The immobilization of recombinant human tropoelastin on metals using a plasma-activated coating to improve the biocompatibility of coronary stents

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1. Introduction

In the last two decades stents have become the dominant metallic vascular implant, with 90–95% of percutaneous coronary interventions (PCI) involving the deployment of a stent [1]. Bare metal stents (BMS) not only suffer restenosis (vessel re-narrowing due to smooth muscle cell hyperproliferation), they are inherently thrombogenic demonstrating occlusion rates up to 24% in the absence of pharmacological intervention [2]. Drug eluting stents (DES) releasing anti-proliferative agents from polymer platforms coated onto metallic scaffolds. These drugs have been shown to delay healing and re-endothelialization [6], and polymer instability has been linked with inflammatory and hypersensitivity reactions [7] exacerbating their inherent bio-incompatibility. Additionally, stent polymer coatings need to withstand the process of crimping and high-pressure expansion during implantation and subsequently pulsatile flow. The plastic deformation of the metal that occurs upon stent manufacture and deployment causes cracking and peeling of the polymer. This is due to the mismatch in mechanical properties between the metal and the coating polymer and the poor adhesion between the layers. Current polymer-coated DES display surface cracking, peeling and flaking [8], exposing the underlying thrombogenic metallic substrate. Hence, all current polymer-coated metallic vascular implants have inherently sub-optimal biocompatibility, which impairs their clinical performance.

Current endovascular stents have sub-optimal biocompatibility reducing their clinical efficacy. We previously demonstrated a plasma-activated coating (PAC) that covalently bound recombinant human tropoelastin (TE), a major regulator of vascular cells in vivo, to enhance endothelial cell interactions. We sought to develop this coating to enhance its mechanical properties and hemocompatibility for application onto coronary stents. The plasma vapor composition was altered by incorporating argon, nitrogen, hydrogen or oxygen to modulate coating properties. Coatings were characterized for 1) surface properties, 2) mechanical durability, 3) covalent protein binding, 4) endothelial cell interactions and 5) thrombogenicity. The N2/Ar PAC had optimal mechanical properties and did not delaminate after stent expansion. The N2/Ar PAC was mildly hydrophilic and covalently bound the highest proportion of TE, which enhanced endothelial cell proliferation. Acute thrombogenicity was assessed in a modified Chandler loop using human blood. Strikingly, the N2/Ar PAC alone reduced thrombus weight by ten-fold compared to 316L SS, a finding unaltered with immobilized TE. Serum soluble P-selectin was reduced on N2/Ar PAC finding unaltered with immobilized TE. Serum soluble P-selectin was reduced on N2/Ar PAC and N2/Ar PAC + TE (p < 0.05), consistent with reduced platelet activation. We have demonstrated a coating for metal alloys with multifaceted biocompatibility that resists delamination and is non-thrombogenic, with implications for improving coronary stent efficacy.

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Achieving biocompatibility has traditionally been limited to the development of inert materials eliciting no adverse host response. While this is appropriate in some instances, certain applications like stents, could benefit from biomimetic material-facilitated physiological responses [9]. Attempts to improve the biocompatibility of stents in this way have focused on improving only one aspect of vascular compatibility. For example, the heparin-coated stent reduced thrombosis, but not restenosis [10]. Stents coated with immobilized anti-CD34 antibodies aim to enhance endothelialization by capturing circulating endothelial progenitor cells, but are associated with a significantly higher rate of restenosis [11]. In contrast, we propose a multifaceted strategy to address the three major aspects of vascular biocompatibility by combining a coating that is: 1) mechanically superior to robustly adhere to the underlying metallic stent; 2) hemocompatible to reduce the thrombogenic nature of metallic alloys, and 3) able to covalently bind a bioactive molecule to enhance endothelialization and reduce restenosis.

There are few molecules that achieve the desired physiological responses of increasing endothelialization and reducing restenosis, whilst having low thrombogenicity. A potential candidate is elastin, an important vascular matrix protein. It has been shown to interact favorably with endothelial cells [12], inhibit smooth muscle cell proliferation [13] and displays low thrombogenicity [14]. The use of elastin has been limited by its extreme insolubility. However, an Escherichia coli expression system allows us to produce recombinant human tropoelastin [15], the monomer of elastin.

Covalent immobilization of proteins in their bioactive form on metal surfaces has been a challenge in the past. We recently developed a plasma deposition technology based on plasma enhanced chemical vapor deposition for producing organic interlayers capable of linker-free covalent immobilization of biomolecules [16–18]. This allows surface modification of metallic alloys with a plasma-activated coating (PAC) that is smooth and strongly adhered to the underlying metal by energetic ion stitching deposition. The coating is wear resistant under pulsatile flow and is able to covalently bind proteins in their bioactive conformation [16–18]. In a proof of principle study, we showed that binding tropoelastin (TE) to PAC enhances endothelial cell attachment and proliferation [19]. However these studies did not address the issues of hemocompatibility, mechanical stability and delamination, which are essential if this technology is to be applied as a viable stent coating.

In this work we have expanded on these initial findings and specifically sought to demonstrate a plasma-activated coating for metals, with 1) an interface engineered to withstand the plastic deformation of stent expansion and eliminate coating delamination, and 2) a surface optimized for hemocompatibility to reduce stent thrombosis. This work demonstrates a robust, proactive, non-thrombogenic plasma coating technology for surface modification of intravascular stents with implications for improved clinical performance of these devices.

2. Methods

2.1. Synthesis of plasma-activated coating

The pulsed plasma deposition system has been previously described [17,18]. The substrates were 316L stainless steel foil (SS) 25 μm thick (Brown Metals); silicon (p-type (100) double side polished) or 3.0 x 10 mm 316LVM stainless steel stents (Laserage, CA, USA). Plasma-activated coatings on 316L stainless steel or stents were generated from acetylene in argon alone (Ar), nitrogen alone (N2), argon mixed with nitrogen (N2/Ar), hydrogen (H2/Ar) or oxygen (O2/Ar).

2.2. Surface analysis

Photoelectron spectroscopy (XPS), (SPECS-XPS, Germany) was used to analyze the elemental compositions of the PAC surfaces. The intrinsic stress was analyzed using a Tencor P-11 profilometer by determining the curvature change before and after deposition of the PAC on a silicon substrate. The refractive index of the PAC layers was determined using spectroscopic ellipsometry (J.A. Woollam M-2000). Surface contact angle measurements were performed at 23 ± 1 °C using a Kruss DSA10-MB2 contact angle analyzer. Sessile water drops of 10 μl were used for advancing contact angle. Recombinant human tropoelastin was expressed and purified as previously described [15]. Metal samples were incubated overnight at 4 °C with 30 μg/ml tropoelastin. SDS treatment and ELISA for characterization of the bound tropoelastin were carried out as previously described [19].

2.3. Cell culture

Human umbilical vein endothelial cells (HUVECs) were harvested enzymatically from umbilical cords as previously described [20]. Cell assays were performed in triplicate in 24-well plates containing either SS, PAC or PAC + TE, with cells between passages 2 and 4. Cell proliferation was quantified by staining with crystal violet and measurement of absorbance at 570 nm [21].

2.4. Thrombogenicity assessment

Whole blood was obtained from healthy, non-smoker, male volunteers with informed consent in accordance with the Declaration of Helsinki, who had not taken aspirin 2 weeks prior to donation. Approval for this work was granted by The University of Sydney, Human Research Ethics Committee (protocol 05-2009/11668). Experiments were conducted 3–4 times with different donor’s blood.

2.5. Whole blood adhesion

Samples of SS, PAC or PAC + TE were incubated with heparinized whole blood (0.5 U/ml) for up to 60 min at 37 °C whilst rocking. Samples were processed for scanning electron microscopy (SEM) as described below.

2.6. Modified Chandler loop

Thrombogenicity under flow conditions was investigated using a modified Chandler loop [22]. Samples were balloon expanded into 28 cm lengths of Tygon S-50-HL tubing (SDR, Australia), connected into loops using 1 cm silicone connectors and filled with heparinized whole blood (0.5 U/ml, 2.5 ml). The loops were rotated at 34 rpm at 37 °C for 30 min, achieving a flow rate of 85 ml/min (approximating coronary flow [23]). The thrombus and steel from each loop were removed for imaging and weighing. The blood from each loop was combined with 10% (v/v) acid citrate dextrose (ACD) and centrifuged at 1000 rpm for 15 min to obtain serum. Soluble P-selectin was detected via an ELISA (R&D Systems, USA).

2.7. Scanning electron microscopy

Samples were fixed in 2.5% (w/v) glutaraldehyde, post-fixed with 1% (v/v) osmium tetroxide, and dehydrated in ascending grades of ethanol before drying with hexamethyldisilasane. The samples were sputter coated with 20 nm gold and imaged with a Philips XL 30 CP scanning electron microscope. Stents were hand crimped onto 3 mm diameter balloon catheters and expanded to 3 mm with 9 atm pressure air. Stents were coated with 5 nm gold prior to imaging.

2.8. Statistical analysis

Data are expressed as mean ± SE and indicated in figures as *p < 0.05, **p < 0.01 and ***p < 0.001. Groups were compared by a one-way analysis of variance (ANOVA) with post-hoc analyses for pairwise comparisons (Bonferroni post-test). Statistical significance was inferred at a 2-sided value of p < 0.05. GraphPad Prism version 4.00 (Graphpad Software, San Diego, California) for Mac was used for statistical analysis.

3. Results

3.1. Incorporation of different elements into the plasma-activated coating

Modulation of the elemental composition of the coating directly influences its mechanical and biological properties. We deposited plasma-activated coatings on 316L stainless steel (the stainless steel alloy used in commercial stents) using an acetylene precursor, incorporating various gases: argon alone (Ar), nitrogen alone (N2), argon mixed with nitrogen (N2/Ar), hydrogen (H2/Ar) or oxygen (O2/Ar). Incorporation of gases for each coating, together with some atmospheric oxygen incorporated post-deposition [17,18], was confirmed by XPS analysis of the carbon, nitrogen and oxygen content on the surface (Fig. 1).
3.2. Deposition of a mechanically stable plasma-activated coating

To test the coating integrity we measured the mechanical properties of the PACs. Given a constant strength of adhesion, the relative likelihood of coating delamination can be inferred by comparing the compressive stress. Coatings with high compressive stress values are more likely to delaminate because of the energy release associated with relieving the strain. Nitrogen containing PACs (N$_2$/N$_2$/Ar) had the lowest compressive stress of 0.14 ± 0.05 GPa, which was not significantly different to the Ar PAC (Fig. 2A). The compressive stress of the N$_2$/N$_2$/Ar and Ar PACs was significantly lower than H$_2$/Ar ($p < 0.01$) and O$_2$/Ar samples ($p < 0.001$). Dense, hard materials have high refractive indices and are also more susceptible to cracking and delamination. The Ar, H$_2$/Ar and O$_2$/Ar PACs had significantly higher refractive indices compared with the N$_2$ and N$_2$/Ar PACs ($p < 0.001$, Fig. 2B).

3.3. Plasma-activated coated stent

One of the nitrogen containing PACs was selected on the basis of its favorable mechanical properties and applied to a stent. The N$_2$/Ar PAC was deposited on a 3.0 × 10 mm laser cut 316LVM stainless steel stent. Coating stability was assessed in comparison to a 3.0 × 12 mm Taxus$^6$ Liberté stent, which is also a laser cut 316L stainless steel stent, coated with SIBS polymer (Boston Scientific). Following balloon expansion to 3 mm (9 atm), SEM of the Taxus$^6$ Liberté stent revealed substantial SIBS polymer peeling (Fig. 3A). Delamination was concentrated at the strut intersections with up to 200 μm areas of exposed stainless steel strut (Fig. 3B black arrow). Ten strut intersections were randomly imaged and peeling had occurred at each one. Extra polymer spanning between stent struts was also observed (Fig. 3A white arrow). In contrast, deployment of our N$_2$/Ar PAC stent under the same conditions displayed no peeling of the coating (Fig. 3D–F). Small cracks up to 5 μm in length and less than 500 nm in width were observed, with no exposure of the underlying stainless steel. This cracking was representative of 9 randomly imaged strut intersections. 3 PAC stents were tested.

3.4. Surface properties of plasma-activated coatings

We next measured the contact angle of the plasma-activated coatings to determine wetability, a key property that can influence biocompatibility. Surfaces that are extremely hydrophilic or hydrophobic interact poorly with proteins and cells and are thrombogenic [24]. The N$_2$/Ar, N$_2$, and O$_2$/Ar coatings were found to be mildly hydrophilic, and Ar, H$_2$/Ar more hydrophobic (Fig. 4A). The contact angle of the PACs was compared to the contact angle of currently available DES which have all been shown to be more hydrophobic (Fig. 4B) [25].

The amount of TE covalently bound to different PACs was determined using an elastin-specific ELISA. Stringent SDS washing was used to remove non-covalently bound protein [17]. 316L SS only retained 24 ± 0.3% of TE bound to its surface after SDS washing (Fig. 4C). The N$_2$/Ar, N$_2$ and Ar PACs retained 89 ± 0.1% and 85 ± 0.2% and 61 ± 1.1% of the tropoelastin, respectively ($p < 0.001$ compared to 316L SS). H$_2$/Ar retained 35 ± 6.9% of bound TE ($p < 0.05$ compared to 316L SS). The O$_2$/Ar PAC peeled off the metal during the assay and was thus excluded. To assess endothelial cell interactions, covalently immobilized tropoelastin on the N$_2$/Ar PAC was subsequently shown to enhance endothelial cell growth by 133 ± 7% compared to 316L SS and the N$_2$/Ar PAC alone ($p < 0.05$, Fig. 5).

3.5. Screening for a low thrombogenic plasma-activated coating

To assess surface thrombogenicity, all PAC samples and 316L SS were incubated with heparinized whole blood. 316L SS was the least hemocompatible, indicated by deposition of fibrin and adhesion of red blood cells and platelets (Fig. 6A arrows). The platelets were spread and bound fibrin, indicating that they were activated [26]. All the PACs showed a marked reduction of fibrin deposition.
and blood cell adhesion compared to 316L SS. N2/Ar and N2 surfaces were the least thrombogenic (Fig. 6B–F). O2/Ar samples showed the formation of buckling likely to lead to delamination, consistent with previous data (Results 3.2. and Fig. 4).

Overall characterization showed the N2/Ar PAC to have the best combination of features desirable for use as a stent coating. It was mildly hydrophilic, had the lowest compressive stress and refractive index and was free of delamination after expansion when applied to a stent. It retained the highest proportion of covalently bound tropoelastin, enhancing endothelial cell proliferation. Moreover, initial screening demonstrated it was the least thrombogenic material. Based on these properties, the N2/Ar surface was used for all further experiments.

3.6. N2/Ar PAC demonstrates reduced thrombogenicity

The thrombogenicity of the N2/Ar PAC with or without TE bound was aggressively tested in both static and flow assays, using whole blood. Temporal analysis revealed minimal blood component adhesion to N2/Ar PAC or N2/Ar PAC + TE at all time points compared to 316L SS (Fig. 7). By 10 min, red blood cells, fibrin and activated platelets were observed on 316L SS. After 30 min there

![Fig. 3. Representative SEM images of an expanded Taxus stent (A–C) and an expanded N2/Ar PAC stent (D–F). Arrows indicate the Taxus SIBS polymer (white arrow, A) and underlying stainless steel strut (black arrow, B).](image)

![Fig. 4. (A) Water contact angles of the PACs. (B) ELISA to detect the percentage of tropoelastin covalently bound to the PACs after SDS treatment, compared to the amount of tropoelastin passively bound. (C) Schematic representation of the contact angle of commercial stents compared to the PACs [21].](image)
were multiple layers of fibrin bound blood cells, which developed into a layer of thrombus by 1 h.

Acute thrombogenicity under flow conditions demonstrated formation of large thrombi in the presence of 316L SS, while N2/Ar PAC and N2/Ar PAC + TE were free of thrombus after 30 min (Fig. 8A). Thrombus weight was significantly reduced by 94 ± 0.9% and 93 ± 1.2% (*p* < 0.05) for N2/Ar PAC and N2/Ar PAC + TE respectively compared to 316L SS (Fig. 8B). Observation of thrombus formation over time (Fig. 8C) revealed that time to thrombus formation on N2/Ar PAC and N2/Ar PAC + TE was increased approximately 3-fold (95 ± 5 and 88 ± 3 min respectively) compared to 316L SS (32 ± 3 min, *p* < 0.01).

In addition, we assessed the contribution of platelet activation on thrombus formation. The level of sP-selectin rose by 65 ± 11% in the presence of 316L SS compared to the basal level in the blood with no implant (*p* < 0.001). Strikingly, the levels of sP-selectin in the blood exposed to N2/Ar PAC and N2/Ar PAC + TE samples were not significantly different to the basal level with no implant (Fig. 8D).

4. Discussion

The salient findings of this study are: 1) the demonstration of a plasma-activated coating (N2/Ar PAC) for metal alloys that resists delamination after expansion on a stent; 2) the N2/Ar PAC strikingly reduced the thrombogenicity of metal alloys in vitro; 3) the N2/Ar PAC enables a high degree of covalent binding of tropoelastin on metal surfaces and 4) the bound tropoelastin on N2/Ar PAC retains non-thrombogenic whilst simultaneously enhancing endothelial cell proliferation. These data demonstrate a biomimetic approach for improving the vascular compatibility of stent coatings.
Current endovascular stents exhibit poor biocompatibility due to the poor intrinsic properties of metals and polymer coatings, manifesting most seriously with late stent thrombosis [4]. In response, DES design has evolved to incorporate numerous modifications aimed at improving clinical outcome. These include strut thickness, polymer type, polymer thickness, stent design, drug type and drug elution kinetics. Despite incremental improvements, a significant benefit in clinical outcome remains to be seen.

Accentuating the incompatibility of DES, all DES polymers are spray- or dip-coated and delaminate after implantation, as shown recently for a range of commercially available stents [8]. Delamination exposes the underlying metal platform increasing stent thrombogenicity.

Our approach has focused on creating a stent coating to address these issues, incorporating enhanced coating adhesion to eliminate delamination, whilst employing biomimicry to enhance biocompatibility. Nitrogen incorporation into the PAC was found to be a critical modulator of mechanical properties and capacity for covalent coupling of biological molecules.

The chemical composition of the PAC was varied to influence key surface properties relevant to stent biocompatibility. The mechanical properties are an important consideration as polymers used to coat DES are susceptible to cracking and delamination [8], resulting in reduced biocompatibility. The process of crimping and expansion of a stent during implantation causes plastic deformation of the metal substrate and will inevitably introduce delamination of an overlying polymer coating with weak adhesion. Excessive compressive stress and a high refractive index make a coating more prone to delamination. Our results illustrate the typical positive correlation between compressive stress and refractive index observed in thin films. The Ar, H2/Ar, and O2/Ar surfaces all had a high compressive stress and refractive index, with the O2/Ar coating frequently found to peel off from stainless steel substrate. Conversely, the nitrogen containing surfaces had the lowest compressive stress and the lowest refractive index, making them least likely to peel from a stent surface. The presence of nitrogen in the coating is critical as it will increase the sp² hybridization and elasticity of the PAC structure [27,28].

To facilitate the future assessment of PAC in vivo, the N2/Ar PAC was applied to a custom stent platform. The deposition was graded from stainless steel to PAC creating an integrated, ion-stitched robust interface to the underlying metal [18]. In accordance with its good mechanical properties, the N2/Ar PAC demonstrated exceptional integrity after stent expansion. There was no detectable peeling and minimal cracking, with no apparent exposure of underlying metal. The manufacture process did not result in any visible areas of excess coating.

The hydrophobicity of PAC was another important consideration given that it greatly influences the interactions of proteins and blood components with the surface. Our results show that the nitrogen and oxygen PACs are more hydrophilic than current DES polymers. Mildly hydrophilic surfaces have been shown to have the most favorable interactions with cells and hydrophilic modification of stents has shown decreased platelet adhesion [24,29].
To facilitate the bio-functionalization of our PAC, the surface coating was required to covalently bind bioactive molecules. Previous attempts to functionalize stents have failed due to the poor attachment of the biomolecule to the surface. We chose to immobilize tropoelastin, the monomer of the important vascular matrix protein, elastin. Tropoelastin has been shown to interact favorably with endothelial cells [12,19] and elastin inhibits smooth muscle cell proliferation [13] and displays low thrombogenicity [14]. All the PAC formulations covalently bound tropoelastin, retaining significantly more protein than bare 316L SS. The extent of covalent attachment and thus the amount of tropoelastin retained on each of the surfaces was varied and demonstrates that this could be modulated based on the amount of protein required for a specific application. Endothelial cell growth was significantly enhanced on the N$_2$/Ar PAC + TE when directly compared to N$_2$/Ar PAC alone and 316L SS. For stent applications, rapid re-endothelialization is beneficial in the context of coronary stents as an intact endothelium is associated with reduced restenosis [6].

Given the complexity of assessing biomaterial thrombogenicity [30], an initial screen was conducted using heparinized whole blood adhesion. All PACs showed reduced thrombogenicity compared to 316L stainless steel, with nitrogen containing surfaces again showing the best results. Based on the combined results of the surface properties of the PAC variations, the N$_2$/Ar PAC was chosen for further characterization. It had optimal characteristics including mild hydrophilicity, low compressive stress and refractive index, high covalent binding of tropoelastin, and low initial thrombogenicity. Compared to bare stainless steel, our N$_2$/Ar PAC is significantly less thrombogenic. This was demonstrated in an extended whole blood adhesion assay over the course of an hour, during which the 316L SS had developed a surface thrombus layer, whilst no such thrombus was present on the N$_2$/Ar PAC, either alone, or with tropoelastin bound. This trend continued when the material was tested under flow conditions using a modified Chandler loop to assess acute thrombogenicity. The results showed a reduction in thrombus weight by more than 10-fold after 30 min of flow. It was further established that the N$_2$/Ar PAC and N$_2$/Ar PAC + TE surfaces delayed the time to thrombus formation by approximately 3-fold compared to 316L SS in this assay of acute thrombogenicity.

It is recognized that platelet-mediated mechanisms are the major contributor to stent thrombosis [31]. Activated platelets express higher levels of the cell surface marker P-selectin and its release into the blood correlates with biomaterial thrombogenicity [32]. Unlike bare stainless steel, the N$_2$/Ar PAC does not increase sP-selectin expression, either alone or with tropoelastin bound. While there are many other contributing factors to thrombosis including activation of complement, coagulation factors, inflammatory

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**Fig. 7.** Representative SEM images or photographs of samples after static incubation with heparinized whole blood for 0, 10, 30 or 60 min. Scale bars indicate 20 μm in SEM images.
factors and thrombin, the low platelet activation observed by N2/Ar PAC is promising and may partially explain the reduction in thrombogenicity that we observe. Additional characterization is required to deduce the specific mechanism of thrombosis in this model [30].

Metal oxides cause denaturation of adsorbed proteins, triggering stent thrombosis and inflammation, clinically manifesting as restenosis [33]. We propose that the reduction in thrombogenicity that we have observed is due to the combination of surface properties of the N2/Ar PAC. It is chemically uniform, hydrophilic, can potentially retain the bioactive state of bound plasma proteins such as albumin and fibrinogen preventing activation of thrombosis [24,33], and the covalent binding of tropoelastin represents a coating that remains non-thrombogenic and can enhance endothelialization. Promising initial in vitro results indicate the strong potential of PAC as a non-thrombogenic stent coating, while issues such as immune response and thrombogenicity in vivo remain to be assessed.

5. Conclusions

We report on a platform coating technology for metallic stents that has optimal mechanical properties and low thrombogenicity. The plasma-activated coating has exceptional adhesion to metallic substrates, withstanding expansion without delamination when applied to a stent. The plasma-activated coating is profoundly less thrombogenic than 316L stainless steel. When the coating is used to bind recombinant human tropoelastin, the surface retains its non-thrombogenic property and also enhances endothelialization. This coating technology is potentially applicable to BMS or DES platforms to significantly enhance stent biocompatibility.

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Appendix

Figures with essential color discrimination. Figs. 5, 7 and 8 in this article are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.07.062.