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Nitric oxide-generating silicone as a blood-contacting biomaterial

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Abstract

Coagulation upon blood-contacting biomaterials remains a problem for short and long-term clinical applications. This study examined the ability of copper(II)-doped silicone surfaces to generate nitric oxide (NO) and locally inhibit coagulation. Silicone was doped with 3-micron copper (Cu(0)) particles yielding 3 to 10 weight percent (wt%) Cu in 70-μm thick Cu/Silicone polymeric matrix composites (Cu/Si PMCs). At 3, 5, 8 and 10 wt% Cu doping, the surface expression of Cu was 12.1 ± 2.8%, 19.7 ± 5.4%, 29.0 ± 3.8%, and 33.8 ± 6.5% respectively. After oxidizing Cu(0) to Cu(II) by spontaneous corrosion, NO flux, J_{NO} (mol*cm^{-2}*min^{-1}), as measured by chemiluminescence, increased with surface Cu expression according to the relationship $J_{NO} = (1.63\ \%SA_{Cu} - 0.81) \times 10^{-11}$, $R^2 = 0.98$ where $\%SA_{Cu}$ is the percentage of surface occupied by Cu. NO flux at 10 wt% Cu was 5.35± 0.74 ×10^{-10} mol*cm^{-2}*min^{-1}. The clotting time of sheep blood exposed to these surfaces was 80 ± 13s with pure silicone and 339 ± 44s when 10 wt% Cu(II) was added. SEMs of coatings showed clots occurred away from exposed Cu-dendrites. In conclusion, Cu/Si PMCs inhibit coagulation in a dose-dependent fashion related to the extent of copper exposure on the coated surface.

Keywords

Nitric oxide; Cu(II); silicone; platelets; coagulation

Introduction

Over 200 million blood-contacting biomaterials are presently used in clinical applications such as catheters, vascular grafts, heart valves, extracorporeal membrane oxygenation (ECMO), cardiopulmonary bypass circuits, artificial kidneys, ventricular assist devices, glucose sensors, and stents [1]. In all these applications, it is important to reduce surface-induced blood activation in order to increase the useful life of the device and reduce thromboembolic complications. Thrombosis is especially problematic in long-term clinical applications such as artificial lungs (arteriovenous CO_2 removal, ECMO, thoracic artificial lungs) where the need for efficient gas transfer requires a larger surface area [2]. Currently, the administration of conventional systemic anticoagulants such as heparin, low-molecular-weight heparin, warfarin, aspirin, and clopidogrel is standard clinical practice to inhibit blood coagulation. Usage of newer GPIIb/IIIa blockers like reopro, aggrastat and integrilin to achieve the same goal are also on the rise [3]. Unfortunately, these anticoagulants act

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systemically and have long half-lives. Thus, they limit coagulation at the artificial surface, at surgical sites, and within native blood vessels. The result is longer device lifespan but also increased risk of bleeding complications [3 – 5].

A relatively new approach to reduce clot formation on biomaterials is through surface release or generation of NO [6,7]. NO is a free radical gas produced by the endothelium to maintain hemostasis [8 –11]. The gas reduces platelet activation by inhibiting agonist binding to their surface receptors. It freely diffuses into platelets to initiate the NO/cGMP [10,11] pathway that in turn phosphorylates G protein-coupled surface receptors, changing their conformation to decrease binding affinities of agonists. Commonly known G protein-coupled receptors on the platelet include thrombin, thromboxane A2, and adenosine diphosphate receptors. The gas also reduces secondary activation of circulating platelets by inhibiting the release of platelets' intracellular granules. This is achieved by blocking the release of their calcium stores needed for actin-myosin interaction that is required for platelets to change their shape and release their granules.

Unlike other platelet inhibitors, NO has a very short half-life (milliseconds), as it is quickly taken up by RBCs, platelets, and other NO scavengers. Thus, the anticoagulant effect occurs near the surface that releases or generates NO and has no effect on coagulation downstream.

NO generation from polymeric materials impregnated with Cu and other transition metal particles has been described [6,7]. In brief, these metallic particles catalyze the release of NO from NO-donors in blood. In the case of Cu, the metal exists mostly as Cu(II) on the surface of the polymer after oxidation. Reducing agents convert Cu(II) to Cu(I), which then interacts with NO-donors (Figure 1a) to produce thiolate ions, Cu(II), and NO gas. The thiolate ions then combine to form disulfides by reducing Cu(II) back to Cu(I). The entire reaction repeats to continuously generate NO. Once NO is generated, numerous reactions affect its concentration in blood (Figure 1b). In addition to binding and inhibiting platelets, NO can form nitrites by oxidative species, including ceruloplasmin,[12,13] and bind to hemoglobin and form methemoglobin. Plasma deoxyhemoglobin can also reduce NO_2^- back to NO. Plasma deoxyhemoglobin is normally associated with blood trauma caused by extracorporeal circulation during CPB or ECMO.

These modified polymers could be used to coat blood contacting surfaces or the blood-contacting device could be constructed wholly out of NO-generating materials. For example, the artificial lung's gas exchange membrane could be made wholly from silicone that is doped with Cu. To this end, the following study characterizes the relationship between the surface exposure of Cu surface NO flux, and the resultant anticoagulant effect from a silicone-Cu polymeric matrix composite (Cu/Si PMC). The aim is to provide a guide to their future use in any device with significant blood-biomaterial interactions.

Materials and Methods

In Vitro Test System

An *in vitro* test system was created by coating standard Hemochron tubes (P214 flip-top, ITC, Edison, NJ) with PMC at various weight percents of Cu. The tubes were emptied of their kaolin activator, rinsed with deionized water, and dried before coating. Three micron particles of elemental Cu (Sigma Aldrich Chemical Co. St. Louis, MO) were blended into part A of a two-part R21-2615 silicone resin (Nusil Silicone Technology, CA). An equal volume of silicone resin Part B was added followed by one mL of acetone per two mL of blend. The mixture was then stirred and sonicated (Branson 5510 Sonicator Bath, Norwich NY) for 60 minutes to create a uniform suspension. Cu microparticles were then added to create 0, 3, 5, 8, and 10 weight percent (wt%) Cu/Si PMCs. The hemochron tubes were

loaded with 0.3 mL of the mixture and rolled over a flat surface to eliminate uncoated patches. The tubes were then loaded horizontally into a rotisserie (Figure 2) and cured by rotating the tubes in the x-y plane at 1.5 rev min^{-1} for 24 hours at ambient temperature.

The resulting, cured coatings had thicknesses of 70 μm approximately. These coatings were oxidized at 37°C for 24 hours by bathing them in PBS at pH 7.4 using the Innova 4000 incubator-shaker. Bathing in PBS oxidizes Cu to Cu(II) by spontaneous corrosion of metallic copper [6], thus creating a Cu(II)/Si PMC. The hemochron tubes were then rinsed for approximately 2 minutes with deionized water and dried for testing.

PMC Surface Analysis

The presence of Cu on the Cu/Si PMC was verified with energy dispersive x-ray spectroscopy (EDAX Inc. Mahwah NJ). The Cu/Si PMC layer was coated on to tubes as described above, but peeled and sectioned into two cm^2 pieces prior to oxidation and further testing. Twelve surface locations per wt% were then analyzed with EDS. The following settings were used: input count rate = 1000cps, energy resolution = 135eV, peak/background = 100, and the scanned surface area = 4 mm^2. SEMs of surfaces analyzed with EDS were then imported into Image J (NIH, Bethesda, MD) for quantifying the percentage of Cu exposed on the surface. The images were converted into a two-dimensional black and white binary map and then processed using the particle analysis package to quantify the percentage of total surface area occupied by Cu.

Measurement of NO Flux and Clotting Times

NO generation from 0, 3, 5, 8, and 10 wt% PMC-coated tubes (N = 6 each) was measured by gas-phase chemiluminescence. Specifically, 2.75 ± 0.5 cm^2 coated pieces of the hemochron tube wall were introduced into a 1 μM S-nitrosoglutathione (GSNO) to activate a chemiluminescence reaction in a standard setup as shown in Figure 3. The NO generation profiles are decaying, left-skewed Gaussian curves (see Figure 4). A peak NO flux is reached initially; thereafter the GSNO concentration decreases and NO flux decreases. NO generation was measured for at least 30 minutes from each sample, and the peak flux was recorded for presentation.

Clotting times of sheep blood were measured for the 0, 3, 5, 8, and 10 wt% Cu-doped PMC-coated tubes (N = 6 each) using the coated hemochron tubes. Non-heparinized sheep blood (0.4ml) was dispensed into the tubes immediately after being drawn. Unlike typical activated clotting time assays, the tubes did not contain any procoagulant activator. Thus, the artificial surface was the only activator present. All other methods, including detection of a formed clot, followed standard Hemochron activated clotting time protocols. Thus, clotting time was measured as the time elapsed between loading the Hemochron tube with blood and when the Hemochron detected solid clot. All in vitro clotting time experiments were performed in atmospheric level of PO_2, or approximately PO_2 = 150 mmHg

Clot Distribution on NO generating Cu(II)/Si PMCs

To examine the mechanism of anticoagulation, Hemochron tubes were coated with 0 or 10 wt% Cu-doped silicone and filled with approximately 5 ml of blood. The tubes were placed on an Eberbach model EL687 shaker (Eberbach Labtools, Ann Arbor, MI) at 50 cycles/min within a 37° C incubator for 30, 60, 90, 240, 360, and 480 seconds. After incubation, the surfaces were gently rinsed with saline solution until the effluent was clear and fixed with 2% gluteraldehyde (Sigma Aldrich Chemical Co. ST. Louis, MO). The samples were imaged with a Philips XL30 FEG SEM to examine protein adsorption and platelet adhesion to the surface.

Statistical Analysis

One-way ANOVA with Tukey's post hoc tests was performed to compare the NO flux, clotting times and surface area of Cu using the weight percent of copper as the independent variable. A p-value < 0.05 is regarded as significant. All data is expressed as mean ± standard deviation.

Results

PMC's Surface Microstructure and Composition

In Figure 5, the surfaces of control and Cu(II)/Si coatings show different surface morphologies. As expected particle aggregates are not present on control surfaces, whereas dendrites of Cu microparticles are present on the non-control surfaces. The presence of Cu dendrites, as verified by EDS microanalysis, is shown in Figure 6. SEMs of the Cu/Si PMC showing Cu dendrites were first analyzed with energy dispersive spectroscopy. The result showed a mapping of Cu in Figures 6B and 6C similar to the Cu dendrite pattern of the SEM in Figure 4A. The energy spectra in Figure 6D also shows the corresponding Cu peaks.

The percentage of the surface area of membranes occupied by Cu was quantified with image J to be 12.1 ± 2.8%, 19.7 ± 5.4%, 29.0 ± 3.8% and 33.8 ± 6.5% at 3, 5, 8 and 10 wt% Cu doping respectively (see Figure 7). These results are significantly different from 0 wt% group and from each other ($p < 0.05$). The only exception is that the percentage of Cu/Si surface area occupied by Cu on the 10 wt% Cu surface was not significantly difference from that of the 8 wt% Cu group.

Relationships Between Surface Expression of Cu, NO Flux, and Clotting Times of PMCs

The peak NO flux increased with surface expression of Cu from $2.13 \times 10^{-10} \pm 0.38 \times 10^{-10}$ mol cm^{-2} min^{-1} at 12.1 ± 2.8% expression to $5.35 \times 10^{-10} \pm 0.73 \times 10^{-10}$ mol cm^{-2} min^{-1} at 33.8 ± 6.5% expression (Figure 8). The average peak flux of each group is significantly different from the control ($p < 0.05$) and from each other ($p < 0.05$). The one exception is that flux from the 33.8 ± 6.5% surface expression group was not significantly different from that of the 28.9 ± 3.7 % surface expression group ($p = 0.4$). These fluxes were all greater than published human endothelial NO flux in the range of $(0.5–4) \times 10^{-10}$mol cm^{-2} min^{-1} [12]. A linear fit of the relationship between NO flux and surface expression of Cu yielded $J_{NO} = (0.16(\%SA_{CU}) - 0.34) \times 10^{-10}$, $R^2 = 0.99$.

Accordingly, clotting times also increased with Cu surface expression from 80 ± 13 seconds at 0% Cu surface expression to 339 ± 44.5 seconds at 33.8 ± 6.5% Cu surface expression (see Figure 8). The average clotting time of each group is significantly different from control and from each other ($p < 0.05$). However the clotting time of the 33.8 ± 6.5% Cu surface expression group was not significantly different from that of the 28.9 ± 3.7% Cu surface expression group ($p = 0.29$). A linear fit of the relationship between clotting time in seconds (CT) and surface expression yielded $CT = 8.08(\%SA_{CU}) + 55.71$, $R^2 = 0.94$.

Clots on Coated Surfaces

Scanning electron micrographs of clots on control and NO-releasing surfaces are shown in Figure 9. Following exposure to blood for different durations, an adsorbed protein layer was present at all times, indicating that NO had no significant effect on total protein adsorption. However, Cu-doped surfaces had a smaller number of adhered platelets. In particular, it can be seen that areas around exposed Cu dendrites are free from platelets. Thus, the effect of NO on coagulation appears to be primarily keeping platelets from adhering to the adsorbed protein layer and perhaps keeping platelets that adhere from activating.

Discussion

Overall, these results indicate that Cu catalyzes NO formation effectively in these Cu/Si composites, and the NO formation leads to significant inhibition of clotting. Clotting times were 1.33 and 4.24 times longer at 3 and 10 wt%, respectively, than without Cu. Furthermore, the NO flux and resultant increase in clotting time is dependent primarily on the amount of copper exposed on the surface of silicone. At higher Cu weight percents, however, there are diminishing returns to adding more copper. The surface concentration of copper does not increase significantly between 8 and 10 wt% (Figure 7), as increasing amounts of Cu get trapped inside the bulk material. Thus, there is little improvement in NO flux and clotting time at higher Cu concentrations.

Thus, at greater than 8%, a significant amount of copper is buried within the bulk material and not assisting in NO generation. If greater NO generation were desired, surface processing would be necessary. A base layer of pure silicone could be laid down first, followed by a second, far thinner layer with a high Cu concentration. The thin layer would keep Cu from being subsumed within the bulk material. In the case of artificial lung membranes, this technique would reduce overall copper content and might better maintain the gas permeability of the membrane.

These results also indicate that each wt% surface was capable of NO fluxes on the same order as endothelial cells [14]. However, *in vivo* NO flux will depend on the concentration of a variety of NO-donors in present in blood and the reaction rate between each donor and Cu(I). In blood, the total concentration of GSNO and other low molecular weight NO donors such as *S*-Nitrosocysteine (CysNO), *S*-nitrosohomocysteine (HCysNO), and *S*-nitrosocysteinylglycine (CysGlyNO) in healthy subjects ranges across several orders of magnitude, from nanomolar to micromolar concentrations [15, 16]. Thus, predicting NO fluxes in blood are difficult.

In this study, 1μM of GSNO was used as the substrate for *in vitro* studies. The concentration of GSNO in blood is about one-third of that used here, 320 ± 60 nM, but this is augmented by the presence of low molecular weight NO donors. In addition, the rate of reaction between Cu(I) and these donors is faster than between Cu(I) and GSNO [17]. Thus, NO generation and the rate of clot formation may be different *in vivo* and vary across test subjects.

Generation of NO will also vary with time. If not supplemented, concentrations of NO donors will decrease as these molecules are cleaved to generate NO. In situations in which small surface area devices such as stents and arterial and venous lines are used, consumption would be minimal. In large surface area devices such as artificial lungs, consumption would be rapid, as there will be more catalytic sites for NO generation and widely studied synthetic RSNOs supplements such as S-Nitrosoglutathione and S-Nitroso-N-acetylpenicillamine (SNAP) would likely have to be infused into the device inlet to maximize the local NO generation. SNAP will be better suited for infusion, as it has been shown to be more stable than other S-nitrosothiols that lead to inconsistency due to their highly unstable nature [18, 19].

Ultimately, choosing an appropriate level of copper for a biomaterial depends greatly on the application. First, the minimum amount of Cu should be used to avoid any potential risk of high blood Cu concentrations. The amount of leached copper was not measured here because of the short blood exposure times. In this study, at 10 wt%, 60 mg of copper was used per coating over a surface area of 33 cm^2. If a similar coating were applied to a stent (surface area = 6 cm^2) and adult sized oxygenators (surface area up to 25,000 cm^2), the total amount of Cu that could potentially leach would be 10 mg and 45,000 mg respectively.

Thus, even if all of the Cu leached from stents, the amount would be negligible compared to the recommended daily intake. The amount that could leach from adult size oxygenators is higher than the tolerable daily intake, but this amount would leach slowly over the device's lifespan. Moreover, it is likely that only surface exposed copper would leach in significant amounts. The rate Cu leaching into plasma has been studied in a 4 h, rabbit model of extracorporeal life support [20]. There, a 10 wt% Cu circuit with a surface area of 0.02 m^2 was used in 2–3 kg rabbits The long term Cu leaching of these surfaces was also addressed by measuring the Cu content over seven days in a similar, saline filled extracorporeal circuit. There was a two-fold increase in plasma Cu to 5.9 ± 0.2 μg/dl after 4h. After 7 days, Cu levels in saline was 23.4 ± 8 μg/dl. This indicates a very slow rate of copper corrosion to copper ions within the coatings containing copper nanoparticles.

Additionally, the level of Cu that should be used will depend on the application and its duration. Large bore cannulae with relatively small surface area to volume ratios and high blood flow velocities will require lesser amounts of NO release, whereas artificial lung surfaces, with high surface area to volume ratios and low blood flow velocities will require far more NO release. Moreover, the longer the application, the greater anticoagulation required. This study indicates that weight percents between 5% and 8% would be the most likely therapeutic range if the copper were in the bulk polymer.

Despite these positive results, this study is limited by the short periods of blood biomaterial contact and the relatively static setting. Future long-term *in vivo* studies are needed to determine the effectiveness of the Cu/Si PMC on coagulation for periods of hours to days under blood flow. The hemochron tube wall velocity is 0.08 m/min, whereas the average blood flow velocity is anywhere from 0.4 m/min in an artificial lung [21] to 35 m/min in an ECMO cannula [22]. The static setting of this study results in pooling of reactants, including activated procoagulant molecules and possibly Cu leaching from the surface into the bulk fluid. In a dynamic system, both would be washed into circulation, reducing their effect at the biomaterial surface.

Conclusion

Results from this study indicate that Cu catalyzes NO formation effectively in silicone-Cu composites, and the NO formation leads to significant inhibition of clotting. Both NO flux and clotting time increase linearly with surface copper exposure. Copper exposure increases with the weight percent of copper in the PMC, but reaches a point of diminishing returns above 8 wt%. At 8 and 10 wt%, 29 and 34% of the surface is copper, respectively, and the clotting time is 3.61 and 4.24 times greater than with pure silicone, respectively. Future long-term *in vivo* studies are still needed to determine the effectiveness of this surface on coagulation for periods of hours to days under blood flow.

Acknowledgments

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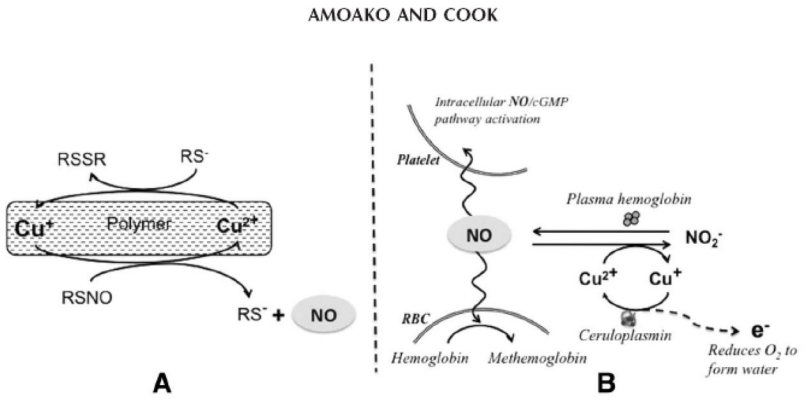

Figure 1.

Cu-mediated NO generation model (6). The in vitro NO generation from these surfaces can be measured accurately however the amount of NO generated in vivo from these surface still remains in question. Ceruloplasmin, a multicopper oxidase synthesized and secreted by the liver into plasma catalyzes the oxidation of NO into NO2 concomitantly with cupric (Cu2+) to cuprous (Cu+) reduction (13, 14). Perhaps, with other NO scavengers in plasma, only a fraction of the NO generated diffuses into platelets to activate the NO/cGMP pathway to inhibit platelet activation.

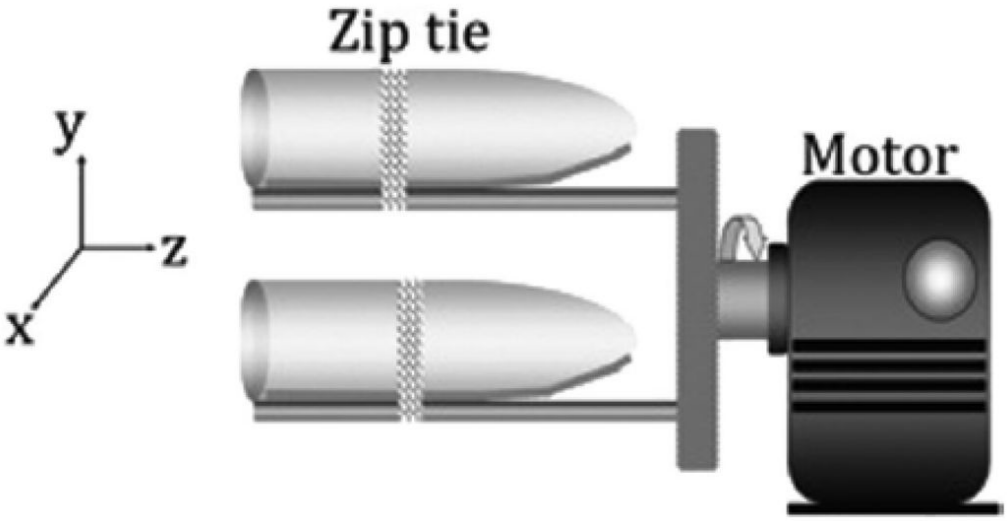

Figure 2.
Curing of coated hemochron tubes at ambient temperature and 1.5 rev min^{-1}. All tubes ware capped with their flip tops during curing.

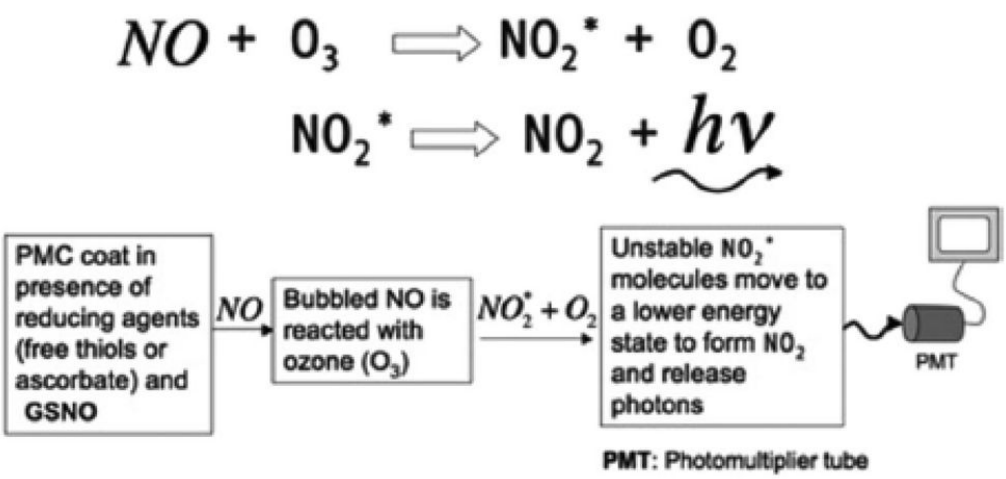

Figure 3.
Chemiluminescence reaction and reaction setup

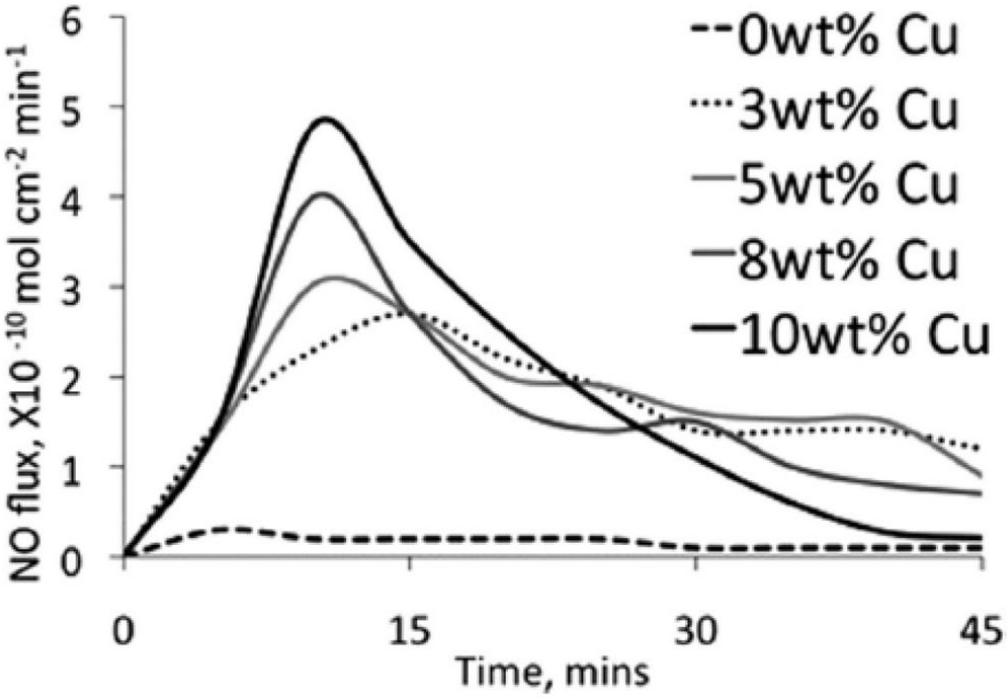

Figure 4.
Representative NO generation profiles for 0 (Control), 3, 5, 8, and 10 wt% Cu PMC coated surface.

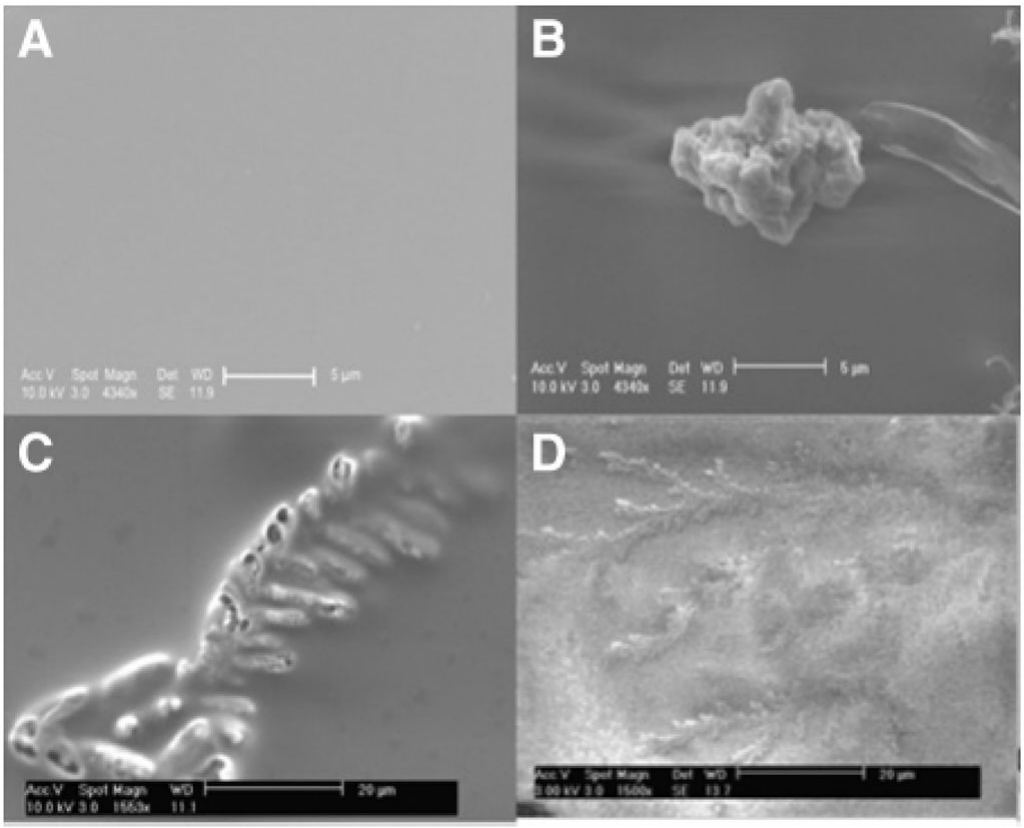

Figure 5.
Surface of silicone coated control (A), and surfaces of Cu(II)/Si PMC coatings at 3 wt% Cu (B), 5 wt% Cu (C), and 10 wt% Cu (D). The treated surfaces all show exposed Cu dendrites necessary for nitric oxide generation.

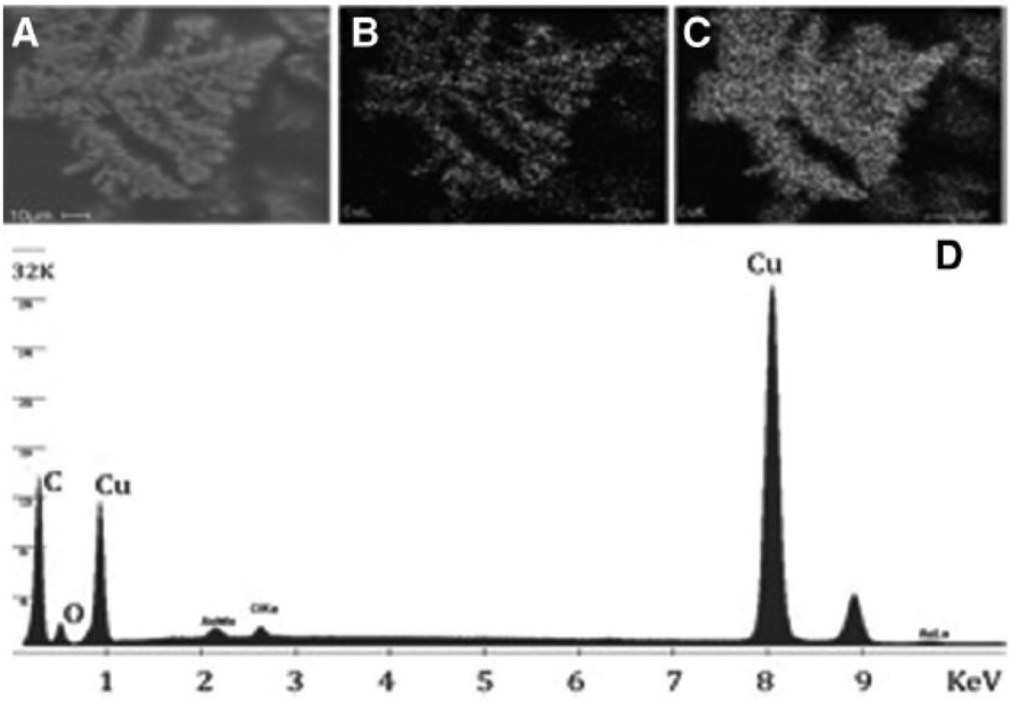

Figure 6.
Energy dispersive X-ray microanalysis results confirming the presence of Cu on Cu/SI PMC coated surfaces: SEM of Cu/Si PMC showing Cu dendrites (A) were analyzed with energy dispersive spectroscopy. The result showed a mapping of Cu in the L (B), and K (C) shells similar to the Cu dendrite pattern of the SEM. The energy spectra in D) shows the corresponding Cu peaks.

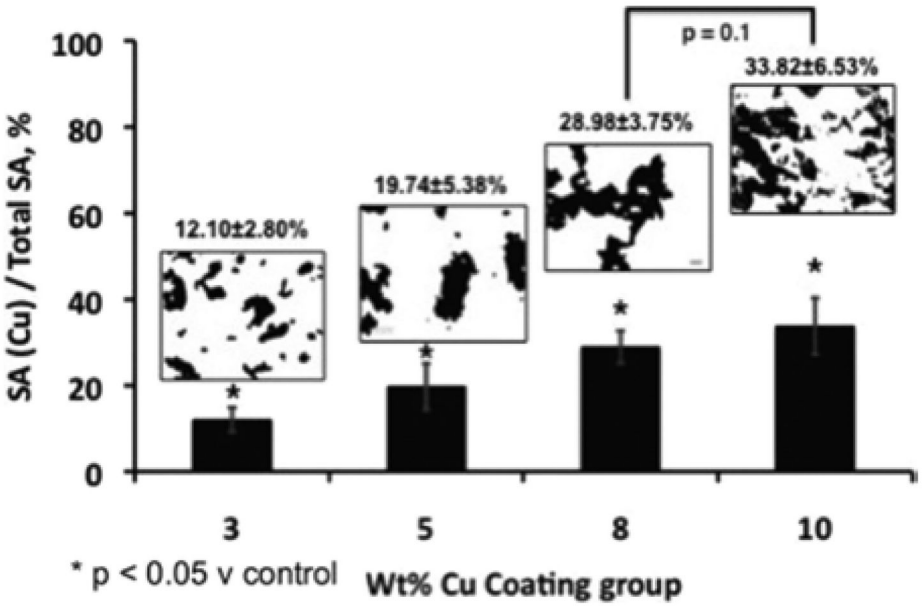

Figure 7.

The percentage of silicone surface-Cu as function of wt% Cu increased from 12.1% at 3wt% Cu to 33.82% at 10wt%Cu. The percentage of surface Cu in each wt% Cu coating group was significantly different from control and all the other groups ($p < 0.05$) with one exception. The 10 wt% Cu group was not significantly different from the 8 wt% Cu ($p=0.1$).

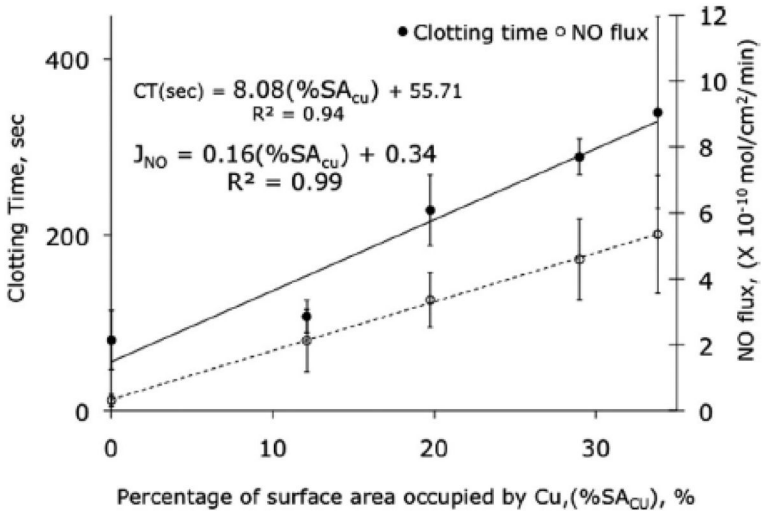

Figure 8.

Average clotting time and NO flux from PMC coated surfaces for 0 (Control), 3, 5, 8, and 10 wt% Cu polymeric matrix composites. The average clotting time and average peak NO flux of each group is significantly different from control and from each other ($p < 0.05$). However the clotting time and peak flux of the 10wt% Cu group was not significantly different from that of the 8wt% Cu group ($p = 0.29$ and $p = 0.4$ respectively).

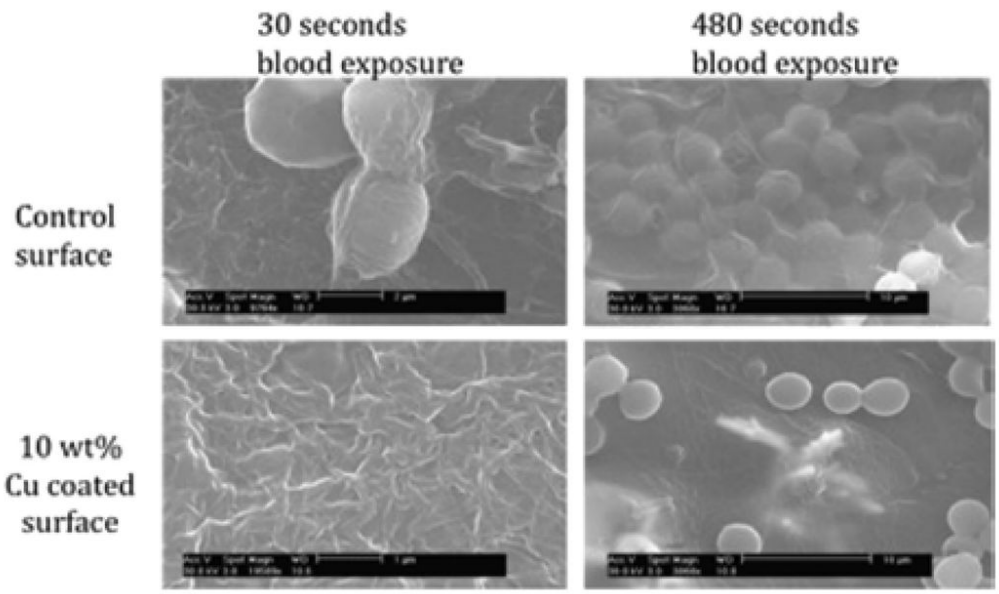

Figure 9.
SEMs demonstrating platelet adherence to control (0 wt% Cu) and 10 wt% Cu materials after 30 and 480 seconds.