Novel ''plum pudding'' gels as potential drug-eluting stent coatings: Controlled release of fluvastatin

F.C. McGillicuddy,[1] I. Lynch,[2,3] Y.A. Rochev,[4] M. Burke,[4] K.A. Dawson,[3] W.M. Gallagher,[1] A.K. Keenan[1]
[1]*UCD School of Biomolecular and Biomedical Science, Conway Institute, University College Dublin, Ireland*
[2]*Physical Chemistry1, Centre for Chemistry and Chemical Engineering, Lund University, Sweden*
[3]*Irish Centre for Colloid Science and Biomaterials, UCD School of Chemistry and Chemical Biology, University College Dublin, Ireland*
[4]*National Centre for Biomedical Engineering Science, National University of Ireland, Galway, Ireland*

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Abstract: This study evaluated novel structural motifs known as ''plum pudding'' gels as potential drug-eluting stent coatings. Controlled delivery of a HMG-CoA reductase inhibitor (statin) from the intravascular stent surface represents a potential therapeutic modality for prevention of in-stent restenosis (ISR). In this study, gels were comprised of fluvastatin-loaded thermoresponsive microgel particles containing the relatively hydrophilic *N*-isopropylacrylamide (NiPAAm), mixed with the more hydrophobic *N*-*tert*-butylacrylamide (NtBAAm) in different wt/wt ratios: 85/15, 65/35, and 50/50, randomly dispersed in a 65/35 or 85/15 NiPAAm/NtBAAm copolymer matrix. Fluvastatin release from 5 μm copolymer films was greatest from the most hydrophilic systems and least from the more hydrophobic systems. Release from hydrophobic matrices appeared to be via Fickian diffusion, enabling use of the Stokes–Einstein equation to determine diffusion coefficients. Release from hydrophilic matrices was non-Fickian. Eluted drug retained its bioactivity, assessed as selective inhibition of human coronary artery smooth muscle cell proliferation. When stainless steel stent wires were coated (25 μm thickness) with fluvastatin-loaded 65/35 microgels in an 85/15 copolymer matrix, drug elution into static and perfused flow environments followed similar elution profiles. In contrast to elution from copolymer films cast on flat surfaces, diffusion from stent wires coated with hydrophilic and hydrophobic systems both followed Fickian patterns, with slightly larger diffusion coefficients for elution from the flow system. We conclude that manipulation of the relative hydrophobicities of both microgel and matrix components of ''plum pudding'' gels results in tightly regulated release of fluvastatin over an extended time period relevant to initiation and propagation of ISR. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 923–933, 2006

Key words: NiPAAm/NtBAAm; microgels; stent wires; local drug delivery; fluvastatin

INTRODUCTION

Percutaneous transluminal coronary angioplasty (PTCA) has largely replaced coronary artery bypass grafting for the treatment of acute coronary syndromes. While deployment of an intracoronary stent during PTCA has greatly reduced the incidence of restenosis, 15–20% of patients with ''ideal'' coronary lesions require repeat procedures, due to in-stent restenosis (ISR). Patients with more complex lesions are at a higher risk, with ISR rates of 30–60%.[1]

Correspondence to: A.K. Keenan; e-mail: alan.keenan@ucd.ie
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Drug-eluting stents have made a major contribution to reducing the incidence of restenosis (partial or total reocclusion of the artery) following PTCA. Local drug delivery from the stent surface represents a means of delivering therapeutic doses of drug directly to the target site. Controlled drug release can be achieved using polymeric materials within which the active agent is stored in reservoirs.[2]

One such group of agents are the HMG-CoA reductase inhibitors or statins, which are primarily employed to lower cholesterol in hypercholesterolemic patients. However, statins are now known to have many actions potentially beneficial in protection against ISR: inhibition of vascular smooth muscle cell migration and proliferation, anti-inflammatory properties, enhancement of endothelial function, antithrombotic, and antiplatelet effects.[3–9] Statins have also been found to increase the number of cir-

culating endothelial progenitor cells,[10] resulting in accelerated re-endothelialization in a rat model of carotid balloon injury.[11] In addition, statins may influence plaque stability by inhibiting macrophage activation.[12]

The need to expand the range of candidate drugs for local delivery is coupled with a requirement for novel coatings with desirable drug-releasing properties. Our group has previously investigated the potential of a range of *N*-isopropylacrylamide (NiPAAm)/ *N-tert*-butylacrylamide (NtBAAm)-derived copolymers to act as vehicles for the delivery of small drug molecules and therapeutic proteins.[13–15] These copolymers are comprised of the hydrophilic, temperature-sensitive poly(NiPAAm) and the more hydrophobic, non-temperature-sensitive poly(NtBAAm). In studies with the antimitotic agent colchicine, a range of copolymers with varying hydrophobicity incorporating variable (85/15, 65/35, and 50/50) wt/wt ratios of NiPAAm/ NtBAAm was synthesized. Examination of drug release profiles over a 48-h period showed a much greater rate and extent of release of the hydrophobic molecule colchicine from the most hydrophilic 85/15 copolymer, in contrast to the much slower and less extensive release from the most hydrophobic 50/50 copolymer.[15]

An alternative approach is to incorporate drugs into polymer pockets or ''plums,'' which are intelligent responsive microgel particles. Incorporation of such drug-loaded microgels into a ''background'' copolymer matrix could then lead to drug release in a diffusion-controlled manner. This strategy has several merits beyond those of drug-loaded polymeric films. First, in separating the functional role (reservoirs or plums) from the support matrix one can simultaneously release several different drugs with quite distinct physiochemical properties using the plum motif. Second, elution does not degrade the supporting film in the same way as drug-loaded films, which leads to a more stable long-term elution profile, without problems such as the shedding of polymeric particles from the film.

In our illustrative example, NiPAAm-derived plum-pudding gels have recently been prepared and shown to elute candidate solutes of variable hydrophobicity.[16,17] In the present study, we used NiPAAm/ NtBAAm copolymers (85/15 and 65/35) as background matrices and synthesized NiPAAm/ NtBAAm (85/15, 65/35, and 50/50) crosslinked microgels. We examined the potential of three different microgel combinations (85/15, 65/35, and 50/50) dispersed in either 85/15 or 65/35 copolymer matrix to incorporate and release fluvastatin. Drug release from six different systems was thus investigated. The copolymer system comprising 65/35 microgels dispersed in an 85/15 copolymer matrix was subsequently coated onto stent wires and release compared from the wires into a static environment and a perfusion circuit. Bioactivity of fluvastatin was evaluated in terms of effects on human coronary artery smooth muscle cell (HCASMC) proliferation, as were effects of drug-containing coated wires ''*in vitro*'' on smooth muscle cell number, morphology, and viability.

MATERIALS AND METHODS

Materials

N-isopropylacrylamide (NiPAAm; Acros Organics, USA) and *N-tert*-butylacrylamide (NtBAAm; Fluka, Switzerland) were recrystallized twice from *n*-hexane and dried at room temperature under vacuum. *N*, *N*-Azobisisobutironitrile (AIBN; Phase Separation, UK) was recrystallized from methanol. Acryloyl chloride, *N*, *N*-dimethylacrylamide, triethylamine, and 4-aminobenzophenone were obtained from Aldrich and used as supplied. Solvents were reagent grade and were purified by conventional methods. [^{14}C]-fluvastatin (specific activity 46 μCi/mmol) was generously donated by Novartis, Hanover. HCASMC together with the corresponding growth-medium (Medium 231) were purchased from Cascade Biologics (Belgium). 5-Bromo-2′-deoxyuridine (BrdU) ELISA was obtained from Roche Diagnostics (Germany). Alamar Blue was purchased from Biosource UK. All other chemicals and reagents were of the highest grade commercially available.

Copolymer preparation

Copolymers were prepared by free-radical polymerization of the corresponding monomers in benzene with AIBN as the initiator. Appropriate measures were taken to ensure proper handling of benzene at all stages of the preparation. Mixtures of NiPAAm and NtBAAm monomers in the desired ratio (wt/wt) were dissolved in benzene (10% wt/vol). AIBN (1:100 molar ratio AIBN:monomers) was then added. Oxygen was removed by bubbling N_2 gas through the solution for 30 min. Polymerization proceeded at 60°C for 24 h. Benzene was then evaporated and the copolymer was dissolved in acetone and precipitated into *n*-hexane. This procedure was repeated three times. Purified copolymer was dried at room temperature under vacuum.

Microgel preparation

NiPAAm/ NtBAAm microgels were synthesized by dispersion polymerization according to the method of Li and Bae.[18] NiPAAm and NtBAAm in the desired wt/wt ratios (85/15, 65/35, 50/50, total 0.2 g) and bisacrylamide (0.02 g) were dissolved in 36 mL of water and 1 mL of 0.1 wt % Triton solution was added. The solution was heated to 70°C and degassed by bubbling with N_2. Ammonium persulfate (0.02 g) was dissolved in 4 mL of water, degassed, and added slowly to the stirring monomer solution, under an atmosphere of N_2. The reaction was left for 12 h at

70°C and the resulting microgel dispersion (1 wt % in water) was cleaned by dialysis and freeze-dried before use. Particular attention was paid to cleaning copolymer and microgels, since it is known that very small amounts of residual monomer or catalyst may lead to anomalies in work with living cells.

Drug incorporation into microgel particles

Microgel particles (5 mg) were incubated with 1 mL of an ethanolic solution of fluvastatin (5 m*M*) containing tracer levels of ^{14}C-fluvastatin for 24 h at 4°C. The microgel/drug suspension was then centrifuged at 5000 rpm for 10 min, the supernatant containing unincorporated drug was removed, and the microgels were resuspended in 0.5 mL ethanol.

Microgel incorporation into copolymer films

Matrix copolymer solutions (0.5 mL of 10% (wt/vol) 85/15 and 65/35 NiPAAm/NtBAAm) were prepared in ethanol. The resuspended drug-loaded microgel particles were subsequently added to the matrix solutions, resulting in six different matrix/microgel combinations (85/15 microgels in 85/15 matrix, 65/35 microgels in 85/15 matrix, 50/50 microgels in 85/15 matrix, 85/15 microgels in 65/35 matrix, 65/35 microgels in 65/35 matrix, and 50/50 microgels in 65/35 matrix). Aliquots (27 μL) of the resulting suspensions were applied evenly to the wells of 24-well tissue culture plates and were allowed to dry overnight at room temperature. The resulting collapsed copolymer films were 5 μm thick [calculation based on the equation: film thickness = copolymer weight/(well area × solution density)].

Manipulation of copolymers

Microgel-containing copolymer solutions for both film casting and stent wire coating were prepared in ethanol. Cast films and coated wires were allowed to dry at room temperature prior to initiation of the experiment. The dried copolymer existed in its collapsed state, which was maintained throughout all release experiments by incubation with aqueous media at 37°C (above the transition temperature of the copolymers).

Drug elution from films

Dried films were washed three times with prewarmed PBS (37°C) to retain the collapsed state of the copolymer, prevent its dissolution, and remove any surface-bound drug. PBS (1 mL) was added to the surface of each film, plates were left to incubate at 37°C, and at indicated time points the overlying solution was removed, stored, and replaced with fresh solution. The amount of ^{14}C-fluvastatin eluted into the overlying solution was determined by scintillation counting against a suitable standard curve, from which total eluted fluvastatin was then determined. All drug amounts released were initially expressed as nmol eluted into 1 mL overlying solution.

Dip coating of stent wires

A 10% copolymer solution was first prepared using the 85/15 copolymer matrix and drug-loaded 65/35 microgel combination. Stainless steel 304L stent wires were then coated using a dip-coater (NIMA Technologies). Wires were dipped eight times, resulting in a 25 μm copolymer film coating on the wire. The thickness of the coating surrounding the wire was measured using a micrometer (diameter of the wire measured before and after coating).

Drug elution from coated wires into static and perfused environments

Elution from drug-coated stent wires was examined under both static and perfused flow conditions over a 60-day period. Under static conditions, the wires were placed in a 12-well tissue culture plate (1 wire/well) and incubated with 3 mL of PBS at 37°C (wires were arranged in the well in a manner ensuring complete immersion in PBS). The plates were kept at 37°C and at indicated time points the overlying solution was removed, stored, and replaced with fresh solution.

Elution was also examined in a perfusion circuit system (CELLMAX^{TM} QUAD Artificial Capillary Cell Culture System; Cellco, Germantown, MD) where wires were constantly perfused with PBS. The apparatus consisted of an enclosed bundle of capillaries through which PBS (at 37°C) was pumped from a reservoir via silicone rubber tubing, at a flow rate of 23 mL/min. Two drug-loaded coated wires were inserted into each capillary and perfused with PBS for 24 h. Drug elution from wires was tracked by measuring ^{14}C-fluvastatin release into the circulating PBS. Release was measured daily for 24 days and thereafter every third day for a total of 60 days.

Scanning electron microscopy

The surface topography of copolymer films coated onto stent wires was examined using a Hitachi S-4700 scanning electron microscope. Because of the nonconductive nature of the copolymer, samples were gold-coated using an Emitech K550 gold coater prior to imaging. Samples were viewed under low magnification and images were captured at ×1000 magnification.

Calculation of release kinetics

Release mechanisms for drug incorporated into the different systems were investigated by plotting log $M(t)/M(\infty)$ against log Kt and determining n, the slope of the line obtained, where $M(t)/M(\infty)$ is the fraction of dissolved drug released at time t, $M(t)$ is the amount released at time t, and $M(\infty)$ is the total amount initially present in the matrix. The effective slope ''n'' determines the release mechanism.[19] Once the release kinetics for a particular drug have been established, it is possible to determine the diffusion coefficient for the drug at the temperature used. Where the release kinetics conform to Fickian diffusion

($n < 0.5$), the diffusion coefficient can be obtained from the Stokes–Einstein equation $M(t)/M(\infty) = 2(Dt/\pi l^2)$, where D is the diffusion constant of the dissolved drug, and l is the thickness of the gel film (5 μm). The same set of calculations was carried out to determine the diffusion mechanism and diffusion coefficients of fluvastatin from plum-pudding films coated onto stent wires. The latter were coated with 85/15 matrix incorporating fluvastatin-loaded 65/35 microgels, and for these calculations the film thickness l was 25 μm.

Cell culture

HCASMC (passage 3) were obtained from a 21-year-old Caucasian male. Cells were cultured in Medium 231 supplemented with fetal bovine serum (4.9%), human epidermal growth factor (hEGF; 0.5 ng/mL), human basic fibroblast growth factor (hFGF; 2 ng/mL), heparin (5 ng/mL), insulin (5 μg/mL), BSA (0.2 μg/mL) plus penicillin G (100 U/mL), streptomycin sulfate (100 μg/mL), and amphotericin-B (0.25 μg/mL) in a humidified atmosphere of 5% CO_2 and 95% air. Cells of passage numbers 5–9 were used in experiments.

Assays of cell number and proliferation

BrdU ELISA

HCASMC (2000 cells/well) were seeded onto 96-well plates and left to adhere overnight. Cells were then incubated with increasing concentrations of fluvastatin (0.01–1 μ*M*) in complete medium for 48 h. Proliferation was subsequently assessed by measurement of BrdU incorporation into the DNA of proliferating cells using a colorimetric ELISA.

Effects of coated wires on HCASMC number

HCASMC were seeded into Falcon dishes at a density of 3×10^4 cells/mL, allowed to adhere, and left for 24 h at 37°C. Cells were subsequently incubated with the following: (i) bare stent wire alone; (ii) copolymer-coated stent wire (85/15 matrix, 65/35 microgels); (iii) drug-loaded copolymer-coated stent wire (85/15 matrix, drug-loaded 65/35 microgels); or (iv) complete medium alone as a control, for 48 h at 37°C. Alamar Blue (10% (vol/vol) in complete medium) was added to the cells and 3 h later the Alamar Blue-containing medium was removed and absorbance at 545 nm measured on a Wallac Victor-1420 Multilabel HTS counter. Cell proliferation was expressed as a percentage of control.

Measurement of cell viability

The methylthiazol tetrazolium (MTT) assay, adapted from the method of Mosmann,[20] was used. HCASMC grown in Falcon dishes were allowed to reach confluence and subsequently growth-deprived in serum-free medium (SFM). Cells were incubated with the following: (i) copolymer-coated stent wire alone (no microgels); (ii) copolymer-coated stent wire (85/15 matrix, 65/35 microgels); (iii) drug-loaded copolymer-coated stent wire (85/15 matrix, 65/35 microgels); or (iv) SFM alone as a control, for 48 h at 37°C. Following aspiration and washing with PBS, each dish was incubated with 2 mL MTT (0.45 mg/mL) in medium 231 for 3 h at 37°C. ''Negative control'' cells were treated with 10 m*M* H_2O_2 for 30 min prior to incubation with MTT. The overlying solution was aspirated and cells solubilized using 1 mL DMSO. The absorbance of the solutions at 600 nm was measured and their viability expressed as a percentage of control.

Data analysis

Data are presented as means ± SEM of the indicated number (n) of determinations. Statistical analysis of differences between groups was performed by ANOVA, followed by Bonferroni or Dunnett's post-tests as appropriate. The statistical package PRISM was used for all analyses. Differences between means were considered significant when $p < 0.05$.

RESULTS

Drug incorporation into copolymer films

The total amount of drug loaded into copolymer films in each case was calculated after release experiments had been concluded. For each microgel/matrix combination, six copolymer films were cast ($n = 6$). Films were redissolved in ethanol following removal of the final aliquot and the amount of drug remaining within the film determined. The total amount eluted was added to that remaining within the film, giving the total amount initially loaded. While equivalent amounts of drug were incorporated into 65/35 and 50/50 microgels, a smaller quantity was incorporated into 85/15 microgels (Table I). It is likely that the relatively hydrophilic nature of the latter microgels rendered them resistant to incorporation of the relatively hydrophobic fluvastatin molecule.

Drug elution from copolymer films

For normalization purposes, all release data were graphed as a percentage of total drug incorporated into copolymer films. As before, for each microgel/matrix combination, six copolymer films were cast ($n = 6$). Greatest percentage elution within the first 24 h was observed from the most hydrophilic combination (85/15 microgels, 85/15 matrix) with 32.40% ± 0.97% of total drug eluted, while the most hydrophobic combination tested (50/50 microgels, 65/35 ma-

TABLE I
Release of Fluvastatin from Films of the Compositions Shown

	Microgel/Matrix Combination	Total Drug/ Film (nmol)	Amount Eluted After 24 h (nmol)	% Total Drug Eluted by 24 h	Amount Eluted at Day 14 (nmol)	% Total Drug Eluted by Day 24
A	85/15 microgels/85/15 matrix	7.89 ± 0.11	2.56 ± 0.08	32.39 ± 0.97	0.11 ± 0.003	74.19 ± 1.72
B	65/35 microgels/85/15 matrix	17.25 ± 0.67	3.52 ± 0.09	20.99 ± 0.58	0.194 ± 0.01	65.63 ± 1.72
C	50/50 microgels/85/15 matrix	17.42 ± 0.29	2.73 ± 0.18	15.67 ± 1.03	0.209 ± 0.01	53.41 ± 1.75
D	85/15 microgels/65/35 matrix	8.12 ± 0.21	1.11 ± 0.04	13.59 ± 0.53	0.057 ± 0.002	35.01 ± 0.75
E	65/35 microgels/65/35 matrix	19.57 ± 0.56	0.52 ± 0.04	2.66 ± 0.21	0.103 ± 0.01	14.53 ± 0.89
F	50/50 microgels/65/35 matrix	14.11 ± 0.33	0.39 ± 0.04	2.76 ± 0.25	0.049 ± 0.003	12.55 ± 0.62

trix) eluted only 2.76% ± 0.25% of total drug within the same period. This difference was more pronounced after 24 days, with 74.19% ± 1.72% and 12.55% ± 0.62% of total drug eluted, respectively (Fig. 1, Table I). Examination of Table I shows that the matrix within which the microgels were dispersed played a major role in regulating release: this was greater in all cases from the more hydrophilic 85/15 copolymer matrix incorporating 85/15, 65/35, and 50/50 microgels than from the corresponding 65/35 copolymer matrix, that is the more hydrophilic matrix expelled the hydrophobic drug at a faster rate.

While greatest percentage elution was, as expected, observed from the more hydrophilic 85/15 microgels, the greatest *amount* of drug was eluted from the 65/35 microgels, into which a higher amount of drug had been initially incorporated. After 24 h, 2.56 ± 0.08 and 3.52 ± 0.09 nmol drug eluted from the 85/15 and 65/35 microgels embedded within the 85/15 copolymer matrix, respectively. This difference was maintained at day 14 (0.10 ± 0.003 and 0.19 ± 0.01 nmol respectively, Table I).

Release kinetics and diffusion coefficient calculations of copolymer films

We next determined whether drug elution followed Fickian kinetics. According to the Yasuda model,[19] when $n < 0.5$, release of incorporated drug is by Fickian diffusion, when $0.5 < n < 1.0$ the release is by non-Fickian diffusion, and when $n = 1$ there is continuous zero-order release. The n-values for each of the six microgel–matrix combinations studied are listed in Table II. It can be noted that in the combinations containing the more hydrophobic 65/35 matrix, the n-values indicated or approxi-

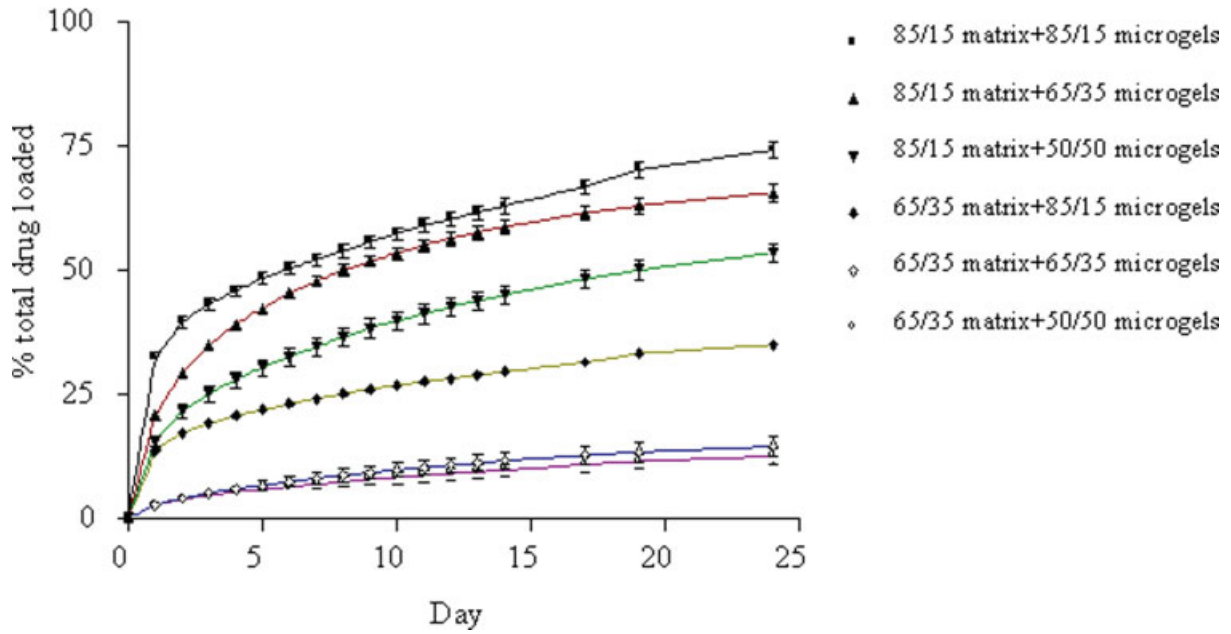


Figure 1. Cumulative release of fluvastatin from 85/15 and 65/35 NiPAAm/NtBAAm matrices incorporating drug-loaded 85/15, 65/35, and 50/50 NiPAAm/NtBAAm microgels over 24 days ($n = 6$). Results are expressed as percentage of total drug amounts incorporated into microgels. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE II
Release Kinetics (*n*-Values) and Diffusion Coefficients (m^2/s) for Fluvastatin from Films of the Compositions Shown

System	*n*-Value	Diffusion Coefficient (25 days)
50/50 microgels/65/35 matrix	0.363	2.36×10^{-17}
65/35 microgels/65/35 matrix	0.448	2.79×10^{-17}
85/15 microgels/65/35 matrix	0.665	6.11×10^{-17}
50/50 microgels/85/15 matrix	1.226	N/A
65/35 microgels/85/15 matrix	1.219	N/A
85/15 microgels/85/15 matrix	1.280	N/A

N/A, not applicable.

mated to Fickian diffusion, whereas in the combinations containing the more hydrophilic 85/15 matrix, the *n*-value was greater than 1, a case not accounted for in the Yasuda model. The Stokes–Einstein equation was used to gain an estimate of the diffusion coefficients in systems showing Fickian diffusion. At $t = 25$ days and taking $M(t)/M(\infty)$ to be the amount released after 25 days, the diffusion coefficients from the three microgel–65/35 matrix combinations were found to be comparable, with the highest coefficient determined for the 85/15 microgel–65/35 matrix combination (Table II).

Effects of fluvastatin on HCASMC proliferation

Fluvastatin (0.1–1 μ*M*) concentration-dependently inhibited proliferation of HCASMC, with values reduced to 72.06% ± 2.46% and 50.05% ± 3.25% of control after treatment with 0.1 and 0.2 μ*M* fluvastatin, respectively (***$p < 0.001$, $n = 8$ in each case, Fig. 2). None of the concentrations tested were cytotoxic to cells, as assessed by MTT assay (data not shown).

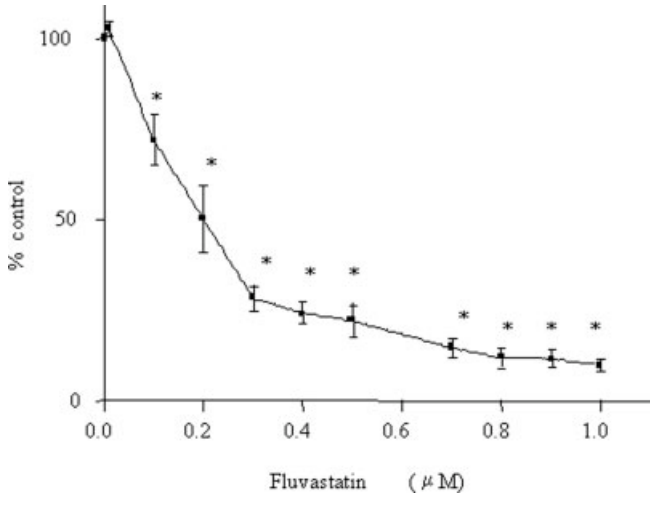
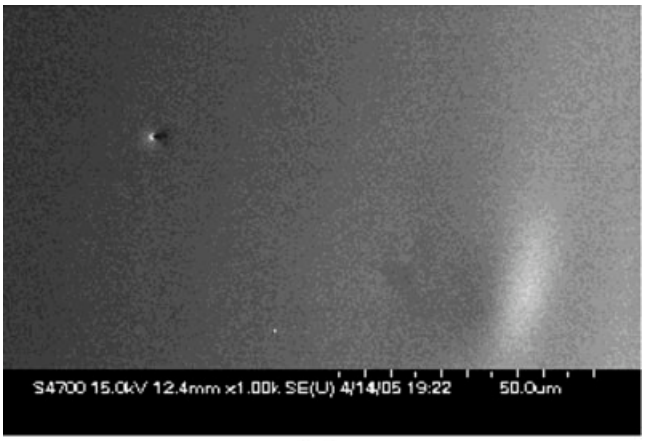


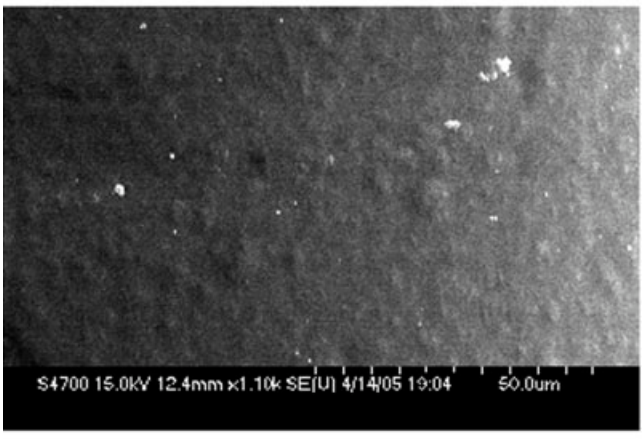
Figure 2. The effects of fluvastatin (0.1–1 μ*M*) on HCASMC proliferation assessed by BrdU incorporation into proliferating cells. Data are presented as percentage control proliferation ± SEM of eight determinations (**$p < 0.01$, ***$p < 0.001$).

Surface topography of coated wires

The surface topography of copolymer-coated stent wires (65/35 microgels-85/15 matrix combination) was assessed by scanning electron microscopy. Wires coated with the copolymer alone had a smooth appearance [Fig. 3(a)], while the insertion of microgels into the copolymer matrix resulted in generation of a rougher surface topography [Fig. 3(b)] consistent with the presence of microgel particles within the copolymer matrix. Distribution of microgels within the matrix was also confirmed by scanning confocal laser microscopy (data not shown).



(a)



(b)

Figure 3. Surface topography of copolymer-coated stent wires. Wires were coated with 85/15 copolymer alone (a) or with 85/15 copolymer incorporating 65/35 microgels (b).

Elution of drug from copolymer-coated stent wires

Elution of drug from copolymer-coated stent wires (65/35 microgels–85/15 matrix combination) was compared for up to 60 days under both static and perfused flow conditions. Data presented are from four determinations in each case. After 60 days, the wires were stripped of copolymer by incubation in ethanol and sonication. The amount of drug remaining on the wires was determined and added to the amount eluted, giving the total amount of drug initially loaded onto wires. A greater release was initially observed under static conditions where 0.50 ± 0.03 nmol drug was eluted within the first 24 h compared with 0.22 ± 0.02 nmol eluted into the perfusion circuit system. This corresponded to 1.47% ± 0.08% and 0.71% of total drug coated onto wires, respectively. However, by day 21 there appeared to be no significant difference between release from wires incubated under the different conditions, with 8.37% ± 0.36% and 8.05% ± 0.22% of total drug eluted from wires under static and flow conditions, respectively; this was still the case by day 60 where cumulative releases of 13.54% ± 0.38% and 13.83% ± 0.42% of drug were observed, respectively, (Fig. 4).

Having observed that drug elution profiles from stent wires under static and flow conditions followed comparable time courses, we next compared release kinetics and diffusion coefficients for both cases. In contrast to the non-Fickian diffusion kinetics determined for release from 65/35 microgel–85/15 matrix 5 μm films, drug release from coated stent wires under both static and flow conditions appeared to occur by Fickian diffusion. As can be seen using $t = 24$ days, the diffusion coefficient

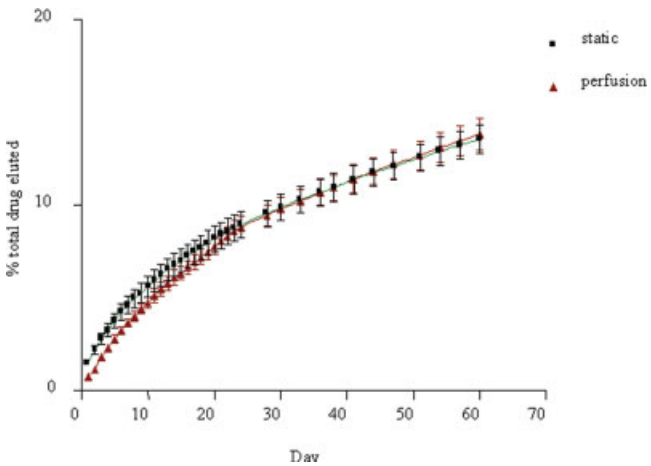


Figure 4. Comparison of cumulative drug release from copolymer-coated wires (drug-loaded 65/35 microgels dispersed in an 85/15 matrix) incubated under either static or perfused flow conditions over a 60-day period ($n = 4$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE III
Comparison of Release Kinetics (*n*-Values) and Diffusion Coefficients (m^2/s) for Fluvastatin from Films (65/35 Microgels in an 85/15 Matrix) Coated onto Stent Wires, Under Static and Perfusion Conditions

System	*n*-Value	Diffusion Coefficient (24 days)
Static conditions	0.150	4.17×10^{-16}
Perfusion conditions	0.156	8.45×10^{-16}

under flow conditions was approximately double than that under static conditions (Table III).

Effects of copolymer-coated wires on HCASMC viability

Wires were coated with the following: (i) 85/15 copolymer alone; (ii) 85/15 copolymer incorporating 65/35 microgels (nondrug-loaded); and (iii) 85/15 copolymer incorporating drug-loaded 65/35 microgels. Incubation with all copolymer-coated wires had no effect on HCASMC viability after 72 h incubation with cells (data not shown).

Effects of copolymer-coated wires on HCASMC proliferation

Bioactivity of drug eluted from coated wires was assessed as the ability to inhibit HCASMC proliferation. No effect on cell number was observed after incubation with bare wires alone or copolymer-coated wires. However, a significant reduction in HCASMC number to 50.93% ± 3.94% of control was observed after incubation with drug-loaded copolymer-coated wires ($p < 0.001$, $n = 4$) [see Fig. 5(i,ii)]. This reduction in cell number was attributed to an inhibition of proliferation, as no toxic effect had been observed after wires were incubated with the cells. The concentration of fluvastatin to which cells had been exposed in this experiment was 0.39 ± 0.03 μ*M*, determined from amounts of ^{14}C fluvastatin in the overlying medium. (This was a bioactive concentration, see Fig. 2.)

DISCUSSION

This study has established the principle that NiPAAm-derived plum-pudding gels can be employed to rigorously manipulate the rate and extent of release of a statin. It further demonstrates the adaptability of such systems to targeting restenosis injury over time courses relevant to its initiation and propagation.

Fluvastatin was chosen for the present study because of both its relative lipophilic and active na-

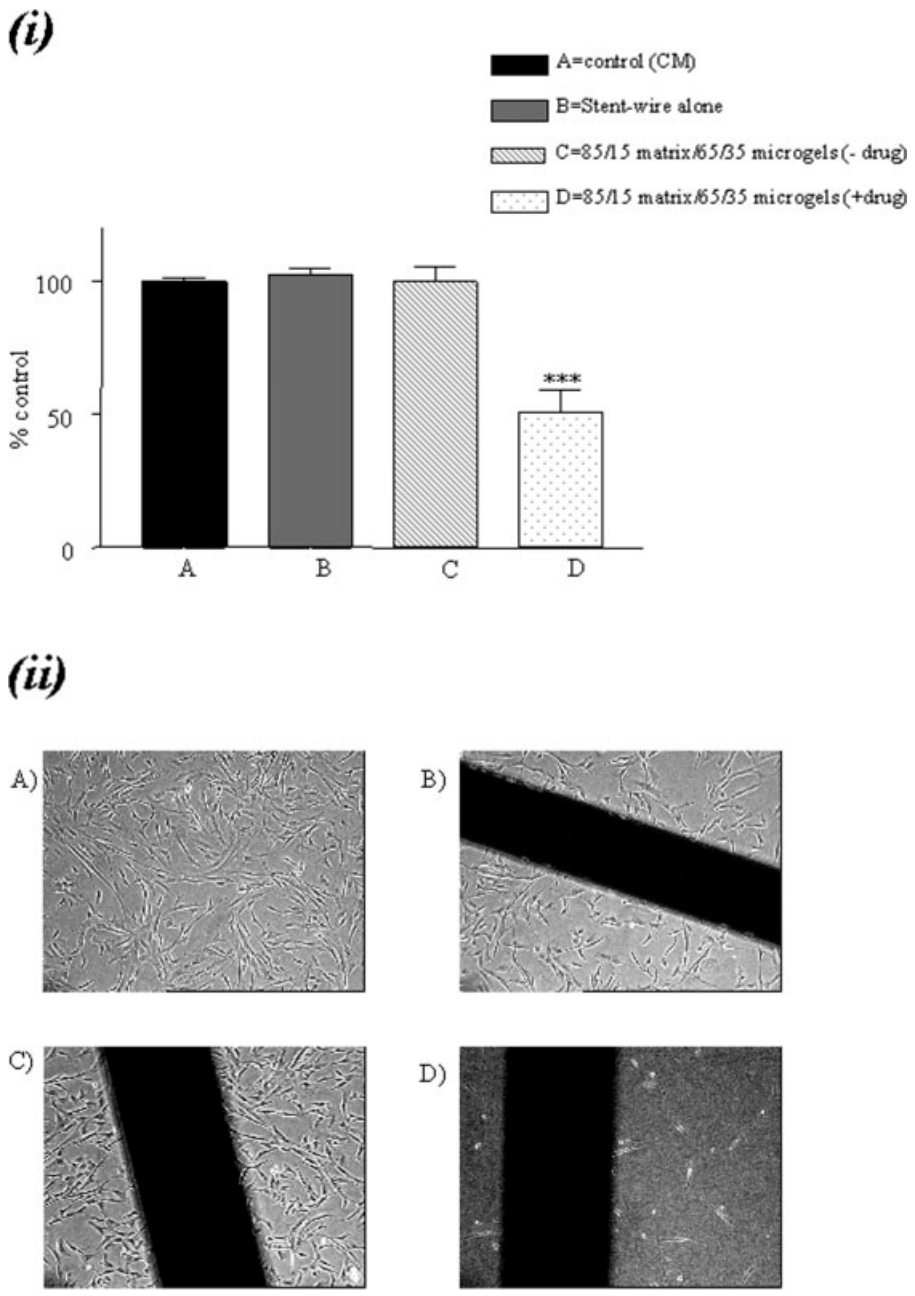


Figure 5. Effects of copolymer-coated wires on HCASMC proliferation, determined (i) as effects on cell number and (ii) from phase contrast images. Cells were incubated with (A) complete medium alone, (B) wire alone, (C) nondrug loaded copolymer-coated wire (85/15 copolymer incorporating 65/35 microgels), and (D) drug-loaded copolymer-coated wires (85/15 copolymer incorporating drug-loaded 65/35 microgels) (***$p < 0.001$, $n = 4$).

ture. Many currently available lipophilic statins require hepatic activation, which limits their use as agents for local drug delivery.[21] The lipophilic character of fluvastatin would be expected to facilitate rapid uptake by target cells after release from the stent surface. Such a property is desirable, since it is important to minimize blood flow-induced reductions of drug concentration at the target site. It may also be desirable that release of bioactive drug amounts be maintained over extended periods and this was achieved in the first part of the present study with the more hydrophobic copolymer films (Fig. 1, Table I). It follows that alteration of system hydrophilicity/hydrophobicity can allow manipulation of drug release profiles. If a higher initial release of drug is required, for example over a 24–72 h period post stent implantation, a hydrophilic system could be used, thus allowing a burst release, followed by a lower therapeutic maintenance dose. One could in theory incorporate drug-loaded microgels into any background matrix, thus making an infinite number of different microgel/matrix combinations.

Our choice of NiPAAm/NtBAAm copolymers for generation of microgels and matrices was based on earlier experience with such systems.[13–17] We have previously exploited their thermoresponsive nature for incorporation and elution of a therapeutic protein from copolymer matrices and of selective solutes from plum-pudding gels.[13,16] In earlier studies, Okano et al.[22] investigated release patterns of the model drug indomethacin from crosslinked copolymer hydrogels of pNiPAAm-co-butylmethacrylate (BMA) and found the initial shrinkage of the gels above the transition temperature (temperature at which PNiPAAm changes from a hydrophilic to a hydrophobic structure[23]) resulted in the formation of a dense outer skin layer that altered release kinetics. Yoshida et al. took advantage of this skin layer formation of NiPAAm-co-BMA using it as an ''on–off'' switch for thermally regulating indomethacin release; at 20°C, indomethacin readily diffused out of the porous polymer network but at 30°C, formation of a skin layer prevented any further release.[24] Formation of such a skin-layer would be undesirable for a stent coating, as at physiological temperature it would act as a barrier preventing elution into the local environment.

Both the Okano and Yoshida studies exploited the thermoresponsive nature of PNiPAAm-based hydrogels for controlling release. In contrast, our study investigated drug release from novel thermoresponsive microgel particles embedded in a collapsed PNiPAAm/NtBAAm copolymer matrix at physiological temperature. We achieved manipulation of drug release by exploiting the varying degrees of hydrophobicity of the different microgel/matrix systems employed.

A recent study by Zhang et al.[25] examined the effects of glycerol poly(ε-caprolactone) microspheres embedded within a thermoresponsive PNiPAAm hydrogel and found that the presence of microspheres enhanced the mechanical properties of the resulting network as assessed by compression modulus calculations. This increased mechanical strength was attributed to the ability of microspheres to act as reinforced nodes for the host matrix. This study also found that incorporation of ovalbumin into microspheres embedded within the matrix prolonged release from 2 days (ovalbumin in hydrogel alone) to 55 days (ovalbumin in microspheres).

Evidence for drug elution by Fickian diffusion from our copolymer films was sought using the Yasuda model.[19] Using this model, we have previously provided evidence that the extent to which release of a solute from plum-pudding gels occurs by Fickian diffusion is determined by relative hydrophobicity of the solute and microgel particles into which it is incorporated.[26] By extension, it could be anticipated that kinetics of release into a solvent would also be determined by the hydrophobicity of the matrix. Previous evidence shows that both these steps influence the diffusion coefficient.[27]

Elution from films incorporating the more hydrophobic matrix appeared to be via Fickian diffusion. However, since an n value >1 was determined for elution from the more hydrophilic matrices, we conclude that diffusion in this case was non-Fickian. We speculate that elution from the film in the latter case results from diffusion out of the microgels, followed by rapid expulsion of the hydrophobic drug from the hydrophilic matrix. Diffusion from microgels into the more hydrophobic matrix results in more classical Brownian motion in an environment compatible with the hydrophobic drug molecule.

In the present study, optimal release was observed from 65/35 microgels dispersed in the 85/15 matrix combination, which we then used to coat stainless steel 304L stent wires. This combination was regarded as optimal because even though greatest percentage release was observed from the more hydrophilic 85/15 microgels, a greater *amount* of drug was loaded into and eluted from the 65/35 microgels (Table I).

Results obtained with drug elution from the 65/35-85/15 microgel–matrix combination coated onto stent wires require two main comments. The delivery system that we used, while it has several practical advantages and is more flexible than some, is somewhat more complex than those that have been studied before. For example, elution profiles under static and flow conditions showed little difference but there still appeared to be a slightly higher diffusion coefficient (measured at 24 days) for the flow system. One should exercise caution in over interpreting such modest differences. We also exercise caution in the interpretation of other observations. For example, the fact that elution from the 85/15 matrix followed Fickian kinetics when the system was coated onto stent wires may have reflected the 5-fold larger film thickness, 25 μm versus the 5 μm films coated onto flat surfaces. Larger sample thickness would indeed tend to lead to a more bulk behavior of the transport properties (almost invariably simple diffusive) as the particles experience more random fluctuations in the material.

One of the main challenges in preventing ISR by local drug delivery lies in the fact that neointimal hyperplasia usually does not manifest itself until ~21 days postprocedure. An effective drug-delivery vehicle would therefore be required to deliver a bioactive concentration of drug for up to and beyond this time point.[28] Our system eluted a continuous amount of bioactive drug from coated wires for more than 60 days (Figs. 4 and 5).

It is worth noting that 86.46% ± 0.38% of the drug remained on the coated wires (copolymer layer 25

μm thick) even after 60 days, while a much smaller proportion of loaded drug (34.37% ± 1.72%) remained in copolymer films (5–10 μm) cast on flat surfaces by day 24. The sirolimus-eluting stent incorporates a coating of 5–10 μm polymer thickness and elutes ~80% of its total drug depot by 29 days[27,29,30]; thus, it is probable that a reduction of copolymer thickness on the stent wires would have resulted in less retention of drug. On the other hand, retention of a large proportion of incorporated statin on the coated wires for extended release may be a desired therapeutic effect. Previous studies have shown that withdrawal of oral statins has a knock-on effect on atherosclerotic progression and endothelial function.[31] Furthermore, statins may not only have protective effects in diseased arterial tissue but may even promote plaque regression.[32]

An important part of the present study was to determine whether exposure of the copolymer coating to physiological flow would modulate drug elution. Two major concerns associated with the use of polymer coatings are that (a) exposure to physiological levels of flow will result in loss of the coating and (b) drug release rates may be substantially altered due to a ''wash-away'' effect in the blood. Figure 4 shows that the fluvastatin release profile was not substantially altered when wires were incubated in a perfusion system where the flow rate was 23 mL/min (although diffusion coefficients were altered, see Table III), providing evidence that the plum pudding gel is a stable drug-eluting system. While we conclude that the control over drug release offered by the plum-pudding gel system appears to be extremely robust, even at the substantial flow rates utilized in our study, we acknowledge that such flow rates do not achieve levels seen in the human coronary arterial system.

In this study, emphasis was placed on fine manipulation of drug incorporation and elution in bioactive form, over an extended period. It was beyond the scope of the work to investigate the hemocompatibility of the plum-pudding gel system. However, preliminary evidence obtained in our laboratory indicates hemocompatible properties; an *in vitro* study has shown a trend towards a decrease in platelet adhesion to such surfaces, depending on copolymer composition (J. Hickey, personal communication).

CONCLUSIONS

This study has demonstrated proof of the concept that novel NiPAAm-derived ''plum pudding'' gels can act as systems for delivery of potential antirestenosis therapy from stent surfaces. The composite nature of the system incorporates release from the microgel population into the matrix, followed by elution from the matrix into the surroundings. In this way, drug release can be uniquely controlled in a manner that depends on the relative hydrophobicities of both microgel and matrix components of these novel systems. To our knowledge, no other delivery system described to date affords this level of control over extended drug delivery.

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