

The inhibition of platelet adhesion and activation on collagen during balloon angioplasty by collagen-binding peptidoglycans

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ABSTRACT

Collagen is a potent stimulator for platelet adhesion, activation, and thrombus formation, and provides a means for controlling blood loss due to injury, and recruiting inflammatory cells for fighting infection. Platelet activation is not desirable however, during balloon angioplasty/stent procedures in which balloon expansion inside an artery exposes collagen, initiating thrombosis, and inflammation. We have developed biomimetic polymers, termed peptidoglycans, composed of a dermatan sulfate backbone with covalently attached collagen-binding peptides. The peptidoglycan binds to collagen, effectively masking it from platelet activation. The lead peptidoglycan binds to collagen with high affinity ($K_D = 24$ nM) and inhibits platelet binding and activation on collagen in both static studies and under flow, while promoting endothelial regrowth on collagen. Application for angioplasty is demonstrated in the Ossabaw miniature pig by fast delivery to the vessel wall through a therapeutic infusion catheter with a proprietary PTFE porous balloon. The peptidoglycan is an approach for locally preventing platelet deposition and activation on collagen. It can be used during angioplasty to prevent platelet deposition on target vessels and could be used in any vessel, including those not amenable to stent deployment.

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1. Introduction

An unintended consequence of angioplasty is damage to the vessel wall at the site of balloon deployment [1]. The layer of endothelial cells covering the internal lumen of the vessel is damaged, and the underlying collagenous connective tissue is exposed. Platelets are well adapted to bind to collagen where they become activated and release or upregulate numerous vasoactive agents and factors that induce coagulation and inflammation [2,3]. The body has evolved these responses as an effective means for controlling blood loss and fighting infection following injury; however, this same collagen-initiated coagulation and inflammatory response occurring inside the artery results in thrombosis and intimal hyperplasia [4–9].

Stents and drug-eluting stents are the current standard for angioplasty, although there are limitations of these devices and a need for a more robust treatment that can be used in all arteries. For example, stent placement in peripheral arteries is limited due to the flexible nature of these vessels and the resulting stresses that can lead to stent crushing or further vessel damage [10–13]. Branched, smaller diameter arteries, or vessels that have stents in place and

require a second procedure are also difficult to address with current stent technologies. Drug-eluting balloons are one promising approach to address the limitation of stents [14,15]. These, like many of the drug-eluting stents, primarily employ the drugs Paclitaxel or Sirolimus [16], which are nonspecific and have cytotoxic or cytostatic effects. The drugs effectively prevent smooth muscle cell proliferation and intimal hyperplasia, but also prevent endothelial cells from regenerating to provide the necessary permanent cover to the underlying collagen. Consequently, underlying collagen remains exposed and this exposure has led to the problem of late-stent thrombosis, particularly in high-risk acute coronary syndrome and in diabetes patients [17–19]. Accordingly, systemic dual antiplatelet therapy is the recommended treatment; though systemic compromise of platelets can cause bleeding complications that increase mortality risk [20–22].

Current approaches for improving the outcome of angioplasty target the downstream effects of inflammation [8,18]. For example, inflammation can be attenuated by systemically depleting monocytes at the time of angioplasty, resulting in inhibition of neointimal formation [23]. This approach requires systemic depletion of monocytes at the time of the invasive angioplasty procedure however, which could potentially carry new risks. Focusing on the initiating event, platelets are responsible for recruiting inflammatory cells to the balloon injured site through expression of p-selectin. The importance of platelets in thrombosis is well known, but less

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appreciated is their role in inflammation, which is fundamental for preventing intimal hyperplasia [23,24]. In a knockout mouse study, inhibition of neointimal formation could be achieved in the absence of platelet p-selectin, not endothelial p-selectin, thus demonstrating the importance of platelets in the adverse inflammation response at the vessel wall [25].

We have targeted the initial platelet binding event on balloon injured vessels by locally masking exposed collagen in the vessel wall following angioplasty. We have developed bioinspired materials termed peptidoglycans, which are composed of a polysaccharide backbone with covalently attached collagen-binding peptides [26,27]. The peptidoglycan binds to collagen through the physical peptide–collagen interactions, and when bound to collagen the peptidoglycan acts as a physical barrier to platelet attachment and subsequent activation as shown in Fig. 1. The peptide sequence is derived from a platelet receptor to collagen, and as such the peptidoglycan directly competes for platelet binding while masking the collagen surface with a hydrophilic barrier [28,29]. Here we demonstrate *in vitro* efficacy of the peptidoglycan, and a clinically relevant method for delivery during angioplasty.

2. Methods

2.1. Reagents

Peptide RRANAALKAGELYKSILYGC (SILY) and a biotin labeled version of the peptide (SILY_{biotin}) were purchased from Genscript (Piscataway, NJ). Dermatan sulfate (DS, MW_{avg} = 46275 Da) was purchased from Celsus Laboratories (Cincinnati, OH). Sodium meta-periodate and crosslinker BMPH were purchased from Thermo Fisher Scientific (Waltham, MA). Fibrillar equine collagen was purchased from Chronolog (Havertown, PA). Local infusion catheters with a proprietary PTFE porous delivery balloons (ClearWay™ Rx) were kindly provided by Atrium Medical (Hudson, NH). All other reagents and supplies were purchased from VWR (West Chester, PA) unless otherwise stated.

2.2. Peptidoglycan synthesis

The peptidoglycan was synthesized as previously described with modifications [26]. DS was oxidized by standard periodate oxidation following manufacturers

protocol, in which the degree of oxidation was controlled by varying amounts of sodium meta-periodate. Oxidized DS was then coupled to the heterobifunctional crosslinker BMPH forming DS-BMPH. Finally, SILY was coupled to DS-BMPH through its terminal cysteine residue forming the final product DS-SILY. Purifications were performed at each step by size exclusion chromatography, and the number of attached peptides was determined by the consumption of BMPH in the second reaction step. The final product DS-SILY_n in which *n* indicates the number of attached SILY peptides was purified in ultrapure water, lyophilized and stored at –20 °C until further testing. A biotin labeled version of the peptidoglycan was also synthesized by reacting 2 mol of SILY_{biotin} per mole of DS-BMPH for 1 h, followed by addition of unlabeled SILY to complete the reaction and form DS-SILY_{n-biotin}.

2.3. Peptidoglycan binding to collagen

Fibrillar collagen was coated onto the surface of a 96-well high bind plate (Greiner, Monroe, NC) at a concentration of 50 µg/mL diluted in isotonic glucose. Plates were incubated overnight at 4 °C. Unbound collagen was removed by rinsing 3 times with 1 × PBS pH 7.4. Plates were then blocked with 1% BSA for 3 h at room temperature. For binding affinity calculation, DS-SILY_{biotin} was dissolved at varying concentrations in 1 × PBS pH 7.4 containing 1% BSA and was immediately added to the collagen surfaces, and allowed to incubate for 15 min at room temperature. Plates were then rinsed 3 times with 1 × PBS pH 7.4 containing 1% BSA. DS-SILY_{n-biotin} was detected using streptavidin-HRP (R&D Systems, Minneapolis, MN). The binding affinity was determined by fitting the saturation binding curve and calculating the inflection point. The peptidoglycan DS-SILY₁₈ which bound with highest affinity while maintaining solubility was then used for all successive experiments.

To determine the diffusion of collagen-bound peptidoglycan over time, peptidoglycan DS-SILY_{18-biotin} was incubated at a concentration of 10 µM as in affinity studies. Plates were incubated at 37 °C with orbital rotation at 300 rpm and wells were rinsed 3 times daily throughout the experiment. Rinsing conditions were 200 µL/well of 1 × PBS pH 7.4 repeated 3 times at each time point. The amount of bound DS-SILY_{18-biotin} was detected by the same methods for affinity testing at varying time points up to 11 days.

2.4. Ex-vivo visualization of bound peptidoglycan

Carotid arteries were harvested from Yorkshire pigs immediately after necropsy from separate studies. Arteries were immediately cut open and rinsed with 1 × PBS pH 7.4. The internal lumen was then gently denuded of the endothelial layer with a rubber policeman, rinsed with 1 × PBS and cut into 4 mm² segments and placed into a 96-well plate. Denuded artery segments were incubated for 15 min with 100 µL of 10 µM peptidoglycan DS-SILY_{18-biotin} at room temperature followed by extensive rinsing with 1 × PBS pH 7.4. Control arteries were treated identically but were not denuded. Arteries were then snap frozen in liquid nitrogen, cut into 7 µm

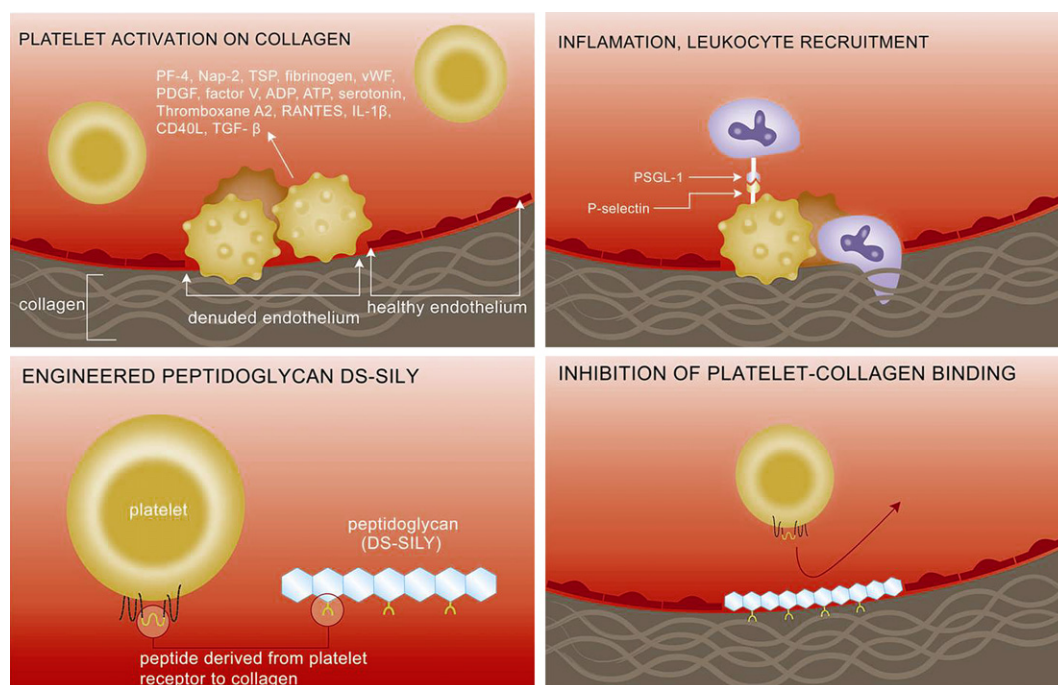


Fig. 1. Masking of collagen by bioinspired peptidoglycan. Platelet binding and activation on denuded arteries initiates coagulation and inflammation. We have developed a bioinspired mask, termed peptidoglycan, which inhibits platelet binding and activation on exposed collagen in denuded arteries.

sections and air dried for 45 min, then stored at -20°C until staining. Tissue was fixed in ice cold acetone, air dried and washed with DI water. Sections were then incubated with streptavidin-HRP for 30 min, washed with DI water, incubated with DAB for 10 min, rinsed and stained with hematoxylin for 5 min. Typical brightfield images were taken at $10\times$. A denuded artery which was incubated with only $1\times$ PBS was used as a control to ensure no nonspecific tissue staining.

2.5. Inhibition of platelet binding and activation

Microplates were coated with fibrillar collagen as described for the binding affinity assays without blocking with BSA. The peptidoglycan was diluted in $1\times$ PBS at concentrations between 0.1 nM and $50\text{ }\mu\text{M}$ and $50\text{ }\mu\text{L}$ solution was added to the collagen coated wells. Controls included DS, peptide, or $1\times$ PBS ($n = 3$ for all treatments and concentrations). Treatments were incubated at 37°C with shaking at 200 rpm for 15 min. Wells were then rinsed of unbound treatment by removing the treatment solution, adding PBS, and shaking the wells for 24 h. During the 24 h, PBS solution was changed 3 times.

Human whole blood was collected from healthy volunteers by venipuncture following the approved Purdue IRB protocol and with informed consent. The first 5 mL of blood was discarded and approximately 15 mL was then collected into citrated glass vacutainers (BD Bioscience). Blood was centrifuged in the glass tube for 20 min at 200g at 25°C . The top layer of the centrifuged blood, the platelet rich plasma, was used for platelet experiments.

Platelet rich plasma ($50\text{ }\mu\text{L/well}$) was added to the microplate for 1 h at room temperature without shaking. After 1 h of incubation, $45\text{ }\mu\text{L}$ of platelet rich plasma was removed from each well and added to a microcentrifuge tube containing $5\text{ }\mu\text{L}$ ETP (107 mM EDTA, 12 mM theophylline, and 2.8 mM prostaglandin E_1) to inhibit further platelet activation [30]. These tubes were spun at 4°C for 30 min at 2000g to pellet the platelets. The supernatant was collected for ELISA studies to test for the presence of platelet activation markers. Sandwich ELISAs were utilized in order to detect each protein. The components for both sandwich ELISAs were purchased from R&D Systems and the provided protocols were followed. It was necessary to dilute the platelet serum 10,000 times in 1% BSA in $1\times$ PBS in order for values to fall within a linear range. Platelet activation was measured through release of platelet factor 4 (PF-4) and β -thromboglobulin (NAP-2).

2.6. Inhibition of whole blood binding to collagen under flow

Flow kits were obtained from Ibidi (Martinsried, Germany). Each channel was coated with fibrillar collagen as described for static microplate studies. Excess collagen was removed from the flow channel by extensive rinsing with $1\times$ PBS through the channel. DS-SILY was incubated in the channel at a concentration of $50\text{ }\mu\text{M}$ for 15 min at 37°C , and unbound peptidoglycan was rinsed with $1\times$ PBS. Control channels consisted of collagen not treated with peptidoglycan.

Whole blood was pushed through the flow channels by a syringe pump at a flow rate of 5.6 mL/h , corresponding to a physiologically relevant shear rate of 1000 s^{-1} [31]. After 5 min of flow, $1\times$ PBS pH 7.4 was pushed through at the same flow rate for 10 min to wash unbound cells. Brightfield images ($n = 3$) were taken of each flow channel with a $10\times$ objective. Images were thresholded and quantified for cellular coverage using ImageJ (NIH, Bethesda, MD) and MatLab (Mathworks, Natick, MA) respectively. Control channels were assumed to have complete cellular coverage.

2.7. Endothelial cell migration

Fibrillar collagen was coated in wells of 96-well Oris Cell Migration Kit (Platypus Technologies, Madison, WI). Stoppers were inserted into the plate to block an inner circular portion of the cell. Human coronary artery endothelial cells (ECs) (Lonza, Walkersville, MD) were seeded at 5×10^3 cells/well and grown to confluence in the outer portion of the well and stained with cell tracker green (Invitrogen, Carlsbad, CA). The stoppers of the wells were removed, and DS-SILY₁₈ solubilized in $1\times$ PBS was incubated on the exposed collagen surface in the inner portion of the well for 15 min at 37°C . Unbound DS-SILY was rinsed from the surface and cell media was returned to the wells. ECs were allowed to migrate from the outer to the inner portion of the wells for 48 h. Fluorescence measurements of the center of each well were measured using a mask provided with the migration kit so that only the treated inner circular portion of the well was measured.

2.8. Ossabaw miniature pig studies

A study was performed on 3 lean Ossabaw miniature pigs to determine the optimal delivery method and peptidoglycan concentration. Ossabaw pigs underwent angioplasty procedures following approved protocols at the Indiana University School of Medicine similar to previous methods described in detail [32–34]. Each animal received preanesthesia with intramuscular injections of 120 mg/kg xylazine (2.2 mg/kg) and telazol (6.6 mg/kg). Following intubation, isoflurane ($2\text{--}4\%$, with oxygen) was administered to maintain stable systemic hemodynamics and a stable level of anesthesia. Under sterile conditions, the right femoral artery was exposed with surgical cut-down technique and an 8F vascular introducer sheath was inserted into the femoral artery followed by administration of heparin (200 Units/kg). An 8F

Amplatz left, or other appropriate guiding catheter (Cordis Corp., Miami, FL) was inserted through the sheath and advanced near the target site.

Arteries (renal and femoral) were intentionally denuded by expansion of an angioplasty balloon to the lumen size without breaking the elastic lamina. While expanded, the balloon was gently pulled back and forth to ensure denudation of the vessel wall. Denudation was verified in separate experiments by the complete loss of endothelium-dependent relaxation of arterial rings *in vitro* (data not shown). Immediately following denudation, treatment was administered to the vessel by therapeutic infusion using a commercially available ClearWay porous PTFE balloon catheter (Atrium Medical, Hudson, NH) sized to the vessel diameter. Treatments included either 2 or 10 mL of peptidoglycan dissolved in $1\times$ PBS pH 7.4. Two concentrations of $10\text{ }\mu\text{M}$ ($n = 3$) and $1.0\text{ }\mu\text{M}$ ($n = 3$) were tested at 2 mL and 10 mL delivered volumes respectively. Saline was delivered as a sham control ($n = 7$). Angiograms with contrast dye were recorded before and after each treatment to monitor balloon positioning and vessel diameters. Three measurements of vessel diameters were recorded pre- and post-injury and the percent constriction was quantified using ImageJ software (NIH, Bethesda, MD). Post-injury measurements were recorded between 5 and 15 min after treatment in order to capture vasospasm.

2.9. Statistics

All experiments were carried out in triplicate unless otherwise specifically stated. Where appropriate, results are presented as mean \pm S.D unless otherwise noted. Statistical analysis was performed by ANOVA using DesignExpert software (StatEase, Minneapolis, MN) and significance determined with $\alpha = 0.05$.

3. Results

3.1. Peptidoglycan DS-SILY characterization

Peptidoglycans DS-SILY₄-biotin and DS-SILY₁₈-biotin were incubated on collagen surfaces at varying concentrations to determine binding affinity to collagen. In Fig. 2 DS-SILY₄-biotin and DS-SILY₁₈-biotin bind to fibrillar collagen with a K_D of 118 nM and 24 nM respectively. By increasing the number of peptides per DS backbone, it is also apparent that more molecules are able to bind to the collagen surface, which is noted by the increased absorbance of DS-SILY₁₈-biotin which does not contain more biotin label than DS-SILY₄-biotin.

Diffusion of bound DS-SILY₁₈-biotin from a collagen surface was measured over 11 days with extensive rinsing and was found to reach equilibrium after 48 h and remain bound at equilibrium levels through 11 days as shown in Fig. 3.

Binding of DS-SILY₁₈-biotin to denuded arteries was measured *ex-vivo* and is depicted in Fig. 4. As shown, DS-SILY₁₈-biotin binds

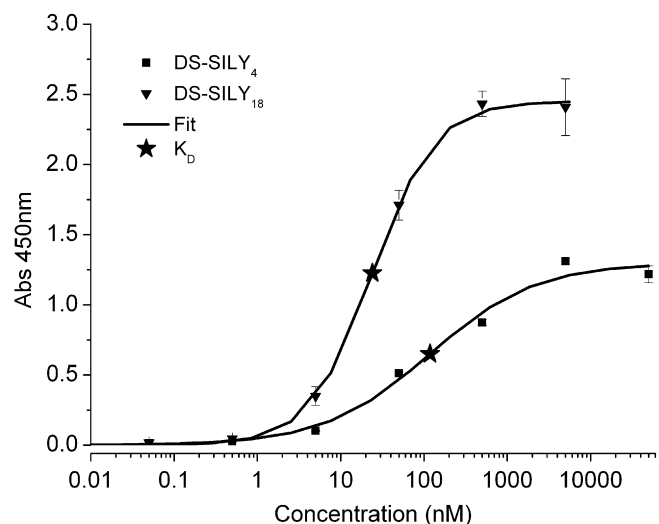


Fig. 2. Peptidoglycan binding affinity. Biotin labeled peptidoglycans DS-SILY₄ and DS-SILY₁₈ were synthesized and incubated on a fibrillar collagen surface. After washing, the bound peptidoglycan was detected and saturation binding curves were fitted to calculate the binding affinities. DS-SILY₄ and DS-SILY₁₈ bind to collagen with $K_D = 118\text{ nM}$ and 24 nM respectively, note DS-SILY₁₈ does not contain more biotin labeled peptides than DS-SILY₄.

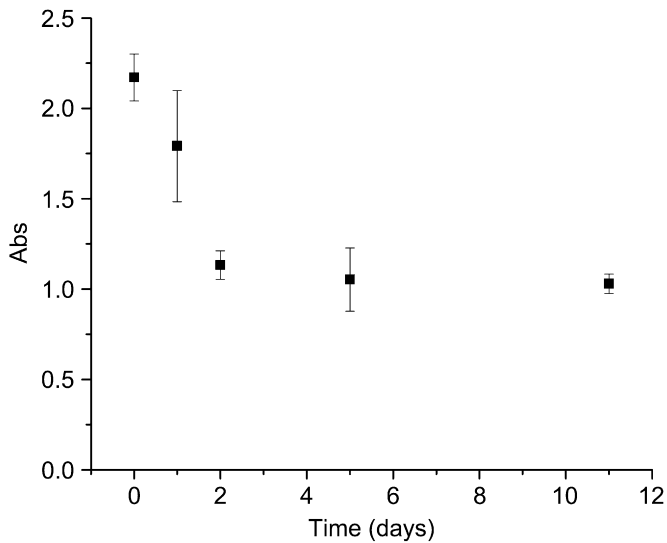


Fig. 3. Diffusion of DS-SILY₁₈ from a fibrillar collagen surface. After 48 h, the amount of collagen-bound peptidoglycan remains constant up to 11 days (the longest time point tested).

specifically to collagen at the surface of denuded arteries and does not diffuse deep into the tissue. It also does not bind to the endothelium of intact arteries.

3.2. Inhibition of collagen-induced platelet binding and activation

Inhibition of collagen-induced platelet activation was measured by the release of platelet activation factors PF-4 and β -thromboglobulin (Nap-2) in static studies. As shown in Fig. 5, platelet

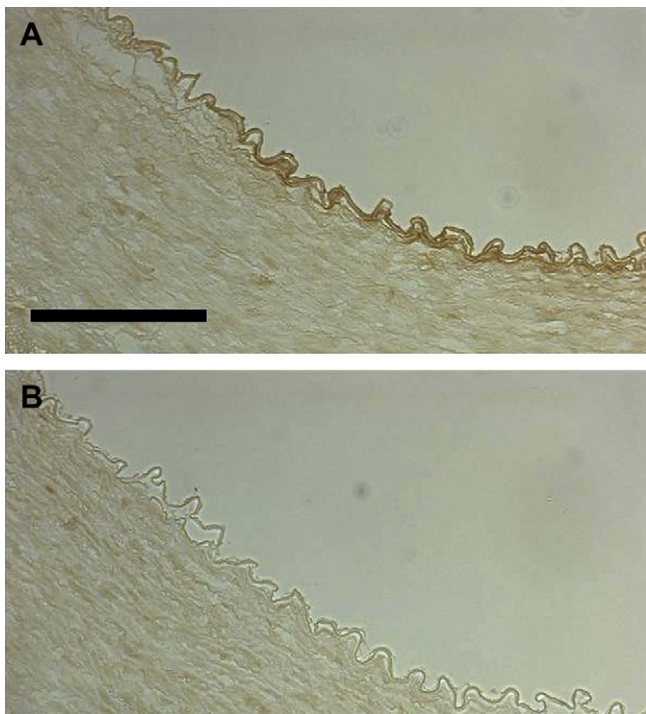


Fig. 4. Ex-vivo binding of peptidoglycan to denuded artery. Peptidoglycan DS-SILY₁₈ was incubated on a denuded pig carotid artery in (A) and on intact control artery (B). The dark brown staining along the luminal surface of the denuded vessel and absence of staining in the uninjured vessel shows specific binding to the underlying collagenous layer and not to the intact endothelium. Scale bar = 250 μ m.

activation was inhibited with increasing concentrations of DS-SILY and near maximal inhibition was achieved at 10 μ M concentrations where more than 80% and 60% decrease in PF-4 and Nap-2 levels were achieved respectively. Whole blood under flow conditions was tested for inhibition of platelet adhesion and thrombus formation, and representative images are compared in Fig. 6. Treated surfaces showed 89% fewer bound cells compared to untreated surfaces. This result was consistent with flow studies performed on PRP in which fewer platelets bound to peptidoglycan treated surfaces (data not shown).

3.3. Endothelial cell migration

Migration of endothelial cells on collagen treated surfaces was evaluated at varying peptidoglycan DS-SILY₁₈ concentrations. As shown in Fig. 7, at higher concentrations of 3.5 μ M and 35 μ M, DS-SILY₁₈ increased endothelial cell migration relative to untreated collagen surfaces.

3.4. In vivo delivery and evaluation of platelet inhibition in Ossabaw miniature pig

Three Ossabaw miniature pigs underwent angioplasty procedures and the peptidoglycan DS-SILY₁₈ was delivered locally to endothelium-denuded arteries through a porous PTFE balloon catheter. Low volume (2 mL) treatment could be delivered within seconds, whereas 10 mL volumes required approximately 1 min. Vessel constriction due to vasospasm, which results from platelet activation on denuded endothelium and release of vasoactive agents [3,35], was quantified and is depicted in Fig. 8. A representative image of DS-SILY₁₈ treated and sham control vessels taken within 15 min post-injury/treatment is shown. It is apparent that sham control vessels responded to balloon injury with severe vasospasm. The peptidoglycan DS-SILY treatment significantly inhibited vasospasm as measured by vessel constriction when delivered in a 2 mL volume at 10 μ M (2% constricted) compared to both sham (66%) and lower concentration delivery 0.1 mg/mL (43%, $p < 0.001$). When DS-SILY was delivered in a volume of 10 mL at a lower concentration of 1 μ M, vasospasm was significantly inhibited

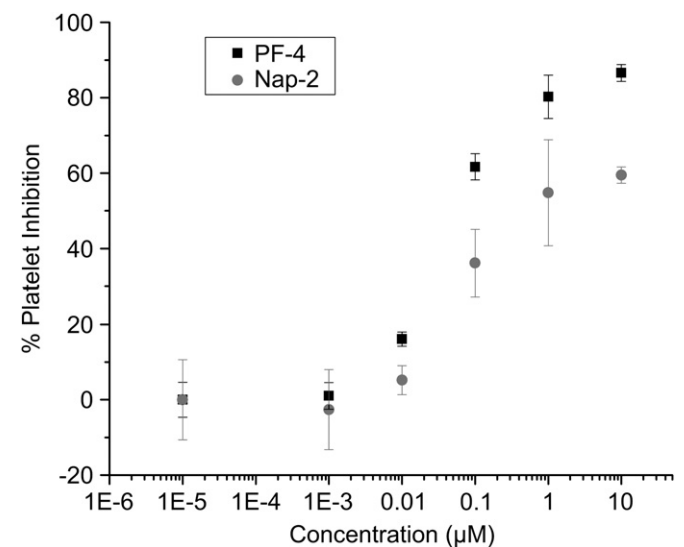


Fig. 5. Inhibition of collagen-induced platelet activation. Human platelets were activated on fibrillar collagen surfaces and release of activation factors PF-4 and Nap-2 was measured. Inhibition of platelet activation was achieved with increasing concentration of peptidoglycan DS-SILY, with near maximal inhibition achieved by 10 μ M concentrations.

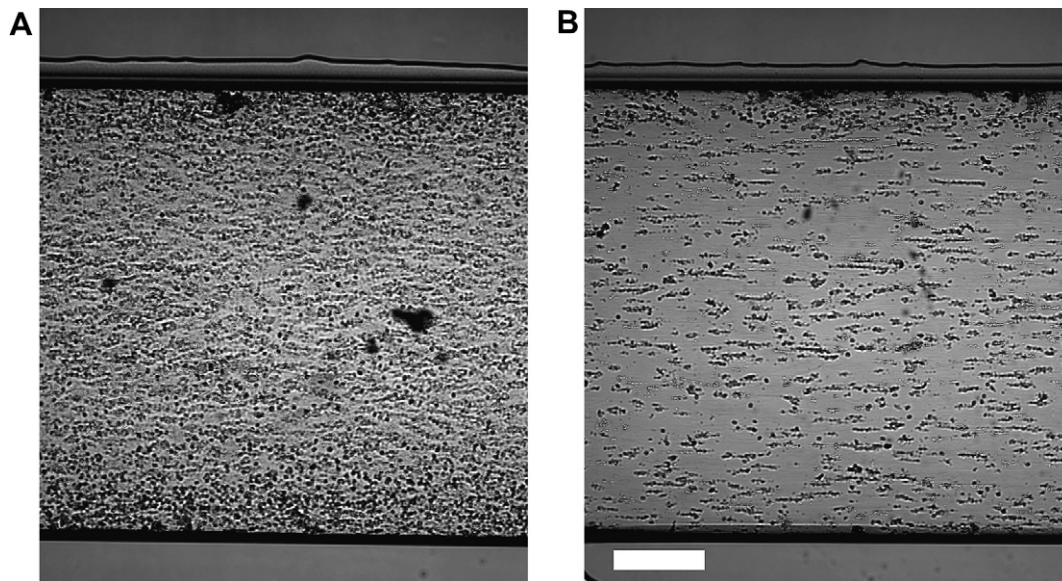


Fig. 6. Whole blood binding to collagen under flow. Human whole blood was tested under flow at a shear rate of 1000 s^{-1} for cellular binding on collagen surfaces. Representative brightfield images show untreated (A) collagen surfaces with significantly more bound cells than DS-SILY treated (B) surfaces. Scale bar = $200 \mu\text{m}$. Bound cells were quantified, showing 89% inhibition on the treated surface.

($p < 0.05$), but the degree of inhibition was diminished compared to the higher concentration at a lower volume.

4. Discussion

An unintended adverse consequence of angioplasty procedures is injury to the vessel wall during balloon expansion, in which the endothelial cell layer is damaged and underlying collagen is exposed [1]. Platelet adhesion and activation on collagen initiates coagulation and inflammation at the balloon injured site, which leads to thrombosis and intimal hyperplasia [2,4,5]. We demonstrate here a method for preventing platelet adhesion and activation on exposed collagen by locally masking the collagen with a bioinspired material, termed collagen-binding peptidoglycan, and the specific

peptidoglycan used here is DS-SILY₁₈. A major strength of masking of collagen and prevention of platelet activation is that it addresses the initiating platelet event, rather than downstream effects, thus providing broad spectrum protection from several diverse vasoactive agents, including nucleotides (e.g. ATP, ADP, UTP), thrombin, platelet-activating factor, serotonin, and thromboxane A₂, and the recruitment of inflammatory cells through p-selectin expression on platelets.

We have previously reported the design and synthesis of collagen-binding peptidoglycans for use in tissue engineering applications as they mimic the native proteoglycans found in collagenous tissues [26,27]. Here, we have engineered our previously designed peptidoglycan molecules for application in balloon angioplasty procedures with the following design criteria: 1. Binds to collagen with high affinity and does not readily detach due to diffusion, 2. Inhibits collagen-induced platelet adhesion and activation, 3. Does not inhibit endothelial cell regeneration, and 4. Can be rapidly delivered to vessels during angioplasty.

The peptidoglycan acts by masking collagen such that platelets do not have access to binding and activation sites along the collagen fibrils. There are several modes of platelet adhesion to collagen including receptors glycoproteins VI and IV, integrin $\alpha_2\beta_1$, collagen specific protein receptor, and through von Willebrand Factor interactions [36]. In the present work, the peptidoglycan was designed to bind to type I collagen through the protein receptor derived type I collagen-binding peptide SILY [29]. The peptidoglycan backbone is composed of highly negatively charged DS, which attracts water and can act as a nonspecific barrier, excluding platelet adhesion through other collagen receptors. Design control of the peptidoglycan allows for modification both in the number of attached peptides as well as the peptide identity, thus allowing for binding to other collagens present in target tissues; however, in the present study only type I collagen was targeted.

Taking advantage of peptidoglycan design control, by increasing the number of attached SILY peptides on the DS backbone, both the binding affinity to collagen and the total amount bound to collagen increase with a higher number of peptides as seen when comparing DS-SILY₄ to DS-SILY₁₈. This result was expected, since more peptides per DS molecule increase the number of potential sites for

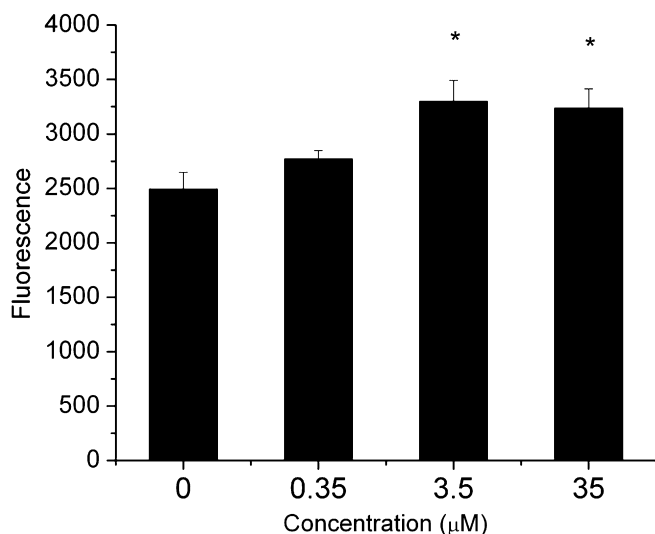


Fig. 7. Endothelial migration on treated collagen surfaces. At higher peptidoglycan concentrations there was a significant increase in endothelial cell migration indicating that the peptidoglycan promotes endothelial cell migration. *indicates significance, $p < 0.05$, $n = 6$.

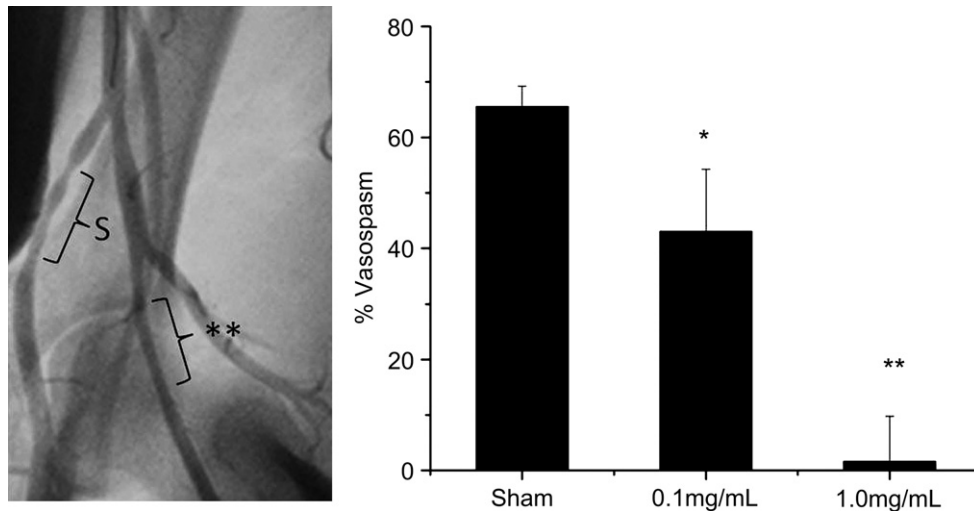


Fig. 8. *In vivo* delivery of peptidoglycan during angioplasty. Vasospasm, resulting from platelet deposition, was evaluated by measuring the % vessel constriction from angiograms collected following balloon injury and treatment. Peptidoglycan treatment significantly inhibited vasospasm at both concentrations evaluated, with increased inhibition at higher concentration. In the image showing two separately treated femoral arteries, S indicates sham control, while ** indicates treatment at 1.0 mg/mL. Sham control was treated first, and partial recovery of vasospasm is observed. Quantification of vessel constriction was performed from angiograms taken between 5 and 15 min of treatment. Data are presented as average \pm S.E., * indicates significance compared to sham ($p < 0.05$), ** indicates significance compared to sham and 0.1 mg/mL ($p < 0.001$).

binding to collagen; thus, the avidity of the peptidoglycan to collagen is increased. While maintaining solubility and maximizing collagen coverage, DS-SILY₁₈ was the lead composition and thus used for all other experiments.

Shown in pig arteries, the peptidoglycan binds specifically to underlying extracellular matrix and does not bind to the intact endothelium. Since the peptide sequence is derived from a platelet receptor to collagen, its specificity to collagen was expected [29].

Inhibition of platelet adhesion and activation on collagen was evaluated by visualizing platelets bound to fibrillar collagen surfaces, and by the release of activation factors platelet factor 4 (PF-4) and β -thromboglobulin (Nap-2). Significant inhibition of platelet activation, up to over 80%, was observed in static studies. In these studies platelet arrest from flow via interaction with von Willebrand Factor is not necessary, therefore increased inhibition was expected under flow conditions [37]. Whole blood experiment performed under physiologically relevant flow conditions showed 89% inhibition of cell binding on peptidoglycan treated collagen surfaces suggesting that the peptidoglycan acts not only as a specific mask to the platelet protein receptor to collagen from which the peptide SILY was derived, but also as a nonspecific inhibitor of molecular and cell receptor binding.

In a damaged artery with flowing blood, the peptidoglycan is susceptible to diffusion from collagen fibrils since its association with collagen is through physical peptide–collagen interactions. It is important for the peptidoglycan to remain bound to exposed collagen until sufficient endothelial regeneration occurs, providing a permanent cover to underlying collagen. Post balloon injury, complete endothelial regeneration can be achieved by 1 week depending on the size of the ballooned area, while the most significant platelet binding occurs during the first 24 h after injury [1]. By covalently attaching 18 collagen-binding peptides per DS backbone, the avidity of the peptidoglycan to collagen is increased and the peptidoglycan does not readily detach from collagen. For example, the peptide binds to collagen thermodynamically and the peptide alone will diffuse from collagen with extensive rinsing. The peptidoglycan on the other hand contains on average 18 peptides and it can be envisioned that if half of the peptides are not bound at any given time, there are 9 other peptides keeping the molecule bound to collagen. Since several peptides remain bound at any given time, it is

unlikely that the peptidoglycan will easily diffuse away from the surface.

Such high avidity of the peptidoglycan is in fact observed where after 48 h of extensive rinsing *in vitro*, approximately half of the bound peptidoglycan remains on the surface, and is maintained at this level up to 11 days. Platelet inhibition was therefore tested after 48 h rinsing, and the degree of inhibition was found to be equivalent to both a 15 min and 48 h rinse (data not shown), thus indicating efficacy over long periods of time.

Endothelial regeneration is essential for restoring the healthy vessel lumen and providing a permanent cover to the exposed collagen. We therefore tested endothelial migration onto collagen surfaces with peptidoglycan treatment. The peptidoglycan accelerated endothelial migration, thus indicating that it could promote more rapid endothelial growth and healing of the vessel wall. This effect may be attributed to the DS backbone of the peptidoglycan, which activates fibroblast growth factor-2 (FGF-2) [38,39]. Bound peptidoglycan may sequester FGF-2 which is known to stimulate endothelial cell proliferation [40,41]. While the peptidoglycan has high avidity to collagen, a migrating endothelial cell, already well anchored in proximity to the collagen and peptidoglycan, would have numerous attachment sites on collagen, thus even higher collagen avidity, and likely competes off the peptidoglycan. The mechanism of endothelial regeneration and whether these cells are competing off peptidoglycan as they grow over treated surfaces is under further investigation.

In vivo proof-of-principle was evaluated in Ossabaw miniature pigs in an effort to demonstrate a clinically relevant delivery method. The peptidoglycan was delivered to balloon injured vessels through a commercially available ClearWay PTFE porous balloon catheter [42]. Delivery of small volume allows for fast delivery to the vessel wall in seconds. Efficacy of acute inhibition of platelet adhesion and activation on exposed collagen was evaluated by quantifying the degree of vasospasm post balloon injury. Vasospasm can occur due to a number of factors relating to endothelial function, and in controlled experiments vasospasm is well documented in pigs as a direct consequence from platelet adhesion, activation, and release of vasoactive agents as a result of endothelial denudation after balloon expansion during angioplasty [3,35,43]. The degree of vessel constriction is a function of

the amount of platelet deposition, and vasospasm was thus used as a metric for quantifying the efficacy of the peptidoglycan treatment [35]. The vessel walls were intentionally denuded without over-expanding the vessel and causing the elastic lamina to break, and therefore the entire ballooned area experienced vasospasm in sham control vessels. Optimal delivery with inhibited vasospasm was achieved at high peptidoglycan concentration in low volume indicating efficacy of inhibited platelet deposition on the denuded artery. The lower concentration of peptidoglycan showed inhibited vasospasm but to a lesser degree, indicating an optimal delivered concentration of at least 10 μM . These results are supported by *in vitro* static studies, in which maximal platelet inhibition is achieved at 10 μM , and inhibition is diminished at lower concentrations. These findings demonstrate a clinically relevant delivery method and indicate efficacy of inhibited platelet activation on the ballooned vessel wall.

The animal studies focused on determining an effective delivery method and providing a guideline for future work, which will further explore the impact of local platelet inhibition on vessel healing including endothelial regeneration, degree of inflammation, and intimal hyperplasia. In addition, while the peptidoglycan is naturally derived and has been shown to be biocompatible in cell culture, any adverse vessel healing as a result of collagen masking should be investigated [27]. There are limitations in healthy animal models, particularly since the composition of healthy and atherosclerotic vessels differs [44]. Collagen is present in both healthy and atherosclerotic vessels however, thus we expect the peptidoglycan to be effective in preventing platelet deposition and activation on balloon treated vessels in both healthy and atherosclerotic models.

5. Conclusions

We demonstrate here a bioinspired material that can inhibit platelet binding and activation on collagen, and a clinically relevant application for use in angioplasty. This is a robust approach for improving angioplasty, specifically targeting the initial platelet response rather than downstream events. The peptidoglycan can be used in any vessel, and could be adopted for use in drug-eluting balloons currently being developed, thus addressing pressing needs in both coronary and peripheral artery disease where stents are limited [14,15].

Disclosure

The technology has been licensed by AMIPurdue. Authors John Paderi, Kate Stuart, Kinam Park, and Alyssa Panitch are inventors on 2 patents for the technology.

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Appendix

Figures with essential colour discrimination. Certain figures in this article, particularly Figs. 1 and 4, are difficult to interpret in black and white. The full colour images can be found in the online version, at doi:10.1016/j.biomaterials.2010.12.025.

Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.biomaterials.2010.12.025.

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