Controlled Delivery of Antisense Oligodeoxynucleotide from Cationically Modified Phosphorylcholine Polymer Films

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Antisense strategy is a promising approach for the prevention of in-stent restenosis if therapeutic agents such as antisense oligodeoxynucleotides (AS-ODNs) can be successfully delivered to the implant site. Optimizing the routes and conditions for controlled loading and release of therapeutic agents from a biocompatible polymer coating is still required. In this study, phosphorylcholine (PC) polymer films bearing different cationic charge densities were deposited onto smooth silicon substrates. The thickness of these films was determined by spectroscopic ellipsometry (SE). Human c-myc AS-ODNs were incorporated into the PC polymer films by immersion in concentrated AS-ODN solution and eluted into PBS under physiological conditions. The elution profile was monitored by UV spectrometry and gel electrophoresis. Cellular uptake of the eluted AS-ODN into vascular smooth muscle cells (VSMCs) was evaluated by fluorescence microscopy. The results showed that ODN loading capacities increased with film thickness and were also strongly dependent on the cationic charge density. AS-ODN release was characterized by a slight initial burst in the first half hour followed by a period of sustained release up to 8 days. Gel electrophoresis demonstrated DNA integrity, and different transfection efficiencies were observed when the eluted ODNs were transfected into VSMCs. These results demonstrated that cationically modified PC polymers are capable of delivery of antisense ODNs in a controlled manner and that they are well suited for specific biomedical devices such as DNA-eluting stents.

1. Introduction

In cardiovascular stent deployment, in-stent restenosis (ISR) is the major drawback of percutaneous coronary interventions, occurring in 15−30% of patients. Recent progress in the pathophysiology of ISR has shown that drug-eluting stents (DESs) can help prevent ISR and has fueled excitement in the interventional cardiology field. Early preclinical work indicated that the DES strategy could reduce neointimal thickening in porcine overstretched models, which were duly translated into startling clinical data for the first commercial products, which include Johnson & Johnson's Cypher stent, Boston Scientific's Taxus stent, and most recently, Medtronic's Endeavor stent.[1]

The currently available DES products deliver potent cytostatic or cytotoxic agents such as rapamycin and its analogues or paclitaxel to arrest smooth muscle cell replication in the vicinity of the stent implantation site. There remains, however, an unease regarding the long-term effects of the delivery of such agents, and long-term follow-up of patients is in progress to gauge these risks. Among the selection of drugs with antiproliferative, antimigratory, antiinflammatory, or prohealing properties, various gene therapy approaches have been already applied and still attract extensive research enthusiasm.[2,3] One aspect of this work is to explore the controlled local delivery of the antisense oligodeoxynucleotides (AS-ODNs) that are complimentary to the messenger RNA (mRNA) so as to inhibit specific gene expression by both antisense and nonantisense mechanisms.[4,5] With advances in ODN chemistry and progress in formulation development, AS-ODNs are becoming widely acceptable.[6] We now understand that inhibition of proto-oncogenes such as c-myc could effectively inhibit vascular smooth muscle cells (VSMCs) proliferation in vitro and experimental neointimal formation in vivo.[7−10]

So far, the major limitation for clinical antirestenosis gene therapy concerns successful local delivery and release. The development of local delivery vehicles is practical, and once successful, such delivery systems will overcome issues related to their safety and efficacy and show attractive advantages over systemic gene therapy. The concept of using stents as vehicles for prolonged and sufficient local gene delivery is appealing compared with balloon- or catheter-based delivery.[11] Moreover, coating materials on the stent's surface not only act as a biologically inert barrier but may also form a reservoir for medications and facilitate prolonged drug release. The coatings can be fabricated to ensure drug retention during deployment and modulate drug-eluting kinetics targeted at distinct phases of the restenosis processes.[12]

In recent years, a number of synthetic polymers, such as poly(methacrylate)-based copolymers, poly(urethane) modified with poly(ethylene oxide), poly(lactide-*co*-glycolide) (PLGA), and their derivatives have been widely used for stent coatings.[13−16] Our own research and recent work carried out by other groups have shown that phosphorylcholine (PC) copolymers perform better than most other types of polymeric materials with respect to biocompatibility and hemocompatibility, both in vitro and in vivo.[17,18] This performance is attributed to the fact that the

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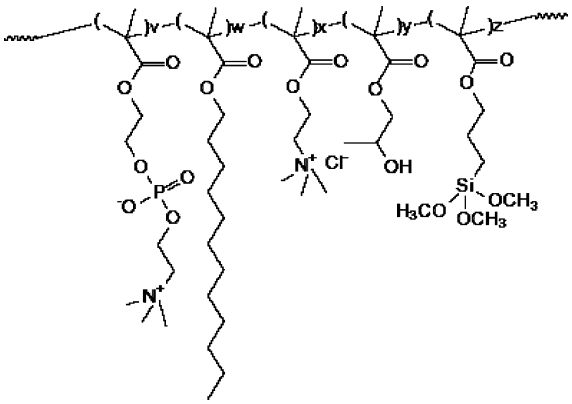

Figure 1. Molecular structure of PC copolymers.

Table 1. Key Physical Parameters of the PC Copolymers

name of PC polymer	MPC mol % (*v*)[a]	LM (*w*)[b]	CM (*x*)[c]	HPM (*y*)[d]	TMSM (*z*)[e]	mol wt
PC 1036 (0% CAT)	29	51	0	15	5	296 400
5% CAT	28	50	5	12	5	204 000
10% CAT	27	46	10	12	5	425 600
20% CAT	24	39	20	12	5	209 200

[a] MPC = 2-methacryloyloxyethyl phosphorylcholine. [b] LM = lauryl methacrylate. [c] CM = choline methacrylate. [d] HPM = 2-hydroxypropyl methacrylate. [e] TMSM = 3-trimethyoxysilysilylpropyl methacrylate.

polymers mimic the natural PC lipid molecules in the cell wall and inherently resist biofouling by proteins.[19,20] Although biologically "neutral" PC polymer coatings are nonthrombogenic, they are not so effective in reducing ISR.[21] Therefore, local delivery of therapeutic AS-ODNs from PC coatings is an attractive option.[22] One key technical limitation is the controlled loading and release of such biomacromolecular drugs from these coatings. In fact, this problem has been seriously overlooked, and to date, little research has been devoted to the understanding of the incorporation of biomacromolecular therapeutics into PC polymers. Bearing in mind that ODN molecules could be large and negatively charged, we propose to introduce cationic choline groups into the PC polymers to enhance drug incorporation through electrostatic attraction, the principle of which has already been well demonstrated by Langer et al. and other workers in their recent study of gene delivery using nanospheres.[23−26] It is hoped that through structural modification of the PC polymers the amount of AS-ODN loaded and the release kinetics can be controlled. It is also important to examine the bioactivity of ODN molecules following interaction with the new coatings together with the assessment of release kinetics. It is thus reasonable to believe that once these issues are resolved our cationically modified PC polymers can have positive effects on the controlled entrapment, sustained release, and stability of ODN therapeutics for further application in DNA-eluting stents.

2. Materials and Methods

2.1. Synthesis of PC Copolymers. Phosphorylcholine (PC)-incorporated copolymers based on 2-methacryloyloxyethyl phosphorylcholine (MPC) were synthesized by Biocompatibles U.K. Ltd., using a free radical polymerization method.[27] The general molecular structure of the copolymers is shown in Figure 1, with the main physical parameters listed in Table 1. The cationically charged group was incorporated in the form of choline methacrylate monomer (CM) in fraction varying from 0%, 5%, 10%, to 20%. The silyl cross-linking agent in the form of 3-trimethoxysilylpropyl methacrylate (TMSM) was fixed at 5%. Incorporation of 2-hydroxypropyl methacrylate (HMP) was necessary to adjust the extent of water intake into the hydrogel material, and incorporation of lauryl methacrylate (LM) was used to tune the hydrophobic feature of the copolymers and to promote nanoporous structure formation within the polymer network upon exposure to aqueous environment.

2.2. Preparation of PC Polymer Films. All PC polymers were dissolved in absolute ethanol under stirring at room temperature overnight with concentrations of 0.2−5 wt %. Polymers were then filtered with 0.22 μm filters to remove possible insoluble particles. Both 13 mm diameter glass coverslips and silicon wafers were cleaned by immersing in dilute basic Decon solution (5%), followed by rinsing with Elgastat ultrapure (UHQ) water. The PC polymer films were prepared by dip coating in PC polymer solutions using a specifically designed coating rig as previously described.[28] Varying solution concentrations together with different motor lifting speeds helped to produce films with required thickness and consistent uniformity. The coated films were then dried in air for at least 30 min at room temperature before being thermally annealed at 150 °C for 3 h under vacuum. The samples were then left to cool to room temperature under vacuum and were stored in vacuum desiccators for subsequent use.[29]

2.3. Spectroscopic Ellipsometry (SE). The thickness and optical constants of polymer films coated on a silicon oxide substrate before and after AS-ODN loading were measured with variable angle spectroscopic ellipsometry (Jobin-Yvon UVISEL, France). The coated wafer was positioned in the central area held by a sample holder. The beam was directed to the sample surface at an incidence angle of 74.5° with respect to the surface normal. The ellipsometric measurement was carried out for the determination of the thickness of the films in open air. Prior to the SE measurements, all films were dried in vacuum for 2 h under ambient temperature. Although the experimental geometry is set up in the same manner as for the normal optical reflection, SE measures the change of the state of polarization of light reflected at the interface instead of light intensity. The change of polarization is usually expressed as two ellipsometric angles, Ψ and Δ, and is dependent on the thickness of any surface layer and its optical constants (refractive indices).[30,31] Simultaneous measurements of Ψ and Δ over a range of wavelengths provide information about the polymer film coated in abstract space, and the usual route to convert such information into layer thickness and refractive indices in the normal space is to perform layer modeling to the pairs of the measured Ψ and Δ. For all the films studied in this work, uniform layer model was found to be adequate for describing them. This was evident from the good agreement between the measured data and calculated fits. To improve the consistency of the data analysis, the refractive indices for the thin polymer films were taken to be the same as obtained for the pure polymer to avoid any uncertainty caused by the correlation between thickness and refractive index when the films were very thin.[28,31] Such treatment should not affect the estimate of the amount of polymer coated in a given surface area. After AS-ODN loading and further drying the total film thickness was again determined assuming that the AS-ODN loading had not altered the refractive indices. The increment in film thickness was then attributed to AS-ODN loading. Ψ and Δ were recorded simultaneously over the wavelengths between 350 and 700 nm. The time required for each scan over this wavelength range was determined by the number of pairs of Ψ and Δ counted. It typically took some 40 s to complete a scan involving some 30 pairs of Ψ and Δ measurements. The experimental data were analyzed using DeltaPsi II software (Jobin-Yvon).

2.4. AS-ODN Loading and DNA Encapsulation Efficiency. Phosphorothioated AS-ODN from the translation initiation region of human c-myc gene was synthesized by Eurogentec Ltd., U.K. The antisense sequence was 5′-AAC, GTT, GAG, GGG, CAT-3′ with the 5′ end labeled with carboxyfluorescein-5-succimidyl ester (FAM). The product was purified by HPLC and lyophilized. AS-ODN was dissolved

Table 2. Determination of the Thickness of PC Polymer Films by Spectroscopic Ellipsometry under Different Coating Conditions before and after ODN Loading[a]

PC polymer	concn (wt %)	dipping speed (mm/s)	thickness(Å) before	thickness(Å) after ODNs loading	thickness(Å) film growth	loading capacity (μg/cm^2)
PC 1036 (0% CAT)	0.2	3	82.2	86.5	4.3	0.043
	0.5	3	202.1	207.7	5.6	0.056
	1	3	429.4	434.7	5.3	0.053
	2	3	921.9	932.2	10.3	0.103
	5	3	2158.3	2177.9	19.6	0.196
	5	6	4453.1	4526.6	73.5	0.735
5% CAT	0.2	3	80.1	90.8	10.7	0.107
	0.5	3	190.4	204.7	14.3	0.143
	1	3	408.6	431.4	22.8	0.228
	2	3	887.4	945.4	58.0	0.58
	5	3	2008.2	2168.0	159.8	1.598
	5	6	3961.4	4255.5	294.1	2.941
10% CAT	0.2	3	80.4	91.7	11.3	0.113
	0.5	3	196.0	216.6	20.6	0.206
	1	3	429.2	480.6	51.4	0.514
	2	3	911.0	1032.9	121.9	1.219
	5	3	2030.4	2329.8	299.4	2.994
	5	6	4128.2	4661.3	533.1	5.331
20% CAT	0.2	3	76.8	94.9	18.1	0.181
	0.5	3	197.1	229.6	32.5	0.325
	1	3	456.3	551.7	95.4	0.954
	2	3	954.3	1176.1	221.8	2.218
	5	3	2321.4	2850.1	528.7	5.287
	5	6	4302.7	5170.1	867.4	8.674

[a] In each sample measurements were made at three different spots, the results were fitted, and the average values are shown.

in distilled water to give a final concentration of 5 mg/mL (1 mmol/L) and stored at −20 °C in the dark. For AS-ODN loading, PC polymer films with different thickness were immersed in 0.5 mL of AS-ODN solution (0.5 mg/mL) at room temperature in the dark for at least 12 h. The films were then washed with PBS for 0.5 h in order to remove the nonadsorbed AS-ODN before elution or vacuum-drying in preparation for SE measurements. The DNA encapsulation efficiency of each PC polymer film was determined by the initial dosage minus the nonadsorbed dosage of AS-ODN, i.e., encapsulation efficiency = (initial dosage − nonadsorbed dosage)/sample surface area (μg/cm^2).

2.5. In vitro Release of AS-ODN from PC Polymer Films. Round glass coverslips of 13 mm diameter coated with PC polymer films and loaded with AS-ODN were placed into 24-well plates and eluted with 0.5 mL of PBS at 37 °C, 150 rpm in a shaking incubator. The elutants were collected separately at designated time points (0 h, 0.5 h, 2 h, 6 h, 1 day, 2 days, 4 days, 6 days, and 8 days), followed by fresh buffer solution replenishment. The concentrations of the eluted AS-ODN solutions were determined by UV spectrometry (Thermo, U.K.) at 260 nm, and the dosages of ODN released from each sample at specific time intervals were calculated as well as the cumulative release.

2.6. Structural Integrity of the Eluted DNA by Gel Electrophoresis. The stability of AS-ODN released from the different PC polymer films was analyzed by agarose gel electrophoresis (1% agarose without ethidium bromide, 100 V, 20 min). In all cases, 25 μL of elutant from each sample was loaded. An amount of 0.01−0.1 μg of intact FAM-labeled AS-ODN was used as control.

2.7. Cell Culture. Porcine vascular smooth muscle cells were primarily cultured by an explant method as previously described.[32] Briefly, the thoracic aorta of adult white pigs was obtained from a local abattoir. After washing with DMEM containing 2× antibiotics, the media was separated from the adventitia and the endothelium was carefully removed by scraping, then the media were cut into 1 mm^2 squares and placed onto the bottom of T25 plates, then incubated with DMEM + 30% FBS (37 °C, 5% CO_2 and humidified air). Primary cells were observed extending from the explants and grown to at least 50% confluence (generally in 10−14 days), the cells were then harvested by trypsinization, and the passaged cells were cultured with DMEM containing 10% FBS. All the cells from each specimen were characterized by immunocytochemical staining for vascular SM α-actin at first and subsequent passages, using FITC-conjugated mouse antihuman SM α-actin IgG1 (DAKO, U.S.A.). Cells at passage 3−5 were used for subsequent experiments.

2.8. ODN Transfection and Fluorescent Microscopy. For transfection of AS-ODN, VSMCs were plated onto gelatin-coated 13 mm diameter coverslips at a density of 2×10^4/well in 24-well plates (Costar, U.S.A.). After 24 h of incubation with DMEM + 10% FBS, the medium was removed and cells were briefly washed with PBS. A volume of 400 μL of eluent from each film mixed with 400 μL of DMEM + 0.8% FBS was added to each well in triplicate for a further 6 h of incubation. Cells were stained with 400 μL of DAPI solution (0.5 μmol/L) for 5 min, then mounted with Prolong Antifade reagent (Molecular Probe, U.S.A.) and observed under fluorescent microscopy (Leica, DM2500 M). Intact FAM-labeled AS-ODN (10 μg/mL in DMEM + 0.4% FBS) was also transfected into VSMCs for 6 h as positive control. Ten random fields with 200× magnification were observed for each sample. The number of both FAM-positive and DAPI-positive cells (representative for total cells) was recorded. The transfection efficiency was defined as the percentage of the ratio of FAM-positive cell number to the DAPI-stained total cell number.

2.9. Statistical Analysis. Statistical analysis of the experimental data was performed by applying the ANOVA one-way test using the software package SPSS 10.0. $P < 0.05$ was considered to be a statistically significant difference.

2.10. Materials. Absolute ethanol, agarose, and DAPI were purchased from Sigma, U.K. Decon 90 and 13 mm glass coverslips were purchased from Fisher, U.K. DMEM, FBS, 100× penicillin−streptomycin, 100× amphotericin, and 1× trypsin−EDTA were products of GibcolBRL, U.K. Silicon wafers were purchased from Compart Technology Ltd., U.K.

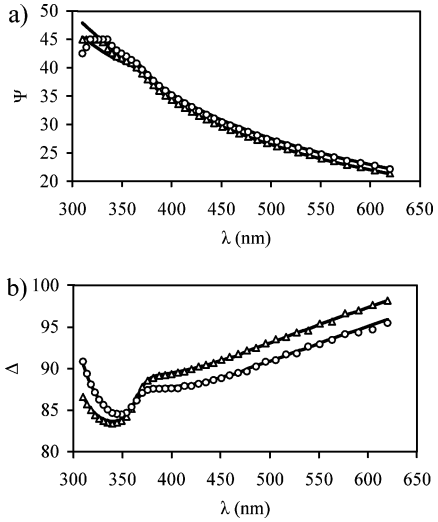

Figure 2. SE measurement of the thickness of the PC film coating using 5% CM PC copolymers. Plots of Ψ (a) and Δ (b) as a function of λ were measured at the air/PC polymer-coated solid interface before (triangle) and after (circle) AS-ODN loading. Measurements were made at an incidence angle of 74.5° with respect to the surface normal at 25 °C. Continuous lines were calculated using the optical matrix formula assuming a polymer layer thickness of 410 Å and thickness after ODNs loading of 434 Å.

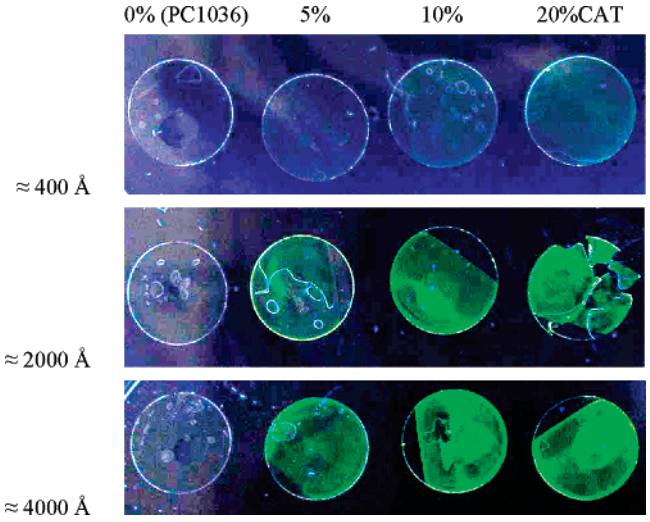

Figure 3. Photographs of FAM-labeled c-myc AS-ODN loaded into different PC polymer films under UV light exposure. The increase in the green intensity with cationic charge density and film thickness indicates the increase in the amount of AS-ODN loaded.

3. Results and Discussion

3.1. PC Polymer Film Formation and DNA Loading. To test the hypothesis that the ability of PC copolymers in incorporating DNA increases with cationic charge density and that the capacity of PC copolymer films in loading DNA is dependent on film thickness, we prepared four PC copolymers with the CM fraction varied from 0% (neutral PC 1036), 5%, 10%, to 20%. The molecular structure of the copolymers is shown in Figure 1, with the fractions of different monomeric constituents given in Table 1. It can be seen from Table 1 that the increase in the fraction of cationic CM is manifested at the expense of MPC and LM, but the ratio of MPC to LM remains almost constant.

PC copolymer films were coated onto optically flat silicon wafers to facilitate the determination of film thicknesses by SE. To obtain different film thicknesses for each copolymer,

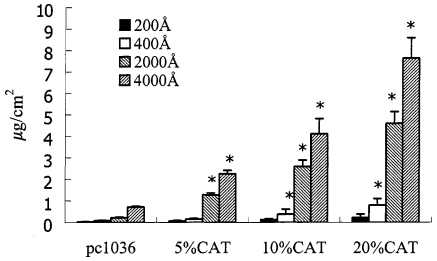

Figure 4. Comparison of loading capacity of AS-ODN in different PC polymers. Glass coverslips (diameter of 13 mm) coated with PC polymer films at ≈200−4000 Å were immersed into 0.5 mg/mL AS-ODN solution overnight and briefly washed with PBS for 30 min. Loading capacity = (initial dosage − uncombined dosage)/surface area (μg/cm^2). Mean ± SD was shown (n = 3). ∗Compared with PC1036 group, $p < 0.05$.

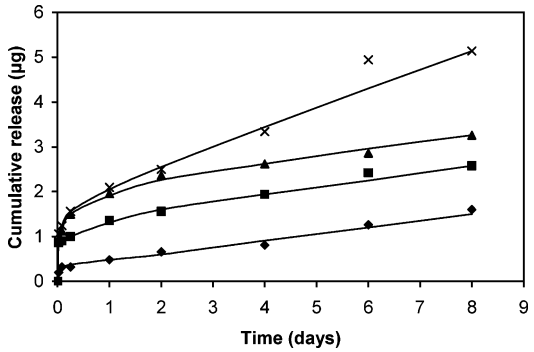

Figure 5. Release profiles of AS-ODN from different PC polymer films (PC1036 (◆); 5% CAT (■); 10% CAT (▲); 20% CAT (×)) into PBS buffer at pH 7.4. Each curve represents the average cumulative release.

different concentrations of PC copolymer solutions were prepared in ethanol. Also different motor lifting speeds were applied when films were dip coated (Table 2). With the use of the combination of these conditions, films with thickness ranging from 80 to 4000 Å were obtained. Figure 2 shows a typical set of plots of Ψ and Δ measured by SE at the air/PC polymer-coated interface using 5% CAT PC polymer. The measurements were made at an incidence angle of 74.5° with respect to the surface normal and at an ambient temperature of 25 °C. The continuous lines represent the optimal fit produced from a fitting program based on the optical matrix formula taking the polymer layer thickness of 410 Å. The wavelength dependence of the refractive index of the polymer film was taken as $n = A + B \times 10^4/\lambda^2$ ($A = 1.475$, $B = 0.48$) following the Cauthy equation. Uncertainty in the refractive index would cause deviation from the true layer thickness but would not affect the relative trend of thickness variation.

The same SE measurement procedures have also been applied to determine film thickness after the films were loaded with AS-ODN. As in the case of the dry films before AS-ODN loading, at least three independent measurements were made on the same surface to check the reproducibility and possible deviation between repeated runs. In the majority of cases, the difference between repeated runs was found to be within ±5 Å, indicating the high degree of smoothness of the outer film surfaces. For comparison, the SE measurements for the dry film shown in Figure 2 after AS-ODN loading are also shown. The subsequent data analysis based on the least-squares fitting routine gave a thickness of 434 Å. Given the uncertainty involved, an increase of 25 Å suggests the loading of AS-ODN into the 5% cationic PC copolymer film.

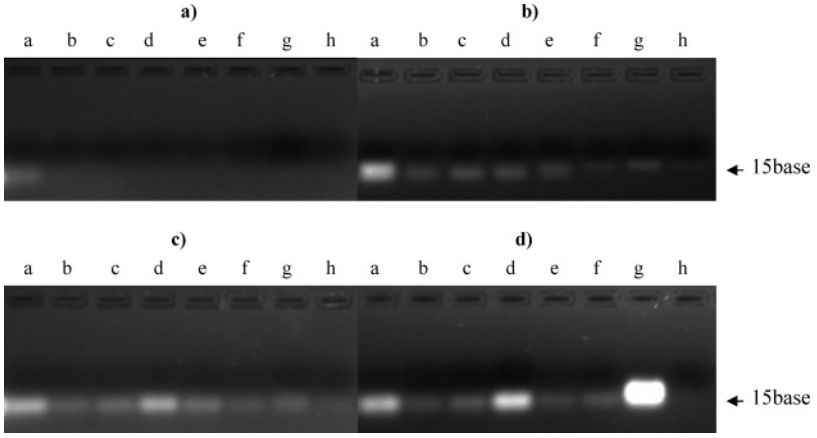

Figure 6. DNA electrophoresis of single release of FAM-labeled c-myc AS-ODN from different PC polymer films into PBS. (a) PC1036; (b) 5% CAT; (c) 10% CAT; (d) 20% CAT. a, 0−1/2 h; b, 1/2−2 h; c, 2−6 h; d, 6 h to 1 day; e, 1−2 days; f, 2−4 days; g, 4−6 days; h, 6−8 days.

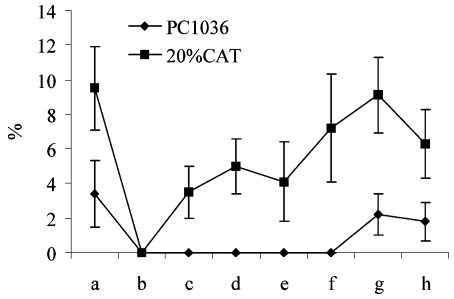

Figure 7. Transfection efficiency of FAM-labeled c-myc AS-ODN in VSMCs eluted from 0% CAT (PC1036, ◆) and 20% CAT PC polymer (■). a, 0−1/2 h; b, 1/2−2 h; c, 2−6 h; d, 6 h to 1 day; e, 1−2 days; f, 2−4 days; g, 4−6 days; h, 6−8 days. $n = 3$ in triplicate.

Table 2 lists similar SE measurements for all four PC copolymers over a representative range of film thickness. The combined film coating conditions enabled us to obtain film thickness of 80, 200, 400, 800, 2000, and 4000 Å for each of the PC copolymers, thereby allowing a systematic characterization of the effects of film thickness and charge density on AS-ODN loading. The AS-ODN loading capacity was estimated assuming the density of polymer and AS-ODN to be 1 g/cm^3. The main conclusion that can be drawn from Table 2 is that the amount of AS-ODN loaded increases with the cationic charge density in the film. It can be seen that while there is little incorporation of AS-ODN into the PC1036 polymer film (without any cationic charge), the extent of AS-ODN loaded in the other three cationic PC copolymer films is approximately proportional to the molar fraction of the charge groups incorporated in the copolymers. A further conclusion that can be drawn from Table 2 is the effect of film thickness; increase in film thickness results in increased loading of AS-ODN. Little AS-ODN is loaded into PC1036 due to the absence of the cationic charge groups, indicating that the electrostatic attraction between anionically charged DNA and cationically charged choline groups is the driving force for DNA loading. This suggestion is supported by the increase of AS-ODN loading with the cationic charge density. However, at a fixed charge density, the extent of AS-ODN loaded appears to increase with film thickness. This trend becomes most obvious for the highest charge fraction of 20%. We attribute this to the boundary effect, an observation that will be discussed further later.

Different mechanistic processes have been described in the literature for entrapment and controlled loading of drug into polymer films.[33,34] In light of the structural and biological stability of AS-ODN, it is useful to optimize the routes and conditions for its controlled loading and release to retain its biological functionality. In general, AS-ODN could be incorporated into PC polymer films in two different ways. One is through mixing DNA with the polymers followed by film coating and processing,[35] and the other is through the formation of films first followed by immersion into a concentrated DNA solution.[36] To avoid thermal deterioration of the AS-ODN when films are thermally annealed, we adopted the second route. As described in the Materials and Methods, each PC polymer film was immersed into aqueous AS-ODN solution at 0.5 mg/mL for a period of 12 h, followed by washing the film to remove the nonadsorbed AS-ODN before SE measurement or controlled release. There are obvious advantages using this method because the total amount of DNA loaded into a given film could be adjusted by varying the loading time and drug concentration used. In addition, the conditions for loading the DNA could be intensified to force more drug molecules into the interior part of the films where necessary.

Although film coating onto optically flat silicon wafers facilitates accurate thickness measurements by SE, cell culture experiments prefer the use of glass coverslips. Following the procedures established from the silicon wafer coatings coupled with the thickness determination from SE, we have chosen a set of coating conditions to dip coat glass coverslips with film thicknesses of 400, 2000, and 4000 Å. The films were then annealed in a manner similar to those coated on silicon oxide wafers. These films were then loaded with AS-ODN by exposing them to the AS-ODN solutions as described previously. Because AS-ODN was tagged with FAM, its loading into the film would produce fluorescent green color. We have observed the AS-ODN loaded coverslips under UV light at 254 nm, and a representative photograph is shown in Figure 3. It can be seen clearly from Figure 3 that the intensity of the green fluorescence varies with film thickness and the extent of cationic charge density. For PC1036 without any cationic charge, the green intensity is weak and varies little with film thickness. However, for PC copolymers with cationic charge groups, fluorescent green color intensifies with the extent of cationic charge density as well as film thickness. At a fixed film thickness, the intensity of the color increases with cationic charge content and reaches the highest at 20% cationic groups. The most intensive fluorescent green color occurs with the thickest film with 20% cationic groups. Although the results shown in Figure 3 are qualitative, they easily reveal the effects of cationic charge and PC copolymer film thickness on AS-ODN loading, a trend consistent with the findings from the SE measurements.

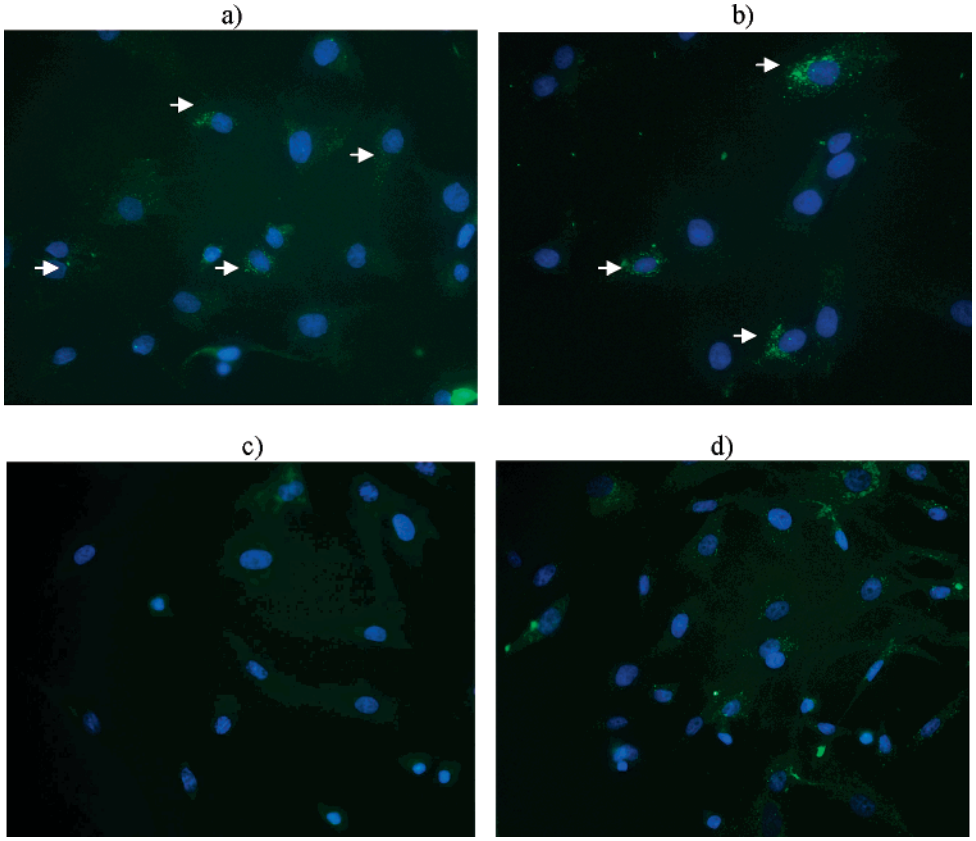

Figure 8. Typical photomicrographs of FAM-labeled c-myc AS-ODN eluted from different PC polymers at $0-{}^{1}/_{2}$ h transfected in VSMCs after 6 h of incubation. Arrowheads indicate FAM-positive cells. (a) 20% CAT; (b) 0% CAT; (c) negative control; (d) positive control.

From the same glass coverslip experiment as described above, it is possible to calculate the exact amount of AS-ODN loaded, thereby making more direct comparison with the SE data. For each glass coverslip, the film was coated on both sides and the total surface area was known. The amount of AS-ODN encapsulated could be determined from the difference between the amount of AS-ODN in the initial solution and the amount left after loading and rinsing. Such an exercise was impractical in the case of silicon wafers, partly because its area was more difficult to estimate and partly because its unpolished backside would obscure the outcome.

Figure 4 shows the amount of AS-ODN loaded into the films coated onto glass coverslips, also plotted in $\mu g/cm^2$ for direct comparison with the SE data. In the group of films with thickness approximate 4000 Å, the amount of AS-ODN loaded from 0% to 20% cationic groups was 0.713, 2.26, 4.12, and 7.64 $\mu g/cm^2$ as compared to 0.74, 2.94, 5.33, and 8.67 $\mu g/cm^2$ on respective wafers obtained from SE, showing strong consistency between the two independent measurements. In the group of cover slips with 20% cationic groups but at different film thicknesses, the loading capacity was 0.23, 0.80, 4.64, and 7.64 $\mu g/cm^2$ as compared to 0.33, 0.95, 5.29, and 8.67 $\mu g/cm^2$, again supporting the high degree of consistency of the independent assessments. These data thus reinforce the outcome from SE measurements, showing a consistent trend of increase of AS-ODN loading with cationic charge density and film thickness.

3.2. Controlled Release of AS-ODN from PC Polymer Films. On the basis of the film formation and AS-ODN loading described above, PC polymer films with a thickness of approximately 4000 Å were chosen for studying in vitro release of AS-ODN. According to the experimental data presented in Figure 4, the loading capacities per glass coverslip with increasing cationic charge density in the film were 1.64 ± 0.35, 5.20 ± 0.69, 9.48 ± 1.24, and 17.58 ± 2.19 μg, respectively. It can thus been seen from Figure 5 that in the case of PC1036 carrying no cationic charge the AS-ODN molecules are almost completely released after 8 days of eluting. With the use of 8 days as the reference time window, the cumulative AS-ODN release profiles from other PC polymer films carrying different cationic charge densities can be compared. It can be seen from Figure 5 that for all the PC copolymers, there is a slight initial burst release over the first half hour, followed by a sustained release in the form of zero-order pattern over the time frame of 8 days studied. The amount of AS-ODN released over the initial burst period is comparable for the three charged PC copolymers, all between 1 and 1.5 μg. The subsequent time dependent release can be approximated to a straight line with its slope increasing with cationic charge density.

An important observation from Figure 5 is that while incorporation of cationic choline groups increases AS-ODN loading, it also extends the period of AS-ODN release. As already mentioned, PC1036 copolymer film incorporated the least amount of the DNA, and with increasing cationic charge density the amount of gene loaded showed an almost linear increase. By 8 days AS-ODN loaded into PC1036 copolymer film was almost completely released. This compares with some 50% release from the 5% cationic PC copolymer and 35% and 25% from the 10% and 20% cationic copolymer films, respectively. It can also be seen from Figure 5 that while the release rate after the initial burst is similar between the three thin films, the release profile from the film with 20% cationic charge density shows faster release rate over the same period. This accelerated release is likely to be associated with the structural disintegration of the copolymer caused by swelling. Given the deviation, the release profile as observed for the 20%

cationic copolymer film is still consistent with a zero-order release profile. It is nevertheless useful to examine whether this type of in vitro release profile could match the time window of restenotic pathological process in vivo.

3.3. In vitro Transfection of AS-ODN into VSMCs. The integrity of eluted AS-ODN molecules is fundamental for retaining their bioactivity as a therapeutic agent. The eluant from each sample in this study was therefore observed for its molecular integrity by gel electrophoresis. It can be seen from the results shown in Figure 6 that all the AS-ODN samples eluted from different polymer films at different time intervals retained their integrity, indicated by no change in molecular size. Meanwhile, because the same volume of each sample was loaded onto the gel, the intensity of each band was proportional to the total AS-ODN amount collected at a given time interval. Thus, in addition to the presence of a single band, the relative intensity of these bands increased with the extent of cationic charge, an observation consistent with results described previously in Figures 3 and 4.

To further examine the transfection efficiency of the AS-ODN molecules released from the different PC copolymer films, the elutants were also used to transfect VSMCs cultured in vitro. It is generally accepted that the abnormal growth of VSMCs accounts for the neointimal formation after stent implantation.[37] During the pathophysiological process of in-stent restenosis, the up-regulation of proto-oncogene, c-myc, may attribute to VSMCs proliferation, migration, and apoptosis.[38] Many approaches targeted at c-myc interference could effectively suppress the incidence of ISR.[39]

In this study, the c-myc AS-ODNs eluted from each PC polymer film were diluted with an equal volume of DMEM containing 0.8% FBS so that the final concentrations of ODN varied from 0.1 to 1 μg/mL. These were subsequently transfected into VSMCs without any auxiliary gene transfer agent. The results of the transfection efficiency from the AS-ODN released from copolymer samples with 0% and 20% cationic charge groups are shown in Figure 7. Although the transfection efficiencies of these samples (0−10%, respectively) were much lower than that of the positive control (88 ± 3% with 10 μg/mL ODN concentration), it is clear from Figure 7 that the percentage of transfected cells from 20% cationically charged PC copolymer film was significantly greater than from PC1036 without any cationically charged group. This is consistent with the release of more AS-ODN from the 20% cationically charged copolymer film. Because we have previously observed the leaching of noncross-linked fragments leached from the swollen 20% cationically charged copolymer films, it is possible that some of the AS-ODN molecules might have been released as condensed vectors formed with the free polymer fragments. Charge condensation on AS-ODN would also increase its internalization into the cells. It should be noted that the intact FAM−c-myc ODN between 0.1 and 1 μg/mL was also used as parallel control. It was found that the transfection efficiencies at different concentrations were consistent with those observed in the experimental groups, demonstrating the possible retaining of full bioactivity of the AS-ODN released from the PC copolymer films. Figure 8 shows the typical photographs of FAM-labeled c-myc AS-ODN transfected in VSMCs after 6 h of incubation. In FAM-positive cells, the spots with green fluorescence were mainly localized around the nucleus within the cytoplasm. It is relevant to note that we have not addressed the antiproliferative and proapoptotic effects of transfected AS-ODNs and the effect of cumulative level of released ODNs on in vitro cell culture in this work.

4. Conclusion

Extensive work has demonstrated the high biocompatibility rendered by PC polymers.[17−22,40−43] The current work has demonstrated that when cationically charged groups are incorporated, PC copolymer films are efficient and effective matrixes for AS-ODN delivery. With the use of PC1036 as a control, we have demonstrated that the amount of AS-ODN loaded is proportional to the molar fraction of cationic choline groups present in the PC copolymers. For all the cationic copolymers with different charge densities, we have shown that the amount of ODN loaded is approximately proportional to the film thickness under the conditions studied.

Through a careful assessment of conditions for film processing and drug loading, we have demonstrated that AS-ODN could be released by predominantly zero-order kinetics and that the time window for efficient AS-ODN dosage has practical significance to controlled release from vascular stent coatings. Studies using cultured VSMCs have demonstrated that the transfection efficiency of AS-ODN released from different PC copolymer films was completely comparable to that of the control ODN samples under identical solution concentrations, therefore confirming the possible full retention of bioactivity.

These results, together with the proven record of high bio- and hemocompatibility demonstrate that cationically modified PC copolymers can work as effective vehicles to deliver antisense ODNs in a controlled manner. These copolymers are well suited for biomedical implant coatings as well as working as controlled DNA delivery matrixes.

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