Reactive & Functional Polymers 72 (2012) 198–205

Contents lists available at SciVerse ScienceDirect

Reactive & Functional Polymers

journal homepage: www.elsevier.com/locate/react

Using a bifunctional polymer for the functionalization of Fe_3O_4 nanoparticles

Quan Zhang [a], Liang Luan [a], Siliang Feng [a], Husheng Yan [b,*], Keliang Liu [a,*]

[a] Beijing Institute of Pharmacology & Toxicology, Beijing 100850, China
[b] Key Laboratory of Functional Polymer Materials, Ministry of Education, Institute of Polymer Chemistry, Nankai University, Tianjin 300071, China

ARTICLE INFO

Article history:
Received 19 October 2011
Received in revised form 11 January 2012
Accepted 12 January 2012
Available online 20 January 2012

Keywords:
Bifunctional polymer
Functionalization of nanoparticles
Fe_3O_4
Nuclear delivery
Tat peptide

ABSTRACT

A bifunctional maleimido-tetra(ethylene glycol)-poly(glycerol monoacrylate) (MAL-TEG-PGA) polymer was synthesized and used as a linker to couple functional biomolecules to iron oxide nanoparticles. The cell-penetrating peptide Tat was chosen as a model ligand and successfully conjugated to the surface of Fe_3O_4 nanoparticles using MAL-TEG-PGA. The Tat-conjugated Fe_3O_4 nanoparticles can be prepared simply by applying the linker to the iron oxide nanoparticles and then coupling the Tat peptide to the maleimide terminus or by coating the nanoparticles with a pre-coupled linker. Cell-uptake studies demonstrated that the Tat peptide was an efficient functional biomolecule to translocate iron oxide nanoparticles into the cell nucleus. Tat-conjugated nanoparticles thus prepared may be useful for drug or gene delivery.

1. Introduction

Magnetic iron oxide nanoparticles (MIONs) have attracted a great deal of attention for their potential applications in biomedical fields such as cellular labeling, tracking, imaging, drug delivery, and tumor treatment using hyperthermia [1–4]. For these applications, the ability to disperse the nanoparticles in water and to modify their surfaces with functional biomolecules (e.g. for targeting and therapeutics) is crucial to their widespread use. The stabilization of MIONs in aqueous solution can be achieved by modifying their surface with small molecular surfactants or polymers [5]. The biocompatibility and toxicity of MIONs are important criteria for their biomedical application. Thus, MIONs are often coated with biocompatible and functional polymers to protect the iron oxide core from agglomeration, to provide chemical handles for conjugation with biomolecules, and to reduce non-specific cell interactions. A large number of biocompatible polymers that might perform these coating functions have been investigated, including natural polymers (dextran [6,7], chitosan [8], starch [9], and gelatin [10], etc.) and synthetic polymers (polyethylene glycol [11,12], polyacrylic acid [13], and other copolymers [14–16], etc.).

Coating MIONs with biocompatible polymers followed by modification with biomolecules improves detection abilities and specific binding to target cells. Many strategies have been developed to conjugate functional biomolecules to the polymeric coatings on MION surfaces. These techniques are a prerequisite for enhancing the functionalized properties of MIONs [17] and can be categorized as either covalent linkage strategies [18] or physical interactions [19]. Compared with physical interactions, covalent linkage strategies are more effective due to their low sensitivity to environmental conditions and high control over the molecular orientation of bound ligands. A number of functional biomolecules have been covalently conjugated to the polymeric coatings on MIONs surfaces, including antibodies [20,21], proteins [22], peptides [23,24], and other targeting ligands [25]. Covalent linkage chemistry techniques include direct nanoparticle conjugation and linker strategies.

Direct conjugation methods are not the preferred approach for the attachment of biomolecules. MIONs may crosslink when a biomolecule, such as a peptide or protein, contains multiple amino functional groups. Many biomolecules are not natively reactive with nanoparticles and require initial modification prior to conjugation, but this modification often leads to a loss of bioactivity [26]. Linker strategies typically involve complicated multi-step synthesis and modification procedures because functionalization requires stepwise MION modification to attach a functional ligand to the surface [23,27–29]. Therefore, the use of a polymer linker that coordinates strongly to the iron oxide surface and also conjugates directly with biomolecules at the desired location would simplify the functionalization process and allow biomolecules immobilized on the nanoparticles to retain their functional characteristics to a large extent.

Here we report the development of a novel bifunctional polymer anchored to the nanoparticle surface via anchoring groups at one end and covalently linked to the functional biomolecule at

* Corresponding authors. Tel./fax: +86 10 68211656.
E-mail addresses: yanhs@nankai.edu.cn (H. Yan), keliangliu55@126.com (K. Liu).

1381-5148/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.reactfunctpolym.2012.01.003

the other end. Poly(glycerol monoacrylate) (PGA) was used as the terminal anchoring block because it has been shown to coordinate tightly to the surface of Fe_3O_4 nanoparticles through its 1,2-diol groups [30,31]. A maleimide at the other terminus can be coupled to thiol-containing ligands such as peptides with cysteine residues. Functionalized iron oxide nanoparticles can be prepared by applying the linker to the iron oxide nanoparticles and then coupling the ligand to the maleimide terminus or by coating nanoparticles with a pre-coupled linker. To demonstrate the effectiveness of the surface modification, we coupled the widely used Tat cell-penetrating peptide to iron oxide nanoparticles. The Tat peptide is a sequence (residues 48–57) derived from the HIV-1 Tat protein that confers the ability to translocate across the plasma membrane [32,33]. A variety of cargoes have been transported using the Tat peptide, including polymers [34], liposomes [35], and nanoparticles [36,37]. The Tat peptide sequence used in the current study was *GRKKRRQRRR*GCG (the italicized amino acids correspond to residues 48–57 of the HIV-1 Tat protein), which features a cysteine residue suitable for conjugation with the linker maleimide. Iron oxide nanoparticles modified with the Tat peptide were efficiently taken up into the cell nucleus.

2. Experimental

2.1. Materials

Solketal (2,2-dimethyl-1,3-dioxane-4-methanol, 97%, Acros Organics), 2-bromoisobutyryl bromide (97%, Acros Organics), 1,1,4,7,7-pentamethyldiethylenetriamine (PMDETA, 98%, Alfa Aesar) and anisole (99%, Alfa Aesar) were used as received. Solketal acrylate (SA) was synthesized as previously described [38]. Cuprous bromide (CuBr, AR, Shanghai National Drug Chemical Plant, China) was purified by overnight treatment with glacial acetic acid followed by washing with absolute ethanol and ethyl ether and overnight drying under vacuum at room temperature. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 without phenol red, and fetal bovine serum (FBS) were purchased from GIBCO (Uxbridge, UK). HeLa cell lines were obtained from the Cell Resource Center (IBMS, CAMS/PUMC). All other chemicals were commercially available and used without further purification.

2.2. Synthesis of the Tat peptide

A modified Tat peptide containing the translocation sequence of the HIV-1 Tat protein was synthesized on a solid-support (Rink amide MBHA resin) using Fmoc chemistry and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/*N*-hydroxybenzotriazole (HOBt) as an activating agent. The sequence of the peptide is *Gly-Arg-(Lys)$_2$-(Arg)$_2$-Gln-(Arg)$_3$*-Gly-Cys-Gly-NH_2 (the italicized amino acids correspond to residues 48–57 of the HIV-1 Tat protein). The peptide was cleaved using trifluoroacetic acid/thioanisole/ethanedithiol/anisole (90/5/3/2) and purified using C18 reversed-phase HPLC. MALDI-TOF MS [M+H$^+$]: 1655.9 (calc.), 1655.0 (found). For fluorescein isothiocyanate (FITC)-labeled Tat peptides, the peptide sequence is FITC-NH-$(CH_2)_5$-*Gly-Arg-(Lys)$_2$-(Arg)$_2$-Gln-(Arg)$_3$*-Gly-Cys-Gly-NH_2. MALDI-TOF MS [M+H$^+$]: 2115.4 (calc.), 2114.7 (found).

2.3. Synthesis of MAL-TEG-PGA (Scheme 1)

Initiator **1** (0.20 g, 0.41 mmol), CuBr (58 mg, 0.41 mmol), SA (3.04 g, 16.3 mmol), and anisole (1.5 mL) were placed in a Schlenk flask. The flask was evacuated and purged with argon for three cycles. PMDETA (87 μL, 0.41 mmol) was added using a syringe that had been purged with argon. The flask was immersed in an oil bath preheated to 65 °C. The polymerization was allowed to proceed for 3 h under stirring. The resulting mixture was diluted with 20 mL of THF and filtered through a column packed with neutral alumina to remove the catalyst. The filtrate was concentrated and precipitated into petroleum ether to give polymer **2**. A solution of **2** (2.0 g) in toluene (20 mL) was heated to reflux. After 7 h, the solvent was removed under reduced pressure to give **3**. Polymer **3** was dissolved in 1.0 M HCl/dioxane (1:3, v/v), and the reaction mixture was stirred at room temperature for 24 h. The product was dialyzed (MWCO 3500) against deionized water and then lyophilized to obtain polymer **4** (MAL-TEG-PGA).

2.4. Synthesis of Tat-TEG-PGA

MAL-TEG-PGA (0.5 g, 0.11 mmol) was dissolved in deionized water, and the Tat peptide (0.23 g, 0.14 mmol) was added under stirring. The reaction mixture was stirred at room temperature for 3 h, dialyzed (MWCO 3500) against deionized water, and lyophilized to obtain the Tat-TEG-PGA.

2.5. Preparation of Fe_3O_4-PGA-TEG-Tat

Path (1): MAL-TEG-PGA (0.12 g) was dissolved in 3.0 mL of deionized water. The solution was purged with argon to remove oxygen. After 30 min, $HClO_4$-stabilized ferrofluid (0.4 mL) was added with vigorous stirring, and the reaction mixture was stirred at room temperature overnight. Fe_3O_4-PGA-TEG-MAL was magnetically recovered and washed three times with deionized water. The Tat peptide (2 mg, 0.0012 mmol) was added to 3 mL of the aqueous dispersion of Fe_3O_4-PGA-TEG-MAL. After sonication for 30 min, the reaction mixture was stirred at room temperature overnight. The polymer-coated nanoparticles were separated with a magnet and washed three times with deionized water. Finally, Fe_3O_4-PGA-TEG-Tat was dispersed in deionized water after being sonicated for 20 min. Path (2): Tat-TEG-PGA (0.12 g) was dissolved in 3.0 mL of deionized water. The pH of the solution was adjusted to ~3 with hydrochloric acid. The solution was purged with argon to remove oxygen. $HClO_4$-stabilized ferrofluid (0.4 mL) was added with vigorous stirring. After sonication for 30 min, the reaction mixture was stirred at room temperature overnight. The polymer-coated nanoparticles were separated with a magnet and washed three times with deionized water. Finally, Fe_3O_4-PGA-TEG-Tat was dispersed in deionized water after being sonicated for 20 min.

2.6. Cell culture

HeLa cells were cultured in DMEM containing 10% FBS and supplemented with penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}) and nonessential amino acids (0.1 mM). The culture was maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.7. Cell uptake of nanoparticles

HeLa cells were seeded in 6-well plates on glass coverslips at a density of 5×10^3 cells/well. After 24 h, nanoparticles at a final iron concentration of 100 μg mL^{-1} were co-cultured with cells at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were washed three times with phosphate-buffered saline (PBS, pH 7.4) at pre-set intervals and subsequently fixed with 4.0% formaldehyde at room temperature for 15 min. The formaldehyde solution was discarded, and the cells were washed twice with 2 mL of PBS. The fixed cells were incubated with 2% potassium ferrocyanide and 6% hydrochloric acid (1:1) for 30 min and counterstained with nuclear fast red. The coverslips were washed twice with PBS