mg/mL) in PBS was added to all wells and incubated in 5% CO2 at 37 °C for 4 h. Then, culture medium with MTT was replaced with DMSO to dissolve formazan crystals. Viable cells were counted at 490 nm on a microplate reader (SUNOSTIK, SPR-960).

CPT-loaded FHNS cytotoxicity was assessed using free CPT as control. Cells were added to 96-well plates and cultured for 12 and 24 h, and then free CPT or CPT-loaded FHNS were added. Cytotoxicity was assayed as described.

3. RESULTS AND DISCUSSION

Synthesis and Analysis of FHNS. Polysaccharides such as glucan and carboxylic Curdlan have been used as both reducing and stabilizing agent to synthesize Au NPs.32,33 Our control experiment indicates that ADA can also be used to reduce Au+ ions into Au NPs with diameters of ca. 10 nm and 15–25 nm according to TEM and dynamic light scattering, respectively (Figure S1). Thus, when FF, ADA and HAuCl4 are mixed, Schiff base covalent bonds are formed when aldehyde groups of ADA react with amino groups of FF, generating rattan flower-like ADA–nFF units, as shown in Scheme 1. Then, Au+ ions are in situ reduced to Au NPs by ADA, directly forming FF–ADA–Au hybrid nanospheres (FHNS) in a “one-pot process” through spacer-driven π–π stacking within or between ADA–nFF chains.34 The trick of introducing Au NPs into the system is to endow the spheres GSH-responsive properties through the ligand exchange reaction of GSH thiols with ADA molecules on Au NP surfaces.

Figure 1a,c shows hybrid spheres with an average diameter of 200 nm and a highly negative charged surface (−48 mV, Figure S2) that ensures stability of FHNS in aqueous solutions. Figure 1b shows that FHNS are assembled by smaller nanoparticles and units. When FHNS are incubated with BSA, little changes in mean size were observed (Figure S2), indicating that the hybrid spheres can repel nonspecific protein adsorption due to highly hydrophilic surfaces.35 Energy-dispersive X-ray spectroscopy (EDS) and TEM elemental mappings (Figure 1d–i) indicate that FHNS contain C, O, N, Au. Among them, Au, and N from FF, are located inside the hybrid spheres. C and O are homogeneously distributed not only within each sphere, but also around the sphere surface, confirming the existence of ADA with amount of COO− groups on the sphere surfaces, consequently causing low ζ-potential.

Figure 2A shows Fourier transform infrared (FTIR) spectra of ADA, FF and FHNS. After assembly into FHNS, a peak at

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1733 cm\(^{-1}\) for the aldehyde symmetric vibration of ADA disappears due to the formation of C\(\equiv\)N bonds, and a peak at 3264 cm\(^{-1}\) for the stretching vibration of FF is weakened, which may contribute to decreases in \(-\text{NH}_2\) groups due to formation of C\(\equiv\)N bonds.\(^{36}\) Although the weak absorption of C\(\equiv\)N stretching at 1633 cm\(^{-1}\) is masked by a typical amino group, it causes the peak of amino I at 1607 cm\(^{-1}\) in FF to shift to 1619 cm\(^{-1}\) in FHNS. In addition, the intensity at 2934 cm\(^{-1}\) in FHNS derived from C\(-\text{H}\) stretching of the Schiff base bond also dramatically increases. Moreover, the formation of Schiff base bonds can be confirmed with X-ray photoelectron spectra (XPS) in Figure 2B, in which the peak at 398.8 eV is attributed to C\(\equiv\)N groups, and the peak at 399.9 eV belongs to O\(-\text{C}\)\(\equiv\)N groups.\(^{36}\)

To understand the molecular arrangement during self-assembly, fluorescent spectra were recorded (Figure 2C). The emission spectrum of FF shows a peak centered at 306 nm, whereas the emission peak of ADA-FF assembly is near 400 nm. The significant red shift from 306 to 400 nm suggests effective \(\pi-\pi\) stacking between the aromatic groups of FF molecules, and indicates that FF may use J-aggregate arrangements during self-assembly after Schiff base reactions.\(^{36,37}\) Another emission peak at 460 nm can be ascribed to the formation of C\(\equiv\)N bonds, which are enhanced due to the presence of Au NPs in the FHNS.

Molecular weight change during the formation of FHNS was identified with MALDI-TOF mass spectrometry (Figure S3). The molecular weight increased from 50 to 120 kDa, indicating that FF was gradually conjugating onto ADA chains via self-assembly of FF within or between ADA chains by way of \(\pi-\pi\) stacking and hydrogen bonding, forming FF-based hybrid nanospheres. FHNS also was autofluorescent due to n\(-\pi^*\) transitions of C\(\equiv\)N bonds in the Schiff base, which was confirmed with fluorescent emission spectra (FL) and confocal laser scanning microscopy (CLSM). When FHNS were excited with a 405 nm laser, multiwave autofluorescence of the FHNS in three bands, blue (430–480 nm), green (500–550 nm) and red (590–640 nm) was observed (Figure S4). This suggests that FHNS can avoid interference due to external addition of fluorochromes.\(^{24,38}\)

**pH- and GSH-Responsive Degradation of FHNS.** The ideal nano drug delivery systems can be not only controlled for drug release in a specific tumor microenvironment but also biodegraded or eliminated easily in vivo.\(^{39}\) Figure 3b–d illustrates the changing morphology of FHNS triggered by pH and GSH. After incubation at pH = 5.0 for 24 h, the mean diameter of FHNS decreases from 200 to 100 nm (Figure S5), due to Schiff base bond breakage (Figure 3a). The assembly is further degraded into nanoparticles (mean size 15 nm) in the presence of 10 mM GSH after 8 h through a ligand exchange reaction of GSH thiol with ADA molecules on Au NP surfaces due to the special aurophilic interaction between Au and GSH thiol groups.\(^{40–43}\)
This pH- and GSH-responsive biodegradation mechanism can be further confirmed by analyzing dissembled samples with MALDI-TOF mass and XPS. The molecular weight decreased from 120 kDa to 30–50 kDa after disassembly, indicating cleavage of a Schiff base bond at pH 5.0 and the presence of Au(I) in XPS validated the formation of covalent bonds of Au–SH (Figure S6).

**In Vitro Stimuli-Responsive Drug Release and Cell Assay.** Most anticancer drugs currently used are hydrophobic, may not cross aqueous environments (e.g., body and tissue fluids) surrounding cells and penetrate cell membranes to reach intracellular targets. And their tendency to aggregate after intravenous administration can cause complications such as emboli or local toxicity. Therefore, it is important to encapsulate hydrophobic drugs in hydrophilic carriers.

We selected CPT, a typical chemotherapeutic, for the model drug. Although CPT has poor aqueous solubility as well as poor solubility in many organic solvents such as chloroform and ethanol, it is fortunately soluble in 1,1,1,3,3,3-hexafluoro-2-propanol which is used as the reaction media for synthesis of FHNS here. This allowed CPT to be added into a suspension of FHNS in aqueous alcohol and incubated at room temperature for 24 h, and then encapsulated in FHNS. Encapsulation was monitored by comparing spectra of free CPT and CPT-loaded FHNS (Figure 4a,b). Note that the encapsulated CPT increases linearly with the increasing CPT concentration and loading efficiency can exceed 95%, which may be due to strong hydrophobic–hydrophobic effects between CPT and FF. Moreover, the mean size of FHNS after being loaded with CPT was negligibly increased (Figure 4c), and the ζ-potential decreased little (ζ-potential = −48.7 mV, Figure S2), indicating stability after drug encapsulation.

Considering that the pH of the circulation is ∼7.4 and subcellular tumor cell compartments such as endosomes and lysosomes are acidic (ca. pH 5.0) with high concentrations of GSH (10 mM), we investigated kinetic release of CPT-loaded FHNS at 37 °C in different release media: (■) pH 7.4 PBS, (▼) pH 7.4 PBS + 10 mM GSH, (▲) pH 5.0 PBS, (◆) pH 5.0 PBS + 10 mM GSH.

This pH- and GSH-responsive biodegradation mechanism can be further confirmed by analyzing disassembled samples with MALDI-TOF mass and XPS. The molecular weight decreased from 120 kDa to 30–50 kDa after disassembly, indicating cleavage of a Schiff base bond at pH 5.0 and the presence of Au(I) in XPS validated the formation of covalent bonds of Au–SH (Figure S6).
and FF in the FHNS can be broken at pH 5.0, and the ligand exchange reaction of GSH thiols with ADA molecules on Au NP surfaces can further disassemble the FHNS, thus presenting excellent pH- and GSH-responsive release of drugs (Figure 3). Such release traits are promising for intracellular drug delivery, because the loaded drugs can remain in nanocarriers without leakage at the bloodstream pH (pH = 7.4) and low concentration of GSH (<0.01 mM), but be quickly released once the nanocarriers meet higher GSH concentration and acidic environments, e.g., the lysosomal compartments of tumor.

The cytotoxicity of blank FHNS and CPT-loaded FHNS in A549 cancer cells was assayed using the MTT cell viability. The blank nanocarriers have negligible influence on cell viability (Figure 5a), suggesting biocompatibility of FHNS even at 600 μg/mL. When the CPT-loaded FHNS with different concentrations are cultured with cancer cells, the cell viability notably declines from 60% to 20% as the CPT concentration increases from 7 to 200 ng/mL after incubation for 24 h (Figure 5b), indicating the concentration-dependent cytotoxicity. As the culture time extends, for example, at 60 ng/mL of CPT, the cell viability is obviously reduced from 75% (12 h) to 25% (24 h), indicating the time-dependent cytotoxicity. In control experiments, the cells cultured with the equivalent dose of free CPT have higher viability than those incubated with CPT-loaded FHNS, indicating the low cytotoxicity and efficiency of free hydrophobic drugs.

The enhanced cytotoxicity of CPT-loaded FHNS can be attributed to high hydrophilicity of nanocarriers and excellent affinity of polysaccharide, i.e., ADA in the FHNS for cell uptake, which can be further validated with CLSM images. After incubation with CPT-loaded FHNS at 37 °C for 8 h, live cells lacking staining are imaged with confocal microscopy exited with a 405 nm laser. Figure 6a,b shows brightly luminescent particles in the cell interior, which can be seen in the overlay of confocal luminescence and bright-field images (Figure 6c). Additionally, almost no luminescent signal beyond the cell is observed. Thus, CPT-loaded FHNS have been completely taken up by cells. These results display that the FHNS have a great potential for hydrophobic drug delivery to cross the aqueous environments and penetrate into cells increasing cancer cell death.

4. CONCLUSIONS

In summary, we synthesized a novel pH- and GSH-dually responsive diphenylalanine-based hybrid nanosphere using a one-pot method with ADA as a cross-linker to induce assembly of diphenylalanine from π-π stacking and as an in situ reducer of encapsulated Au3+ ions into Au NPs. These biocompatible hybrid nanospheres can encapsulate as much as 95% of hydrophobic chemotherapeutic drugs such as CPT and they are stable under normal physiological condition, but can be triggered for quick release of almost 100% of CPT at pH 5.0 and 10 mM GSH which mimic the tumor microenvironment through the breakage of Schiff base covalent bonds between ADA and FF, and the ligand exchange reaction of GSH thiols with ADA molecules on Au NP surfaces. The CPT-loaded hybrid nanospheres can be readily taken up by A549 cancer cells to offer greater higher killing capacity than free drugs even at very low drug concentrations. All these results show that the hybrid nanosphere we present here are very promising as drug loading and delivery systems, which may promote controllable and highly efficient drug delivery systems for real practical application. The idea and one-pot process we present here, using natural ADA as both the cross-linker for the formation of Schiff base covalent bonds and the inducer for the in situ reduction of Au3+ ions into Au NPs, may also be extended for synthesis of other responsive colloidal spheres and drug delivery systems.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemmater.6b02604.

Size-distribution, ζ-potential, MALDI-TOF mass patterns, CLSM images, FLS and XPS of FHNS under different conditions (PDF).

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