Protein Immobilization on Aminated Poly(glycidyl methacrylate) Nanofibers as Polymeric Carriers

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Recently, protein carriers based on nanomaterials have been highlighted in diverse biological applications such as protein extraction, separation, and delivery due to their facile gravimetric sedimentation in the aqueous phase and abundant surface functionalities, which were used as anchoring sites for proteins. From this viewpoint, poly(glycidyl methacrylate) nanofibers (PGMA NFs) can be an excellent candidate for protein support because PGMA NFs possess the activated epoxide functional groups on the surface. In addition, cured PGMA NFs (PGMA−NH_2 NFs) reveal different surface functionalities such as primary amine groups. They can be linked with carboxylated proteins. Ferritin and streptavidin were selected as models of the pristine and biolinker-mediated proteins in this experiment and immobilized onto PGMA NFs and aminated PGMA−NH_2 NFs. The successful conjugations of ferritin and streptavidin were confirmed with transmission electron microscopy and fluorescein-isothiocyanate-tagged molecules. Protein immobilization using the pristine and the cured PGMA NFs could be considered as an outstanding protocol for facile protein delivery.

Introduction

There is considerable current interest in the design of protein carriers for practical purposes to fabricate soluble protein supports. From the viewpoint of polymeric materials for biomolecule supports, poly(glycidyl methacrylate) (PGMA) has attracted great attention in various biofields such as protein extraction, peptide separation, DNA carriers, and drug delivery systems since PGMA has activated epoxide groups in its polymer backbone;[1−8] such surface functional groups in pristine PGMA can be directly coupled with biomolecules via their ring opening reactions and further modified for a variety of biological applications. Also, the cured PGMA resins reveal different amine functionalities on their surfaces that provide binding sites to various organic or inorganic molecules.[9,10] In particular, PGMA nanomaterials are of great interest with respect to protein carriers due to their high surface area followed by abundant surface functionalities: epoxides, hydroxyl groups, amines, and so on. The high density of surface functionalities would enhance the capacity of protein immobilization.

To date, several reports on PGMA-functionalized materials as protein carriers have appeared in the literature.[11,12] Kodaka et al. reported the fabrication of biotinylated and pyruvate-kinase-immobilized carriers using bifunctional latex beads including hydroxy and epoxy groups.[11] Groth and co-workers announced the synthesis of PGMA-coated polystyrene nanoparticles using seeded polymerization.[12] The prepared particles were used to deliver endotoxin proteins. However, most of them were limited to micron size and spherical structures.

One-dimensional (1D) nanostructures have merits derived from their fascinating geometries: unique chemical/physical properties and various potential applications.[13−17] In particular, insoluble polymer nanofibers may allow the facile recovery of biomolecules such as protein and DNA, and their high surface area is capable of providing numerous sites for protein immobilization onto the nanofiber surface. Therefore, there is a growing demand for developing versatile 1D nanostructures. Herein, we report the fabrication of poly(glycidyl methacrylate) nanofibers (PGMA NFs) using a vapor-deposition-mediated initiator-impregnated template method.[18,19] This novel synthetic route gave rise to the fabrication of tailored functionalized vinyl polymer nanofibers. Moreover, the low-temperature curing agent made it possible to produce amino-functionalized polymer nanofibers. This epoxy-rich nanosupport seems to be the versatile system to develop very easy protocols for protein immobilization.

Experimental Section

Materials. Glycidyl methacrylate (GMA) monomer was purchased from Aldrich, and the monomethyl ether hydroquinone (MEHQ) inhibitor was removed using a MEHQ remover column. Anodic aluminum oxide (AAO) membrane with 100 nm pores was received from Whatman. Initiator azobisisobutyronitrile (AIBN) and curing agent diethylenetriamine (DETA) were ordered from Junsei and Aldrich, respectively, and used without further purification. 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT−MM) was synthesized by reacting 2-chloro-4,6-dimethoxy-1,3,5-triazine with *N*-methylmorpholine in tetrahydrofuran (THF) media for 30 min, and precipitates were washed with THF.[20] Completely dried DMT−MM was stored in a freezer under −20 °C. Biotin, ferritin, and several streptavidins were also purchased from Aldrich and dispersed in Tris-buffered saline (TBS) containing 0.1 M NaCl.

Fabrication of PGMA NFs. To prepare PGMA NFs, AIBN was dissolved in methylene chloride (MC) solution. Exactly 0.2 g of AIBN and 2 mL of MC were mixed, and the AAO membrane was soaked into the mixture for 5 s. Rapid solvent evaporation proceeded in the vacuum oven for 3 h at room temperature. This step induced the impregnation of initiator into the AAO membrane pores. The initiator-adsorbed membrane was placed into the reactor equipped with the monomer reservoir and membrane holder. The reactor was placed under vacuum for 20 min up to 10^{-2} Torr. Then, 0.1 mL of GMA was injected

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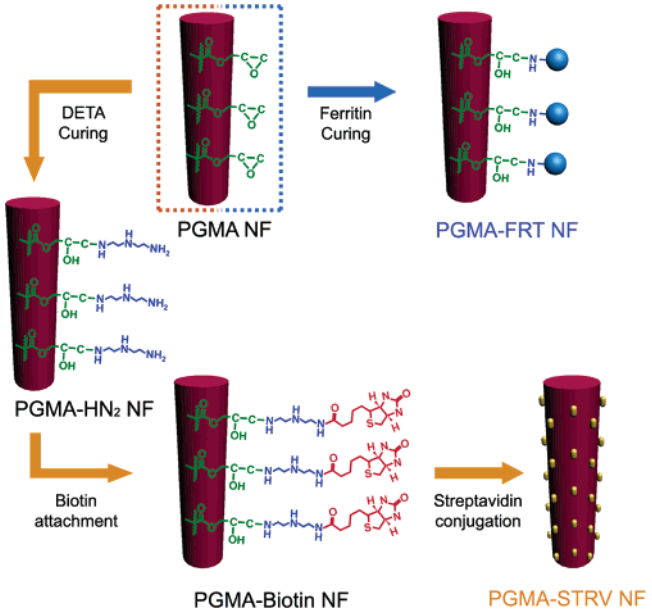

Figure 1. Schematic diagram for the fabrication of protein-immobilized PGMA NFs. Pristine PGMA NFs are emphasized with a dotted line.

into the reactor. The vaporized monomer was adsorbed into the membrane pores and polymerized during 6 h at 70 °C. The membrane template was removed with 0.1 N NaOH solution. The membrane was soaked in the NaOH solution for 2 h and sonicated for 15 min. The resulting PGMA NFs were obtained by washing the products with distilled water and ethanol.

Curing PGMA NFs with DETA. PGMA NF curing was performed with a low-temperature curing agent, namely, DETA. Exactly 10 mL of PGMA NFs dispersed in ethanol (0.4 mg mL^{-1}) and 0.8 mL of DETA were mixed in a 25 mL vial. The PGMA NFs were cured at 40 °C for 12 h with mild stirring, and the unreacted DETA was removed by excess ethanol. Last, the cured PGMA NFs were obtained by centrifuging.

Conjugation of Streptavidin on PGMA−Biotin NFs. Ferritin was readily immobilized onto PGMA NFs (PGMA−FRT NFs) by curing the surface epoxide groups of PGMA NFs with the activated surface amine groups of ferritin. Exactly 1 mL of PGMA NFs dispersed in TBS solution (50 mg mL^{-1}) was reacted with 10 μL of ferritins (10 mg mL^{-1}) at room temperature for 24 h. Streptavidin-functionalized PGMA NFs (PGMA−STRV NFs) were prepared by attaching biotin onto the amino functional groups of PGMA−NH_2 NFs followed by conjugating streptavidins. Exactly 1 mL of PGMA−NH_2 NFs dispersed in TBS solution (50 mg mL^{-1}) were mixed with 0.5 mg of biotin and reacted for 12 h with mild stirring. Then, biotinylated PGMA NFs (PGMA−biotin NFs) were linked with 0.1 mL of streptavidin (1 mg mL^{-1}). Conjugation of streptavidin with PGMA−biotin NFs was carried out by vigorously shaking the mixture and incubating it for 12 h. Residual streptavidin was washed with TBS solution.

Results and Discussion

Figure 1 describes the synthetic procedures for protein-immobilized PGMA NFs. PGMA NFs were prepared using the initiator-impregnated template method. AAO membranes with 100 nm pores were soaked in AIBN-dissolved (3×10^{-2} M) methylene chloride solution. Solvent evaporation induced the impregnation of AIBN onto membrane pores. Initiator-adsorbed membrane and 0.1 mL of GMA monomer were placed into the reactor under vacuum for 20 min up to 10^{-2} Torr. Then, the monomers were vaporized and polymerized within membrane pores for 6 h when the reactor was moved into a 70 °C oven.

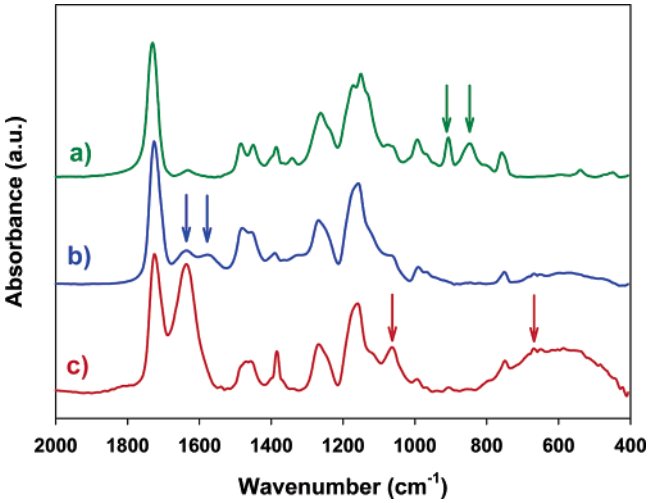

Figure 2. FT-IR spectra of (a) PGMA NFs, (b) PGMA−NH_2 NFs, and (c) PGMA−biotin NFs. Arrows indicate the peaks of the epoxide group (green), amide bond and −NH_2 (blue), and C−O−C and −S (red). These spectra were recorded over 32 scans in absorption mode using a Bomem MB 100 FT-IR spectrometer.

The template membrane was removed using diluted 0.1 N NaOH solution to preserve the surface epoxide groups. The number-average molecular weight of the resulting PGMA NFs was measured using gel permeation chromatography[21] and found to be 1.1×10^4. Subsequently, aminated PGMA NFs (PGMA−NH_2 NFs) were prepared by curing the pristine PGMA NFs with diethylenetriamine at 40 °C for 12 h. Generally, the curing of PGMA is carried out above 70 °C. Therefore, low-temperature curing would facilitate the amino functionalization of the nanofiber surface rather than cross-linking all over the nanofiber body.[9] When the nanofibers were swollen in distilled water at 25 °C for 2 h, the equilibrium water content (EWC) measured was considerably low (4.3). This result may be attributed to the low cross-linking density of the nanofibers.[22] Covalently attached amino functional groups acted as the anchoring sites for carboxylated compounds. To immobilize protein onto the nanofiber surface, the pristine PGMA NFs and the aminated PGMA−NH_2 NFs were dispersed in TBS containing 0.1 M NaCl. Ferritin and streptavidin were selected as model proteins, since ferritins are iron detoxification and mineralizing proteins in vivo that play a central role in sequestering Fe as the mineral ferrihydrite.[23,24] Moreover, streptavidin has been focused on as a versatile molecule tag using a specific biomolecular interaction with biotin.[25] Ferritin-immobilized PGMA NFs (PGMA−FRT NFs) were readily synthesized by curing the surface epoxide groups of PGMA NFs with activated surface amine groups of ferritin. Exactly 1 mL of PGMA NF solution (50 mg mL^{-1}) was reacted with 10 μL of ferritins (10 mg mL^{-1}) at room temperature for 24 h. Streptavidin-functionalized PGMA NFs (PGMA−STRV NFs) were prepared by attaching biotin onto amino functional groups of PGMA−NH_2 NFs followed by conjugating streptavidins. Exactly 1 mL of PGMA−NH_2 NF solution (50 mg mL^{-1}) was reacted with 0.5 mg of biotin for 12 h, and biotinylated PGMA NFs (PGMA−biotin NFs) were linked with 0.1 mL of streptavidin (1 mg mL^{-1}). The procedure for immobilizing biotins and proteins proceeded in TBS solution and condensing agent was used with DMT−MM.

The successful curing of PGMA NFs and attachment of biotin on the nanofibers were confirmed using transmittance Fourier transform infrared (FT-IR) spectroscopy. In Figure 2a,

Table 1. CHNS Elemental Analysis of PGMA NFs, PGMA−NH_2 NFs, and PGMA−Biotin NFs[a]

	C	H	N	S	N/C	S/C
PGMA NFs	55.9	6.7	0	0	0	0
PGMA−NH_2 NFs	47.9	7.5	8.3	0	0.16	0
PGMA−biotin NFs	35.7	4.1	15.2	6.2	0.36	0.07

[a] The data were recorded three times with an EA 1110 apparatus (CE instruments), and the averaged values are given.

the characteristic peaks of epoxide groups (green arrows) appeared at 910 and 850 cm^{-1}.[24] During the curing process, the epoxide groups were cleaved and transformed to hydroxy and amine moieties in Figure 2b. Thus, the epoxide peaks were diminished, and a new primary amine band (blue arrow) arose at 1640 cm^{-1}.[27] In addition, the stretching band of −CNH− (blue arrow) at 1580 cm^{-1} confirmed that the nanofiber surface was functionalized via a covalent bond (amide III).[28] In Figure 2c, immobilization of biotin on PGMA−NH_2 NFs remarkably enhanced the peak intensity of the amide bonds at 1640 cm^{-1}. It originated from amide bonds between amine groups of PGMA−NH_2 NFs and carboxylates of biotin as well as carbonyl groups of biotin. The weak and broad stretching vibration band (red arrow) of C−S bonds at 675 cm^{-1} also ascertained the presence of biotin on PGMA−NH_2 NFs.[29] In addition, the intensity of the C−O−C band at 1066 cm^{-1} in PGMA−biotin NFs increased due to the ester bond of biotin. The characteristic vibration of methacrylate was observed at 1720 cm^{-1} and assigned as a carbonyl ester.

The bulk composition of PGMA NFs was investigated through CHNS elemental analysis (EA) in Table 1. During the curing of PGMA NFs, the molar ratio of N to C increased by 0.16 without a change in the molar ratio of S to C. It was caused from the nitrogen element of the curing agent DETA. Furthermore, the EA of PGMA−biotin NFs revealed that the molar ratio of S to C increased by 0.07, compared with PGMA NFs and PGMA−NH_2 NFs. It was thought that the S element resulted from biotin compounds. On the basis of the EA data, the successful attachment of biotin onto PGMA−NH_2 NFs was ascertained. It was consistent with the characteristic peaks of PGMA−NH_2 NFs and PGMA−biotin NFs in FT-IR spectra in Figure 2.

The number of epoxide groups in PGMA NFs was measured by the HCl−dioxane titration method.[30,31] PGMA NFs were dispersed in 1,4-dioxane solution, and the epoxide groups were cleaved by 0.2 N HCl in dioxane at 40 °C for 12 h. The unreacted residual HCl was titrated with 0.1 N NaOH in methanol. The number of epoxides was calculated by subtracting the unreacted number of HCl from the initial number of HCl. Consequently, the number of epoxides on PGMA NFs was 6.8 μmol mg^{-1}; the population of epoxy groups per unit surface area was 1.15×10^{-4} mol m^{-2}. This value means that 96% of the initial epoxides survive. Judging from these data, epoxy functional groups were retained remarkably during template removal.

Figure 3 presents field emission scanning electron microscopy (FE-SEM) and transmission electron microscopy (TEM) images of PGMA NFs and protein-functionalized PGMA NFs. In Figures 3a and 3b, FE−SEM and TEM images describe the typical nanofiber morphology such as a high aspect ratio and an occupied inner space. This morphology would be helpful to potential applications as protein carriers due to their facile gravimetric sedimentation for soluble proteins. In addition, PGMA−NH_2 NFs were also generated without morphological transformation by the low-temperature curing agents. Figures 3c and 3d show TEM images of ferritin-functionalized PGMA NFs and FE-SEM images of streptavidin-immobilized biotinylated PGMA NFs. Ferritins with a diameter of 12 nm containing a 7 nm core were clearly visualized as spherical nanoparticles on nanofibers. The surface epoxides of PGMA NFs were directly cured with activated amine moieties of ferritins. This facile protein conjugation method exhibited that most of the proteins possessing amino groups could be deposited on PGMA NFs without sophisticated surface modification processes or intermediate reagents. Moreover, streptavidin was also precisely immobilized with the aid of a biolinker, namely, biotin. Biotinylated PGMA NFs were employed as a model supporter for biolinker-mediated protein carriers. The FE-SEM image of PGMA−STRV NFs in Figure 3d shows that the streptavidin was successfully decorated on PGMA−biotin nanofibers. There was obviously no aggregation observed. In addition, no conjugation of streptavidin with PGMA−NH_2 NFs was monitored by means of photoluminescence of fluorescein isothiocyanate (FITC)-tagged streptavidin without a biolinker. The specific conjugation of streptavidin molecules with PGMA−biotin NFs produced the typical absorbance at 280 nm in the UV−vis spectrum.[32]

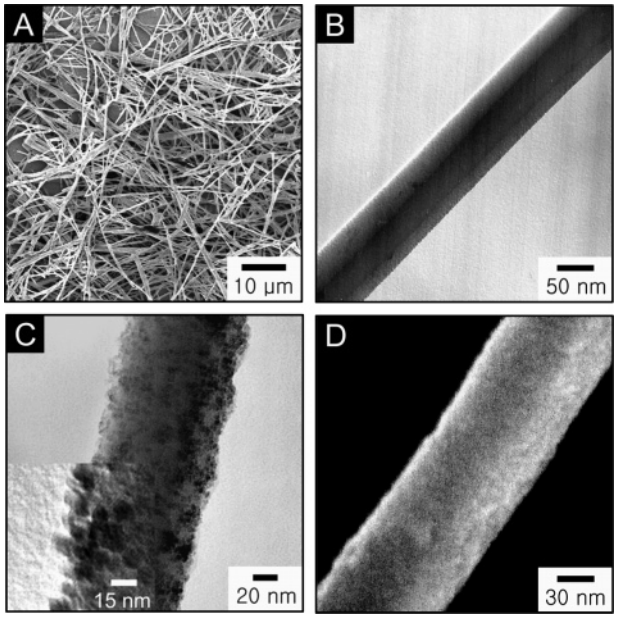

Figure 3. (a) FE-SEM image of PGMA NFs, TEM images of (b) PGMA NFs and (c) PGMA−FRT NFs, and (d) FE-SEM image of PGMA−STRV NFs.

In particular, streptavidin can be labeled with a variety of organic molecules or metal nanoparticles, because it has abundant amine moieties and four subunits, which are specifically conjugated with biotinylated complexes.[33,34] Therefore, various molecule-tagged streptavidins could be coupled with PGMA−biotin NFs. Figure 4 describes PGMA−biotin NFs conjugated with 10 nm gold nanoparticle-tagged streptavidin and FITC-labeled streptavidin. In Figure 4a, 10 nm dark dots clarified the precise conjugation of streptavidin with PGMA−biotin NFs. The confocal laser scanning microscopy (CLSM) image of PGMA−STRV NFs in Figure 4b also presents the accurate conjugation of FITC-labeled streptavidin with PGMA−biotin NFs by the photoluminescence of FITC. In a control experiment, the photoluminescence was not observed in PGMA−NH_2 NFs without biotin. From these results, it was considered that PGMA−biotin NFs could be applied as versatile carriers for streptavidins tagged with metal nanoparticles and organic dyes.

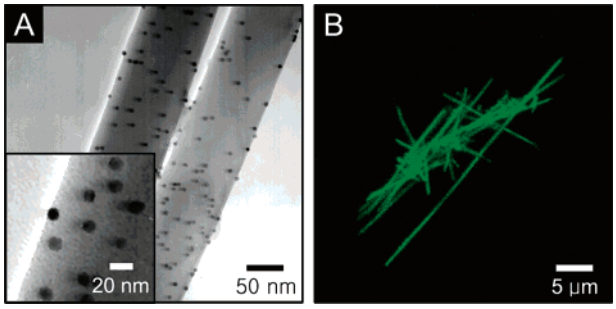

Figure 4. TEM and CLSM images of PGMA−biotin NFs conjugated with streptavidins, which were tagged with various materials: (a) 10 nm gold nanoparticles and (b) FITC.

Conclusion

Pristine PGMA NFs and PGMA−NH_2 NFs were successfully fabricated using vapor deposition polymerization combined with initiator-impregnated template synthesis followed by curing with a low-temperature curing agent. These nanofibers were readily employed as protein carriers, since their surfaces possessed the protein anchoring sites such as the epoxide groups of PGMA NFs and the amine moieties of PGMA−NH_2 NFs, respectively. Ferritins were directly immobilized onto PGMA NFs by curing the surface epoxides with activated amine groups of ferritins, and streptavidins were anchored on nanofibers with the aid of biolinkers. In addition, the labeled streptavidins were immobilized on PGMA−biotin NFs to demonstrate the possibility of biotinylated PGMA NFs as tagged protein supports. From the view point of protein immobilization, it was thought that PGMA NFs, PGMA−NH_2 NFs, and PGMA−biotin NFs demonstrated the possibility to be potential protein carriers for both pristine and tagged proteins.

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