Macromolecular Research, Vol. 17, No. 4, pp 259-264 (2009)

Bioconjugation of Poly(poly(ethylene glycol) methacrylate)-Coated Iron Oxide Magnetic Nanoparticles for Magnetic Capture of Target Proteins

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Received August 16, 2008; Revised September 10, 2008; Accepted September 18, 2008

Abstract: Chemical modification of magnetic nanoparticles (MNPs) with functional polymers has recently gained a great deal of attention because of the potential application of MNPs to *in vivo* and *in vitro* biotechnology. The potential use of MNPs as capturing agents and sensitive biosensors has been intensively investigated because MNPs exhibit good separation-capability and binding-specificity for biomolecules after suitable surface functionalization processes. In this work, we demonstrate an efficient method for the surface modification of MNPs, by combining surface-initiated polymerization and the subsequent conjugation of the biologically active molecules. The polymeric shells of non-biofouling poly(poly(ethylene glycol) methacrylate) (pPEGMA) were introduced onto the surface of MNPs by surface-initiated, atom transfer radical polymerization (SI-ATRP). With biotin as a model of biologically active compounds, the polymeric shells underwent successful post-functionalization via activation of the polymeric shells and bioconjugation of biotin. The resulting MNP hybrids showed a biospecific binding property for streptavidin and could be separated by magnet capture.

Keywords: magnetic nanoparticles, atom transfer radical polymerization, biological applications of polymers.

Introduction

Magnetic nanoparticles (MNPs) have gained a great deal of attention because of their potential applications in biotechnology, including magnetic separation,[1] sensing,[2] drug delivery,[3] hyperthermia,[4] and magnetic resonance imaging (MRI) contrast enhancement.[5] Among various applications, the use of MNPs for rapid detection and separation of target proteins and pathogens has been attempted in order to develop an efficient immunoassay method, because MNPs have an advantage of good separation-capability; the good separation-capability of MNPs could make MNPs one of the promising candidates for sensitive biosensors.[6] However, there are problems to be solved for practically applying MNPs to biomedical areas, such as agglomeration and short shelf-life. Nanoparticles, including MNPs, tend to agglomerate into large clusters, and plasma proteins are easily adsorbed onto them due to high surface area/volume ratio; nanoparticles lose their desirable properties and are quickly sequestered by cells of the reticular endothelial system, such as macrophages, when they agglomerate.[4b,7]

Much effort has been made to modify the surface of MNPs by using molecular,[8] polymeric,[9] and inorganic layers in the aim of minimizing agglomeration of MNPs.[10] These layers also could endow MNPs with additional functions/properties in addition to minimizing agglomeration.[11] For example, silica-coated MNPs showed target specificity after antibody conjugation and passivation with oligo(ethylene glycol) via amide bond-forming reaction.[12] Along with silica layers, passive coating with functional polymers has been used for coating MNPs. Various monomers have been employed for polymerization from MNPs, such as styrene,[13] (dimethylamino)ethyl methacrylate,[14] methyl methacrylate,[15] *N*-isopropyl acrylamide,[16] *N*-hydroxysuccinimide methacrylate,[17] hydroxyethyl methacrylate,[18] and poly(ethylene glycol) methacrylate (PEGMA).[19] Especially, coating of MNPs with poly(PEGMA) (pPEGMA) was reported to reduce the MNP uptake of macrophage cells, indicating good biocompatibility as well as non-biofouling property.[19]

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Poly(ethylene glycol) (PEG) is a well-known and widely-used non-biofouling material for passive coating.[20] Methods for coating MNPs with PEG-based materials are generally categorized as "grafting-onto" and "grafting-from" approaches. In the "grafting-onto" approach, polymers are immobilized directly onto surfaces by physicochemical interactions.[21] For example, a random copolymer of 3-(trimethoxysilyl)propyl methacrylate and poly(ethylene glycol) methyl ether methacrylate (PEGMEMA) was directly grafted onto MNPs.[5c,22] In the "grafting-from" approach—surface-initiated polymerization (SIP), polymers are grown directly from the surface of substrates. PEGMA and PEGMEMA have successfully been used as monomers for PEG-based coating of MNPs via "grafting-from" approach.[19] Surface-initiated polymerization proves advantageous over other grafting methods in the aspect of chemical controllability, because it is capable of controlling thickness (by monomer-concentration and reaction time), grafting density (by initiator-density), and chemical composition of layers.[23] In addition to the PEG-based passive coating that gives MNPs non-biofouling property, the introduction of biologically active moieties onto MNPs is required for their wider applications, such as target-oriented delivery, detection and separation of targets, and biosensors. As far as we know, there has been only one previous report on biologically active molecule-attached, PEG-based layers of MNPs.[24] The functionalization procedures involved multiple, step-wise reactions: 3-(aminopropyl)triethoxysilane coating of MNPs, "grafting-onto" of acryloylpoly(ethylene glycol)-*N*-hydroxysuccinimide via amide bond-forming reaction, and conjugation of a cysteine-containing peptide via Michael addition. While successful, this method introduced a limited number of molecules on surfaces, one molecule per polymer chain, at maximum.[25] For some applications, it is needed to increase the number of bioconjugation sites and to maximize the number of introduced molecules.[26a] In this paper, we demonstrated the conjugation of biologically active molecules onto pPEGMA-coated MNPs in the aim of giving specific binding and magnetic capturing properties to the non-biofouling pPEGMA-coated MNPs.

Experimental

Materials. *N,N'*-Dimethylformamide (DMF, 99.9+%, Aldrich), 2-(3,4-dihydroxyphenyl)ethylamine hydrochloride (dopamine hydrochloride, 98%, Aldrich), triethylamine (99.5%, Fluka), 2-bromo-2-methylpropionyl bromide (98%, Aldrich), copper(I) bromide (CuBr, 99.999%, Aldrich), 2,2'-dipyridyl (99+%, Aldrich), *N,N'*-disuccinimidyl carbonate (DSC, Aldrich), 4-(dimethylamino)pyridine (DMAP, 98%, Fluka), phosphate-buffered saline (PBS, Sigma), tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin (Pierce), 5-(biotinamido)pentylamine (Pierce), 2-aminoethanol (99+%, Aldrich) and methanol (HPLC grade, Merck) were used as received. Poly(ethylene glycol) methacrylate (PEGMA, M_n =360, Aldrich) was used after removing the inhibitor by column chromatography with basic alumina. Magnetic nanoparticles (MNPs, Fe_3O_4, average size: 20 nm) were purchased from Aldrich. Ultrapure water (18.3 MΩ·cm) from the Human Ultra Pure System (Human Corp., Korea) was used.

Instrumentation. FTIR spectra were recorded using NEXUS (ThermoNicolet); 256 scans were averaged to yield the spectra at a resolution of 4 cm^{-1}. Transmission electron microscopy (TEM) images were obtained with CM20 (Philips). The thermal characteristics of pPEGMA-coated MNPs were measured by thermogravimetric analysis (TA instruments, TGA 2050) at a heating rate of 10 °C/min under nitrogen atmosphere. Optical and Fluorescence images were acquired on an IX 71 fluorescence microscope (Olympus, Japan).

Introduction of the Polymerization Initiator onto MNPs. To DMF (5 mL) were added MNPs (50 mg) and dopamine hydrochloride (0.5 g), and the mixture was sonicated for 1 h at room temperature. MNPs were collected by a magnet after immobilization of dopamine hydrochloride. The resulting products were washed with DMF five times and dispersed in DMF (5 mL). To a round-bottom flask containing dopamine-coated MNPs was added triethylamine (0.73 mL), and the mixture was stirred for 1 h at room temperature. 2-Bromo-2-methylpropionyl bromide was then added slowly to the mixture, followed by stirring for 2 h at room temperature. MNPs were collected by a magnet, washed with methanol five times, and dried under reduced pressure.

Surface-Initiated, Atom Transfer Radical Polymerization (SI-ATRP): Formation of pPEGMA-Coated MNPs. The initiator-immobilized MNPs (5 mg) were placed in a Schlenk flask, and the flask was degassed under vacuum and purged with argon. CuBr (7 mg) and 2,2'-dipyridyl (15 mg) were added to another Schlenk flask, and the mixture was degassed under vacuum and purged with argon. Degassed water (3.4 mL) and purified PEGMA (1.6 mL) were added to the Schlenk flask containing the catalysts. The resulting solution was transferred by a syringe to the Schlenk flask containing the initiator-immobilized MNPs. The mixture was sonicated for 30 s and stirred for 1 h at room temperature. pPEGMA-coated MNPs were separated from the suspension by a magnet and washed several times with methanol.

Conjugation of Biotin. The terminal hydroxyl groups of pPEGMA were activated with DSC for the conjugation of pPEGMA-coated MNPs with ligands. The pPEGMA-coated MNPs (1.2 mg) dispersed in DMF (0.5 mL) were placed in a vial, to which a DMF solution (0.5 mL) of DSC (0.1 M) and DMAP (0.1 M) was added. The activation reaction was carried out for 3 h at room temperature. The resulting nanoparticles were separated by a magnet, washed with DMF, and re-suspended in a PBS solution of 5-(biotinamido)pentylamine (0.5 mM) and 2-aminoethanol (0.5 mM) for 3 h at

room temperature. After washing with PBS buffer several times, biotin-conjugated MNPs were incubated with a PBS solution of TRITC-conjugated streptavidin (0.01 mg/mL) at room temperature. After 1 h, the samples were collected by a magnet and washed with PBS several times. As a control experiment, pPEGMA-coated MNPs (the ones not presenting biotin) were incubated with a PBS solution of TRITC-conjugated streptavidin in the same manner.

Results and Discussion

In this work, the introduction of ligands onto pPEGMA-coated MNPs was achieved by selective activation of the terminal hydroxyl groups of the PEG chains with DSC and subsequent bioconjugation via amide bond-forming reaction (Figure 1).[26] We used dopamine as an anchoring moiety, because it had been known to tightly bind onto the surface of MNPs.[27] After immobilization of dopamine, the initiating part for SI-ATRP was attached by using simple S_N2 reaction between the terminal amine group of dopamine and 2-bromo-2-methylpropionyl bromide. The initiator-immobilized MNPs were characterized by FT-IR spectroscopy. New peaks were observed at 1487 ($-NH_3^+$ deformation) and 1540 cm^{-1} (amide band, NH bending) after immobilizations of dopamine and the initiator, respectively (Figures 2(b) and (c)).

The formation of pPEGMA shells on the MNPs was achieved by SI-ATRP. The SI-ATRP of PEGMA was carried out by stirring a mixture of initiator-immobilized MNPs, PEGMA, and catalysts for ATRP (CuBr and 2,2′-dipyridyl) for 1 h at room temperature. After SI-ATRP, the pPEGMA-coated MNPs were characterized by FT-IR spectroscopy, thermogravimetric analysis (TGA), and transmission electron microscopy (TEM). The IR spectrum of the pPEGMA-coated MNPs showed characteristic peaks at 2830-2980

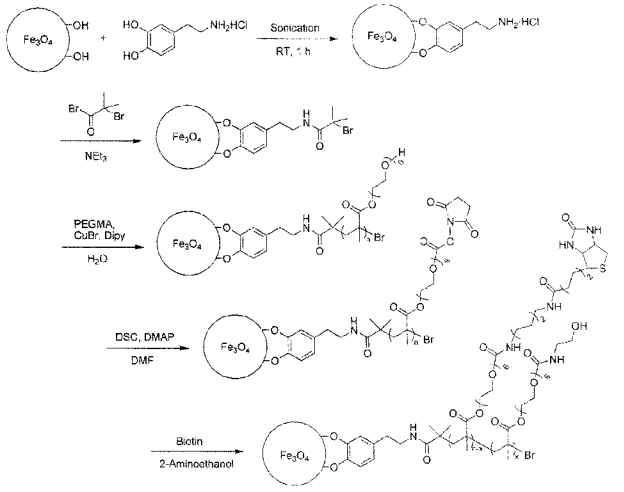

Figure 1. The procedure for formation of pPEGMA-coated MNPs and subsequent conjugation of biotin.

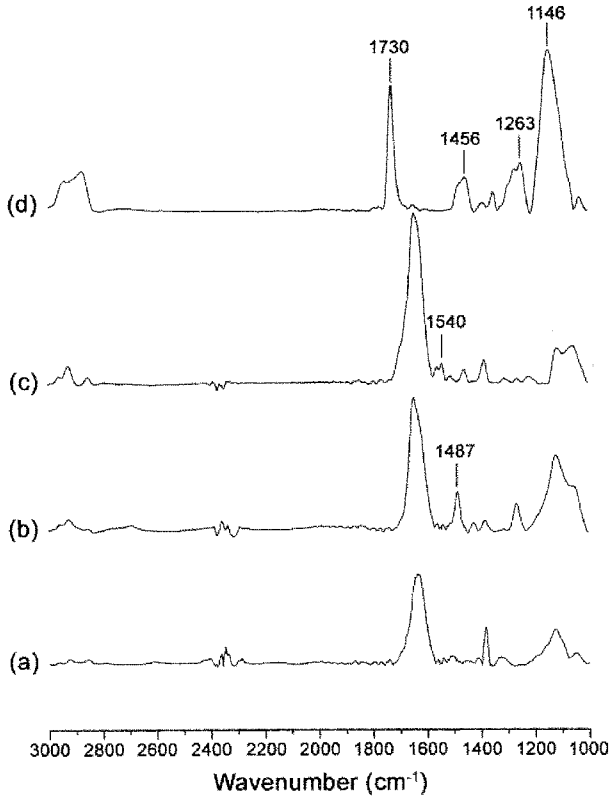

Figure 2. IR spectra of (a) MNPs, (b) dopamine-coated MNPs, (c) initiator-immobilized MNPs, and (d) pPEGMA-coated MNPs.

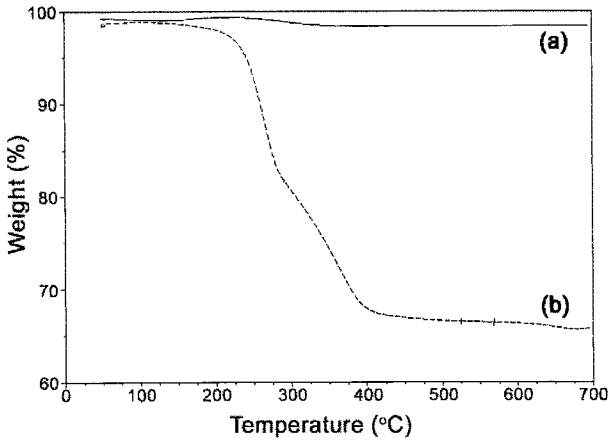

Figure 3. TGA curves of (a) MNPs and (b) pPEGMA-coated MNPs.

(C-H stretching), 1730 (C=O stretching), 1456 (methylene scissoring), 1263 (methylene wagging and methylene twisting-rocking), and 1146 cm^{-1} (C-O-C stretching), which had been absent in the IR spectrum of the initiator-immobilized MNPs (Figure 2(d)).[26a] The appearance of new characteristic peaks confirmed that the polymerization of PEGMA occurred from the MNPs. The presence and content of the pPEGMA layer on MNPs were further confirmed by TGA (Figure 3). TGA is a useful method for verification of the

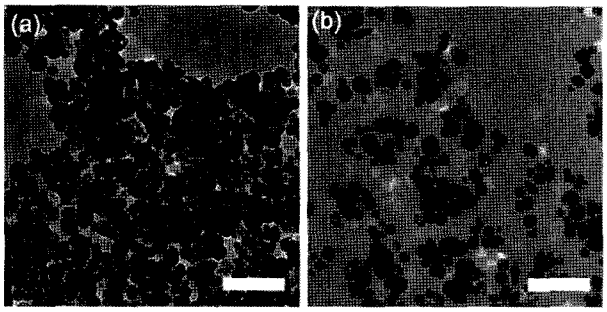

Figure 4. TEM images of (a) MNPs and (b) pPEGMA-coated MNPs (Scale bar: 200 nm).

organic contents, because the thermal degradation of organic contents is usually completed at around 300 °C.[28] In this work, TGA studies were performed for bare MNPs and pPEGMA-coated MNPs. The TGA curve of bare MNPs showed a negligible weight loss after heating up to 700 °C. On the other hand, in the case of pPEGMA-coated MNPs, we observed about 34%-weight loss, indicating that the weight portion of pPEGMA was 34%. There were two weight-loss steps at 283 and 393 °C in the TGA curve. This two-stage weight loss might have resulted from separated elimination for side chains and main chains of polymers,[19] which has often been observed for surface-grafted polymers.[28,29] The TEM images of MNPs and pPEGMA-coated MNPs are shown in Figure 4. Bare MNPs were observed as aggregates over several hundred nanometers, while pPEGMA-coated MNPs were observed as a well-dispersed form. The increased dispersability indicated that the formed pPEGMA layer endowed MNPs well-solubility and stability in the solvents.[30]

The bioconjugation of pPEGMA-coated MNPs was confirmed by FT-IR spectroscopy. The activation of the hydroxyl groups was validated by intense peaks at 1813 and 1789 cm^{-1} (C=O stretching of the NHS group) (Figure 5(a)). After

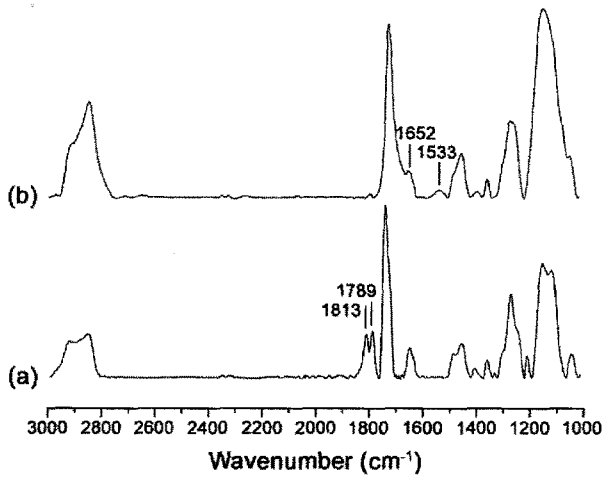

Figure 5. IR spectra of (a) DSC-activated MNPs and (b) biotin-conjugated MNPs.

confirmation of successful activation, subsequent conjugation of activated pPEGMA-coated MNPs with biologically active molecules was investigated. Biotin was used as a model of the biologically active molecules, because biotin has strong and specific interactions with streptavidin (K_D= 10^{-15} M),[31] and the attachment of biotin is easily visualized by fluorescent dye-conjugated streptavidin. A biotin compound bearing a primary amine group, 5-(biotinamido)pentylamine, was conjugated onto the activated pPEGMA shells via amide bond-forming reaction. The appearance of two new IR peaks at 1652 and 1533 cm^{-1} (amide bands) and the disappearance of two peaks from the NHS ester group (at 1813 and 1789 cm^{-1}) indicated that the biotin compounds were conjugated successfully (Figure 5(b)).

The successful introduction of biologically active moieties to non-biofouling pPEGMA-coated MNPs would make it possible to capture target proteins and separate them magnetically. The specific binding and magnetic capture properties of biotin-conjugated pPEGMA-coated MNPs were characterized by optical and fluorescence spectroscopy, after 1 h-incubation in a PBS solution of TRITC-conjugated streptavidin and magnetic capture with a magnet. Because streptavidin is tetrametic, the specific binding between biotin and streptavidin leads to the formation of networks/aggregates. In optical spectroscopy, complexes of streptavidin and biotin-conjugated MNPs were observed as an aggregated form (Figure 6(a)). In contrast, pPEGMA-coated MNPs were well-dispersed in agreement with the TEM image (Figure 6(c)). In the fluorescence micrographs, red fluores-

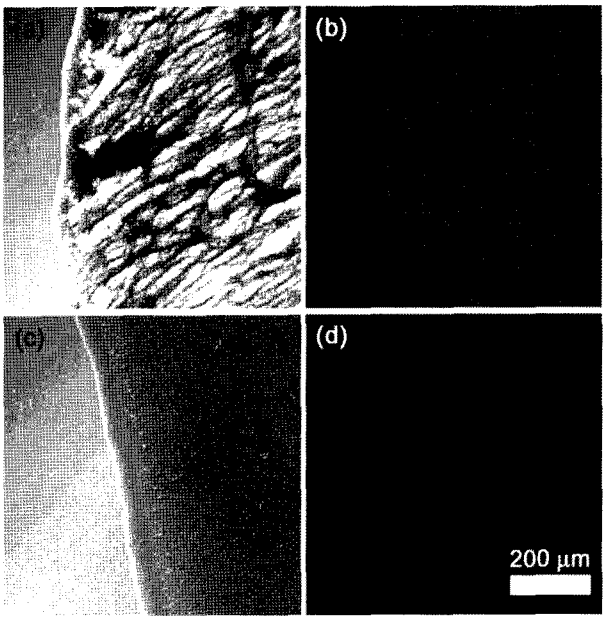

Figure 6. (a) Optical and (b) fluorescence micrographs of biotin-conjugated, pPEGMA-coated MNPs after complexation with TRITC-conjugated streptavidin. (c) Optical and (d) fluorescence micrographs of the control.

cence was detected only when the pPEGMA-coated MNPs were conjugated with biotin (Figures 6(b) and (d)). The results indicated that the pPEGMA shells themselves kept protein-resistant ability after the chemical functionalization, which made the biotin-conjugated pPEGMA shells possess biospecific and selective binding property for streptavidin.

Conclusions

In summary, the preparation and application of pPEGMA-coated MNPs were investigated by the combination of SI-ATRP and subsequent bioconjugation; the hydroxyl groups of the pPEGMA shell were activated by *N,N'*-disuccinimidyl carbonate, followed by conjugation with biotin. The specific binding and capturing properties for streptavidin were observed, when the pPEGMA shell was conjugated with biotin compounds. The method described herein suggested a simple but versatile method for attaching biologically active molecules onto the pPEGMA-coated MNPs, which, we believe, would lead to the generation of multifunctional biosensors that utilize ligand-attached, pPEGMA-coated MNPs. In other words, the pPEGMA-coated MNPs could serve as sensitive probes for capturing bio-entities, such as proteins and cells, with their biospecific binding ability given by suitable bioconjugation.

Acknowledgements. This work was supported by Kyungnam University Foundation Fund, 2008. K-BL gratefully acknowledges the financial support for this project provided by the KBSI grant (N28072).

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