Poly(amidoamine) Dendrimer-Enabled Simultaneous Stabilization and Functionalization of Electrospun Poly(γ-glutamic acid) Nanofibers

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S *Supporting Information*

ABSTRACT: We report a facile and general approach to using generation 2 (G2) poly(amidoamine) (PAMAM) dendrimers for simultaneous stabilization and functionalization of electrospun poly(γ-glutamic acid) nanofibers (γ-PGA NFs). In this study, uniform γ-PGA NFs with a smooth morphology were generated using electrospinning technology. In order to endow the NFs with good water stability, amine-terminated G2.NH$_2$ PAMAM dendrimers were utilized to crosslink the γ-PGA NFs via 1-ethyl-3-(3-dimethylami-nopropyl) carbodii-mide coupling chemistry. Under the optimized crosslinking

G2.NH2-FA-crosslinked γ-PGA nanofibers   FAR-mediated specific cancer cell capture

conditions, G2.NH$_2$ dendrimers partially modified with fluorescein isothiocyanate (FI) or folic acid (FA) were used to crosslink γ-PGA NFs. Our results reveal that G2.NH$_2$−FI is able to simultaneously render the NFs with good water stability and fluorescence property, while G2.NH$_2$−FA is able to simultaneously endow the NFs with water stability and the ability to capture FA receptor-overexpressing cancer cells in vitro via ligand−receptor interaction. With the tunable dendrimer surface chemistry, multifunctional water-stable γ-PGA-based NFs may be generated via a dendrimer crosslinking approach, thereby providing diverse applications in the areas of biosensing, tissue engineering, drug delivery, and environmental sciences.

KEYWORDS: *poly(γ-glutamic acid), electrospinning, nanofibers, dendrimers, functionalization, water stability*
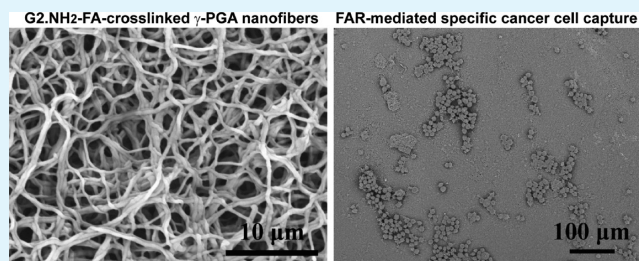
■ INTRODUCTION

Poly(γ-glutamic acid) (γ-PGA) is a microorganism metabolite produced by several *Bacillus* species, consisting of γ-carboxy-linked glutamate residues.[1] With superior biodegradability, biocompatibility, and water retention ability,[2] γ-PGA and its derivatives have been widely used in the fields of drug delivery,[3,4] wound dressing,[5] and tissue engineering.[6,7] One outstanding characteristic of γ-PGA is that it possesses abundant free carboxyl groups, which renders γ-PGA with excellent hydrophilicity and desired functionality via facile modification of the carboxyl residues.[8] With these properties of γ-PGA, it is desirable to develop various γ-PGA-based multifunctional scaffolds for various applications.

Electrospinning has been demonstrated as a powerful technology to generate synthetic or natural polymer fibers with a diameter ranging from tens of nanometers to several micrometers.[9] The attractive advantages of electrospun nanofibers (NFs) including large specific surface area, high porosity, flexibility in surface functionalities, superior mechanical durability, and ability to mimic the topology of natural extracellular matrix enable them to be used as scaffolds for tissue engineering and drug delivery applications.[10−20] However, due to the superior hydrophilicity of γ-PGA, electrospun

γ-PGA NFs have to be crosslinked or stabilized before their further applications. Many different approaches to improving the water stability of electrospun γ-PGA NFs have been extensively explored.[2,7,21−23] For instance, Lee et al. prepared water-insoluble γ-PGA butyl ester for subsequent electrospinning to form water-stable γ-PGA NFs using 1,1,1,3,3,3-hexafluoro-2-propanol as a solvent.[21] Tajima et al. reported the stabilizaion of electrospun γ-PGA NFs using an oxazoline component polymer as a crosslinking agent for a heat-induced ring-opening reaction with γ-PGA carboxyl groups.[2,23] In another study, Yoshida et al. reported the preparation of water-stable electrospun γ-PGA NFs by crosslinking of free γ-PGA carboxyl side groups with amine groups of cystamine via 1-ethyl-3-(3-dimethylami-nopropyl) carbodiimide (EDC) coupling chemistry for tissue engineering applications.[22] In our previous study, we prepared water-stable γ-PGA NFs with controllable diameter and morphology using cystamine as a crosslinker.[7] Except the cystamine crosslinker that can render the γ-PGA NFs with redox-responsiveness,[22] in most of these
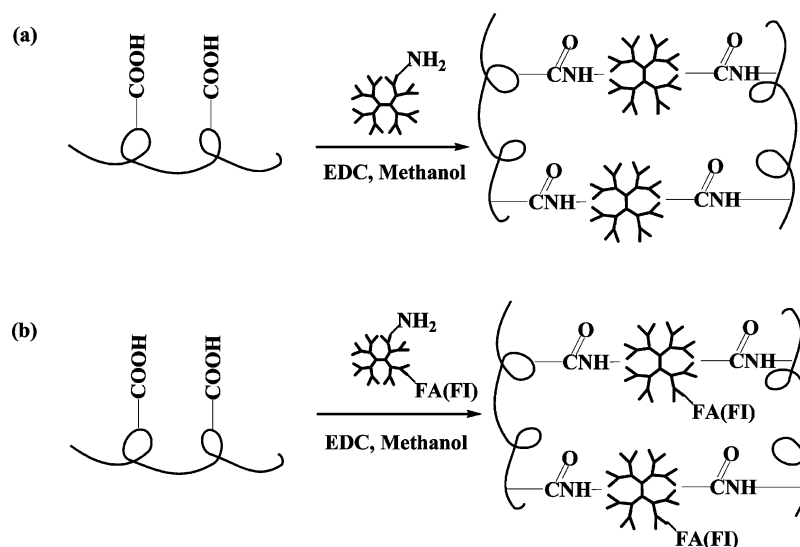
Figure 1. Schematic illustration of the crosslinking reaction between γ-PGA and G2.NH$_2$ dendrimer (a) and between γ-PGA and G2.NH$_2$−FI or γ-PGA−G2.NH$_2$−FA dendrimer (b).

studies, the used crosslinking agents are only able to render the γ-PGA NFs with water stability. It is important to find a crosslinking agent that can simultaneously afford NFs with water stability and other functionalities.

Polyamidoamine (PAMAM) dendrimers are a class of synthetic macromolecules with well-defined structure, composition, and abundant terminal functional groups.[24−26] The unique structural features of PAMAM dendrimers enable them to be used as a multifunctional platform for covalently linking with targeting ligands, fluorescent dyes, and drugs for targeting, imaging, and treatment of biological systems.[27−29] With the tunable surface chemistry, amine-terminated PAMAM dendrimers prefunctionalized with other molecules are expected to be able to react with the carboxyl groups of γ-PGA, thus affording the formed γ-PGA NFs simultaneously with both water stability and other designed functionalities.

In this present study, we first prepared electrospun γ-PGA NFs by dissolving γ-PGA powder into diluted trifluoroacetic acid (TFA) aqueous solution. Then, amine-terminated generation 2 PAMAM dendrimers (G2.NH$_2$) were utilized to crosslink the γ-PGA NFs to render them with good water stability. Under the optimized crosslinking conditions, G2.NH$_2$ dendrimers premodified with fluorescein isothiocyanate (FI) or folic acid (FA) were used as a crosslinking agent to form water-stable γ-PGA NFs with different functionalities. The fluorescence property of the formed γ-PGA−G2.NH$_2$−FI NFs and the specific FA receptors (FAR)-overexpressing cancer cell capturing ability of the γ-PGA−G2.NH$_2$−FA NFs were studied in detail. Finally, the cytocompatibility and hemocompatibility of the formed electrospun γ-PGA NFs were assessed in vitro via rezasurin reduction assay of mouse fibroblast cells (L929 cells) and hemolytic assay of human red blood cells (HRBCs), respectively. To our knowledge, this is the first report attempting to use dendrimers as a crosslinking agent to render electrospun γ-PGA NFs simultaneously with water stability and desired functionalities.

■ EXPERIMENTAL SECTION

Materials. γ-PGA (M_w = 1000 kDa) was purchased from Nanjing Saitesi Co., Ltd (China). TFA and EDC were obtained from J&K Chemical. Co., Ltd (China). Ethylenediamine cored G2.NH$_2$

dendrimers were purchased from Dendritech (Midland, MI). Orange II and FI were purchased from Aldrich (St. Louis, MO). Human epithelial carcinoma cell line (KB cells) and L929 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Roswell Park Memorial Institute-1640 (RPMI-1640), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). All other chemicals were from Sinopharm Chemical Reagent Co., Ltd (China). Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford, MA) with resistivity higher than 18.2 MΩ·cm. Regenerated cellulose dialysis membranes (molecular weight cutoff, MWCO = 1000) were acquired from Fisher.

Electrospinning. Electrospinning was performed according to the procedures reported in our previous study.[7] Briefly, a certain amount of γ-PGA powder was dissolved into TFA aqueous solution (5%) under magnetic stirring for 10 h to form a homogeneous γ-PGA solution with a concentration of 16 wt %. The electrospinning equipment consisted of a 10 mL syringe, a silicone hose, a 16 gauge stainless steel needle, a high voltage power supply, and a thin conductive plate acting as a collector which was positioned vertically and grounded. A clamp was used to connect the high voltage power supply with the needle. The grounded flat collector was covered with an aluminum foil. After electrospinning, γ-PGA NFs were taken off from the aluminum foil and vacuum dried for at least 2 days to remove the residual organic solvent and moisture. The as-prepared γ-PGA NFs were stored in a desiccator before characterization and use.

Synthesis of G2.NH$_2$−FI or G2.NH$_2$−FA Conjugates. G2.NH$_2$ dendrimers were conjugated with FI or FA moieties according to the literature with slight modifications.[30] For the synthesis of G2.NH$_2$−FI conjugates, G2.NH$_2$ dendrimers (33.87 mg, 0.02877 mmol) were dissolved into DMSO (5 mL). Then, FI (10.13 mg, 0.072 mmol) in DMSO solution (5 mL) was dropwise added into the G2.NH$_2$ dendrimer solution under vigorous magnetic stirring at room temperature for 24 h. The reaction mixture was dialyzed against phosphate buffered saline (PBS) (3 times, 4 L) and water (3 times, 4 L) through a dialysis membrane with MWCO of 1000 for 3 days to remove the excess of reactants, followed by lyophilization to get G2.NH$_2$−FI conjugates. For the synthesis of G2.NH$_2$−FA conjugates, FA (22.18 mg, 0.0502 mmol) dissolved in DMSO (4 mL) was first activated with EDC (180.6 mg, 0.942 mmol) under vigorous magnetic stirring at room temperature for 3 h. The resulting solution was then dropwise added to the G2.NH$_2$ solution (61.35 mg, 0.01884 mmol) in DMSO (4 mL) under vigorous magnetic stirring at room temperature

for 3 days. The reaction mixture was treated in a manner similar to the purification of G2.NH$_2$–FI conjugates, and G2.NH$_2$–FA conjugates were finally obtained.

Preparation of Water-Stable γ-PGA NFs. G2.NH$_2$ dendrimers were employed as a crosslinker to react with the carboxyl groups of γ-PGA through EDC coupling (Figure 1a). Briefly, 3 pieces of γ-PGA nanofibrous mats (50 mg each) in parallel were first separately immersed into an EDC solution (75 mg, 25 mL in methanol) under magnetic stirring for 3 h to activate the carboxyl groups of γ-PGA. The activated γ-PGA nanofibrous mats were rinsed with methanol for 3 times, followed by immersion into 25 mL of methanol solution containing 78.75, 26.29, and 15.78 mg of G2.NH$_2$ dendrimers (with molar ratios between the γ-PGA side carboxyl groups and G2.NH$_2$ terminal amine groups (n_{-COOH}/n_{-NH_2} ratio) of 1:1, 3:1, and 5:1), respectively, for 72 h to crosslink the γ-PGA NFs. For a given n_{-COOH}/n_{-NH_2} ratio of 3:1, the crosslinking reaction was performed for different time periods (12, 24, 48, and 72 h, respectively) in order to optimize the stability of the NFs.

The prepared G2.NH$_2$–FI or G2.NH$_2$–FA conjugates were then used to crosslink the carboxyl groups of γ-PGA NFs using the above optimized procedures to realize the simultaneous crosslinking and surface modification of γ-PGA NFs (Figure 1b). Briefly, 50 mg of γ-PGA NFs in methanol were first activated with EDC for 3 h, rinsed with methanol for 3 times, and then immersed into 25 mL of methanol solution containing 15.78 mg of G2.NH$_2$–FI or G2.NH$_2$–FA conjugates for 72 h, which was the optimized crosslinking time (see below).

After crosslinking reaction, all γ-PGA NFs were washed with methanol and water, followed by lyophilization. The formed γ-PGA–G2.NH$_2$, γ-PGA–G2.NH$_2$–FI, or γ-PGA–G2.NH$_2$–FA NFs were then stored in a desiccator before characterization and use. The water stability of the crosslinked γ-PGA NFs was checked by immersing them in PBS for one month.

Characterization Techniques. The formed γ-PGA NFs before and after crosslinking with G2.NH$_2$ were characterized using Nicolet Nexus 670 Fourier transform infrared (FTIR) spectroscopy (Nicolet-Thermo, Waltham, MA). All spectra were recorded using a transmission mode with a wavenumber range of 650 to 4000 cm^{-1}. The surface primary amine density of γ-PGA–G2.NH$_2$ NFs was determined according to the procedure reported in the literature.[31] Briefly, a 10 mg γ-PGA–G2.NH$_2$ nanofibrous mat was immersed in an Orange II dye solution (2 mg/mL, 2 mL) in hydrochloric acid (pH = 3) at 40 °C for 30 min. The sample was then intensively rinsed using hydrochloric acid (pH = 3) for 3 times to remove the unbound dye. After being air-dried, the colored mat was immersed in an aqueous NaOH solution (1 mL, pH = 12). Then, the solution containing the desorbed dye was adjusted to pH 3 by adding concentrated hydrochloric acid. The absorbance of the solution was then monitored at 484 nm by a Lambda 25 UV-Vis spectrophotometer (Perkin Elmer, USA), and the concentration of desorbed dye, which is proportional to the density of the surface primary amine groups of NFs, was calculated via a standard calibration curve. The morphology of γ-PGA, γ-PGA–G2.NH$_2$, γ-PGA–G2.NH$_2$–FI, and γ-PGA–G2.NH$_2$–FA NFs was observed using a scanning electron microscope (SEM, JEOL JSM-5600LV, Tokyo, Japan) with an accelerating voltage of 10 kV. Fiber diameters were measured using Image J 1.40 G software (http://rsb.info.nih.gov/ij/down-load.html). At least 100 NFs from different SEM images for each sample were randomly selected and analyzed. UV-Vis spectra of G2.NH$_2$–FA and G2.NH$_2$–FI were collected using a Lambda 25 UV-Vis spectrophotometer. Samples were dissolved in water at a concentration of 0.25 mg/mL before measurements. ^{1}H NMR spectra of G2.NH$_2$–FA and G2.NH$_2$–FI were recorded using Bruker AV-400 NMR spectrometer. Samples were dissolved in D$_2$O at a concentration of 6.0 mg/mL before measurements. A confocal laser scanning microscope (CLSM, Carl Zeiss LSM 700, Jena, Germany) was used to observe the fluorescence emission of the formed γ-PGA–G2.NH$_2$–FI NFs. Before measurements, a fibrous mat was attached onto a clean glass coverslip with a diameter of 14 mm. Samples were imaged using a 10× objective lens.

Cell Culture. KB cells or L929 cells were continuously cultured in 25 cm^2 tissue culture flasks with 5 mL of RPMI-1640 or DMEM medium containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator with 5% CO$_2$ at 37 °C.

Specific Cell Capture Experiment. For specific capture of cancer cells overexpressing FAR, γ-PGA–G2.NH$_2$ and γ-PGA–G2.NH$_2$–FA NFs on coverslips were fixed in a 24-well tissue culture plate (TCP) with stainless steel rings and sterilized by exposure to 75% alcohol solution for 2 h. After that, samples were washed 3 times with PBS and soaked in RPMI-1640 overnight before cell seeding. Then, 1.5×10^5 KB cells were seeded into each well. After incubation in a humidified incubator with 5 % CO$_2$ at 37 °C for 4 h, the medium was gently shaken and transferred to eppendorf centrifuge tubes to collect all the KB cells that were not captured by the NF mats. The KB cell density in the medium was counted using a Counter Star cell counter. The captured cells can be deduced from the difference of the initial seeding cells and the unattached cells. Besides the quantitative analysis, the cell capture capacity was also qualitatively assessed via visualization of the residual cells in the medium using a Leica DM IL LED inverted phase contrast microscope and SEM observation of NFs with the captured cells. For the inverted phase contrast microscope observation, 100 μL of medium with residual cells was transferred into a clean 48-well TCP before visualization. For the SEM observation, NFs with the captured cells were first fixed with 2.5 wt % glutaraldehyde at 4 °C for 2 h and dehydrated through a series of gradient ethanol solutions of 30%, 50%, 70%, 80%, 90%, 95%, and 100% and air dried overnight. SEM was performed at an accelerating voltage of 10 kV. Samples were sputter coated with a 10 nm thick gold film before measurements.

Cytocompatibility Assay. The cytocompatibility of γ-PGA–G2.NH$_2$ NFs was assessed using a rezasurin reduction assay of the viability of L929 cells according to our previous study.[32] In brief, γ-PGA–G2.NH$_2$ NFs on coverslips were fixed, sterilized, and washed in a manner similar to the cell capture experiment and finally soaked in DMEM overnight. Then, L929 cells were seeded at a density of 2×10^4 cells/well. TCP without NFs was used as control. The viability of cells cultured onto different substrates was quantitatively evaluated after incubation of the cells for 3 days. Then, the medium was replaced with 900 μL of fresh DMEM and 100 μL of resazurin solution (1 mg/mL in PBS). The plate was incubated for an additional 4 h, and the fluorescence intensity (λ_{ex} = 530 nm, λ_{em} = 590 nm) reflecting the viability of cells adhered onto the γ-PGA NFs was measured using a microplate reader (BioTek, Synergy, USA). Mean and standard deviation for the triplicate wells for each sample were reported.

Hemolysis Assay. The hemolytic effect of the formed γ-PGA–G2.NH$_2$ NFs was tested by hemolysis assay of HRBCs. Fresh human blood stabilized with heparin was kindly provided by Shanghai First People's Hospital (Shanghai, China) and approved by the ethical committee of Shanghai First People's Hospital. HRBCs obtained by removing the serum via centrifugation and washing with PBS were 10 times diluted with PBS according to the literature.[33−37] Then, 0.3 mL of the diluted HRBC suspension was transferred to a 1.5 mL sample vial containing 10 mg of γ-PGA–G2.NH$_2$ NFs suspended within 1.2 mL of PBS. The diluted HRBC suspension (0.3 mL) was also added into 1.2 mL of water and PBS, which was set as positive and negative control, respectively. The above mixtures were incubated at 37 °C for 2 h, followed by centrifugation (10 000 rpm, 1 min). The absorbance of the supernatants related to hemoglobin was recorded with a Lambda 25 UV-Vis spectrophotometer at 541 nm. The hemolytic percentage (HP) was calculated using the following equation,[34]

$$HP\ (\%) = \frac{(D_t - D_{nc})}{(D_{pc} - D_{nc})} \times 100\% \tag{1}$$

where D_t is the absorbance of the test samples and D_{pc} and D_{nc} are the absorbance of the positive and negative control, respectively.

Statistical Analysis. Statistical analysis was performed by the analysis of variance (ANOVA) method. In all evaluations, $p < 0.05$ was considered as statistically significant, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.
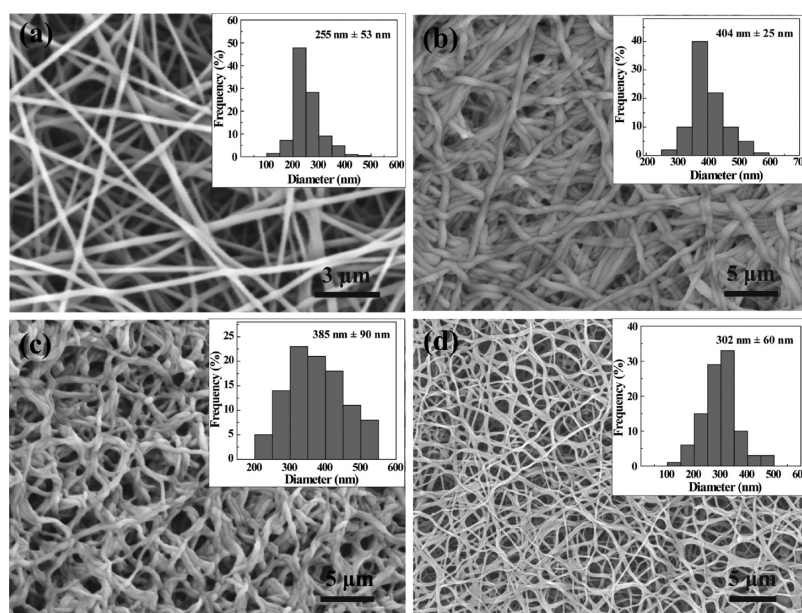
Figure 2. SEM images and fiber diameter distribution histograms of γ-PGA NFs before (a) and after crosslinking with G2.NH$_2$ at n_{-COOH}/n_{-NH_2} ratios of 1:1 (b), 3: 1 (c), and 5: 1 (d) for 72 h, followed by immersion in PBS for one month.

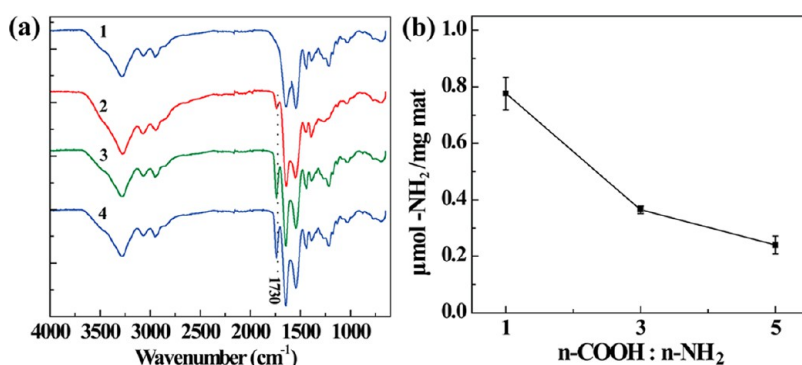**Figure 3.** (a) FTIR spectra of γ-PGA NFs before (1) and after crosslinking with G2.NH$_2$ at n_{-COOH}/n_{-NH_2} ratios of 5:1 (2), 3:1 (3), and 1:1 (4) for 72 h. (b) Surface primary amine density of crosslinked γ-PGA$-$G2.NH$_2$ nanofibers as a function of the n_{-COOH}/n_{-NH_2} ratio.

■ RESULTS AND DISCUSSION

G2.NH$_2$ Dendrimer-Crosslinked Stable γ-PGA NFs. In our previous study, we prepared water-stable γ-PGA NFs using cystamine as a crosslinking agent and systematically investigated the correlation between processing variables and the morphology of the electrospun γ-PGA NFs.[7] In this present study, we employed amine-terminated G2 PAMAM dendrimers as a novel crosslinking agent to render γ-PGA NFs with water stability or simultaneously with desired surface functionality (Figure 1). The selection of G2 dendrimer as a crosslinker is based on the fact that (1) G2 dendrimer has a sufficient amount of terminal amine groups that can be partially functionalized with small molecular ligands such as FI and FA and (2) the molecular weight of G2 dendrimer is small enough, enabling efficient penetration inside the single fibers for effective crosslinking of γ-PGA carboxyl groups.

G2.NH$_2$ dendrimers were first used as a crosslinker to react with the carboxyl groups of γ-PGA through EDC coupling (Figure 1a). Similar to our previous study,[7] we first fixed the n_{-COOH}/n_{-NH_2} ratio at 3:1 to optimize the crosslinking time. Under the optimized electrospinning conditions reported in

our previous work,[7] smooth and intact γ-PGA NFs with random orientation were formed with a mean fiber diameter of 255 ± 53 nm (Figure 2a). After crosslinking for 72 h and being immersed into PBS for one month, smooth and intact NFs were formed (Figure 2c), suggesting that a reaction time period of 72 h is able to render γ-PGA NFs with excellent water stability and smooth fibrous morphology. In contrast, at a short crosslinking time of 12 and 24 h (Figure S1, Supporting Information), the fiber morphology was totally damaged in PBS solution (Figure S1a,b, Supporting Information). Although the fibrous morphology was able to be partially reserved at the crosslinking time of 48 h, the fibers displayed serious adhesion between each other (Figure S1c, Supporting Information).

Besides the crosslinking time, the amount of G2.NH$_2$ also impacted the morphology and crosslinking behavior of the γ-PGA NFs. At the optimal crosslinking time, we then altered the n_{-COOH}/n_{-NH_2} ratios to 1:1, 3:1, and 5:1, respectively. As can be seen in Figure 2b–d, similar to the case of 3:1 (Figure 2c), smooth and intact NFs with random orientation can be formed under the other two cases (1:1 and 5:1), implying that, under the reasonable n_{-COOH}/n_{-NH_2} ratios, crosslinking time plays a

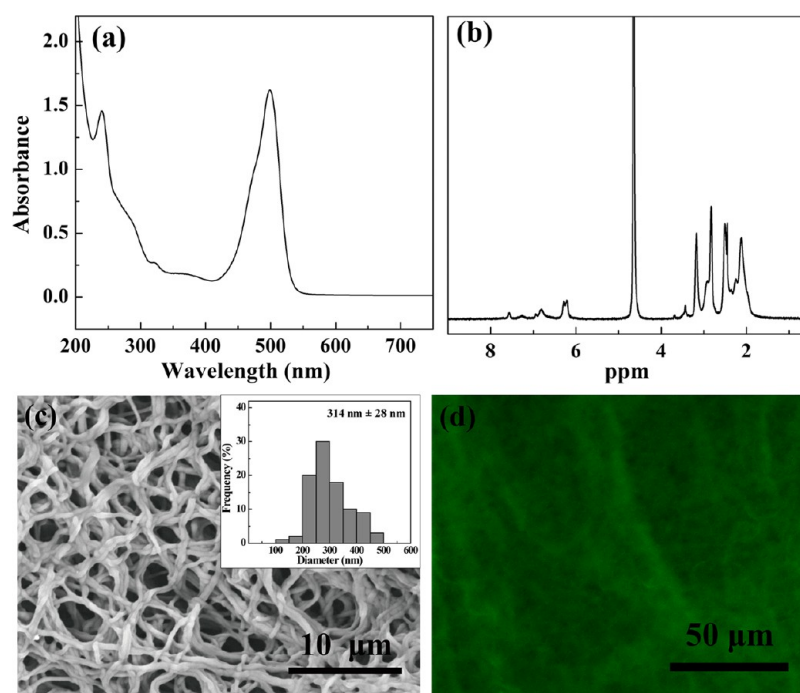**Figure 4.** (a) UV-Vis and (b) ^{1}H NMR spectra of G2.NH$_2$−FI conjugates. (c) SEM image, fiber diameter distribution histogram, and (d) CLSM image of γ-PGA−G2.NH$_2$−FI NFs.

dominant role to affect the fiber morphology. After cross-linking, the fiber diameter increased from 255 ± 53 nm (Figure 2a) to 404 ± 25 nm (Figure 2b), to 385 ± 90 nm (Figure 2c), and to 302 ± 60 nm (Figure 2d), respectively, under the $n_{-\mathrm{COOH}}/n_{-\mathrm{NH_2}}$ ratio at 1:1, 3:1, and 5:1. This may be due to the swelling of the NFs during the crosslinking reaction process and the immersion in PBS solution, similar to our previous report.[7] Notably, the γ-PGA NFs displayed serious adhesion between each other (Figure 2b) at $n_{-\mathrm{COOH}}/n_{-\mathrm{NH_2}}$ ratio of 1:1, which may be ascribed to the fact that a high amount of G2.NH$_2$ likely results in strong binding between adjacent fibers. Since the $n_{-\mathrm{COOH}}/n_{-\mathrm{NH_2}}$ ratio at 3:1 or 5:1 was sufficient to form uniform NFs with good water stability, we then selected the $n_{-\mathrm{COOH}}/n_{-\mathrm{NH_2}}$ ratio at 5:1 for subsequent studies.

FTIR spectroscopy was then carried out to further characterize the G2.NH$_2$ dendrimer-crosslinked γ-PGA NFs (Figure 3a). The peaks at 1216 and 1071 cm^{-1} in the four different curves belong to the stretching vibration of C−N and C−O, respectively, and peaks at 1437 cm^{-1} in all curves can be assigned to the deformation vibration of C−H.[7] The two strong peaks of each curve at 1636 and 1527 cm^{-1} can be assigned to the amide I and amide II band, respectively.[38] The broad peak at around 3274 cm^{-1} in all curves may be attributed to the overlap of N−H and O−H stretching vibrations of γ-PGA skeleton. In contrast to γ-PGA NFs before crosslinking (Figure 3a, Curve 1), new peaks emerged at 1730 cm^{-1} in Curves 2, 3, and 4 after crosslinking, which can be assigned to the amide I band arising from the amido group generated from reaction of amine group of G2.NH$_2$ and the side carboxyl group of γ-PGA NFs.[39,40] It seems that, at a lower $n_{-\mathrm{COOH}}/n_{-\mathrm{NH_2}}$ ratio, the peak intensity at 1730 cm^{-1} is higher, indicating that more G2.NH$_2$ dendrimers have been involved in the crosslinking reaction. FTIR spectra qualitatively confirmed the successful crosslinking reaction of γ-PGA NFs with G2.NH$_2$ dendrimers.

The crosslinking of γ-PGA NFs with G2.NH$_2$ dendrimers enabled the NFs with surface amine functionality. The surface amine density of the γ-PGA−G2.NH$_2$ NFs was determined using Orange II dye, which can be absorbed by the primary amine groups of the γ-PGA NFs at a lower pH (pH = 3) and desorbed from γ-PGA NFs at a higher pH (pH = 12). Accordingly, the surface amine density of the NFs can be calculated from the concentration of adsorbed orange II dye. As shown in Figure 3b, the surface primary amine density decreased with the ratio of $n_{-\mathrm{COOH}}/n_{-\mathrm{NH_2}}$ from 0.78 ± 0.06 to 0.24 ± 0.03 μmol/mg NFs. This further confirmed the successful crosslinking reaction between G2.NH$_2$ dendrimers and the γ-PGA NFs. The amine functionality may provide many possibilities for further functionalization of the NFs for different applications.

G2.NH$_2$−FI Dendrimer-Crosslinked Stable γ-PGA NFs. Amine-terminated G2.NH$_2$ dendrimers prefunctionalized with different ligands were also used to crosslink γ-PGA NFs in order to simultaneously render the NFs with both water stability and desired functionality. To prove our hypothesis, we synthesized FI-modified G2 dendrimers to crosslink the γ-PGA NFs. The formation of G2.NH$_2$−FI conjugates was characterized via UV-Vis spectroscopy (Figure 4a). It is clear that G2.NH$_2$−FI conjugates show a strong absorption peak at 500 nm, which is associated with the typical FI absorbance feature, indicating that FI has been successfully conjugated onto the surface of G2.NH$_2$ dendrimers. Further ^{1}H NMR spectrum of the G2.NH$_2$−FI conjugates (Figure 4b) reveals the existence of the aromatic protons of FI moieties in the chemical shift region of 6−8 ppm. By integration of the proton peaks related to the signals of dendrimer and FI, we were able to estimate the number of FI moieties attached onto each dendrimer to be 2.8.

G2.NH$_2$−FI conjugates were then used to crosslink the γ-PGA NFs under the optimized conditions. After being immersed into PBS for one month, the formed γ-PGA−G2.NH$_2$−FI NFs still maintained the smooth and uniform
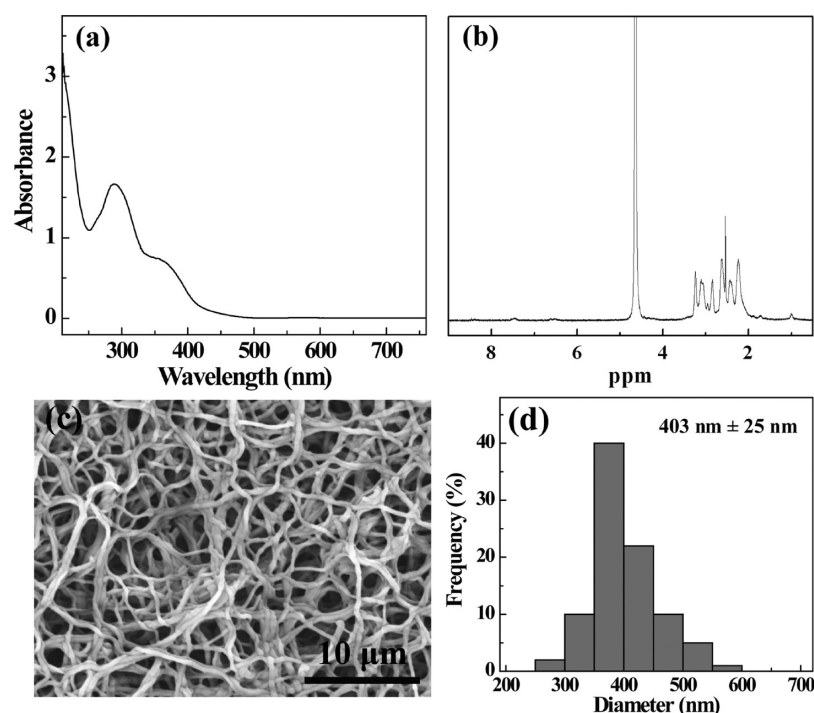
Figure 5. (a) UV-Vis and (b) ^{1}H NMR spectra of G2.NH$_2$−FA conjugates. (c) SEM image and (d) fiber diameter distribution histogram of γ-PGA−G2.NH$_2$−FA NFs.
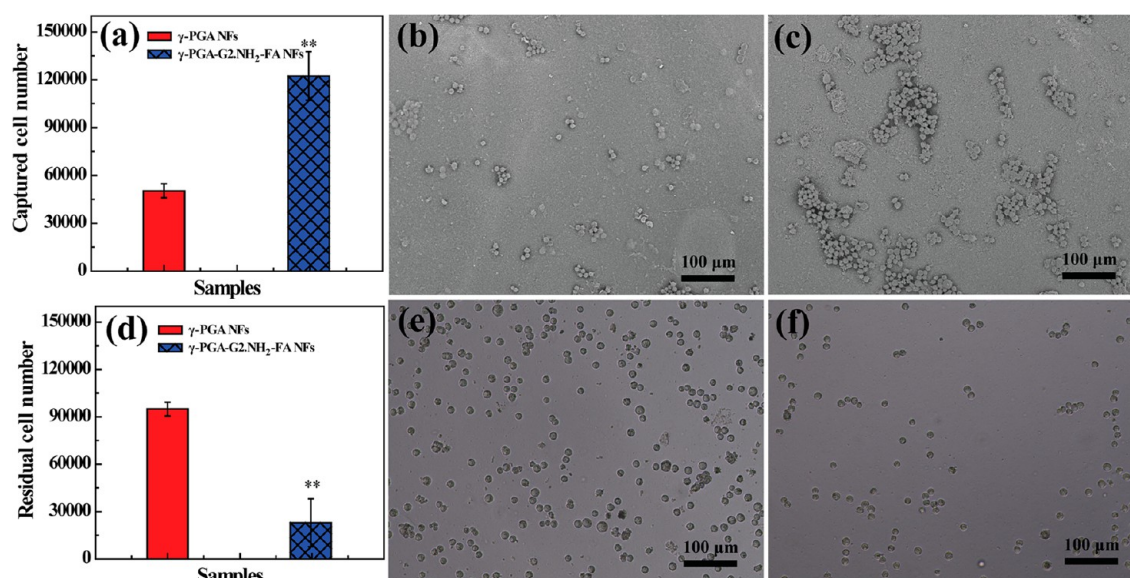
Figure 6. Cell capture capacity of γ-PGA−G2.NH$_2$ and γ-PGA−G2.NH$_2$−FA NFs. (a) shows the captured cell number onto γ-PGA−G2.NH$_2$ and γ-PGA−G2.NH$_2$−FA NFs; (b) and (c) show SEM images of γ-PGA−G2.NH$_2$ and γ-PGA−G2.NH$_2$−FA NFs with captured KB cells, respectively; (d) shows the residual cell number in the medium after cell capture; (e) and (f) show the phase contrast photomicrographs of cells in the medium of γ-PGA−G2.NH2 and γ-PGA−G2.NH2 NFs-FA NFs, respectively.

fibrous morphology (Figure 4c), except that some adjacent fibers attached together and the fiber diameter increased as compared to that of NFs before crosslinking (Figure 2a, 314 ± 28 nm versus 255 ± 53 nm), which is due to the swelling behavior of γ-PGA NFs during the crosslinking process and the immersion in PBS solution. Besides the rendered water stability, the crosslinking of γ-PGA NFs with G2.NH$_2$−FI conjugates also enabled the NFs with green fluorescence property, which is associated with the conjugated FI moieties. This can be confirmed by CLSM image of the formed γ-PGA−

G2.NH$_2$−FI NFs (Figure 4d). Our results clearly suggest that, via the crosslinking of FI-functionalized G2 dendrimers, γ-PGA NFs can be simultaneously rendered with both water stability and fluorescent property in one step.

G2.NH$_2$−FA Dendrimer-Crosslinked Stable γ-PGA NFs. As reported in the literature, FA has been extensively investigated for targeting various cancer cells overexpressing FAR.[30,41−45] It is reasonable to speculate that via the crosslinking of FA-functionalized G2 dendrimers, water-stable γ-PGA NFs may be generated to have an ability to specifically

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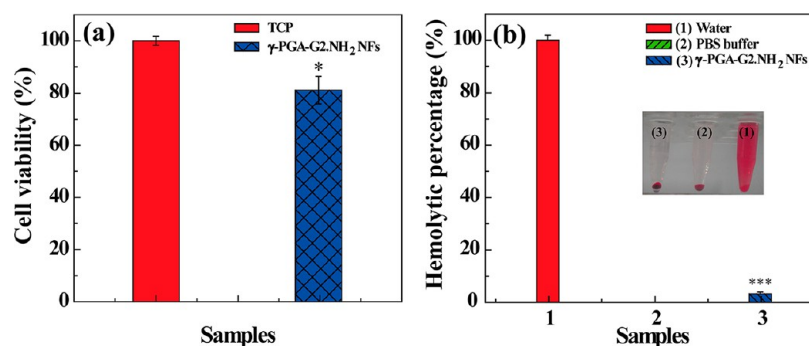dx.doi.org/10.1021/am405273v | ACS Appl. Mater. Interfaces 2014, 6, 2153−2161

Figure 7. (a) Rezasurin reduction assay of the metabolic activity of L929 cells cultured onto TCP and γ-PGA–G2.NH$_2$ NFs after a 3 day culture (mean $\pm$ SD, n = 3). (b) Hemolytic assay of HRBCs after treatment with water, PBS, and γ-PGA–G2.NH$_2$ NFs for 2 h. The inset shows the photograph of HRBC suspensions exposed to (1) water, (2) PBS, and (3) PBS suspended with γ-PGA–G2.NH$_2$ NFs for 2 h, followed by centrifugation.

capture cancer cells overexpressing FAR via ligand–receptor interaction, which is important for cancer diagnosis applications. To prove our hypothesis, G2.NH$_2$–FA conjugates were synthesized and characterized using UV-Vis and ^{1}H NMR spectroscopy (Figures 5). The strong absorption peak at 280 nm associated with the typical FA absorbance feature clearly indicated the successful conjugation of FA moieties onto the G2 dendrimer surface (Figure 5a). Similarly, the appearance of the aromatic proton peaks related to the FA moieties in the NMR spectrum also indicated the successful conjugation of FA onto the dendrimer surface (Figure 5b). By comparison of the NMR peak integration of the dendrimer and the FA, the number of FA moieties attached onto each G2 dendrimer was estimated to be 1.0. The prepared G2.NH$_2$–FA conjugates were used as a crosslinker to stabilize the γ-PGA NFs. After immersion of the formed γ-PGA–G2.NH$_2$–FA NFs into PBS for one month, the NFs still remained intact with a smooth and uniform fibrous morphology (Figure 5c), except that the fiber diameter was swelled to 403 $\pm$ 25 nm (Figure 5d), similar to the crosslinking reaction using G2.NH$_2$–FI conjugates as a crosslinker.

To confirm the specific cancer cell capturing ability of the formed γ-PGA–G2.NH$_2$–FA NFs, KB cells overexpressing FAR were cultured using the functional NFs as a substrate. γ-PGA–G2.NH$_2$ NFs without FA moieties were used as control. As shown in Figure 6a, after culturing for 4 h, the amount of captured KB cells by γ-PGA–G2.NH$_2$–FA NFs was significantly higher than that by γ-PGA–G2.NH$_2$ NFs (p < 0.01). The measurement of the residual KB cells in the culture medium showed that the number of cells cultured onto the γ-PGA–G2.NH$_2$–FA NFs was significantly lower than that onto the γ-PGA–G2.NH$_2$ NFs (p < 0.01, Figure 6d). Further qualitative SEM imaging results revealed that much more KB cells were able to be captured by γ-PGA–G2.NH$_2$–FA NFs (Figure 6c) than that by γ-PGA–G2.NH$_2$ NFs (Figure 6b). Moreover, phase contrast microscopy results also showed that the residual KB cell density in the culture medium of γ-PGA–G2.NH$_2$ NFs (Figure 6e) was consistently higher than that of γ-PGA–G2.NH$_2$ NFs-FA NFs (Figure 6f). Taken together, our results suggest that γ-PGA–G2.NH$_2$–FA NFs are able to specifically capture KB cells via ligand–receptor interaction. Since other kinds of cancer cells such as ovary, kidney, uterus, testis, brain, colon, lung, and myelocytic blood cancer cells also overexpress FAR,[46−49] the formed γ-PGA–G2.NH$_2$–FA NFs may be used as a general scaffold to capture different types of FAR-overexpressing cancer cells. It is worth noting that quite a

small amount of KB cells can also be captured by γ-PGA–G2.NH$_2$ NFs, which may be due to the non-specific interaction of the aminated surface of γ-PGA–G2.NH$_2$ NFs and the negatively charged cell membranes.

Cytocompatibility and Hemocompatibility of γ-PGA–G2.NH$_2$ NFs. Cytocompatibility and hemocompatibility are two key concerns for the materials to be used for biomedical applications. We next explored the cytocompatibility of γ-PGA–G2.NH$_2$ NFs using L929 cells as a model cell line (Figure 7a). After a 3 day culture, the in vitro rezasurin reduction assay reveals that the formed γ-PGA–G2.NH$_2$ NFs show a slight toxicity to L929 cells when compared with the TCP control (p < 0.05). However, the cell viability was still kept at a relatively high level (81.1 $\pm$ 5.2%). The slight toxicity of γ-PGA–G2.NH$_2$ NFs should be due to the electrostatic interaction between the positively charged NF surface and the negatively charged cell membranes.[50−52] Although the surface primary amine groups have turned out to be cytotoxic to cells in this study, further modification of the NF surface amine groups via acetylation or carboxylation to neutralize the surface amines may be able to solve this issue.[50,53]

The hemocompatibility of the formed γ-PGA–G2.NH$_2$ NFs was tested via a hemolysis assay (Figure 7b). In contrast to the positive control, where HRBCs exposed to water were totally damaged (inset of Figure 7b, vial 1), HRBCs treated with γ-PGA–G2.NH$_2$ NFs did not show any visible hemolysis effect (inset of Figure 7b, vial 3), similar to the negative PBS control (inset of Figure 7b, vial 2). The HP of the γ-PGA–G2.NH$_2$ NFs was measured to be 3.3 $\pm$ 0.8%, much lower than the threshold value of 5%[34] and significantly lower than the positive control (p < 0.001), suggesting the good hemocompatibility of the formed γ-PGA–G2.NH$_2$ NFs.

CONCLUSION

In summary, we report a facile approach to using amine-terminated G2 PAMAM dendrimers as a crosslinker to simultaneously render electrospun γ-PGA NFs with good water stability and desired functionality. Via a proper optimization, water-stable γ-PGA NFs were formed using G2.NH$_2$ dendrimers as a crosslinker. Further uses of FI- or FA-functionalized amine-terminated G2 dendrimers as a crosslinker enabled the generation of water-stable γ-PGA NFs with green fluorescence property or the ability to specifically capture FAR-overexpressing cancer cells. With the combination of the versatile dendrimer surface chemistry and the powerful electrospinning technology, water-stable multifunctional γ-

PGA-based NFs may be fabricated for diverse applications in the fields of biosensing, tissue engineering, drug delivery, and environmental sciences.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional SEM images of γ-PGA NFs crosslinked with G2.NH$_2$ dendrimers at different crosslinking times, followed by immersion in PBS for one month. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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