

## Development of a Functionalized Polymer for Stent Coating in the Arterial Delivery of Small Interfering RNA

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Received July 1, 2009; Revised Manuscript Received September 1, 2009

In patients receiving drug eluting stents, there is a growing concern about both the long-term toxicity/degradability of the polymers used for the coating, and the nature of the therapeutic agents. We hypothesized that the use of a functionalized biocompatible polymer for a stent coating could be appropriate for local arterial therapy. A cationized pullulan hydrogel was thus prepared to cover bare metal stents that could be further loaded with small interfering RNA (siRNA) targeted at MMP2 for gene silencing in vascular cells. The efficient coverage of the stent struts by a smooth polymeric layer, which can withstand the crimping of the stent on a balloon-catheter and its deployment, was demonstrated by fluorescence microscopy, scanning electron microscopy, and atomic force microscopy. The release of siRNA from the stents was modulated by the presence of the cationic groups, as compared to noncationized pullulan hydrogel. In vivo implantation of coated stents was successful and cationized pullulan-based hydrogels loaded with siRNA in rabbit balloon-injured carotid arteries induced an uptake of siRNA into the arterial wall and a decrease of pro-MMP2 activity. These results suggest that cationized pullulan-based hydrogel could be used as a new biocompatible and biodegradable stent coating for local gene therapy in the arterial wall.

### Introduction

Atherosclerosis and its complications are the main cause of mortality in the world.<sup>1</sup> Coronary stent implantation has now become a common practice in interventional cardiology. However, restenosis remains by far the main complication of this technique. To overcome this problem, numerous studies reported a stent coating by a polymeric layer that included a therapeutic agent. This strategy was transferred into the clinical practice with antimitotic agents released from coated stents to reduce vascular cell proliferation.<sup>2–4</sup> Despite very promising early results, increased late events have been observed recently at 3–4 years postimplantation in patients receiving drug eluting stents compared with bare metal stents, thus leading to a growing concern about both the long-term toxicity/degradability of the polymers used for the coating and the therapeutic agents.<sup>5–7</sup> We hypothesized that the use of a functionalized biocompatible polymer as stent coating could be appropriate for local arterial therapy.

In this context, various studies have shown that small interfering RNA (siRNA) could be used for sequence-specific gene silencing in vascular cells.<sup>8,9</sup> These results suggested that RNA interference technology could be of particular interest in the prevention or the treatment of cardiovascular diseases. In particular, siRNA delivery into vascular smooth muscle cells to reduce their proliferation and migration could be an alternative

strategy to reduce intrastent restenosis. The potential use of siRNA-based therapy for the treatment of arterial diseases requires efficient local siRNA delivery in the arterial wall and siRNA uptake by vascular cells. Indeed, the intravenous application of siRNA may lead to undesirable accumulation of siRNA in nontarget organs.<sup>10</sup> Recent studies have shown successful transfer of plasmid DNA or oligonucleotides from coated stents into the arterial wall in vivo.<sup>11–16</sup> This strategy represents an attractive option for local delivery of siRNA in vascular cells in vivo.

To obtain a safe, efficient, and local gene delivery system, an important issue is related to the nature of the polymer. Pullulan is a natural polysaccharide, and due to its nontoxic, nonimmunogenic, and biodegradable properties, it is considered as a promising material for biomedical applications.<sup>17</sup> Pullulan can be cross-linked into biocompatible and biodegradable hydrogels.<sup>18,19</sup> Hydrogels are particularly suitable for the delivery of therapeutic agents because they can absorb a wide range of molecules and further release them by diffusion.<sup>20</sup> To use hydrogel matrices for the delivery of genetic materials such as oligonucleotides, plasmid DNA or siRNA, one possibility to increase the interaction of the hydrogel with these negatively charged molecules is to incorporate cationic charges into the polymer.<sup>21</sup> For this purpose, pullulan can be easily modified by chemical grafting of amino groups to obtain cationized pullulan.<sup>22,23</sup> In previous studies, we demonstrated that cationized pullulan 3D matrices could deliver plasmid DNA into vascular cells in vitro. We also showed that the complexation of DNA into these matrices could be modulated by changing the density and the nature of amino groups grafted on the pullulan backbone.<sup>23,24</sup> According to these results, and taking into account the intrinsic film-forming properties of pullulan

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and peculiar elasticity of this type of polymer, we designed a functionalized stent coating for gene delivery based on cationized pullulan. The aim of the present work was to prepare and characterize this new stent coating and to evaluate the use of stents coated with cationized pullulan-based hydrogel for local delivery of siRNA into rabbit arterial walls as a new device for *in vivo* gene silencing.

## Experimental Section

**Materials.** Pullulan ( $M_n$  78000 g/mol) was purchased from Hayashibara Inc. (Okayama, Japan). Cationized pullulan was synthesized by chemical grafting of diethylaminoethylamine (DEAE) groups, as previously described.<sup>24</sup> The presence of amino groups on the pullulan backbone was evidenced by FTIR (ThermoNicolet AVATAR 370 FTIR) and 500 MHz <sup>1</sup>H NMR (VARIAN Unity Inova 500 MHz spectrometer; 30 mg/mL in D<sub>2</sub>O). Nitrogen elemental analysis (CNRS, Vernaison, France) and acid–base titration (DL 53 Titrator, Mettler Toledo) gave the amount of amino groups. Molecular weights were determined by size-exclusion chromatography coupled online to a multiangle laser light scattering (MALLS) detector (DAWN-F, Wyatt Technology). Trisodium trimetaphosphate (STMP), fluorescein-isothiocyanate-dextran 500 (FITC-dextran), and pullulanase were obtained from Sigma (St Louis, MO). Phosphate buffer saline (PBS) was obtained from Gibco-BRL. The siRNA sequence was synthesized and tagged with tetramethylrhodamine (Tamra) by Eurogentec and siRNA stock solution was stored at –20 °C.<sup>25</sup> Multilink Mini Vision coronary stents and stainless steel coupons (10 mm diameter) were obtained from Guidant and Abbott Lab, respectively. Rabbit MMP2-siRNA (5'-UCAUCGUCGUAGUUGGUUG99-3' sense strand and 3'-99A-GUAG-CAGCAUCAACCAAC-5' antisense strand) and scramble siRNA (5'-AACACACCUACG-GCAAAGU99-3' sense strand and 3'-99UUGU-GUGGAUGCCGUUUC-5' antisense strand) sequences were determined with the Ambion Web-based algorithm (accession number = D63579). The MMP2-siRNA sequence was designed to inhibit matrix metalloproteinase (MMP)2 gene expression while scramble siRNA is a negative control with the same nucleotide composition as MMP2-siRNA but which lacks sequence homology to the genome. Both sequences were synthesized and tagged with tetramethylrhodamine (Tamra) by Eurogentec.

**Preparation of Cationized Pullulan-Coated Stents and Stainless Steel Coupons.** Hydrogels were obtained from a solution or a mixture at 2.5% in water (w/v) of pullulan, pullulan/FITC-dextran (96/4 w/w), pullulan/cationized pullulan (52/48 w/w), or pullulan/cationized pullulan/FITC-dextran (48/48/4 w/w/w). Chemical cross-linking was carried out with STMP under alkaline conditions. Briefly, a total amount of 25 mg of polysaccharide was dissolved in 900  $\mu$ L of 0.9 M NaOH. After 30 min, under magnetic stirring, 100  $\mu$ L of STMP (110 g/L in water) was added and the mixture was kept under magnetic stirring for an additional 10 min. Bare metal stents were dip coated with this mixture and placed at 50 °C for 1 h. The samples were then washed extensively with PBS and water to remove residual salts. They were allowed to air-dry overnight and submitted to UV exposure (30 min) prior to use.

**Characterization of the Polymer Coating.** FITC-dextran was added into the coating mixture to use fluorescence microscopy as a simple and nondestructive quality control of stent coatings. Fluorescence microscopy was performed with a Nikon Y-FL fluorescence microscope. The morphology of coated stents was characterized by SEM using a Leica-Zeiss LEO 440 scanning electron microscope with an accelerating voltage of 15 kV. Samples were coated with a thin layer of gold/palladium using a sputterer (7.5 nm) prior to imaging. For experiments designed to evaluate the effects of stent expansion on the coating, coated stents were mounted on a balloon and balloon expansion at nominal pressure was performed. Surface profiles of bare metal stents and cationized pullulan-coated stents were visualized with a Digital Instruments Dimension 3100 atomic-force microscope (Veeco Metrology Group). Imaging was performed in the intermittent noncontact

(tapping) mode by using oscillating MPP-21100 tips (Veeco probes) with a resonance frequency range of 50–100 Hz. Each data was collected over a scan area of  $5 \times 5 \mu\text{m}^2$  at a scanning frequency of 1.001 Hz. Roughness analyses were performed at three randomly chosen locations to determine the average surface roughness ( $R_a$ ). To determine the mass of the coating, the polymeric layer was removed from the stent struts by enzymatic degradation. In brief, each coated stent was incubated with 1 mL of pullulanase at 40 units/mL during 3 h. Fluorescence intensity of FITC-polymer in the incubation medium was then measured using a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo Electron Corporation) with excitation and emission wavelengths of 485 and 538 nm, respectively. The mass of FITC-polymer in the incubation medium was deduced from a calibration curve obtained from the incubation medium of a fixed volume of cross-linked mixture. Contact angles were obtained from static contact angle measurements of deionized water droplets. Contact angles of noncoated stainless steel coupons and stainless steel coupons coated with cationized pullulan-based hydrogel were measured with a video contact angle system (KrTiss, model DSA 10-Mk2) applying a 1  $\mu$ L droplet of deionized water on the samples' surface. For each droplet, the contact angle was determined at 15 s with the Drop Shape Analysis software. Four measurements were performed on each sample and three samples per group were used.

**siRNA Loading onto Coated Stents and In Vitro Retention.** For these experiments, stents coated with pullulan or cationized pullulan-based hydrogels without FITC-dextran were used. For each group, 10 mm long coated stents ( $n = 3$ ) were immersed into 150  $\mu$ L of an aqueous solution of Tamra-tagged siRNA at 10  $\mu$ M during 1 h at room temperature. The supernatant of siRNA solution remaining on the stents after immersion was carefully removed by aspiration and siRNA-loaded coated stents were then allowed to dry for 1 h at room temperature with protection from light. The total amount of Tamra-tagged siRNA loaded onto the stents was calculated as the difference between the siRNA content of the dipping solution before and after immersion, measured by fluorescence spectroscopy with a fluorescence spectrophotometer (Fluoroskan Ascent, Thermo Electron Corporation) with excitation and emission wavelengths of 510 and 590 nm, respectively. The presence of Tamra-tagged siRNA in the polymeric layer was then assessed by fluorescence microscopy. Coated stents loaded with siRNA were immersed in 500  $\mu$ L of PBS in a 24-well plate and placed at 37 °C under gentle shaking. The supernatants were collected with replacement every 10 min during 1 h. The siRNA concentration of the incubation solutions and in the supernatants of the elution experiment was measured by fluorescence spectroscopy as described above.

**In Vivo Implantation of siRNA-Loaded Cationized Pullulan-Coated Stents.** The animal protocol was approved by the Bichat University Institutional Animal Care and Use Committee. A total of 11 New Zealand white rabbits (3.5–4.0 kg) were fed with a 0.3% cholesterol diet during 3 weeks. Before angioplasty, animals were premedicated with intramuscular 1% acepromazine, then anesthetized with intravenous 0.1% pentobarbital. A balloon catheter was introduced percutaneously via a 5-French femoral sheath and advanced in the right carotid artery under fluoroscopic guidance (BV Endura, Philips). Right carotid artery balloon abrasion (3.5 mm balloon diameter, three inflations at 10 atm for 30 s) was performed and then the skin wound was repaired. After 15 days, two cationized pullulan-coated stents were implanted per rabbit in the balloon-injured carotid arteries. In five animals, one coated stent without siRNA and one coated stent with Tamra-tagged siRNA were implanted. In the six others, one coated stent loaded with Tamra-tagged MMP2-siRNA and one coated stent loaded with Tamra-tagged scramble-siRNA were implanted. All the implanted stents were coated with a polymeric mixture containing FITC-dextran and a quality control of the coating was performed by fluorescence microscopy prior to use. The siRNA loading on the cationized pullulan-coated stents was performed as described above, except that the incubation of coated stents into the siRNA solution was completed overnight at 4 °C. After 24 h, the animals were euthanized by intravenous pentobarbital overdose and

the carotid arteries were harvested. The stents were carefully separated from the arteries of the first series of five animals, and the latter were flushed with PBS and frozen in liquid nitrogen. Fluorescence microscopy was performed on arterial 7  $\mu\text{m}$  thick cross sections cut with a cryostat (Leica CM 1900). The arteries of the second series of 6 animals were incubated for 48 h in 200  $\mu\text{L}$  of serum-free medium at 37  $^{\circ}\text{C}$  in 95% air–5%  $\text{CO}_2$ . The conditioned media were collected and stored at  $-20$   $^{\circ}\text{C}$  until gelatinolytic activities were measured as described previously.<sup>26</sup> Densitometric analysis of scanned gelatinolytic bands per mg of sample is performed with NIH software, release beta 3b (National Institutes of Health, Bethesda, MD) and expressed in arbitrary units (AU).

**Statistical Analysis.** Values are expressed as means  $\pm$  SD. A two-way ANOVA test was used to compare the overall effects of treatment and time followed by post hoc Fisher test for intergroup comparisons when appropriate (StatView 4.5). Differences were considered statistically significant at  $p < 0.01$ .

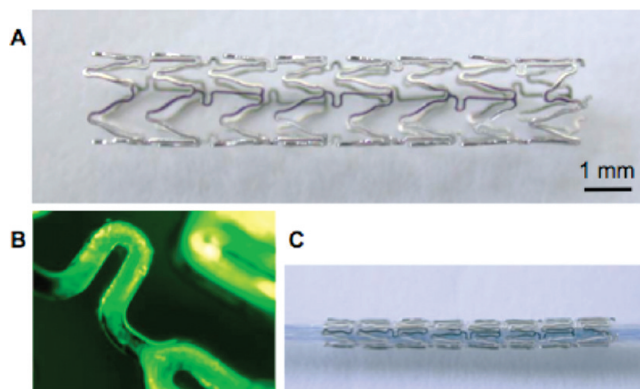
## Results

**Coating of Bare Metal Stents with Cationized Pullulan-Based Hydrogels.** To develop a new functionalized biocompatible stent coating, an aqueous solution of pullulan, cationized pullulan and STMP as a cross-linker was dip-coated onto bare metal stents. The functionalization of pullulan with DEAE was chosen because DEAE-pullulan is able to form complexes with DNA. The amount of grafted amine was related to the feeding ratio of DEAE in the reaction mixture. A DEAE-pullulan was obtained with a  $M_n$  of 81000 g/mol (polydispersity index = 1.5) and 2.1 mmol/g of DEAE. This polymer was previously demonstrated to complex plasmid DNA.<sup>24</sup> Then the cross-linking of the pullulan chains was obtained by simply placing the coated stents at 50  $^{\circ}\text{C}$  for 1 h, and no organic solvent was used. By adjusting the concentrations of reagents, we were able to obtain an efficient covering of the stent struts. Note that the choice of polymer concentration (2.5%) for the gelling mixture was carefully adapted since the use of lower concentrations resulted in an incomplete covering of the stent struts, while higher concentrations induced the formation of webbings between the stent struts. When this protocol was used, various geometries of stents could be used, regardless of manufacturers (stents from Guidant and Abbott were used for instance in this study with identical results). The cross-linking of the polymer chains with STMP for hydrogel formation, as described by Lack et al.,<sup>27</sup> allowed to obtain a stable polymeric coating that was stable in a dry state for months. Previous studies from our laboratory have already demonstrated the biocompatibility and biodegradability of such hydrogels.<sup>18,19</sup>

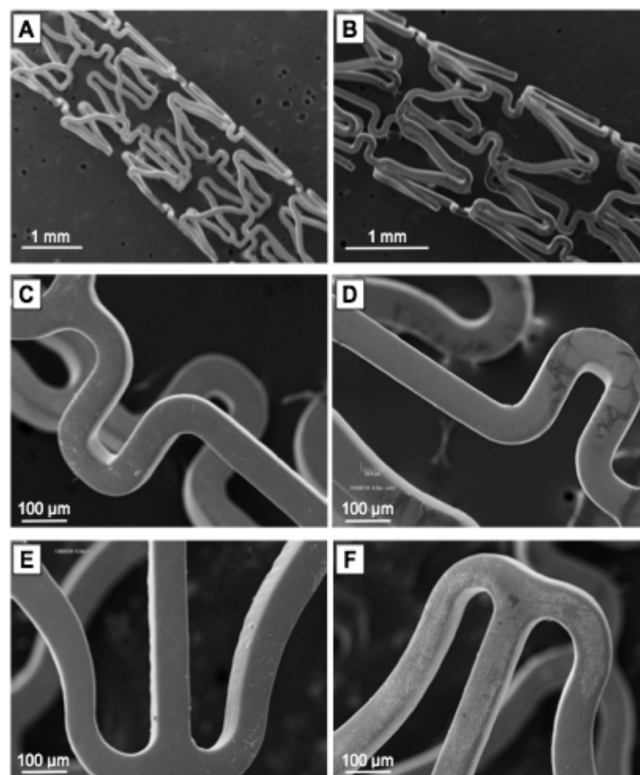
**Characterization of the Coating Before and After Deployment of the Stent.** The polymer efficiently covered the stents (Figure 1A,B). Interestingly, there was no crack on coated stent surface after crimping onto a balloon catheter (Figure 1C).

The coating was smooth in straight portions of the struts and even in the bends (Figure 2). SEM images of the coated stents did not show crack or webbing in the polymeric layer. These surface characteristics were maintained after full expansion of the stent by the angioplasty balloon (Figure 2F), indicating that the polymer coating can withstand the compressive strains applied during this procedure.

Tapping mode AFM images of the surface of bare metal stents and cationized pullulan-coated stents are shown in Figure 3. Topography AFM images strongly suggested the covering by a uniform and smooth thin layer on the surface of the coated stents. The average surface roughness ( $R_a$ ) obtained from the AFM analyses was very low for both types of stents, with  $R_a =$



**Figure 1.** Appearance of a cationized pullulan-coated stent. The coated stent before crimping on a balloon-catheter is shown in (A) for gross appearance and in (B) for fluorescence microscopy. (C) Gross appearance after crimping.

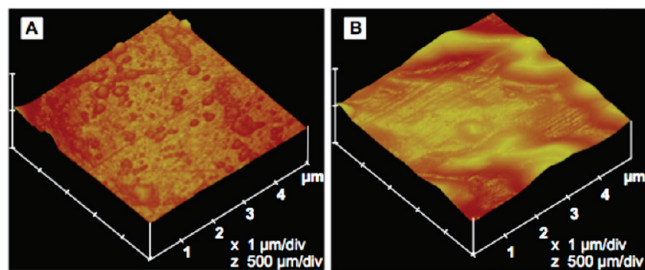


**Figure 2.** SEM images at different magnifications of semiexpanded (A–D) and fully expanded (E, F) stents. Bare metal stents are displayed on the left panel (A, C, E) and cationized pullulan-coated stents on the right panel (B, D, F).

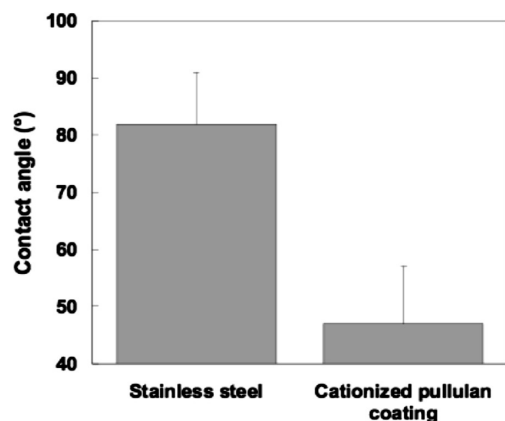
$11 \pm 3$  nm for bare metal stents and  $19 \pm 2$  nm for cationized pullulan-coated stents ( $n = 3$  per group).

Static contact angle measurements of water droplets were then carried out on noncoated stainless steel coupons and stainless steel coupons coated with cationized pullulan-based hydrogel. Contact angles were significantly different ( $p < 0.001$ ) with values of  $82 \pm 9$  and  $47 \pm 10$  degrees measured for noncoated and cationized pullulan-coated samples, respectively (Figure 4).

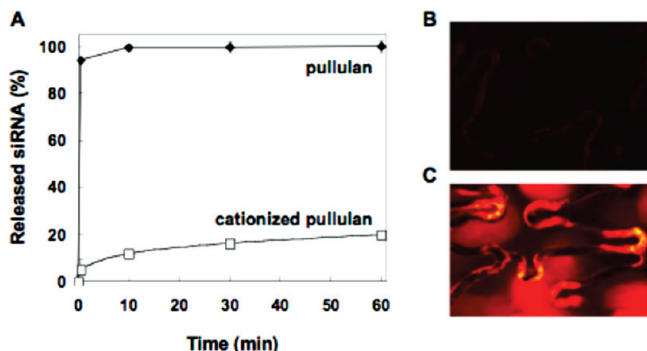
After enzymatic degradation of the biopolymer coating, the calculated mass of the polymer coated on 10 mm long stents was  $1.4 \pm 0.2$  mg. Overall, these physical characterizations indicated the successful coating of stents by a functionalized biomacromolecule. The coating was stable for days and no modification was observed after immersion in saline solutions.



**Figure 3.** Topography AFM images ( $5 \times 5 \mu\text{m}^2$ ) of the surface of bare metal stent (A) and cationized pullulan-coated stent (B).

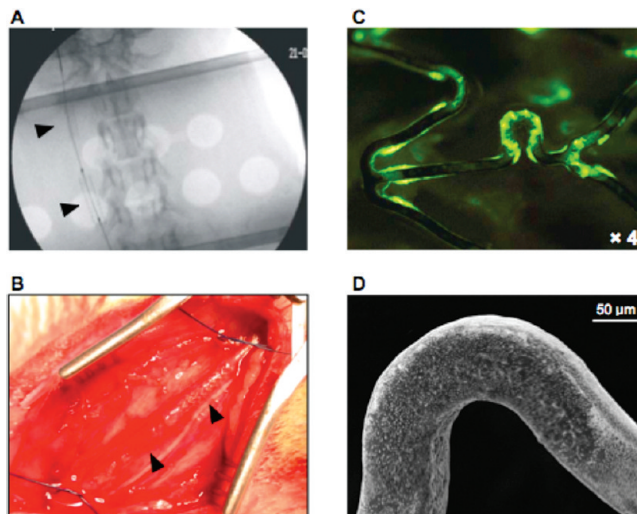


**Figure 4.** Water contact angles measured on uncoated stainless steel coupons, or coated with cationized pullulan hydrogel. Contact angles were significantly different ( $p < 0.001$ ) between the two groups.



**Figure 5.** In vitro release profile of Tamra-tagged siRNA from hydrogel-coated stents. (A) In vitro release profile of Tamra-tagged siRNA from plain pullulan or cationized pullulan-coated stents. Fluorescence microscopy images after 1 h elution are shown on the right for pullulan-coated stents (B) or cationized pullulan-coated stents (C).

**siRNA Loading onto Cationized Pullulan Coating and In Vitro Retention.** Immersion of 10 mm long coated stents into a  $10 \mu\text{M}$  solution of siRNA resulted in an uptake of  $0.9 \pm 0.3 \mu\text{g}$  and  $2.3 \pm 0.5 \mu\text{g}$  of siRNA by pullulan and cationized pullulan coatings, respectively, clearly visible by the red fluorescence from Tamra-tagged siRNA. Loaded cationized pullulan-coated stents were then submitted to an in vitro release study. The kinetics study indicated only  $20 \pm 2\%$  of siRNA release in 1 h from cationized pullulan-coated stents, whereas siRNA was fully released from noncationized pullulan-coated stents in an early burst of less than 10 min (Figure 5A). After the 1 h elution test, siRNA was no longer visible by fluorescence microscopy on noncationized pullulan-coated stents (Figure 5B), whereas Tamra-tagged siRNA was still clearly visible on cationized pullulan-coated stents (Figure 5C). In Figure 5C, red fluorescent light emitted from the Tamra-tagged siRNA remain-



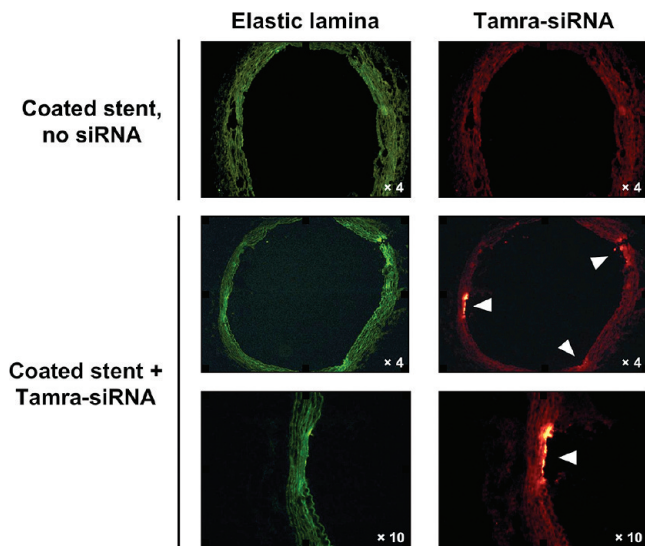
**Figure 6.** In vivo implantation of cationized pullulan-coated stents. (A) Angiographic image obtained during the implantation procedure evidenced the two implanted stents (arrows). Macroscopic view of implanted coated stents (arrows) in (B), fluorescence microscopy image (green fluorescence of FITC-tagged coating) in (C), and scanning electron microscopy in (D).

ing on the stent surface can be observed clearly in the foreground and as diffused light from the background.

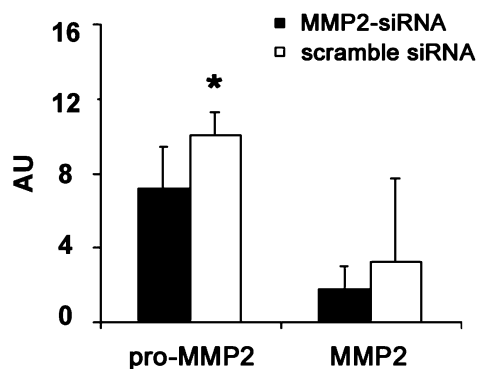
**Implantation of Cationized Pullulan-Coated Stents into Rabbit Carotid Arteries.** Implantation of the cationized pullulan-coated stents into rabbit carotids was then carried out (Figure 6A). The procedure was performed successfully in all animals ( $n = 11$ ). All animals were alive after implantation and no adverse effects were observed on the implanted rabbits. After explantation, no macroscopic inflammation or thrombus was seen around the implanted stents (Figure 6B). The polymeric layer was still clearly visible at 24 h on the struts of the explanted stents as shown by fluorescence microscopy and SEM analysis (Figure 6C and D, respectively), although some cracks were observed by SEM in the polymeric layer. In fluorescence microscopy images, some autofluorescence from fibrin deposition could appear in addition to the fluorescence light emitted by the FITC-polymer (Figure 6C). Only few platelets were observed on SEM images of the explanted stents (Figure 6D).

**In Vivo Delivery of Tamra-Tagged siRNA into the Arterial Wall by Cationized Pullulan-Coated Stents.** Rabbits were submitted to a balloon angioplasty two weeks before stent implantation, an experimental model used to induce neo-intima formation.<sup>28</sup> Fluorescence microscopy was performed on arterial cross sections of rabbit carotid arteries after implantation of cationized pullulan-coated stents loaded or not with Tamra-tagged siRNA (Figure 7). Despite the autofluorescence of arteries, images clearly showed an uptake of siRNA when siRNA-loaded stents were implanted (Figure 7, bottom), as compared to rabbit carotids in which coated stents without siRNA were used (Figure 7, top).

**MMP2 Activity in Rabbit Carotid Arteries after Implantation of Cationized Pullulan-Coated Stents Loaded with MMP2-siRNA.** Gelatin zymography of the conditioned media of explanted segments of carotid arteries after implantation of siRNA-loaded cationized pullulan-coated stents showed the presence of 60, 70, and 98 kDa gelatinolytic bands corresponding to MMP2, pro-MMP2, and proMMP9, respectively.<sup>29</sup> Although a decrease in MMP2 activity was observed but not statistically significant, a significant ( $p < 0.005$ ) decrease in pro-MMP2 activity after incubation for arteries implanted with



**Figure 7.** In vivo delivery of Tamra-tagged siRNA into balloon-injured rabbit carotid arteries via cationized pullulan-coated stents. Cross sections of carotid arteries were obtained after implantation of cationized pullulan-coated stents without siRNA (top) or loaded with Tamra-tagged siRNA (bottom). Despite the green autofluorescence of elastic arteries (left panels), areas of red fluorescence (right bottom panels) showed an uptake of Tamra-tagged siRNA by the arterial tissue that was in contact with the stent struts during the implantation.



**Figure 8.** MMP2 activity in the conditioned media of siRNA-transfected stented hypercholesterolemic carotid arteries. Bar graphs show pro-MMP2 and MMP2 activities after implantation of cationized pullulan stents coated with Tamra-tagged MMP2-siRNA or Tamra-tagged scramble siRNA. Densitometric analysis of scanned gelatinolytic bands is expressed in arbitrary units (AU). \* $P < 0.005$  for MMP2-siRNA vs scramble siRNA.

MMP2-siRNA-loaded stents versus scramble siRNA-loaded stents (Figure 8). The inhibition in pro-MMP2 activity reached  $28 \pm 13\%$  for MMP2-siRNA-loaded stents versus scramble siRNA-loaded stents. In contrast, pro-MMP9 activity was not modified by MMP2-siRNA-loaded stents, suggesting that the inhibitory effect of MMP2-siRNA was gene specific.

## Discussion

Several studies have demonstrated the therapeutic potential of RNA interference for the treatment of cardiovascular diseases.<sup>28,30</sup> However, the intravenous application of siRNA may lead to siRNA degradation by circulating enzymes and undesirable accumulation of siRNA in nontarget organs. To overcome these problems, the local delivery of siRNA from a polymeric stent coating may be useful. For this purpose, biopolymers have emerged as promising materials for new gene

delivery systems because of their low toxicity and their biodegradability.<sup>31,32</sup> In the present study, a cationized pullulan as a potential polymeric stent coating was investigated for siRNA delivery.

Pullulan is a well-characterized, biodegradable polymer, which has achieved wide regulatory acceptance with its proven safety record.<sup>33,34</sup> Moreover, pullulan can be modified by chemical grafting such as in our case amino-groups to obtain cationized pullulan with high affinity for nucleotides.<sup>22,23</sup> In a recent study, we have also shown that cationized pullulan-based hydrogels can be used for transfection of smooth muscle cells in vitro.<sup>23</sup>

In the present work, bare metal stents were coated by dip-coating of a cationized pullulan-based gelling mixture that forms a homogeneous layer onto the stent struts by chemical cross-linking of the polysaccharide with STMP. Although dip-coating is not the best method to cover bare metal stents,<sup>35</sup> this simple method was useful as proof-of-principle. Gross appearance of cationized pullulan-coated stents was very encouraging since no webbings were observed between the struts (Figure 1A). To ascertain the efficient covering of the stent struts with the cationized pullulan-based hydrogel, 4% of FITC-polymer was added to the gelling mixture. This allowed nondestructive quality control of the coatings using by fluorescence microscopy (Figure 1B). The surface topography of cationized pullulan-coated stents was not affected by the presence of this small amount in the formulation (data not shown). Although the mass of the stent coating is reported in few publications by weighting the stent before and after coating,<sup>36</sup> this method appeared not enough accurate in our hands. Instead, FITC was used to determine the mass of the coating after complete enzymatic degradation by pullulanase of the polymeric layer. In the present study, a polymeric layer of  $1.4 \pm 0.2$  mg was coated on 10 mm long bare metal stents (corresponding to approximately  $0.1 \text{ mg/mm}^2$ ), which fall in the same range of the  $3.6 \pm 1.4$  mg of polyurethane coated on 12 mm long stents by Takahashi et al.<sup>13</sup> SEM observations of cationized pullulan-coated stents confirmed the efficient covering of the stent struts by the polymeric layer. A smooth surface was visualized on the coated stents. The polymeric layer was not affected by the implantation procedure. Indeed, the coating was not affected by crimping on the balloon catheter (Figure 1C), passing through the introducer valve, and deployment by balloon expansion (Figure 2F). AFM analyses showed that the surface of cationized pullulan-coated stents was smooth, with a roughness average of 19 nm (Figure 3). For comparison, AFM analyses of the luminal surface of electropolished nitinol and stainless steel stents, performed with a scan area of  $5 \times 5 \mu\text{m}^2$  by Thierry and co-workers, showed an average surface roughness in the range of 30 nm, which was considered as relatively smooth and did not increase stent thrombogenicity.<sup>37</sup> The activation of platelets and the adsorption of proteins such as fibronectin or fibrinogen have been shown to play a role in the development of thrombi and immunologic reactions.<sup>38,39</sup> In general, polysaccharide coatings hinder non-specific adsorption of proteins and cells, which are known to lower the functions of blood-contacting materials.<sup>40,41</sup> One important parameter influencing the interaction between implanted devices and the blood components is the hydrophilic character of the surface. It is generally accepted that hydrophilic materials are particularly adapted for in vivo implantation.<sup>42</sup> For example, it has been reported that fibroblast cell adhesion was suppressed by alginate coating,<sup>43</sup> and a reduction of platelet adhesion on alginate-coated stainless steel was attributed to the hydrophilic character of hydroxyl groups.<sup>44</sup>

These results confirmed previous reports of the low affinity of platelets and proteins for hydroxyl group-bearing materials.<sup>45,46</sup> We consider that the hydrophilic nature of pullulan-based coating (Figure 4) is an interesting property for in vivo applications.

The presence of the coating on the stent struts was assessed after implantation in balloon-injured rabbit carotid arteries. Only a few cracks were observed in the polymeric layer after explantation. We observed the presence of platelets adherent to the polymer coating, a response to stent implantation that in the present study may be also increased by the procedure used to harvest the stented arteries, which required to tie the artery, thus enhancing platelet adhesion. A follow up of implanted cationized pullulan-coated stents over longer periods will be necessary, in particular, to study the hemostasis response in vivo and biodegradation of the cationized pullulan-based stent coating.

In a recent study, Chan et al. demonstrated successful local delivery of antisense oligonucleotides (AS-ODN) to the transcription factor *c-myc* from cationized phosphorylcholine-coated (PC) stents in porcine carotid arteries in vivo. Despite a rapid release of the AS-ODN-*c-myc* from the stent resulting in a maximal uptake of only 0.29  $\mu\text{g}$  of AS-ODN-*c-myc* into the stented segments, the authors showed a reduction in *c-myc* protein expression at 6 h. Significant increase of the lumen cross-sectional area and decrease of the neo-intimal thickness in the AS-ODN-*c-myc* stent group compared to a control stent group was also evidenced.<sup>15</sup> In the present work, the study of Tamra-tagged siRNA loading on coated stent and its release in vitro demonstrated that 10-mm long coated stents immersed into Tamra-tagged siRNA solution could retained at least 2  $\mu\text{g}$  of siRNA, corresponding to  $0.17 \pm 0.07 \mu\text{g}/\text{mm}^2$ . A total of 80% of the siRNA content was retained after 1 h from cationized pullulan-coated stents, whereas noncationized pullulan-coated stents fully released their siRNA content in 10 min (Figure 5). After replacement of the incubation medium at 24 h followed by 4 days of additional incubation in PBS, the amount of released siRNA from the cationized pullulan-coated stents only increased by 1%. Even though the drug release from the polymer coating in vitro could not reflect the in vivo kinetics, this result showed that the functionalized coating could act as a reservoir for sustained siRNA delivery from an endovascular prosthesis.

Cationized pullulan-coated stents were then successfully deployed in balloon-injured rabbit carotid arteries (Figure 6). A large variety of rabbit models have been used to study arterial drug or gene delivery using intravascular (balloon or stent) or extra-vascular (collar model)<sup>47–49</sup> approaches, with or without previous injury of artery. The hypercholesterolemic rabbit model has been commonly used to study potential treatments for the inhibition of restenosis. In this model, rabbits are usually submitted to a hypercholesterolemic diet for 3–4 weeks and undergo arterial denudation and stent deployment in the iliac or carotid arteries.<sup>50–52</sup> In the double injury model used in the present study, animals were fed with a high cholesterol diet and their carotid arteries were submitted to balloon abrasion to develop an atherosclerotic lesion that would undergo the second injury, namely, the stent implantation. Stents were implanted 2 weeks after balloon injury, when an upregulation of pro-MMP2 expression and an increased MMP2 activity is observed.<sup>25</sup> Fluorescence microscopy evidenced well-defined areas of siRNA uptake by the arterial tissue that was in contact with the stent struts during the implantation period (Figure 7). In the present study, stents were implanted in vivo for only 24 h, which is a short period of time to evaluate the biological response.

However, it was long enough to allow the transfer of siRNA molecules into the arterial wall and to induce a significant decrease of pro-MMP2 activity for arteries implanted with MMP2-siRNA loaded stents, as compared to arteries implanted with scramble-siRNA loaded stent (Figure 8). We have recently demonstrated that the delivery in 1 h of a solution of MMP2-siRNA in rabbit injured carotid arteries resulted in internalization of MMP2-siRNA in the neo-intimal cells leading to inhibition of MMP2 activity.<sup>25,53</sup> However, several studies have shown that siRNA alone could not reach the intracellular space without the use of a transfection agent (either viral or cationic compound). In a previous work, we have demonstrated the ability of cationized pullulan hydrogels loaded with plasmid DNA to induce a significant transgene expression by vascular smooth muscle cells in vitro by direct contact.<sup>23</sup> As hypothesized in the case of these cationized pullulan hydrogels loaded with plasmid DNA, the efficient transfection of MMP2-siRNA into the vascular cells may result from the sustain release of MMP2-siRNA from the cationized pullulan-coating and/or the release of cationized-pullulan/siRNA complexes from the coating. In a recent work, Raemdonck et al. reported the release of siRNA from cationic dextran microgels and showed that siRNA encapsulation was depending on the number of cationic charges incorporated in the gel network, and that the release of siRNA was governed by the degradation of the cross-links in the gel network.<sup>54</sup> Similarly, the siRNA loading capacity of the cationized pullulan coated-stents described in the present study could be modified by changing the content as well as the nature of cationic charges within the polymer. The decrease of pro-MMP2 activity of the stented arteries, after only 24 h incubation, demonstrates that siRNA molecules were not only delivered into the arterial wall, but also efficiently transfected into the vascular cells. Although the exact mechanism of the transfection process still have to be studied in great detail and the combined follow up of the biodegradation of the coating and transfection efficiency in vivo deserves to be carried out, these promising results showed that cationic pullulan-coated stents represent an efficient functionalized polymeric device for siRNA delivery into the arterial wall of rabbits.

## Conclusions

In conclusion, we have reported here the preparation of a new cationized pullulan coating that can be applied on metallic stents to enable siRNA loading on the stent surface. The coating method is aqueous-based, and we evidenced that a smooth polymeric layer of approximately 0.1  $\text{mg}/\text{mm}^2$  could be obtained. This coating was not affected by crimping on the balloon catheter or by deployment by balloon expansion in vitro and it was also clearly visible on the stent struts after implantation in balloon-injured rabbit carotids in vivo. Cationized pullulan-coated stents were shown to retain 80% of the loaded siRNA after a 1 h elution test in vitro and in vivo experiments demonstrated efficient delivery of Tamra-tagged siRNA into the arterial wall. These cationized pullulan-coated stents could therefore, with further development, represent an interesting device for the study of local RNAi as a therapeutic approach for cardiovascular diseases.

**Acknowledgment.** This work was supported by INSERM, University Paris 7 and University Paris 13, IFR Paris-Nord Plaine de France, the Fondation de l'Avenir (ET4-368 and ET6-421) and the Fédération Française de Cardiologie. We are grateful to Guidant and Abbott for providing the uncoated stents. We thank Liliane Louedec (INSERM U698) for her help in animal studies.

## References and Notes

- (1) Lopez, A. D.; Mathers, C. D.; Ezzati, M.; Jamison, D. T.; Murray, C. J. *The Lancet* **2006**, *367*, 1747–1757.
- (2) Moses, J. W.; Leon, M. B.; Popma, J. J.; Fitzgerald, P. J.; Holmes, D. R.; O'Shaughnessy, C.; Caputo, R. P.; Kereiakes, D. J.; Williams, D. O.; Teirstein, P. S.; Jaeger, J. L.; Kuntz, R. E. *N. Engl. J. Med.* **2003**, *349* (14), 1315–23.
- (3) Stone, G. W.; Ellis, S. G.; Cox, D. A.; Hermiller, J.; O'Shaughnessy, C.; Mann, J. T.; Turco, M.; Caputo, R.; Bergin, P.; Greenberg, J.; Popma, J. J.; Russell, M. E. *Circulation* **2004**, *109* (16), 1942–7.
- (4) Takahashi, H.; Letourneur, D.; Grainger, D. W. *Biomacromolecules* **2007**, *8* (11), 3281–93.
- (5) Virmani, R.; Kolodgie, F. D.; Farb, A. *Am. Heart Hosp. J.* **2004**, *2* (2), 85–88.
- (6) Nordmann, A. J.; Briel, M.; Bucher, H. C. *Eur. Heart J.* **2006**, *27* (23), 2784–2814.
- (7) Holmes, D. R., Jr.; Moses, J. W.; Schofer, J.; Morice, M. C.; Schampaert, E.; Leon, M. B. *Eur. Heart J.* **2006**, *27* (23), 2815–2822.
- (8) Dwarakanath, R. S.; Sahar, S.; Reddy, M. A.; Castanotto, D.; Rossi, J. J.; Natarajan, R. *J. Mol. Cell. Cardiol.* **2004**, *36*, 585–595.
- (9) Hou, R.; Liu, L.; Anees, S.; Hiroyasu, S.; Sibinga, N. E. *J. Cell Biol.* **2006**, *173*, 417–429.
- (10) Schifflers, R. M.; Mixson, A. J.; Ansari, A. M.; Fens, M. H.; Tang, Q.; Zhou, Q.; Xu, J.; Molema, G.; Lu, P. Y.; Scaria, P. V.; Storm, G.; Woodle, M. C. *J. Controlled Release* **2005**, (109), 5–14.
- (11) Klugherz, B. D.; Jones, P. L.; Cui, X.; Chen, W.; Meneveau, N. F.; DeFelice, S.; Connolly, J.; Wilensky, R. L.; Levy, R. J. *Nat. Biotechnol.* **2000**, *18* (11), 1181–1184.
- (12) Perlstain, I.; Connolly, J. M.; Cui, X.; Song, C.; Li, Q.; Jones, P. L.; Lu, Z.; DeFelice, S.; Klugherz, B.; Wilensky, R.; Levy, R. J. *Gene Ther.* **2003**, *10* (17), 1420–1428.
- (13) Takahashi, A.; Palmer-Opolski, M.; Smith, R. C.; Walsh, K. *Gene Ther.* **2003**, *10* (17), 1471–8.
- (14) Wu, Y. X.; Johnson, T.; Herdeg, C.; Baumbach, A.; Newby, A. C.; Karsch, K. K.; Oberhoff, M. *Di Yi Jun Yi Da Xue Xue Bao* **2003**, *23* (12), 1263–1265.
- (15) Chan, K.; Armstrong, J.; Withers, S.; Malik, N.; Cumberland, D.; Gunn, J.; Holt, C. *Biomaterials* **2007**, *28*, 1218–1224.
- (16) Fishbein, I.; Alferiev, I.; Bakay, M.; Stachelek, S. J.; Sobolewski, P.; Lai, M.; Choi, H.; Chen, I. W.; Levy, R. J. *Circulation* **2008**, *117* (16), 2096–103.
- (17) Rekha, M. R.; Sharma, C. P. *Trends Biomater. Artif. Organs* **2007**, *20* (2), 116–121.
- (18) Chaouat, M.; Le Visage, C.; Autissier, A.; Chaubet, F.; Letourneur, D. *Biomaterials* **2006**, *27* (32), 5546–5553.
- (19) Autissier, A.; Letourneur, D.; Le Visage, C. *J. Biomed. Mater. Res., Part A* **2007**, *82* (2), 336–342.
- (20) Storrie, H.; Mooney, D. J. *Adv. Drug Delivery Rev.* **2006**, *58*, 500–514.
- (21) Palmer, R. R.; Lewis, A. L.; Kirkwood, L. C.; Rose, S. F.; Lloyd, A. W.; Vick, T. A.; Stratford, P. W. *Biomaterials* **2004**, *25*, 4785–4796.
- (22) Jo, J.-I.; Ikai, T.; Okazaki, A.; Yamamoto, M.; Hirano, Y.; Tabata, Y. *J. Controlled Release* **2007**, *118*, 389–398.
- (23) San Juan, A.; Hlawaty, H.; Chaubet, F.; Letourneur, D.; Feldman, L. J. *J. Biomed. Mater. Res., Part A* **2007**, *82* (2), 354–362.
- (24) San Juan, A.; Ducrocq, G.; Hlawaty, H.; Bataille, I.; Guenin, E.; Letourneur, D.; Feldman, L. J. *J. Biomed. Mater. Res., Part A* **2007**, *83A* (3), 819–27.
- (25) Hlawaty, H.; San Juan, A.; Jacob, M. P.; Vranckx, R.; Letourneur, D.; Feldman, L. J. *Am. J. Physiol. Heart Circ. Physiol.* **2007**, *293* (6), H3593–601.
- (26) Badier-Commander, C.; Verbeuren, T.; Lebard, C.; Michel, J. B.; Jacob, M. P. *J. Pathol.* **2000**, *192* (1), 105–12.
- (27) Lack, S.; Dulong, V.; Picton, L.; Le Cerf, D.; Condamine, E. *Carbohydr. Res.* **2007**, *342* (7), 943–53.
- (28) Santel, A.; Aleku, M.; Keil, O.; Endruschat, J.; Eshe, V.; Fisch, G.; Dames, S.; Löffler, K.; Fechtner, M.; Arnold, W.; Giese, K.; Klippel, A.; Kaufmann, J. *Gene Ther.* **2006**, *13*, 1360–1370.
- (29) Feldman, L. J.; Mazighi, M.; Scheuble, A.; Deux, J. F.; De Benedetti, E.; Badier-Commander, C.; Brambilla, E.; Henin, D.; Steg, P. G.; Jacob, M. P. *Circulation* **2001**, *103* (25), 3117–3122.
- (30) Tang, Y.; Ge, Y.-Z.; Yin, J. Q. *Acta Pharmacol. Sin.* **2007**, *28* (1), 1–9.
- (31) Kean, T.; Roth, S.; Thanou, M. J. *Controlled Release* **2005**, *103* (3), 643–653.
- (32) Lv, H.; Zhang, S.; Wang, B.; Cui, S.; Yan, J. J. *Controlled Release* **2006**, *114* (1), 100–109.
- (33) EFSA. *EFSA J.* **2004**, *85*, 1–32.
- (34) Gupta, M.; Gupta, A. K. *J. Pharm. Pharmacol. Sci.* **2004**, *7* (1), 38–46.
- (35) Okner, R.; Oron, M.; Tal, N.; Mandler, D.; Domb, A. J. *Mater. Sci. Eng., C* **2007**, *27* (3), 510–513.
- (36) Pan Ch, J.; Tang, J. J.; Weng, Y. J.; Wang, J.; Huang, N. J. *Controlled Release* **2006**, *116* (1), 42–49.
- (37) Thiery, B.; Merhi, Y.; Bilodeau, L.; Trépanier, C.; Tabrizian, M. *Biomaterials* **2002**, *23*, 2997–3005.
- (38) Offermanns, S. *Circ. Res.* **2006**, *99* (12), 1293–1304.
- (39) Kumar, R.; Beguin, S.; Hemker, H. C. *Thromb. Haemostasis* **1994**, *72* (5), 713–721.
- (40) Frazier, R. A.; Matthijs, G.; Davies, M. C.; Roberts, C. J.; Schacht, E.; Tendler, S. J. B. *Biomaterials* **2000**, *21*, 957–966.
- (41) Österberg, E.; Bergström, K.; Holmberg, K.; Schuman, T. P.; Riggs, J. A.; Burns, N. L.; Van Alstine, J. M.; Harris, J. M. *J. Biomater. Res.* **1995**, *29*, 741–747.
- (42) Abraham, G. A.; de Queiroz, A. A.; Roman, J. S. *Biomaterials* **2001**, *22* (14), 1971–1985.
- (43) Morra, M.; Cassinelli, C. J. *Biomater. Sci., Polym. Ed.* **1999**, *10*, 1107–1124.
- (44) Yoshioka, T.; Tsuru, K.; Hayakawa, S.; Osaka, A. *Biomaterials* **2003**, *24*, 2889–2894.
- (45) Lin, J. C.; Chuang, W. H. *J. Biomed. Mater. Res., Part A* **2000**, *51*, 413–423.
- (46) Lestelius, M.; Liedberg, B.; Tengvall, P. *Langmuir* **1997**, *13*, 5900–5908.
- (47) Laitinen, M.; Pakkanen, T.; Donetti, E.; Baetta, R.; Luoma, J.; Lehtolainen, P.; Viita, H.; Agrawal, R.; Miyahara, A.; Friedmann, T.; Risau, W.; Martin, J. F.; Soma, M.; Yla-Herttuala, S. *Hum. Gene Ther.* **1997**, *8* (14), 1645–50.
- (48) Turunen, M. P.; Hiltunen, M. O.; Ruponen, M.; Virkamaki, L.; Szoka, F. C., Jr.; Urtti, A.; Yla-Herttuala, S. *Gene Ther.* **1999**, *6* (1), 6–11.
- (49) Bhardwaj, S.; Roy, H.; Heikura, T.; Yla-Herttuala, S. *Eur. J. Clin. Invest.* **2005**, *35* (11), 669–76.
- (50) Nakayama, Y.; Ji-Youn, K.; Nishi, S.; Ueno, H.; Matsuda, T. *J. Biomed. Mater. Res.* **2001**, *57* (4), 559–66.
- (51) Walter, D. H.; Cejna, M.; Diaz-Sandoval, L.; Willis, S.; Kirkwood, L.; Stratford, P. W.; Tietz, A. B.; Kirchmair, R.; Silver, M.; Curry, C.; Wecker, A.; Yoon, Y. S.; Heidenreich, R.; Hanley, A.; Kearney, M.; Tio, F. O.; Kuenzler, P.; Isner, J. M.; Losordo, D. W. *Circulation* **2004**, *110* (1), 36–45.
- (52) Cooney, R.; Hynes, S. O.; Sharif, F.; Howard, L.; O'Brien, T. *Gene Ther.* **2007**, *14* (5), 396–404.
- (53) Hlawaty, H.; San Juan, A.; Jacob, M. P.; Vranckx, R.; Letourneur, D.; Feldman, L. J. *J. Gene Med.* **2009**, *11* (1), 92–9.
- (54) Raemdonck, K.; Van Thienen, T. G.; Vanderbroucke, R. E.; Sanders, N. N.; Demeester, J.; De Smedt, S. C. *Adv. Funct. Mater.* **2008**, *18*, 993–1001.

BM900740G