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Simultaneous Release of a Hydroxy-Methylglutaryl Coenzyme A Reductase Inhibitor and a Glycoprotein IIb/IIIa Antagonist from a Thermoresponsive NiPAAm/NtBAAm Copolymer System

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ABSTRACT

While deployment of intracoronary stents has been shown to reduce restenosis, stenting can also damage the endothelial monolayer lining the vessel wall, leading to possible in-stent thrombosis. Local drug delivery from stent surfaces represents a means of delivering therapeutic doses of drug directly to the target site. The aim of this study was to elute fluvastatin, which can inhibit vascular smooth muscle cell proliferation, and xemilofiban, which prevents platelet adhesion and aggregation, together in bioactive concentrations from the same copolymer system. Combined elution from thermoresponsive N-isopropylacrylamide (NiPAAm)/N-tert-butylacrylamide (NtBAAm)-derived copolymer systems was achieved using microgels (NiPAAm/NtBAAm 65/35 wt/wt) randomly dispersed in 85/15 matrices. Fluvastatin elution from 5 μm films over a 14-day period showed initial burst release, which leveled off. Of the total incorporated (8.33 ± 0.21 nmol, n = 4), 68.5% was eluted during this period. Xemilofiban release was measured in terms of its ability to inhibit platelet adhesion, using a microfluidic system. To investigate the influence of location and hydrophobicity on elution of bioactivity, three separate systems were employed. While elution of anti-adhesive activity from the system containing xemilofiban-loaded matrices was more dramatic in the short term, a more sustained level of inhibition was achieved when xemilofiban had been incorporated into microgels. All samples investigated for anti-adhesive activity also decreased human coronary artery smooth muscle cell proliferation. Therefore xemilofiban has potential as an agent for preventing in-stent thrombosis. Our study has demonstrated the feasibility of using this novel matrix/microgel system to regulate simultaneous release of both agents in bioactive concentrations.

Keywords*: *Fluvastatin, Xemilofiban, Nipaam/Ntbaam, Microgels, Local Drug Delivery

1. Introduction

The use of intravascular coronary stents during angioplasty procedures was first reported by Ulrich Sigwart in 1986 [1]. With the utilization of a stent there is the possibility of increased neointimal hyperplasia, as the metallic stent can cause physical injury to the endothelium, which can result in in-stent restenosis (ISR) and in-stent thrombosis (IST). Those higher risk patients with complex lesions (small vessel, bifurcation lesions) have an in-stent restenosis (ISR) occurrence rate of 30-60%, while in those with less complex lesions the rate is reduced to 15-20% of patients [2]. IST has an occurrence rate of ≈ 1% [3]. Drug-eluting stents (DES) have had a major influence on the reduction of in-stent restenosis. To prevent restenosis, a clear understanding of events responsible for growth factor- or cytokine-mediated vascular smooth muscle cell (VSMC) proliferation and migration (intimal hyperplasia) has led to a precise selection of potential therapeutic candidates [4]. However, the current leading agents, sirolimus and paclitaxel, can inhibit endothelial cell as well as VSMC proliferation,

thereby affecting the re-endothelialisation process which is central to vessel recovery. HMG CoA reductase inhibitors (statins) could be an option for the inhibition of neointimal hyperplasia, since these agents inhibit VSMC proliferation by a mechanism independent of cholesterol synthesis [5]. In an attempt to prevent the problem of IST, patients are placed on a strict regime of anti-platelet agents. However despite these adjunct therapies, stent thrombosis still occurs in 1% of patients [6]. The residual occurrence of IST may be caused by lack of penetration of such agents in adequate concentrations to the desired site of action following oral or systemic administration. Local delivery of agents designed to inhibit VSMC proliferation and platelet aggregation via a copolymer-coated stent platform is an attractive strategy for offsetting the incidence of ISR and IST.

Poly (n-isopropylacrylamide) (NiPAAm) is a polymer that can become inversely soluble upon heating. This change in state occurs at what is known as the lower critical solution temperature (LCST) [7]. Below the LCST the polymer chains are extended, separated and surrounded by water, but above the LCST the polymer becomes insoluble and precipitates out of solution [8]. The temperature range at which the soluble-insoluble shift occurs in relation to NiPAAm is between 31-34˚C. The addition of N-*tert*-butyl-acrylamide **(**NtBAAm) alters the relative hydrophobicity/hydrophilicity of NiPAAm thereby giving a means for regulating the elution of drugs for local delivery on the basis of their polarity. In this study, a range of copolymers exhibiting increasing hydrophobicities were synthesised in varying (w/w) ratios of NiPAAm/NtBAAm (85/15, 65/35 and 50/50).

The potential of NiPAAm/NtBAAm-derived copolymers to act as drug delivery vehicles for small molecules like colchicine [9-10] and therapeutic proteins like vascular endothelial growth factor (VEGF) [11] has been previously investigated. Another approach is to cross-link NiPAAm/NtBAAm copolymer microgel particles and disperse these through a bulk copolymer matrix. At their LCST the network volume changes from an expanded water-containing network, to a collapsed network where the water has been expelled. As the temperature increases the particles shrink and become hard and sphere shaped. [12] These microgels are then dispersed through the non cross-linked NiPAAm/NtBAAm matrices and can be used for drug loading and elution. Our group has previously shown that fluvastatin could be eluted from these matrix/microgel copolymer systems for up to 60 days with retention of bioactivity.

In this study, dual drug release from 3 different systems was investigated. System A was comprised 65/35 microgels containing fluvastatin embedded in a 50/50 matrix containing xemilofiban, while System B comprised 65/35 microgels containing fluvastatin embedded in an 85/15 matrix containing xemilofiban. Finally, System C contained 65/35 microgels incorporating xemilofiban, embedded in an 85/15 copolymer containing fluvastatin. Bioactivity of fluvastatin was assessed in terms of effects on human coronary artery smooth muscle cell (HCA-SMC) and human coronary artery endothelial cell (HCA-EC) proliferation while the bioactivity of xemilofiban was evaluated in terms of effects on platelet adhesion under conditions of flow.

2. Materials and Methods

2.1. 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-Azobisisobutyronitrile (AIBN; Phase Separation, UK) was recrystallized from methanol. Solvents were reagent grade and were purified by conventional methods. [^{14}C]-fluvastatin (specific activity 56.9 Ci/mmol) was generously donated by Novartis, Hanover. Xemilofiban (SC-54701) was kindly donated by Dr. Dermot Cox, RCSI. HCASMC with the corresponding growth medium (Medium 231) were purchased from Cascade Biologics (Belgium). HC-AEC with the corresponding medium (Endothelial Cell Growth Medium) were purchased from Promocell. 5-Bromo-2'-deoxyuridine (BrdU) was obtained from Roche Diagnostics (Germany). All other chemicals and reagents were of the highest grade commercially available.

2.2. Preparation of NiPAAm/NtBAAm Copolymers

The copolymers were synthesized through free radical polymerisation of the corresponding monomers with AIBN used as an initiator. The methodology was as outlined by McGillicuddy *et al*. [13] Briefly, mixtures of NiPAAm and NtBAAm monomers in different ratios were dissolved in benzene with AIBN then added. The removal of oxygen was achieved by bubbling N_2 gas through the solution for 30 min. Polymerisation then proceeded at 60˚C for 24 h. Benzene was evaporated off and the copolymer was dissolved in acetone and precipitated in *n*-hexane. This whole procedure was repeated three times. The purified copolymer was dried at room temperature under vacuum.

2.3. Preparation of Microgels

Microgels were synthesized using the method of dispersion polymerisation. [14] In their desired w/w ratios

(85/15, 65/35, 50/50, total 0.2 g) NiPAAm/NtBAAm and bisacrylamide (0.02 g) were dissolved in 36 ml of water. One ml of 0.1 wt% Triton solution was added and heated to 70˚C. The solution was degassed by bubbling with N_2. Ammonium persulphate (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) cleaned by dialysis, and freeze-dried before use.

2.4. Preparation of, and Drug Incorporation into Matrix/Microgel Copolymer Systems

Fluvastatin was incorporated into 65/35 microgel particles (5 mg) by incubation with 1 mL of an ethanolic solution of 5mM drug 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 resuspended in 0.5 ml ethanol. Drug-loaded microgel suspensions were subsequently added to 0.5 mL of a 10% 50/50 or 85/15 matrix copolymer solution containing 2.5 mM xemilofiban. Alternatively xemilofiban was incorporated into 65/35 microgel particles which were subsequently incorporated into a 50/50 matrix containing fluvastatin. 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 in an ethanolic atmosphere. All manipulations in aqueous medium were done at 37˚C which is above the LCST and therefore all copolymer systems were in the collapsed/insoluble state.

2.5. Drug Release from Copolymer Films

Dried films were washed with prewarmed PBS (37˚C) to retain the collapsed state of the copolymer, prevent its dissolution, and remove any surface-bound drug. Drug elution from the resulting films at 37˚C was monitored (as [^{14}C]-fluvastatin) every 24 h by removing, storing and replacing the PBS 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 determined.

2.6. Cell Culture

HCASMC that had been isolated from a 21-year old Caucasian male by Cascade Biologics were received as cryopreserved cultures (passage 2) from Cytotech Ltd. (Denmark). The cells were maintained in their appropriate medium, Medium 231, supplemented with foetal bovine serum (4.9%), basic fibroblast growth factor (2ng/ml), epidermal growth factor (0.5g/ml), heparin (5ng/ml), insulin (5µg/ml), BSA (0.2µg/ml), gentamicin (10µg/ml) and amphotericin B (0.25µg/ml). The cells were grown to confluency in 75cm^2 filter top tissue culture flasks and were maintained at 37˚C in a humidified atmosphere containing 95% O_2 and 5% CO_2. Subcultures were created by passaging using a trypsin/EDTA (T/E) (0.025 %/0.01%) mixture in phosphate buffer saline (PBS), harvested by centrifugation (3 min at 433 × g) and seeded at stated densities. Cells of passages 4-10 were used for experiments.

HCAEC were purchased from Promocell (Germany). The cryopreserved cells (passage 2) received had been isolated from a 63-year old Caucasian male. The appropriate endothelial cell growth medium was used to maintain the HCAEC. The medium was supplemented with 20 % heat-inactivated foetal calf serum (FCS), endothelial cell growth supplement/heparin (2 ml), human recombinant epidermal growth factor (5 µg/500 µl), hydrocortisone (500 µg/500 µl), gentamicin (10 µg/ml) and amphotericin B (0.25 µg/ml). The cells were grown to confluency in 75 cm^2 filter top tissue culture flasks and were maintained at 37˚C in a humidified atmosphere containing 95% O_2 and 5% CO_2. Subcultures were created by passaging using a T/E (0.025%/0.01%) mixture in PBS, harvested by centrifugation (3 min at 433 × g) and seeded at stated densities. Cells of passages 4-10 were used for experiments. Cells were also routinely tested for the presence of mycoplasm.

2.7. Measurement of Cellular Proliferation (BrdU ELISA)

HCASMC or HCAEC (3×10^4 cells/ml/well) were seeded into 96-well plates and left to adhere overnight. Cells were then incubated with samples of fluvastatin eluted from the various copolymer systems and left for 48h. Proliferation was subsequently assessed by measurement of BrdU incorporation into the DNA of proliferating cells using a colorimetric ELISA.

2.8. Measurement of Platelet Adhesion under Conditions of Flow

A novel microflow system consisting of a novel syringe pump with microfluidic biochip and flow sensor controlled by a PC using dedicated software was used to assess the effects of eluted samples of xemilofiban. Blood was collected from healthy volunteers who were not taking any medication and were free from aspirin and other anti-platelet agents within the previous 2 weeks. The blood was drawn by venupuncture into tubes containing a 1:10 volume of 3.8% (wt/vol) trisodium citrate and gently mixed. Each microgel channel of the novel

microflow system was coated overnight in humid conditions at 4˚C with fibrinogen (2 μg/ml). All channels were then coated with BSA (10 μg/ml) to saturate non-specific binding sites and left for approximately 30 min. Prior to the shear experiments all channels were washed through with JNL. Aliquots of whole blood were incubated for 5 or 10 min with the day 1, 3, 9 and 14 samples released from the following copolymer films:

A) 65/35 microgels containing fluvastatin embedded in a 50/50 matrix containing xemilofiban

B) 65/35 microgels containing fluvastatin embedded in an 85/15 matrix containing xemilofiban

C) 65/35 microgels containing xemilofiban embedded in an 85/15 copolymer containing fluvastatin.

Whole blood and sample were together infused into the fibrinogen-coated channels under a shear stress of 32 dyne cm^{-2} for 3 min. Phase contrast images at the indicated shear stress levels were captured using MetaMorph imaging software for analysis at a later time. The images were then used to quantify platelet adhesion by counting the number of platelets within each illustrated quadrant.

2.9. 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's multiple comparisons post-test. The statistical package PRISM was used for all analyses. Differences between means were considered significant when $p < 0.05$.

3. Results

3.1. Drug Elution from Copolymer Films

For normalization purposes, all release data were graphed as a percentage of total drug incorporated into copolymer films. The elution of fluvastatin from fluvastatin-loaded 65/35 microgels embedded in a xemilofiban-containing 85/15 matrix saw a release of 12.69 ± 0.74% on day 1 with a total release of 68.55 ± 1.54% by the final day (**Figure 1(a)**). After the 14-day elution a distribution graph was calculated to show the amounts eluted, remaining and total amount incorporated in the system (**Figure 1(b)**). A total of 8.33 ± 0.21 nmol was incorporated, 5.71 ± 0.13 nmol were eluted and 2.58 ± 0.12 nmol remained upon completion.

3.2. Effect of Native and Eluted Xemilofiban on Platelet Adhesion as Assessed Using a Microfluidic System

Xemilofiban had a statistically significant effect on

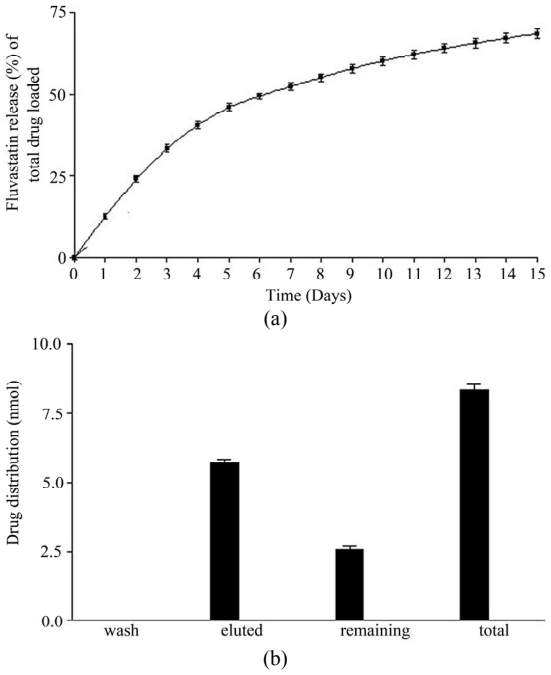

Figure 1. (a) Cumulative release of fluvastatin from fluvastatin-loaded 65/35 microgels embedded in an 85/15 copolymer matrix containing xemilofiban over a 14-day period. The microgels were pre-incubated with 5 mM fluvastatin while the matrix was pre-incubated with 2.5 mM xemilofiban and left overnight. Copolymer films were then cast in 24-well plates and after 24 h PBS was added to the wells. On the final day the copolymer films were dissolved and total drug incorporated was calculated. Cumulative release was subsequently expressed as a % of total drug incorporated into the films. Data are representative of mean ± S.E.M. of 4 individual copolymer films. (b) Distribution graph of drug- eluting films releasing fluvastatin. The total amount of drug initially loaded into the copolymer films was determined after dissolving the copolymer films. Data are representative of mean ± S.E.M. of 4 individual copolymer films.

platelet adhesion at concentrations of 0.01 and 0.1 μM, with adhesion being reduced to 56.06 ± 7.8% and 3.44 ± 1.43% of controls, respectively (**Figure 2**). A group of controls were used in the experiment with a "high" xemilofiban concentration (1 μM), a "low" xemilofiban concentration (0.01 μM) and a "low" concentration of fluvastatin (0.1 μM). All samples were mixed with whole blood and subjected to shear. Of the controls the "high" xemilofiban concentration significantly inhibited platelet adhesion, reducing it to 5.81 ± 1.59%. "Low" xemilofiban reduced platelet adhesion to 65.25 ± 9.91% and "low" fluvastatin to 57.83 ± 19.13%. The phase contrast images from which these results were extrapolated, by quantifying the number of adhered platelets, also show clearly the decrease in platelet adhesion using the high xemilofiban concentration (**Figure 3**). System A (the

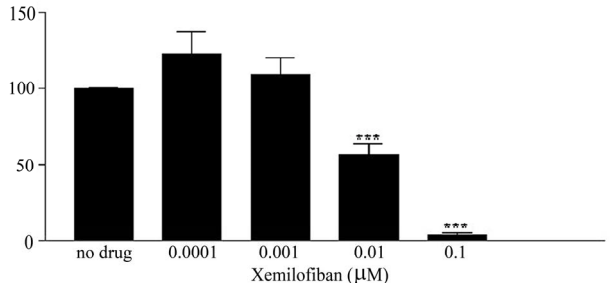

Figure 2. Effect of xemilofiban on platelet adhesion as assessed using a microfluidic system. Whole blood which had been treated with increasing concentrations of xemilofiban was passed through microcapillary channels on a biochip under shear stress. Numbers of platelets adhered were counted using MetaMorph. Results were calculated as a % of control (no drug) and values are presented as mean ± S.E.M., n = 3, ***p < 0.001 w.r.t. control.

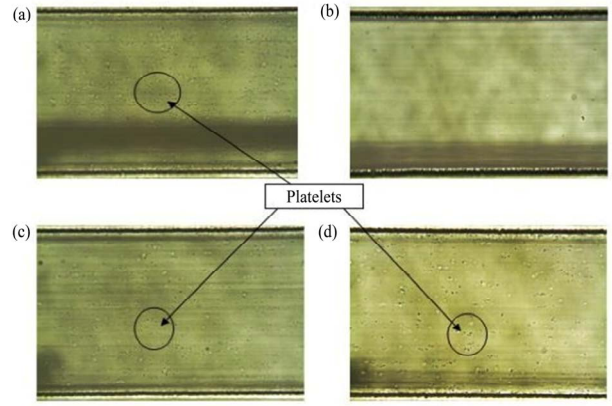

Figure 3. The effects of xemilofiban and fluvastatin on whole blood assessed using the microfluidic system. Whole blood was mixed with either drug and then passed through the fibrinogen coated biochannels and subjected to shear rate, phase contrast images were captured using MetaMorph. Images are as follows: (a) whole blood alone; (b) high xemilofiban, 1 μM; (c) low xemilofiban, 0.01 μM; (d) fluvastatin 0.1 μM.

most hydrophilic system of the 3) showed significant decreases for day 1 and 3 with adhesion reduced to 7.18 ± 3.02% and 15.46 ± 5.62%, respectively; thereafter platelet adhesion increased to 62.43 ± 20.4% and 82.08 ± 24.97% respectively. System B (the more hydrophobic system) showed a significant decrease for day 1 with a reduction to 8.74 ± 4.57%. On days 3, 9 and 14, there was no significant difference in reductions; however there was an apparent reduction in platelet adhesion to half that of the control on day 14. Finally, with system C in which xemilofiban was incorporated into the microgels, there was a significant decrease seen for days 1, 3, 9 and 14, with reductions to 8.96 ± 5.14%, 47.97 ± 11.89%, 50.54 ± 15.37% and 51.11 ± 10.26% respectively (**Figure 4**).

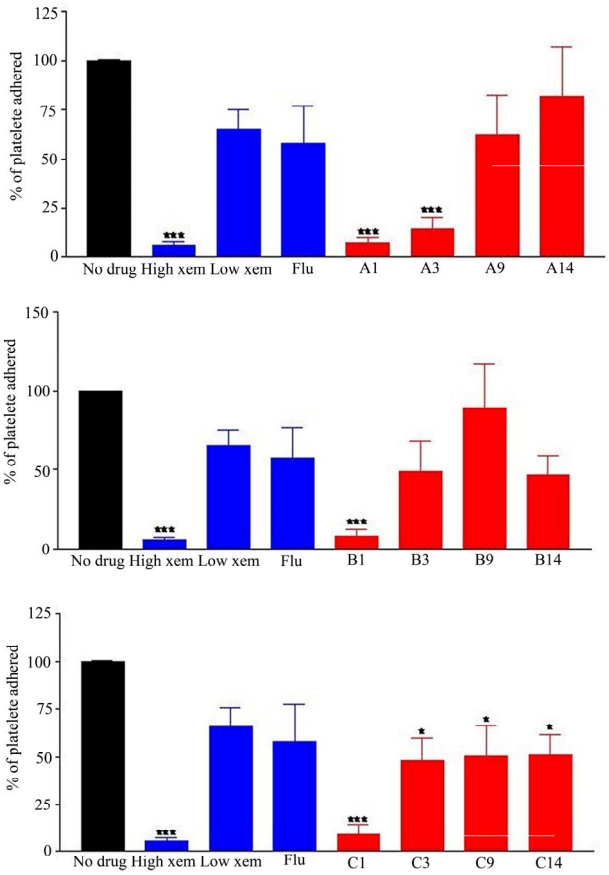

Figure 4. Effect of samples eluted from copolymer films on platelet adhesion assessed using the microfluidic system. System A composition was as follows 65/35 microgels containing fluvastatin embedded in a 85/15 matrix containing xemilofiban, System B was 65/35 microgels containing fluvastatin embedded in an 85/15 matrix containing xemilofiban and System C was comprised of 65/35 microgels containing xemilofiban embedded in an 85/15 copolymer containing fluvastatin Copolymer films were cast into 24-well plates and overlaid with PBS and eluted samples were collected daily. Whole blood was treated with these eluted samples and passed through the microfluidic system. The amount of platelets adhered was counted using MetaMorph. Results were calculated as a % of control (no drug) and values are presented as mean ± S.E.M., n = 5, ***p < 0.001 w.r.t. control.

3.3. Effect of Eluted Fluvastatin on HCASMC Proliferation

All samples eluted from each system significantly inhibited HCASMC proliferation (**Figure 5**). It could be seen that while proliferation was inhibited in the presence of each sample, a steady loss in anti-proliferative activity was seen over time. Samples eluted from System A decreased proliferation to 12.86 ± 4.55% of control, using the Day 1 sample, with proliferation recovering to 62.29

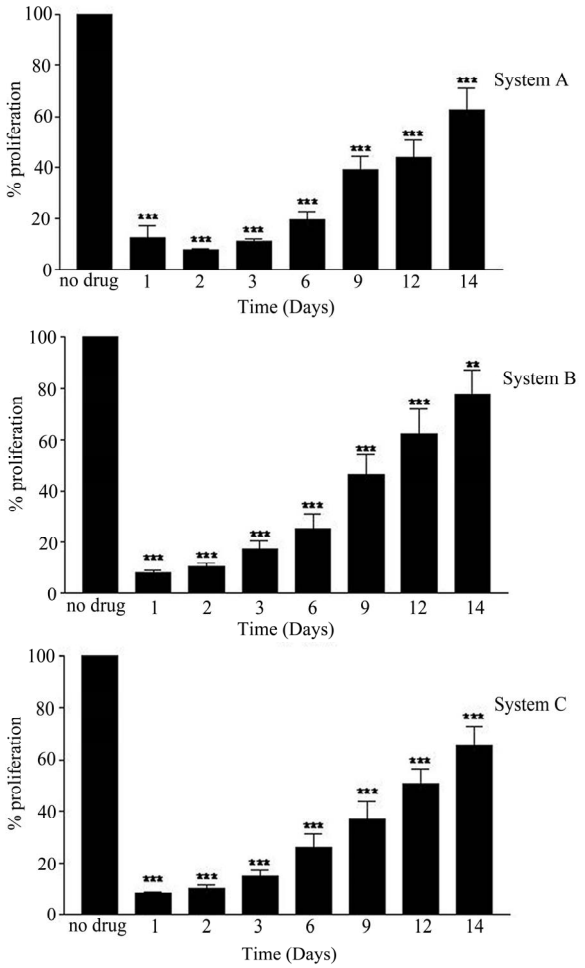

Figure 5. Effects of samples eluted from system A-C on HCASMC proliferation as assessed using the BrdU assay. Each sample was incubated with smooth muscle cells for 48 h. Results were calculated as % of control (no drug) and values are presented as mean ± S.E.M., n = 3, ***p < 0.001 w.r.t. control.

± 8.17% by day 14. With regard to Systems B and C, proliferation was reduced to 8.17 ± 0.6% and 8.32 ± 0.6 % of control respectively, on day 1. By day 14 HCA-SMC proliferation was 77.74 ± 9.44% with System B, and 65.22 ± 7.69% with System C. Upon comparison of anti-proliferative activity with that obtained with native fluvastatin, it can be approximated that fluvastatin was present in concentrations between 0.01 and 1.4 μM in samples tested from these systems.

3.4. Effect of Eluted Fluvastatin on HCAEC Proliferation

When endothelial cells were treated for 48 h with the eluted samples, there was no statistically significant effect on proliferation seen in the presence of any sample from any of the 3 systems (**Figure 6**).

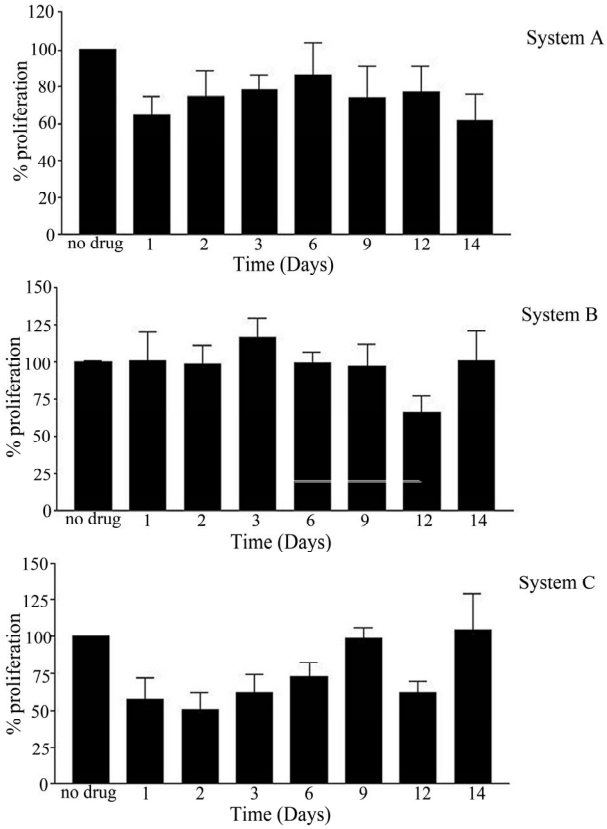

Figure 6. Effects of samples eluted from system A-C on HCAEC proliferation as assessed using the BrdU assay. Each sample was incubated with endothelial cells for 48 h. Results were calculated as % of control (no drug) and values are presented as mean ± S.E.M., n = 3, N.S. w.r.t. control.

4. Discussion

The overall aim of this study was to control release and deliver bioactive concentrations of a HMG-CoA reductase inhibitor (fluvastatin) and glycoprotein IIb/IIIa antagonist (xemilofiban) from a NiPAAm/NtBAAm matrix/microgel drug delivery system. Using the thermoresponsive properties of the matrices and microgels, the potential of such systems for the elution of these drugs has been demonstrated.

The anti-proliferative nature of statins has been extensively studied. Statins have been reported to inhibit smooth muscle cell proliferation both *in vitro* and *in vivo* [15]. Lovastatin, simvastatin and cerivastatin were found to significantly inhibit VSMC proliferation when treated with concentrations of 0.1-50 μM. [16,17].

Takeda *et al.* [18] found that simvastatin significantly inhibited cell proliferation in bronchial VSMC while having no effect on cell viability. An *in vitro* study by Corpataux *et al.* [19] found that the reduction in smooth muscle cell proliferation produced by fluvastatin was

significantly greater than that with other statins such as simvastatin, lovastatin, atorvastatin, cerivastatin and pravastatin. Jaschke *et al.* [17] showed that after treatment with cerivastatin (0-100 nM) only the highest concentration had a significant effect on endothelial cell proliferation. Thus, although fluvastatin was used to treat both HCAEC and HCASMC in this study, the latter were much more sensitive to these anti-proliferative effects. Therefore fluvastatin was selected as the statin of choice in the present project for local drug delivery due to its active and lipophilic nature, both essential requirements for a potential candidate for local drug delivery.

We have previously reported that NiPAAm/NtBAAm copolymers can elute fluvastatin for up to 60 days while retaining its bioactivity, as assessed under both static and perfusion conditions [20]. The present study has further shown that it is possible to co-elute bioactive fluvastatin with a second drug type incorporated in the system. In System A fluvastatin was eluted from 65/35 microgels and then released through a hydrophilic 85/15 matrix. Upon completion of this release experiment there was < 20% of the drug remaining in the system. It is important to note here that the pH value of blood may have an effect on drug elution *in vivo*; however such an investigation *in vitro* was beyond the scope of the present work.

Also, while every step in the preparation of the microgel/copolymer systems was carefully carried out, there is the possibility that small quantities of drug were lost. However, such losses were deemed minimal, since subsequent experiments showed comparable amounts of activity eluted in replicate experiments. While the inhibition of vSMC proliferation steadily decreased with each of the daily samples used, proliferation at 48 h was still inhibited. Samples eluted from the other two systems showed an inhibition of proliferation resembling that of System A.

In Systems A and B, fluvastatin was incorporated into the microgels and then eluted through hydrophilic (System A) or hydrophobic (System B) matrices. This may explain the slight differences between these systems *i.e.* System A samples showed slightly greater inhibiton of proliferation and the drug was eluted faster. With regard to System C fluvastatin was dispersed in the matrix and thus only had one barrier to pass through; the results here were similar to those for System A. This could be due to the fact that hydrophobic drug was eluted from microgels into a hydrophilic matrix in one instance and straight from a hydrophobic matrix in the other. It is possible that the time course of elution from a hydrophobic matrix is comparable to that of elution from microgels and a hydrophilic matrix together. However it is also important to note that major differences may only be seen with extension of the 14-day period (**Figure 6**). The comparable levels of overall inhibition seen between systems could also be due to the fact that each of the systems is eluting a similar concentration of fluvastatin on the relevant days.

None of the samples eluted from system A-C had a significant effect on HCAEC proliferation after 48 h, which suggests that re-endothelialisation *in vivo* would not be impeded up to this point.

The rationale for using the GpIIb/IIIa antagonist xemilofiban in this study was based on the fact that GpIIb/IIIa antagonists have been used successfully as intravenous anti-platelet agents post-surgery (PTCA) [21] following their development of oral agents, mixed results coronary syndromes and long-term management of patients [22]. However with 5 large-scale trials completed by 2001, which included over 42,000 patients (EXCITE, [23] OPUS, [24] SYMPHONY 1 and 2, [25,26] BRAVO [27]), it was consistently found that the GpIIb/IIIa agents xemilofiban, orbofiban, sibrafiban and lotrafiban were no more effective than aspirin when given post-surgery. However, another study showed that their use during PCA procedures was effective and in fact improved in-hospital survival rates [28]. Heer *et al.* [29] have also offered evidence of their effectiveness during primary angioplasty. Therefore their effectiveness as an intravenous treatment in conjunction with PCA gives an indication that these agents could be used for local delivery from a stent platform.

The rationale for elution of an anti-platelet agent has been strengthened by reports demonstrating elution of GpIIb/IIIa receptor monoclonal antibody from polymer-coated stents. Yin *et al.* [30] showed that the GpIIb/IIIa receptor antibody eluted from I-PLA polymer-coated stents inhibited platelet aggregation. Aggarwal *et al.* [31] showed that GpIIb/IIIa antibody could be eluted for up to 14 days, with antibody still remaining after that time under conditions of flow.

Our study firstly showed that xemilofiban concentrations of 0.01 and 0.1 μM had a statistically significant effect on platelet adhesion (**Figure 3**). This gives a good indication that xemilofiban is capable of exerting its effects under conditions of flow and that platelets in circulating blood are more susceptible to the anti-adhesive action of xemilofiban in this model.

Under conditions of flow System A showed that on day 1 and 3, samples decreased platelet adhesion significantly, and the results were in fact on a comparable level with the control "high xemilofiban" concentration; however day 9 and 14 samples had only a minor effect on adhesion equivalent to that of the "low xemilofiban" concentration (**Figures 4 and 5**). As this was a hydro-

philic matrix it could be inferred that the hydrophobic xemilofiban was expelled rapidly and therefore only had a significant effect at day 1 and 3. It may be possible to prolong the elution of the drug from this matrix type by incubating the matrix or microgels with a higher concentration of xemilofiban. Alternatively using a more hydrophobic matrix could also potentially prolong the release of xemilofiban. It should be noted here that measurement of the exact concentration of xemilofiban released was beyond the scope of the present study.

System B had 65/35 microgels containing fluvastatin embedded in a 50/50 matrix containing xemilofiban; this is the more hydrophobic system and therefore it could be anticipated that elution would be prolonged. With this system a statistically significant effect was seen on day 1 with a reduction to a level similar to "high xemilofiban". This correlated well with the burst release that is usually seen on Day 1 with all systems. While the remaining samples did not elicit a statistically significant effect it could be seen that there was a trend towards continued inhibition of platelet adhesion (**Figure 5**). Thus this system gave an indication that the inhibitory effect would have continued beyond 14 days.

System C saw the incorporation of xemilofiban into 65/35 microgels, which were then embedded in a 50/50 matrix. This final system showed release of bioactive drug over an extended time interval. In this case drug would have firstly been released from the microgels and then passed through the hydrophobic matrix. Each of the daily samples had a significant effect on platelet adhesion, with the day 1 sample having the greatest effect (**Figure 5**).

The contrasts between the profiles of each of the delivery systems can be explained in terms of their composition. With regard to Systems A and B, xemilofiban is contained in a hydrophilic and hydrophobic matrix respectively. The drug appears to be eluted faster from System A as a greater effect can be seen on Days 1 and 3, after which the anti-adhesive effect is reduced. The hydrophobic matrix of System B retards the release of xemilofiban slighty, thereby resulting in a greater anti-adhesive effect being seen at Day 14 compared with System A. Another point to note with these two systems is that as xemilofiban is contained in the matrix, it only has one diffusion barrier prior to release, thereby resulting in faster elution. With System C however, xemilofiban is contained in the microgel component, and therefore has two diffusion barriers prior to release. Comparing System C with Systems A and B, it can be noted that a steady elution rate appears to be maintained, which may reflect more tightly controlled release. With all three systems the Day 1 eluate had the most significant anti-adhesive activity, which is characteristic of the burst release seen with these systems [20].

5. Conclusions

Overall, this study showed that it is possible to elute two bioactive drug types from one copolymer system, resulting in three points of information. Firstly alteration of the composition of the copolymer systems *i.e.* the hydrophobic or hydrophilic nature of the matrix or microgels can affect elution. Also placement in either matrix or microgels can alter the period of elution *i.e.* the incorporation of drug into the microgels can prolong the effect of the drug. The study finally shows that xemilofiban remains bioactive and can affect platelet adhesion under flow conditions. The use of thermoresponsive copolymers is relevant as they allow for the incorporation of drug into a soluble mixture followed by precipitation above the LCST onto, potentially, a stent. Therefore the thermoresponsive properties coupled with the relative hydrophobicities and hydrophilicities of the NiPAAm/NtBAAm copolymers adds to their potential for the delivery of numerous drugs for extended periods. Therefore this study has shown that two drugs eliciting different actions can be eluted for up to 14 days in bioactive concentrations with the potential for extended release. Also fluvastatin did not alter platelet adhesion at concentrations eluted nor did xemilofiban affect cell proliferation at estimated concentrations eluted.

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REFERENCES

[1] U. Sigwart, J. Puel, V. Mirkovitch, F. Joffre and L. Kappenberger, "Intravascular Stents to Prevent Occlusion and Restenosis after Transluminal Angioplasty," *New England Journal of Medicine*, Vol. 316, No. 12, 1987, pp. 701-706. doi:10.1056/NEJM198703193161201

[2] V. A. Voudris, J. S. Skoularigis, Y. K. Dimitriou, G. N. Grapsa, J. S. Malakos, G. S. Pavlides, *et al.*, "Diabetes Mellitus and Unstable Coronary Artery Disease: Improved Clinical Outcome of Coronary Artery Stenting in an Era of Glycoprotein IIb/IIIa Inhibitors and Lipid-Lowering Therapy," *Coronary Artery Disease*, Vol. 15, No. 6, 2004, pp. 353-359. doi:10.1097/00019501-200409000-00009

[3] A. T. Ong and P. W. Serruys, "Drug-Eluting Stents: Current Issues," *Texas Heart Institute Journal*, Vol. 32, No. 3, 2005, pp. 372-377.

[4] B. L. Hiatt, F. Ikeno, A. C. Yeung and A. J. Carter, "Drug-Eluting Stents for the Prevention of Restenosis: In

Quest for the Holy Grail," *Catheterization and Cardiovascular Interventions*, Vol. 55, No. 3, 2002, pp. 409-417. doi:10.1002/ccd.10161

[5] S. Park and S. Lee, "Optimal Management of Platelet Function after Coronary Stenting," *Current Treatment Options in Cardiovascular Medicine*, Vol. 9, No. 1, 2007, pp. 37-45. doi:10.1007/s11936-007-0049-7

[6] R. M. da Silva, J. F. Mano and R. L. Reis, "Smart Thermoresponsive Coatings and Surfaces for Tissue Engineering: Switching Cell-Material Boundaries," *Trends in Biotechnology*, Vol. 25, No. 12, 2007, pp. 577-583. doi:10.1016/j.tibtech.2007.08.014

[7] E. S. Ron and L. E. Bromberg, "Temperature-Responsive Gels and Thermogelling Polymer Matrices for Protein and Peptide Delivery," *Advanced Drug Delivery Reviews*, Vol. 31, No. 3, 1998, pp. 197-221. doi:10.1016/S0169-409X(97)00121-X

[8] F. Eeckman, K. Amighi and A. J. Moes, "Effect of Some Physiological and Non-Physiological Compounds on the Phase Transition Temperature of Thermoresponsive Polymers Intended for Oral Controlled-Drug Delivery," *International Journal of Pharmaceutics*, Vol. 222, No. 2, 2001, pp. 259-270.

[9] K. B. Doorty, T. A. Golubeva, A. V. Gorelov, Y. A. Rochev, L. T. Allen, K. A. Dawson, W. M. Gallagher and A. K. Keenan, "Poly(N-isopropylacrylamide) Co-Polymer Films as Potential Vehicles for Delivery of an Antimitotic Agent to Vascular Smooth Muscle Cells," *Cardiovascular Pathology*, Vol. 12, No. 2, pp. 105-110. doi:10.1016/S1054-8807(02)00165-5

[10] S. J. Wilson, A. V. Gorelov, Y. A. Rochev, F. C. McGillicuddy, K. A. Dawson, W. M. Gallagher and A. K. Keenan, "Extended Delivery of the Antimitotic Agent Colchicine from Thermoresponsive N-isopropylacrylamide-Based Copolymer Films to Human Vascular Smooth Muscle Cells," *Journal of Biomedical Materials Research*, Vol. 67, No. 2, 2003, pp. 667-673.

[11] C. A. Kavanagh, T. A. Gorelova, I. I. Selezneva, Y. A. Rochev, K. A. Dawson, W. M. Gallagher, A. V. Gorelov and A. K. Keenan, "Poly(N-isopropylacrylamide) Copolymer Films as Vehicles for the Sustained Delivery of Proteins to Vascular Endothelial Cells," *Journal of Biomedical Materials Research*, Vol. 72, No. 1, 2005, pp. 25-35. doi:10.1002/jbm.a.30192

[12] I. Lynch and K. A. Dawson, "Synthesis and Characterization of an Extremely Versatile Structural Motif Called the "Plum-Pudding" Gel," *Journal of Physical Chemistry*, Vol. 107, No. 36, 2003, pp. 9629-9637.

[13] F. C. McGillicuddy, "Evaluation of N-isopropylacrylamide/N-tert-butylacrylamide Copolymer Microgel/Matrix Systems as Anti-Restenotic Drug Delivery Vehicles," Ph.D. Thesis, 2006.

[14] Y. Li and Y. Bae, "Volume Phase Transition of Submicron-Sized NIPAM/BAm Particles by Photon Correlation Spectroscopy," *Journal of Applied Polymer Science*, Vol. 67, 1998, pp. 2088-2092.

[15] B. R. Kwak, F. Mulhaupt and F. Mach, "Atherosclerosis: Anti-Inflammatory and Immunomodulatory Activities of Statins," *Autoimmunity Reviews*, Vol. 2, No. 6, 2003, pp. 332-338. doi:10.1016/S1568-9972(03)00049-1

[16] R. Riessen, D. I. Axel, M. Fenchel, U. U. Herzog, H. Rossmann and K. R. Karsch, "Effect of HMG-CoA Reductase Inhibitors on Extracellular Matrix Expression in Human Vascular Smooth Muscle Cells," *Basic Research in Cardiology*, Vol. 94, No. 5, 1999, pp. 322-332.

[17] B. Jaschke, C. Michaelis, S. Milz, M. Vogeser, T. Mund, L. Hengst, *et al.*, "Local Statin Therapy Differentially Interferes with Smooth Muscle and Endothelial Cell Proliferation and Reduces Neointima on a Drug-Eluting Stent Platform," *Cardiovascular Research*, Vol. 68, No. 3, 2005, pp. 483-492. doi:10.1016/j.cardiores.2005.06.029

[18] N. Takeda, M. Kondo, S. Ito, Y. Ito, K. Shimokata and H. Kume, "Role of RhoA Inactivation in Reduced Cell Proliferation of Human Airway Smooth Muscle by Simvastatin," *American Journal of Respiratory Cell and Molecular Biology*, Vol. 35, No. 6, 2006, pp. 722-729. doi:10.1165/rcmb.2006-0034OC

[19] J. M. Corpataux, J. Naik, K. E. Porter and N. J. London, "The Effect of Six Different Statins on the Proliferation, Migration, and Invasion of Human Smooth Muscle Cells," *Journal of Surgery Research*, Vol. 129, No. 1, 2005, pp. 52-56. doi:10.1016/j.jss.2005.05.016

[20] F. C. McGillicuddy, I. Lynch, Y. A. Rochev, M. Burke, K. A. Dawson, W. M. Gallagher and A. K. Keenan, "Novel "Plum Pudding" Gels as Potential Drug-Eluting Stent Coatings: Controlled Release of Fluvastatin," *Journal of Biomedical Materials Research*, Vol. 79, No. 4, 2006, pp. 923-933. doi:10.1002/jbm.a.30839

[21] J. F. Granada and N. S. Kleiman, "Therapeutic Use of Intravenous Eptifibatide in Patients Undergoing Percutaneous Coronary Intervention: Acute Coronary Syndromes and Elective Stenting," *American Journal of Cardiovascular Drugs*, Vol. 4, No. 1, 2004, pp. 31-41.

[22] C. Patrono, F. Bachmann, C. Baigent, C. Bode, R. De Caterina, B. Charbonnier, *et al.*, "Expert Consensus Document on the Use of Antiplatelet Agents. The Task Force on the Use of Antiplatelet Agents in Patients with Atherosclerotic Cardiovascular Disease of the European Society of Cardiology," *European Heart Journal*, Vol. 25, No. 2, 2004, pp. 166-181. doi:10.1016/j.ehj.2003.10.013

[23] W. W. O'Neill, P. Serruys, M. Knudtson, G. A. van Es, G. C. Timmis, C. van der Zwaan, *et al.*, "Long-Term Treatment with a Platelet Glycoprotein-Receptor Antagonist after Percutaneous Coronary Revascularization. EXCITE Trial Investigators. Evaluation of Oral Xemilofiban in Controlling Thrombotic Events," *New England Journal of Medicine*, Vol. 342, No. 18, 2000, pp. 1316-1324.

[24] C. P. Cannon, C. H. McCabe, R. G. Wilcox, A. Langer, A. Caspi, P. Berink, *et al.*, "Oral Glycoprotein IIb/IIIa Inhibition with Orbofiban in Patients With Unstable Coronary Syndromes (OPUS-TIMI 16) Trial," *Circulation*, Vol. 102, No. 2, 2000, pp. 149-156.

[25] L. K. Newby, "Long-Term Oral Platelet Glycoprotein IIb/IIIa Receptor Antagonism with Sibrafiban after Acute Coronary Syndromes: Study Design of the Sibrafiban Versus Aspirin to Yield Maximum Protection from Ischemic Heart Events Post-Acute Coronary Syndromes (SYMPHONY) Trial," Symphony Steering Committee, *American Heart Journal*, Vol. 138, 1999, pp. 210-218. doi:10.1016/S0002-8703(99)70104-3

[26] SYMPHONY, "Comparison of Sibrafiban with Aspirin for Prevention of Cardiovascular Events after Acute Coronary Syndromes: A Randomised Trial. The SYMPHONY Investigators. Sibrafiban Versus Aspirin to Yield Maximum Protection from Ischemic Heart Events Post-acute Coronary Syndromes," Lancet, Vol. 355, 2000, pp. 337-345. doi:10.1016/S0140-6736(99)11179-6

[27] E. J. Topol, J. D. Easton, P. Amarenco, R. Califf, R. Harrington, C. Graffagnino, *et al.*, "Design of the Blockade of The Glycoprotein IIb/IIa Receptor to Avoid Vascular Occlusion (BRAVO) Trial," *American Heart Journal*, Vol. 139, 2000, pp. 927-933.

[28] V. S. Srinivas, B. Skeif, A. Negassa, J. Y. Bang, H. Shaqra and E. S. Monrad, "Effectiveness of Glycoprotein IIb/IIa Inhibitor Use during Primary Coronary Angioplasty: Results of Propensity Analysis Using the New York State Percutaneous Coronary Intervention Reporting System," *American Journal of Cardiology*, Vol. 99, 2007, pp. 482-485. doi:10.1016/j.amjcard.2006.08.061

[29] T. Heer, U. Zeymer, C. Juenger, A. K. Gitt, H. Wienbergen, R. Zahn, *et al.*, "Beneficial Effects of Abciximab in Patients with Primary Percutaneous Intervention for Acute ST Segment Elevation Myocardial Infarction in Clinical Practice," *Heart*, Vol. 92, 2006, pp. 484-489.

[30] T. Yin, G. Wang, C. Ruan, R. Guzman and R. Guidoin, "*In-Vitro* Assays of Polymer-Coated Stents Eluting Platelet Glycoprotein IIb/IIIa Receptor Monoclonal Antibody," *Journal of Biomedical Materials Research*, Vol. 83, 2007, pp. 861-867. doi:10.1002/jbm.a.31369

[31] R. K. Aggarwal, D. C. Ireland, M. A. Azrin, M. D. Ezekowitz, D. P. de Bono and A. H. Gershlick, "Antithrombotic Potential of Polymer-Coated Stents Eluting Platelet Glycoprotein IIb/IIIa Receptor Antibody," *Circulation*, Vol. 94, No. 12, 1996, pp. 3311-3317.

