Controlled Gene Delivery System Based on Thermosensitive Biodegradable Hydrogel

Zhenhua Li,[1] Wen Ning,[2] Jumei Wang,[3] Augustine Choi,[2] Pui-Yan Lee,[1] Pradeep Tyagi,[1] and Leaf Huang[1,4]

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Purpose. Currently, most pDNA delivery systems based on synthetic polymers are either nonbiodegradable or not sensitive to the release environment. The primary objective of this study was to develop and evaluate an aqueous-based, thermosensitive, biodegradable and biocompatible triblock copolymer to control pDNA delivery *in vitro* and *in vivo*.

Methods. The triblock copolymers, poly[ethylene glycol-b-(D, L-lactic acid-co-glycol acid)-b-ethylene glycol] (PEG-PLGA-PEG), were synthesized as previously described. The molecular weight and polydispersity of PEG-PLGA-PEG were monitored by gel permeation chromatography (GPC). The cytotoxicity of PEG-PLGA-PEG was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The release of ^{32}P-labeled pDNA entrapped in aqueous dispersion of PEG-PLGA-PEG in 0.1 mol/L sodium phosphate buffer solution (pH 7.4) was studied at 37°C under agitation. Gene transfection efficiency was evaluated in a skin wound model in CD-1 mice.

Results. The aqueous dispersion of PEG-PLGA-PEG flows freely at room temperature but form a gel at 37°C body temperature. The *in vitro* degradation of PEG-PLGA-PEG lasted for more than 30 days. The cytotoxicity of PEG-PLGA-PEG evaluated in HEK 293 cells was significantly lower than that of poly-L-lysine hydrochloride. The release profile of supercoiled pDNA from the polymer followed the zero-order kinetics up to 12 days. Maximal gene expression of luciferase was at 24 h in the skin wound of CD-1 mice and by 72 h, the expression dropped by nearly 94%.

Conclusions. These results suggest hydrogel formed by PEG-PLGA-PEG could be a promising platform for delivery of pDNA, which represents a novel strategy that may serve as a non-viral vector for gene therapy in wound healing.

KEY WORDS: PEG-PLGA-PEG; triblock copolymers; thermosensitive polymer; wound healing; gene transfection.

INTRODUCTION

The success of gene therapy is largely dependent on the delivery vector system. Development of vectors characterized by maximum efficiency and minimal toxicity will define the success of gene therapy and its chances of being accepted by the public and clinicians. Viruses fulfilled the first requirement, but their use in humans raised concerns over their safety (1). Of late, biodegradable polymers have been extensively studied to serve the role of safe DNA-delivery systems (2,3). Hydrogels represent an improvement as a class of biodegradable polymeric materials with excellent biocompatibility, which can swell by taking up large amounts of water without dissolving to allow protracted localized gene expression, and improve the pDNA release kinetics (4).

Thermosensitive hydrogels are a newer class of hydrogels that have many advantages over "traditional" hydrogel because they can exhibit sol-gel transition at both room and elevated temperatures. These changes corresponding to swelling or deswelling of the polymer can be controlled to release of encapsulated pDNA in response to external temperature changes (5,6). Recent work has demonstrated that biodegradable triblock copolymers PEG-PLA-PEG and PEG-PLGA-PEG (poly[ethylene glycol-b-(D, L-lactic acid-co-glycol acid)-b-ethylene glycol]) can undergo sol-gel transition (7,8). The primary propose of the present work was to evaluate the feasibility of using biodegradable thermosensitive hydrogel based on PEG-PLGA-PEG as a carrier for controlled pDNA release *in vitro* and local gene transfection *in vivo*. Our study helps to define the promise and limitation of the pDNA-delivery system based on thermosensitive biodegradable hydrogel.

MATERIALS AND METHODS

Materials

D,L-lactide (DLLA) and glycolide (GA; Boehringer Ingelheim, Petersburg VA, USA), monomethoxy poly(ethylene glycol) (mPEG, Mw = 750 Da, Aldrich, Milwaukee, WI, USA), hexamethylene diisocyanate (HMDI; Sigma, St. Louis, MO, USA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained as molecular biology grade (Sigma). Poly-L-lysine hydrochloride (PLL; Mw 19 kDa) was from Sigma. Plasmid pNGVL3-Luc (pDNA), which contains the cDNA of firefly luciferase inserted into a pNGVL-Luc vector, was custom prepared by Bayou Biolabs (Harahan, LA, USA). CD-1 female mice (approx. 40 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were kept at the University of Pittsburgh Central Animal Facility. All experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee.

Synthesis and Characterization of Triblock Copolymers

Triblock copolymers were prepared by ring-opening polymerization as described elsewhere (7,8). Briefly, ring-opening polymerization of DLLA and GA onto mPEG 750 was performed, followed by coupling of the resulting diblock copolymer (mPEG 750-PLGA) using HMDI as a coupling agent. The resulting PEG-PLGA-PEG triblock copolymers were dried under reduced pressure. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 30°C with a Bruker DPX-300 NMR spectrometer operating at 300 MHz by using $CDCl_3$ as solvent. The molecular weight and molecular weight distribution of the triblock copolymers were monitored by GPC system with Waters 1515 isocratic HPLC pump and Waters 2410 refractive index detector. The GPC data were calibrated with polystyrene standards with molecu-

[1] Center for Pharmacogenetics, School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania 15213
[2] Division of Pulmonary, Allergy & Critical Care Medicine, UPMC, University of Pittsburgh, Pittsburgh, Pennsylvania 15213.
[3] Department of Neurobiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261.
[4] To whom correspondence should be addressed. (e-mail: HuangL@msx.upmc.edu)

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lar weights in the range of 640 to 1,010,000. Styragel® HR 4 and HR 2 columns (Waters) were used in series. The molecular weights were calculated using monodispersed polystyrene standard (Waters). All of the determinations were conducted in tetrahydrofuran (THF) at a flow rate of 1.0 mL/min at 35°C.

Degradation of Polymer *in Vitro*

Previously weighed PEG-PLGA-PEG triblock copolymers were placed in individual test tubes containing 1.0 mL of 0.1 M sodium phosphate (pH 7.4; phosphate buffer) to form a clear 30% (w/v) aqueous dispersion. The tubes were kept at 37 ºC. One milliliter of phosphate buffer was added 5 min later to the formed gel and shaken at 50 rpm. The samples were taken at designated time intervals, supernatant was removed, and the remaining samples were freeze-dried for 36 h. The degree of degradation was estimated from the decrease of molecular weight of the polymer and decrease of pH of the solution. The morphology of post-degradation hydrogel in phosphate buffer at 37ºC was examined by a scanning electron microscopy (JOEL Series 300).

Cytotoxicity Assay

Cytotoxicity of PEG-PLGA-PEG triblock copolymers following incubation for 48 h with HEK293 cells was evaluated in comparison with PLL (MW 19kD) using MTT assay as described by Fischer et al. (9).

Release of pDNA from Hydrogel *in Vitro*

pDNA was labeled with a random primers DNA labeling system (Invitrogen) in the presence of α-^{32}P dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) according to the vendor's protocol. Free radioactive nucleotide was removed by using a Bio-Spin® 30 column (Bio-Rad Laboratories, Hercules, CA). Ten microliters of ^{32}P-labeled pDNA and 10 μl (2.0 μg/μL) pDNA were mixed with PEG-PLGA-PEG triblock copolymers solutions in a 5-ml test tube to form a homogeneous clear solution at room temperature. Tubes were incubated at 37ºC to form a clear gel. After 5 min, 1 ml of phosphate buffer (pH 7.4) containing NaN_3 (0.02%, w/v) was added to the tubes as release medium under shaking. The amount of pDNA released in each time interval was determined by counting the radioactivity in the supernatant.

Integrity of Released pDNA and Encapsulated pDNA within the Hydrogel

pDNA was extracted from hydrogel for agarose gel electrophoresis by a chloroform/TE extraction method. Samples of control and recovered pDNA were separated on 1% (w/v) agarose gel. The gel was electrophoresed for 40 min at 100 V/cm in a TAE buffer system (pH 8.0) and the pDNA was visualized using ethidium bromide staining (10).

Gene Delivery to Skin Wound *in Vivo*

Mice were anesthetized by inhalation of Isoflurane. Two full-thickness square skin wounds (7×7 mm diameter) were produced on the dorsum of each mouse with a standard biopsy scissors and left undressed. After surgery, the wound was treated with 200 μg of luciferase pDNA in either 50 μL of 30% PEG-PLGA-PEG aqueous solution, or in phosphate buffer using a sterile pipette. At days 1, 2, and 3 of postapplication, mice were sacrificed, and the wound biopsies were collected and the luciferase activity in the homogenized tissue was measured by using a luminometer. The activity is presented as relative light units per mg soluble tissue protein (RLU/mg protein).

RESULTS

Characterization and Degradation of PEG-PLGA-PEG Triblock Copolymer

PEG-PLGA-PEG triblock copolymers were synthesized, and its structure was confirmed by ^{1}H NMR. The weight average Mw of triblock copolymer was 12,798 Da with a polydispersity index of 1.56 as determined by GPC. The aqueous solution (30%, w/v) of this polymer flowed freely at room temperature or below, but became a gel at elevated temperature such as 37°C (Fig. 1A). Figure 2A shows the change in

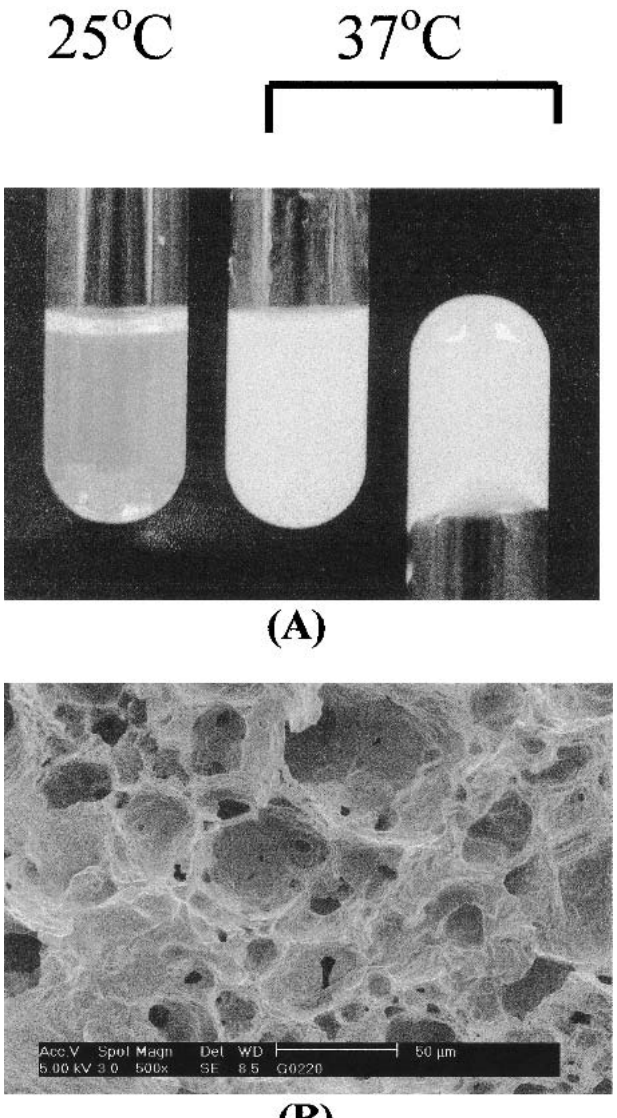

Fig. 1. Photograph of the hydrogel at different temperatures (A) and scanning electron micrograph of the cross section of the hydrogel at 30 days (B).

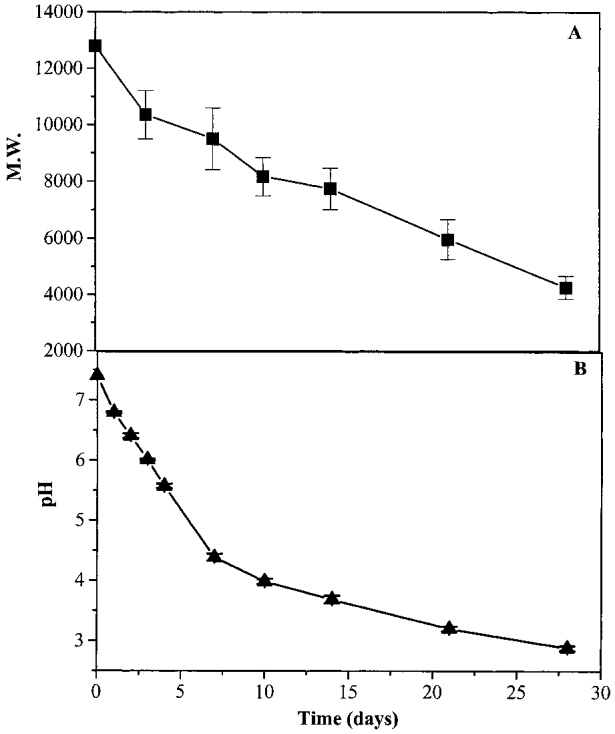

Fig. 2. Degradation of the triblock copolymer. Time course of decrease in the molecular weight of PEG-PLGA-PEG (A) and change of pH of the medium (B) during incubation in 0.1 M phosphate buffer (pH 7.4) at 37°C. Data points indicate mean values ± SD, n = 3.

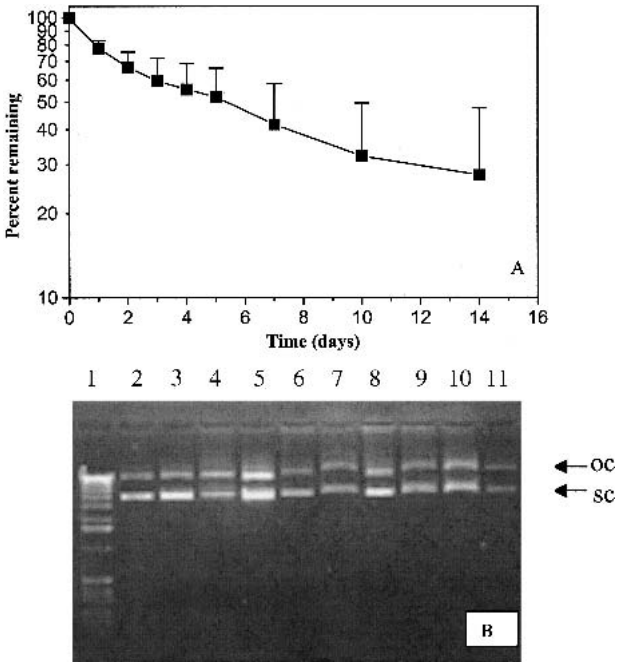

Fig. 3. Release profile of pDNA from 30% PEG-PLGA-PEG hydrogel in phosphate buffer at 37°C. Data points indicate mean values ± SD, n = 3 (A). Electrophoretic mobility analysis of pDNA released into the medium or remained in the hydrogel (B). Lane 1: molecular weight markers (1.0 kb); lane 2: control pDNA; lanes 3–5: free pDNA incubated in phosphate buffer at days 2, 6, and 12; lanes 6–8: pDNA remained in the hydrogel at days 2, 6, and 12; lanes 9–11: pDNA released from the hydrogel at days 2, 6, and 12. oc = open circle; sc = supercoiled.

molecular weight of the remaining gel during degradation at 37°C with an initial concentration of 30 wt%. When the hydrogel was exposed to phosphate buffer, water permeated into gel matrix resulting in random hydrolysis of ester bonds and the decrease of molecular weight. The apparent half-life of degradation was about 15 days. Figure 2B displays the decrease of the medium pH versus incubation time. The pH of triblock copolymer solution reached a final value of 2.87. Thus, PEG-PLGA-PEG hydrogel gel persisted for about 30 days owing to the relatively high hydrophobicity. Scanning electron microscopy analysis of postdegradation hydrogels showed a highly porous morphology with interconnections as seen in the cross section of the hydrogel (Fig. 1B).

Cytotoxicity *in Vitro*

After incubating HEK 293 cell line for 48 h with 500 μg/mL copolymer, the cells showed neither a decrease in population nor a change in morphology, and the cell viability was over 98% compared with untreated control as determined by MTT assay (data not shown). However, cell viability was only 5% after incubation with PLL, apart from granulated appearance of the cells.

In Vitro pDNA Release from Hydrogel

Figure 3A shows the release of pDNA from hydrogel over the incubation time. In this study, pDNA was encapsulated into PEG-PLGA-PEG hydrogel and release profiles of pDNA exhibited an approximately constant rate over the 15 days of incubation at 37°C. The apparent half-life of release was about 5 days. Fig. 3B shows that released pDNA was predominantly supercoiled, although a small amount of open circle pDNA was visible at 12 days. Linearized pDNA was also present after encapsulation, but to a significantly smaller extent when compared to the released pDNA.

Gene Delivery by PEG-PLGA-PEG Hydrogel in Mouse Skin Wound

When the polymer solution was applied to a fresh skin wound, it formed a hydrogel in approximately 45 min. We

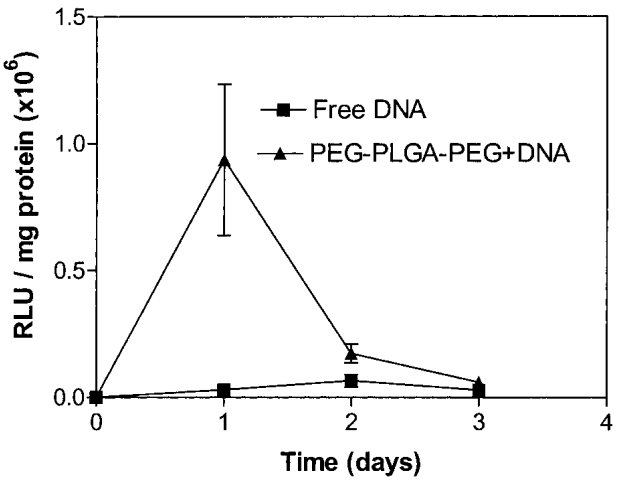

Fig. 4. Luciferase activity in mouse skin wound after treatment with luciferase pDNA in either phosphate buffer or PEG-PLGA-PEG hydrogel. Data points indicate mean values ± SD, n = 3.

studied the expression pattern of the luciferase reporter gene after treatment with PEG-PLGA-PEG hydrogel delivery system. The expression of luciferase reached its maximum at 24 h and declined thereafter. It was reduced to 6.4% of the maximal activity at 72 h (Fig. 4). The level and duration of gene expression were similar to that of the same pDNA delivered by electroporation (Chesnoy & Huang, unpublished result).

DISCUSSION

Efficient delivery of DNA remains an important issue for nonviral gene therapy. The goal of this study was to develop a biocompatible and biodegradable thermosensitive hydrogel as a gene carrier for controlled delivery of pDNA. The transfection efficiencies of nontoxic polymeric carriers, e.g., a biodegradable cationic polymer, were not satisfactory (11,12). The use of thermosensitive biodegradable hydrogel as a delivery system for pDNA may have some important advantages over other technologies. PEG-PLGA-PEG is composed of ester and urethane in the backbone with no pendant functional groups. The PEG-PLGA-PEG hydrogel system is thought to have a core-shell structure in an aqueous environment. The hydrophilic PEG occupies the shell region and hydrophobic PLGA hides into the core in order to reduce the free energy (13).

Because the hydrogel is degradable, both diffusion and degradation could drive drug release. However, pDNA release follows a constant rate with an apparent half-life of 5 days. Because this is shorter than the polymer degradation half-life, which is approximately 15 days, the mechanism of pDNA release must be a combination of diffusion and polymer erosion. It is important that the pDNA is not released immediately upon contact with aqueous medium, which can be the case for release of small hydrophilic molecules from hydrogels. The relatively high degradability of PEG-PLGA-PEG allowed the release of pDNA in a sustained fashion. Ideally, release from such a uniform matrix of pDNA would be completely dependent on polymer degradation, very similar to that described for the zero-order kinetics observed in the release of macromolecules from microspheres (14).

Hydrolysis is the chief process for the degradation of polyester, such as PLGA. The hydrolysis of the polyester follows acid-base catalyzed reaction to generate lactic acid and glycolic acid, which results in the decrease of medium pH. Recently, it was reported that pDNA could be degraded in low pH environment as a result of lactic acid and glycolic acid coming from PLGA degradation (10). In our study, we observed that pDNA released from this hydrogel system in the first 12 days was of supercoiled form, reflecting maintenance of structural integrity of pDNA in the release medium and the hydrogel (Fig. 3), and there was a gradual conversion of supercoiled plasmid to the nicked isoform of pDNA at 14 days (data not shown).

In the concentration range used in our study, the PEG-PLGA-PEG triblock copolymers displayed much lower cytotoxicity and higher degree of biocompatibility than PLL. DNA entrapped in PEG-PLGA-PEG hydrogel can effectively deliver genes into skin cells. The decline to 6% by 72 h of the maximal gene expression level at 24 h suggests that the process of topical delivery of DNA facilitate a transient, high-level expression of the exogenous gene delivered into the skin cells. A similar observation has been reported in a study, in which mouse skin was treated topically with β-galactosidase DNA (15). In preliminary studies, the hydrogel prepared from the triblock copolymer can deliver TGF-β1 cDNA to promote wound healing (data not shown).

Limited study has been reported using hydrogel, such as engineered silk-elastin-like hydrogel as a pDNA delivery system (16); however, it was only limited to *in vitro* studies. There was no cytotoxicity evaluation *in vitro* and gene expression *in vivo*. Furthermore, the hydrogel was not thermosensitive. Poloxamers have been shown to increase gene expression in skeletal muscle possibly by protecting pDNA from enzymatic degradation and by enhancing distribution of pDNA across the tissue (17). However, they are not considered an optimal system for the delivery of pDNA because they are nonbiodegradable and likely to be toxic.

In summary, our results confirm that thermosensitive biodegradable hydrogel provides a useful and novel approach to sustained delivery of pDNA and enables local delivery of pDNA for efficient expression of a recombinant gene in the skin cells. These encouraging results indicate the potential use of this novel non-viral gene delivery system for therapies of skin disorder and wound healing.

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