

The biological effects of copper alloying in Zn-based biodegradable arterial implants

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ABSTRACT

Biodegradable metals based on zinc are being developed to serve as temporary arterial scaffolding. Although the inclusion of copper is becoming more prevalent for grain refinement in zinc alloys, the biological activity of the copper component has not been well investigated. Here, two Zn—Cu alloys (0.8 and 1.5 wt% Cu) with and without thermal treatment were investigated for their hemocompatibility and biocompatibility. The microstructure was examined using scanning electron microscopy and X-ray diffraction. Zn-1.5Cu was found to contain nearly double the amount of second phase (CuZn₅) precipitates as compared to Zn-0.8Cu. Thermal treatment dissolved a portion of the precipitates into the matrix. Since copper is a well-known catalyst for NO generation, the metals were tested both for their ability to generate NO release and for their thrombogenicity. Cellular responses and *in vivo* corrosion were characterized by a 6 months *in vivo* implantation of metal wires into rat arteries. The as-received Zn-1.5Cu displayed the least neointimal growth and smooth muscle cell presence, although inflammation was slightly increased. Thermal treatment was found to worsen the biological response, as determined by an increased neointimal size, increased smooth muscle cell presence and small regions of necrotic tissue. There were no trends in NO release between the alloys and thermal treatments. Corrosion progressed predominately through a pitting mechanism *in vivo*, which was more pronounced for the thermally treated alloys, with a more uniform corrosion seen for as-received Zn-1.5Cu. Differences in biological response are speculated to be due to changes in microstructure and pitting corrosion behavior.

1. Introduction

Biodegradable metal stents that could replace the clinical standard permanent metal vascular stents have been under intensive academic and industrial development for the past several decades [1–3]. These metals would maintain mechanical integrity for ~6–12 months during the arterial healing process [1] as they uniformly and safely degrade into harmless byproducts that the human body can clear. Iron and magnesium-based degradable metal alloys have been widely developed but struggle to meet the required arterial stent degradation rate [4,5]. Zinc (Zn) has recently been introduced as a third metal candidate, with acceptable biocompatibility and a corrosion rate closer to the clinical

requirements [6,7]. However, pure Zn lacks the mechanical strength and microstructural stability for stenting applications. Consequently, a broad spectrum of Zn alloys has been developed to boost the mechanical and structural deficiencies of pure Zn [8–11]. Unfortunately, we have found that the alloying of Zn to improve mechanical performance usually reduces biocompatibility, as measured by *in vivo* neointimal morphometrics and inflammation [8,10–13].

However, Zn—Cu alloys that are being developed to improve the mechanical properties of zinc may pave the way towards a zinc-alloy stent with high performance. Huang et al. developed Zn alloys with Cu weight percents of 1 to 3. They found that increased Cu content and thermal processing strengthen the material compared to pure Zn [18].

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The *in vivo* response of Zn-0.8Cu stents was evaluated in the coronary arteries of pigs for two years without signs of thrombosis, necrosis, severe inflammation [19], or in-stent restenosis after 18 months [20]. Mostaed et al. found that a Zn-1.0Cu alloy exhibited superplasticity [21]. We recently reported an advanced quinary alloy system (Zn-4Ag-0.8Cu-0.6Mn-0.15Zr) with enhanced mechanical and microstructural properties that surprisingly performed similarly to pure Zn in terms of biocompatibility [12,13]. Therefore, Zn-Cu alloys show promise for biodegradable stent applications. But the biological effects of the copper addition remain relatively unexplored.

In order to clarify the biological effects of Cu-containing phases in the Zn matrix, a binary alloy system of Zn and Cu was developed with copper concentrations of 0.8 and 1.5 wt%. Zn-Cu materials of Zn-0.8Cu and Zn-1.5Cu (wt%) were characterized for tensile strength, microstructure and corrosion, both *in vitro* and *in vivo*. Because ionic copper is a strong catalyst for nitric oxide (NO) generation, the thrombogenicity and NO catalyzing properties of the materials were also examined. We implanted Zn-Cu alloy wires into the rat artery to explore the Cu-containing phases' contribution to the biological response, following our murine model [8]. Thermal-treated counterparts were also implanted to compare the same elemental compositions with improved Cu distribution. Results for the binary Zn-Cu alloy were also compared to reference materials (high purity Zn and biostable platinum), as well as the recently reported advanced quinary alloy [12].

2. Methods

2.1. Material preparation, processing and characterization

2.1.1. Casting and processing

Binary Zn-0.8Cu and Zn-1.5Cu were formulated and investigated. The as-cast alloys in the shape of cylindrical billets with a diameter of 28 mm were produced by melting stoichiometric quantities of Zn shots (99.995 %) and pure Cu shot (99.9 % purity-Alfa Aesar) in sealed graphite crucibles at 700 °C inside a resistance furnace. Annealing at 400 °C was conducted for 2 h to homogenize the cast structure, followed by water quenching. Annealed samples were subsequently extruded at 310 °C with an extrusion ratio of 39:1 to obtain cylindrical rods. The extruded rods of 4 mm in diameter were centerless ground to provide a pristine surface finish. They were then multi-pass drawn at room temperature into wires with a diameter of 0.25 mm by Fort Wayne Metals (Fort Wayne, IN, USA) at an extrusion rate of 20 % initial strain rate/min and constitute the as-received condition. Thermal treatment was conducted at 300 °C for samples of both alloys. Zn-0.8Cu was treated for 60 s and Zn-1.5Cu was treated for 150 s.

2.1.2. Microstructure analysis

Wire samples embedded in resin were ground and finely polished as per the standard metallographic procedures and then etched with a 3 % Nital solution (3 mL HNO₃ + 100 mL ethanol). The samples were etched for <30 s to avoid over-etching. The microstructure was recorded using an Olympus PME 3 inverted metallurgical microscope (Center Valley, PA, USA) with brightfield observation at magnifications of 50× and 200×. The average grain size was found using the line intercept method. The embedded and polished wires were also carbon coated and then imaged with the Philips XL 40 ESEM (Amsterdam, Netherlands) for microstructure analysis. Elemental analysis was conducted using energy-dispersive X-ray spectroscopy (EDS).

2.1.3. Phase identification

X-ray diffraction (XRD) was carried out on the Zn-Cu wires for phase identification using a Scintag XDS 2000 diffractometer (College Park, MD, USA). The samples were wound around the zero-background holder for the XRD analysis. A Cu K α radiation of wavelength 1.540562 Å was used to scan in the range of 20° to 90° with a step time of 20 s and step size of 0.03°.

2.1.4. Mechanical testing

Tensile testing of the alloys was performed using a TA Instruments Q800 Dynamic Mechanical Analyzer (DMA) with TA Instruments Universal Analysis 2000 data analysis system (New Castle, Delaware, USA). The tensile tests were carried out on the wire segments and a variable gauge length of 10 ± 1 mm at a strain rate of 10 %/min, with and without thermal treatment. The testing was conducted in triplicates for each alloy and an average was calculated. A higher strain rate was chosen because of the strain rate sensitivity of the Zn alloys [21].

2.2. In vitro corrosion

2.2.1. Corrosion experiment

Wires of each alloy type were cut to ~95 mm in length and ultrasonically cleaned in acetone and 100 % ethanol (n = 5). Additionally, the diameter and starting weight were measured for each sample. They were then placed in 15 mL of balanced Hank's solution (Sigma Aldrich, H1387-10 L) with added sodium bicarbonate (Sigma Aldrich, S6014) with a pH of ~7.4 for a surface-to-volume ratio of 0.2 mL/mm². The samples were incubated at 37 °C with a CO₂ level of 5 % and relative humidity of 90 % for 10 or 24 days. The solution was changed every 2 days to maintain the pH, and discarded solution was stored at 4 °C for later analysis. After the allotted times, the wires were cleaned and dried before weighing, and the corrosion rate was calculated using the following equation:

$$CR = \frac{W \cdot d}{4 \cdot m \cdot t} \quad (1)$$

where CR = corrosion rate (mm/year), W = weight loss (g), d = wire diameter (mm), m = wire mass (g) and t = exposure time (years).

2.2.2. Elemental analysis

ICP-OES was performed using the Perkin Elmer Optima 7000DV (Waltham, MA, USA). The discarded solution from *in vitro* corrosion experiments was acidified with nitric acid to dissolve precipitates before analysis. Each sample was analyzed for Zn and Cu concentration. Fresh Hank's balanced salt solution was also run for comparison.

2.2.3. MicroCT analysis

The region of most extensive corrosion for each wire was imaged with microCT (Phoenix NanoTom M, GE Measurement and Control Solutions, Germany) after 10 days of *in vitro* corrosion. The NanoTom M is equipped with a 180-kV/15 W energy micro/nanofocus X-ray tube and a diamond-coated tungsten target was used for all acquisitions. Images were acquired at an ~1.3 µm isotropic voxel size, with a source voltage of 170 kV and a tube current of 42 µA. The exposure time was 500 ms. Imaging was done in mode 0, and 2400 projections were acquired with an average of 1 and skip 0. All acquisitions took ~15 min in fast scan mode. A copper filter of 0.25 mm thickness was placed between the source and the sample. The microCT datasets were reconstructed with the Datos|x software (GE Measurement and Control Solutions). A beam hardening correction (BHC) of 9 was applied. BHC goes on a scale from 0 (no correction applied) to 10 (powerful correction applied). The micro-CT image-based visualization of the corrosion site was generated using CTvox software (Bruker micro-CT, Belgium). Three different post-processing filters were applied in the reconstruction software: inline median (projections are filtered during reconstruction using a 3 × 3 median filter, which reduces the noise in the volume), ROI-CT filter (increases the resolution of the CT dataset for a selected ROI of the object), and filter volume (volume is filtered in slices after reconstruction using the Unsharp Masking Filter, enhancing object edges). Slices were exported as 16-bit XY slices (.tiff) and then converted to 8-bit (.bmp) images.

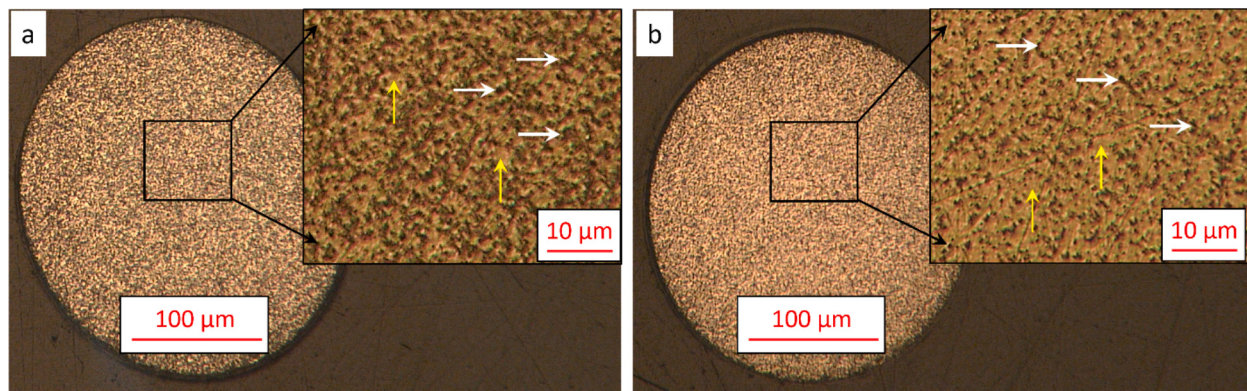


Fig. 1. Optical micrographs of as-received alloy microstructure at low (50 $\times$) and higher (200 $\times$) magnifications; a) Zn-1.5Cu, b) Zn-0.8Cu. Average grain size found via line intercept method is $\sim 1\ \mu\text{m}$ for both alloys. Yellow arrows mark the matrix and white arrows mark the secondary phase.

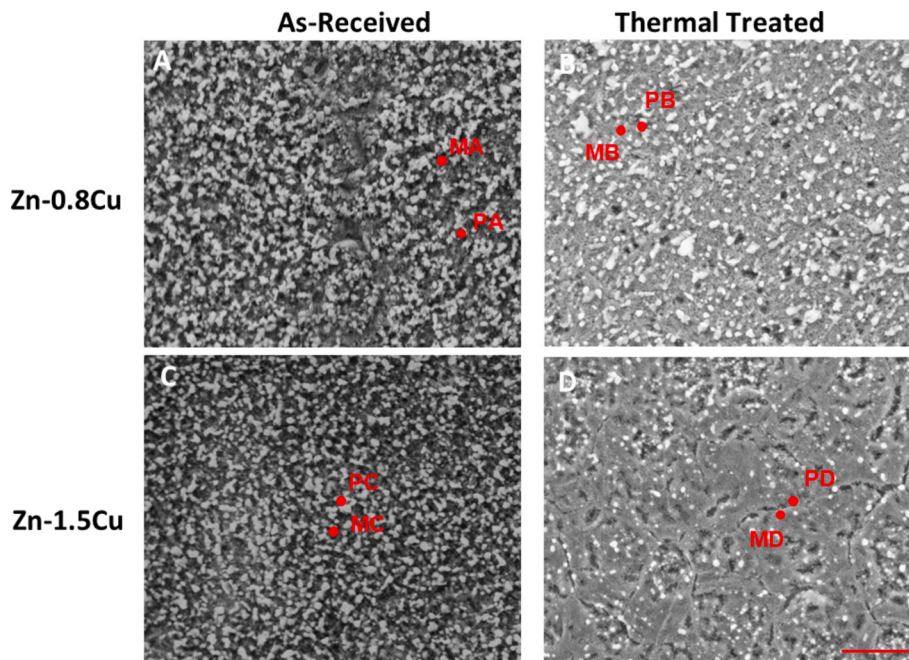


Fig. 2. Representative SEM images of non-implanted wire polished cross sections with magnification of 1950 $\times$. Representative points where EDS spectra were collected are shown for each alloy. “P” stands for precipitate and “M” stands for matrix. Elemental weight percents for each point can be found in Table 1 and correspond to “MA” and “PA”. The scale bar in D is equal to 10 μm .

2.2.4. Nitric oxide analysis

Nitric oxide generation by wires was investigated using the Sieves 280i Nitric Oxide Analyzer (Chicago, IL, USA). Wires of the different alloy compositions and high-purity control wires were suspended in 2 mL of 1 mM SNAP solution for 2 h and the NO level was recorded every second. Wires samples after 10 days of *in vitro* corrosion were also tested for NO generation. High-purity control metals included Zn, Cu, and Pt (Goodfellow).

In all cases, the oxide layer was removed from the wires before placing in the SNAP solution. The oxide layer was removed from Pt by heating in an ethanol flame. The oxide layer was removed using acetic acid for 45 s for pure Cu. To remove the oxide film on Zn and Zn alloys, the wires were washed in 7 % ammonia for 30 s and then washed in deionized water.

2.3. *In vitro* thrombogenicity

2.3.1. Reagents

Pooled human platelet poor plasma (PPP) was acquired from 6

volunteers (3 male, 3 female). All human experiments were approved by the institutional review board (IRB) of Oregon Health and Science University. Blood from volunteers was centrifuged at 2000 $\times g$ for 10 min. Afterwards the top layer was removed and centrifuged under the same conditions. The top layer of plasma was isolated and frozen at $-70\ ^\circ\text{C}$. Aliquots for this experiment were thawed at $37\ ^\circ\text{C}$. The as-received binary alloys (Zn-1.5Cu and Zn-0.8 Cu, 0.25 mm diameter) wires were tested. Controls of pure Zn wire (0.25 mm diameter), pure Cu wire (0.25 mm diameter), and CoCr (Co40-Cr20-Fe15-Ni15-Mo7-Mn2-C-Be, 0.25 mm diameter) were acquired from Goodfellow. S-2302 Factor (F)XIIa chromogenic substrate was acquired from Fisher Scientific (Waltham, MA, USA). HEPES and phosphate-buffered saline (PBS) were acquired from Sigma (St Louis, MO, USA).

2.3.2. FXIIa generation assay

6 mm length wires were added to a 96-well clear polystyrene plate and rinsed twice with room temperature PBS before the addition of PPP. PPP was diluted 1:1 by addition of HEPES before addition to wires. All conditions were given 2.4 mM S-2302. The color change from the Factor

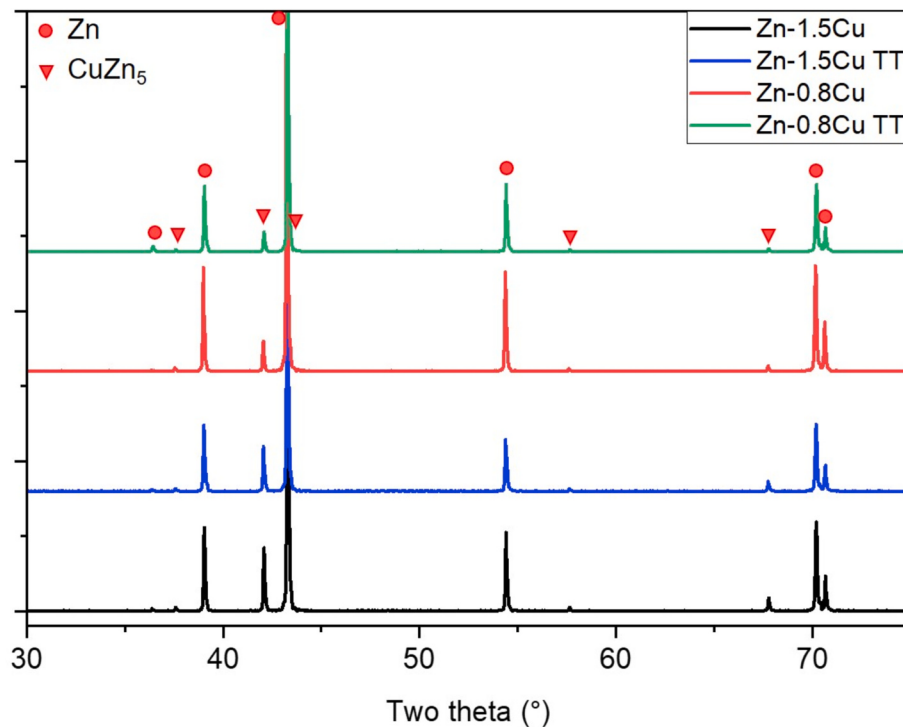


Fig. 3. XRD results of the alloys before and after thermal treatment (TT). All significant peaks of Zn and Cu phases were present. Zn-1.5Cu had increased CuZn₅ peak intensity due to increased Cu weight percent.

Table 1

Representative EDS points and weight percents (wt%) of elements in non-implanted wire cross-sections.

	Label	O (wt%)	Cu (wt%)	Zn (wt%)	Total
Zn-0.8Cu	PA	3.43	5.2	91.37	100
	MA	2.11	2.55	95.34	100
Zn-0.8CuTT	PB	3.64	2.9	93.46	100
	MB	2.79	4.45	92.76	100
Zn-1.5Cu	PC	7.26	12.58	80.16	100
	MC	2.23	1.47	96.3	100
Zn-1.5CuTT	PD	5.86	2.17	91.97	100
	MD	5.46	1.65	92.89	100

Table 2

Weight percentage of individual phases from whole pattern fitting (WPF) analysis of X-ray diffraction (XRD) pattern.

Samples	Zn-phase (wt%)	Cu-Zn phase (wt%)
Zn-1.5Cu, without thermal treatment	92.1	7.9
Zn-1.5Cu, thermal treated at 300 °C	93.1	6.9
Zn-0.8Cu, without thermal treatment	96.2	3.8
Zn-0.8Cu, thermal treated at 300 °C	96.7	3.3

(F)XIIa chromogenic substrate in each well was read by a TECAN infinite-m200 plate reader at 405 nm over the course of a 1-h kinetic cycle, each cycle equal to 60 s. The max slope of the change in absorbance was calculated by Magellan 7.2 software. Each assay was repeated 3 times with 3 different wires resulting in $n = 9$.

2.3.3. Fibrin generation assay

6 mm length wires were added to plate and cleaned, as described above. PPP was diluted 1:1 by addition of HEPES before adding to wires. Afterwards, 25 mM CaCl₂ was added to all wells. The plate was read with the same parameters described above, however, the reading at 405 nm measured the solution's turbidity. The maximum slope of the change in

absorbance and the take-off time for fibrin clotting were calculated by Magellan 7.2 software. This assay was repeated twice in quadruplicate and once in triplicate, resulting in 3 total trials and $n = 11$ samples.

2.4. In vivo studies on biodegradation and biocompatibility

All animal experiments were approved by the Michigan Technological University Institutional Animal Care and Use Committee (IACUC) board.

2.4.1. Implantation and removal

0.25 mm diameter Zn-0.8Cu and Zn-1.5Cu wires (with and without thermal treatment) were implanted into the abdominal aortas of Sprague Dawley rats, as previously reported [8]. After six months, the animals were euthanized, and the aortas were removed. All samples were flash frozen in optimal cutting temperature (OCT) medium (Thermo Fisher Scientific) and liquid nitrogen before being stored at -80°C until cross-sectioning.

All explants were cross-sectioned at a temperature of -25°C and a thickness of 9 μm using an HM525 NX Thermo Fisher Scientific cryomicrotome. Cross-sections were collected once the implant had advanced through the arterial elastic lamina. The cross-sections were placed on Histobond slides (VWR) and stored at -80°C to await staining.

2.4.2. Elemental analysis

In vivo corroded wires were embedded in resin and polished for imaging and elemental analysis with the Philips XL 40 ESEM to determine a degree of biodegradation and identify composition of corrosion products.

2.4.3. Hematoxylin and eosin (H&E) staining/cell morphology

H&E staining was performed to qualitatively analyze neointimal cell composition and distribution. Cross-sections were fixed in 10 % neutral buffered formalin (Sigma Aldrich) and rinsed in 3 changes of phosphate-buffered saline (PBS) for 5 min each. Then, they were rinsed in deionized

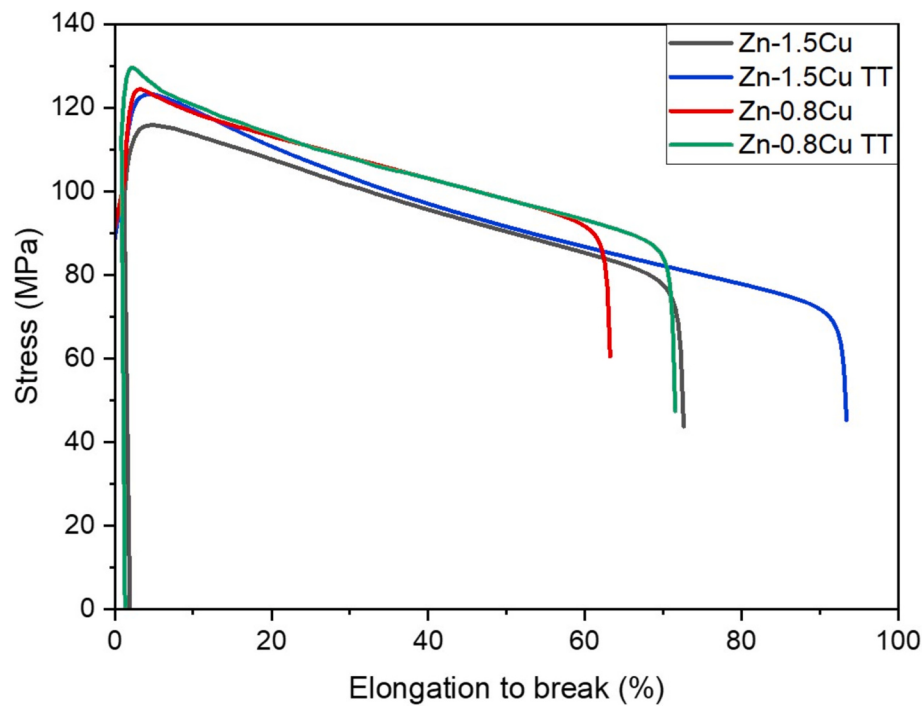


Fig. 4. Engineering stress-strain curve of the alloys found through tensile testing. “TT” stands for thermal treatment.

Table 3
Engineering tensile properties of the alloys with and without thermal treatment.

Samples	Yield strength (MPa)	Ultimate tensile strength (MPa)	Elongation (%)
Zn-1.5Cu, without thermal treatment	98 ± 2	117 ± 2	74 ± 7
Zn-1.5Cu, thermal treated at 300 °C	104 ± 2	125 ± 2	76 ± 15
Zn-0.8Cu, without thermal treatment	106 ± 3	125 ± 1	63 ± 5
Zn-0.8Cu, thermal treated at 300 °C	112 ± 4	130 ± 2	61 ± 8

Table 4
Average corrosion rate (CR) for *in vitro* wires for 10 day and 24 day time points. “TT” stands for thermal treatment.

Average	CR (mm/year) after 10 days	CR (mm/year) after 24 days
Zn-0.8Cu	0.15	0.01
Zn-1.5Cu	0.03	0.06
Zn-0.8CuTT	0.11	0.06
Zn-1.5CuTT	0.02	0.11
Pure Zn	0.11	0.05

water for 5 min and stained with Gill’s Hematoxylin #3 (Sigma Aldrich) for 5 min. The stain was regressed in diluted hydrochloric acid with a pH of 1.8–2.0, followed by “bluing” for 5 min in running tap water. Slides were then washed in two changes of 95 % ethanol for 5 min each, followed by counterstaining in Eosin Y working solution for 30 s. The stain was regressed with 95 % ethanol and then put in two changes of 100 % ethanol for 5 min each. Slides were dunked in used xylene substitute and then placed in two changes of new xylene substitute (Sigma Aldrich) for 5 min each. Then, slides were cover slipped using Eukitt quick hardening mounting medium. Samples were later imaged at 40× magnification using a Zeiss Axioscan Z1 (Oberkochen, Germany).

2.4.4. Verhoeff van Gieson (VVG) staining

VVG staining was performed to quantitatively measure wire-to-lumen thickness (WLT) and neointimal area (NA). Cross-sections were fixed in 10 % neutral buffered formalin for 15 min, followed by three changes of PBS for 5 min each. Next, slides were stained with Verhoeffs for 8 min, and each slide was differentiated separately in ferric acid. The slides were subsequently soaked in deionized water for 5 min and sodium thiosulfate for 1 min. Cross-sections were then “blued” in tap water for 5 min and counterstained in van Gieson stain for 4 min. The stain was regressed by dunking in 95 % ethanol. The slides were dehydrated in two changes of 100 % ethanol for 5 min each, dunked in used xylene substitute and soaked in two changes of xylene substitute before cover slipping. Samples were imaged at 20× using the Zeiss Axioscan.

2.4.5. CD68 labeling for macrophages

Cross-sections were fixed with 100 % ethanol for 2 min, followed by three washes in PBS. Slides were blocked for 30 min in 10 % goat serum (Abcam, ab7481), for 10 min in biotin, and 10 min in avidin (Abcam, ab64212). The slides were incubated in an anti-CD68 primary (Abcam, ab125212) that was diluted 1:100 in PBS for 1 h, followed by incubation in a secondary antibody (Abcam, ab6720) [1:500] for 1 h. Next, the samples were labeled with Streptavidin conjugated Alexa Fluor 488 (Thermo Fisher, S32354) [1:500 in PBS] for 1 h. Finally, the samples were labeled with 4’,6-diamidino-2’-phenylindole dihydrochloride (DAPI, Sigma Aldrich, D8417-1MG), diluted 1:1000 in PBS. Sections were washed twice with PBS between each step. The slides were mounted, and cover slipped using Fluoromount aqueous mounting medium. Images were collected at 40× using the Zeiss Axioscan. Fluorescent signal quantification was performed using Cellsens software (v2.3) and normalized to neointimal area.

2.4.6. α-SMA labeling for smooth muscle cells (SMCs)

Slides were fixed in ice-cold methanol for 5 min followed by 3 PBS washes. Slides were blocked in 10 % goat serum for 30 min before being incubated in anti-alpha smooth muscle Actin (Abcam, ab5694) diluted 1:250 in PBS for 1 h. This was followed by an hour of incubation in fluorescent antibody Alexa Fluor 488 (Abcam, ab150077) diluted 1:300.

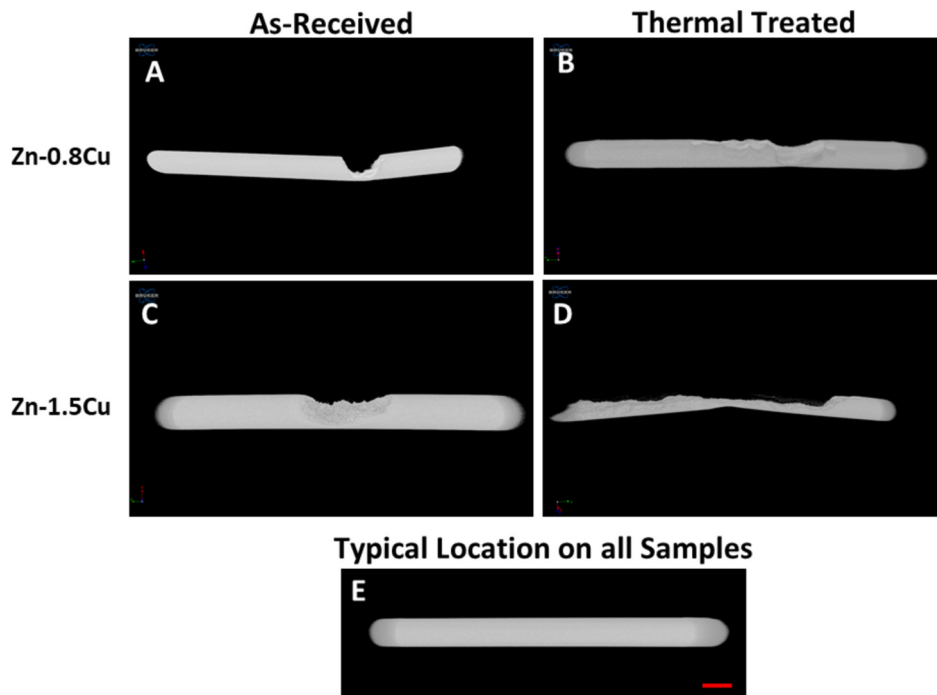


Fig. 5. A–D) Micro-CT images of the most degraded corrosion site for each alloy over an inspected ~ 50 mm wire length, following *in vitro* corrosion experiments for 10 days. E) typical non-corroded location seen for all alloy types. Scale bar is equal to 250 μm .

Table 5

Average Zn and Cu ion presence in *in vitro* corrosion solution after 10 or 24 days for each alloy found via ICP analysis. “TT” stands for thermal treatment.

	10 day		24 day	
	Zn concentration (mg/L)	Cu concentration (mg/L)	Zn concentration (mg/L)	Cu concentration (mg/L)
Zn-0.8Cu	4.96	0.08	7.96	0.10
Zn-0.8CuTT	6.39	0.10	7.52	0.13
Zn-1.5Cu	5.24	0.09	5.82	0.08
Zn-1.5CuTT	5.45	0.09	6.84	0.10
Pure Zn	11.80	0.03	3.70	0.03

Finally, the slides were labeled with DAPI, and cover slipped. Images were collected at $40\times$ using the Zeiss Axioscan and later thresholded for fluorescent signal.

2.5. Statistical analysis

Statistical analysis was performed in R (v. 2023.06.0). Data was tested for normality using a Shapiro-Wilk’s test, and data with a p -value > 0.05 were considered normal. A Bartlett’s test was also used to test for equal variance with a p -value > 0.05 , showing that the variances were equal. A Kruskal-Wallis test followed by a Dunn’s test was used for non-normal data, and ANOVA was used for normal data. Statistical significance was determined if the p -value was < 0.05 .

3. Results

3.1. Materials characterization

The optical micrographs of the alloys illustrate that both samples exhibited ultra-fine grain sized microstructure due to severe plastic

deformation during cold-drawing (Fig. 1). The average grain size for both alloys was close to one micron. The optical micrograph (at higher magnification) also suggests the presence of a dendritic secondary phase with Zn-1.5Cu having more volume fractions. Tang et al. also observed a dendritic secondary phase (CuZn_5) for Zn–Cu binary alloys with its volume fraction increasing with the copper concentration [22].

SEM images of polished metal surfaces showed two phases (Fig. 2), as seen previously for Zn–Cu alloys [21]. The bright features are CuZn_5 precipitates [21] that increase in density with the increasing copper content (as determined from the XRD patterns — Fig. 3). Thermal treatment reduced the weight fraction of precipitates, especially for Zn-1.5CuTT, which had a longer annealing time of 150 s. The movement of copper in the matrix was confirmed with point EDS, with normalized weight percentages presented in Table 1.

Fig. 3 displays the XRD patterns of the alloys before and after thermal treatment at 300 °F. Both alloys exhibited all the significant peaks of the Zn phase. However, the (002) plane in the Zn phase showed a low-intensity peak due to the texturing effect of severe plastic deformation during the cold drawing of the wires. Additionally, the samples exhibited peaks for 2θ values at 37.6°, 42.1°, 43.8°, 57.6°, and 67.8°, which corresponded to the presence of an additional phase called CuZn_5 [23–25]. The intensity of CuZn_5 peaks was higher for Zn-1.5Cu alloy due to the increased weight fraction of Cu. Using whole pattern fitting (WPF) analysis, the compositions of each phase in the alloys before and after thermal treatment studied were identified/calculated (Table 2). The weight percentage of secondary phase in Zn-1.5Cu was nearly two times higher than that of the Zn-0.8Cu alloy, which supports the peak intensity data from XRD pattern and optical micrographs. However, after thermal treatment, there was no additional phase formation or peak shift; while a small change in peak intensity was observed for both the alloys due to the dissolution of secondary phase.

The stress-strain curve from tensile testing is presented in Fig. 4. Even though the alloy with lower copper exhibited a higher strength, the area under stress-strain curve for Zn-1.5Cu was comparatively higher. The tensile test results are summarized in Table 3. The improvement in mechanical properties of the alloys compared with pure Zn is attributed to the solid solution strengthening effect of copper due to its higher

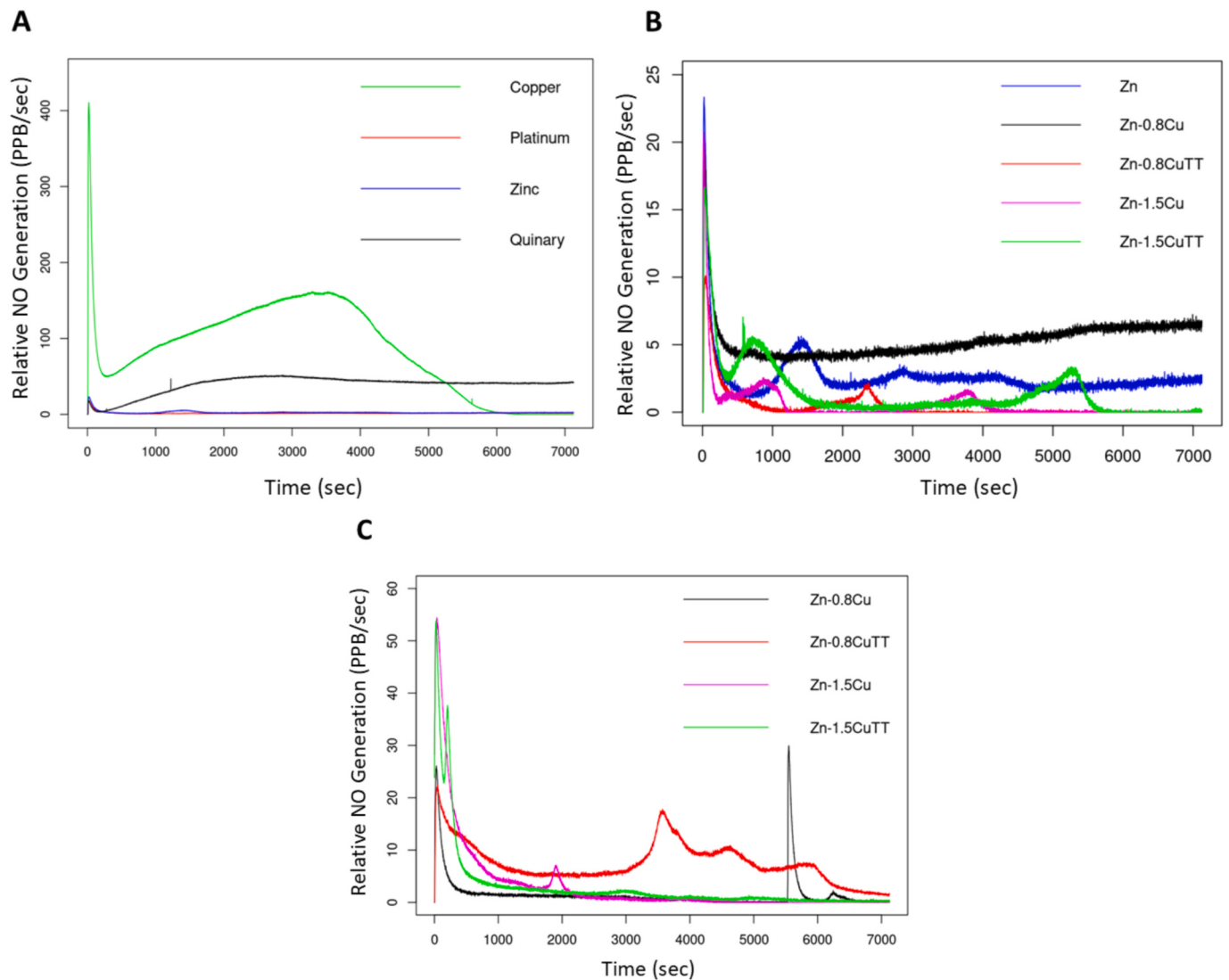


Fig. 6. Ionic Zn and Cu are known to be catalyst for nitric oxide generation. Wires with a diameter of 0.25 mm were placed in 1 mM SNAP solution and NO generation was measured over 2 h using chemiluminescence. Average relative nitric oxide generation from A) pure metal wires and quinary (Zn-4Ag-0.8Cu-0.6Mn-0.15Zr) B) Zn and Zn—Cu alloy wire C) Zn and Zn—Cu alloy wire *in vitro* corroded alloy wires. “TT” stands for thermal treatment.

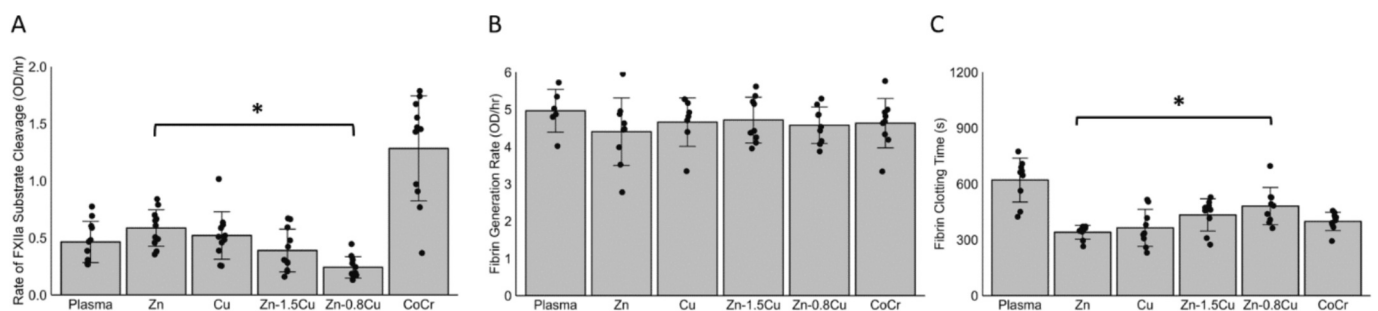


Fig. 7. All metals were tested *in vitro* using PPP to investigate thrombogenicity. (A) The amount of FXIIa generated in the presences of Zn-0.8Cu was significantly lower than pure zinc. All conditions were significantly lower than the CoCr clinical control. This assay was repeated 3 times with 3 wires for $n = 9$. (B) No significant difference was found in fibrin generation rate by the different materials. (C) The initiation time of fibrin generation was significantly faster with the pure Zn than Zn-0.8Cu, suggesting reduced thrombogenicity for Zn-0.8Cu. All materials were significantly faster in take-off time than PPP alone condition. This assay was performed twice in quadruplicate and one in triplicate, giving 3 total trials with $n = 11$. * indicates $p \leq 0.05$. Statistical analysis used one-way ANOVA with Tukey's *post hoc*.

solubility in Zn, as well as uniform dispersion of the secondary phase (CuZn₅). Furthermore, it is evident from the results that thermal treatment led to marginal improvement in the tensile behavior. This improvement can be attributed to the reduction in strain hardening and

better dissolution of the secondary phase during thermal treatment [12].

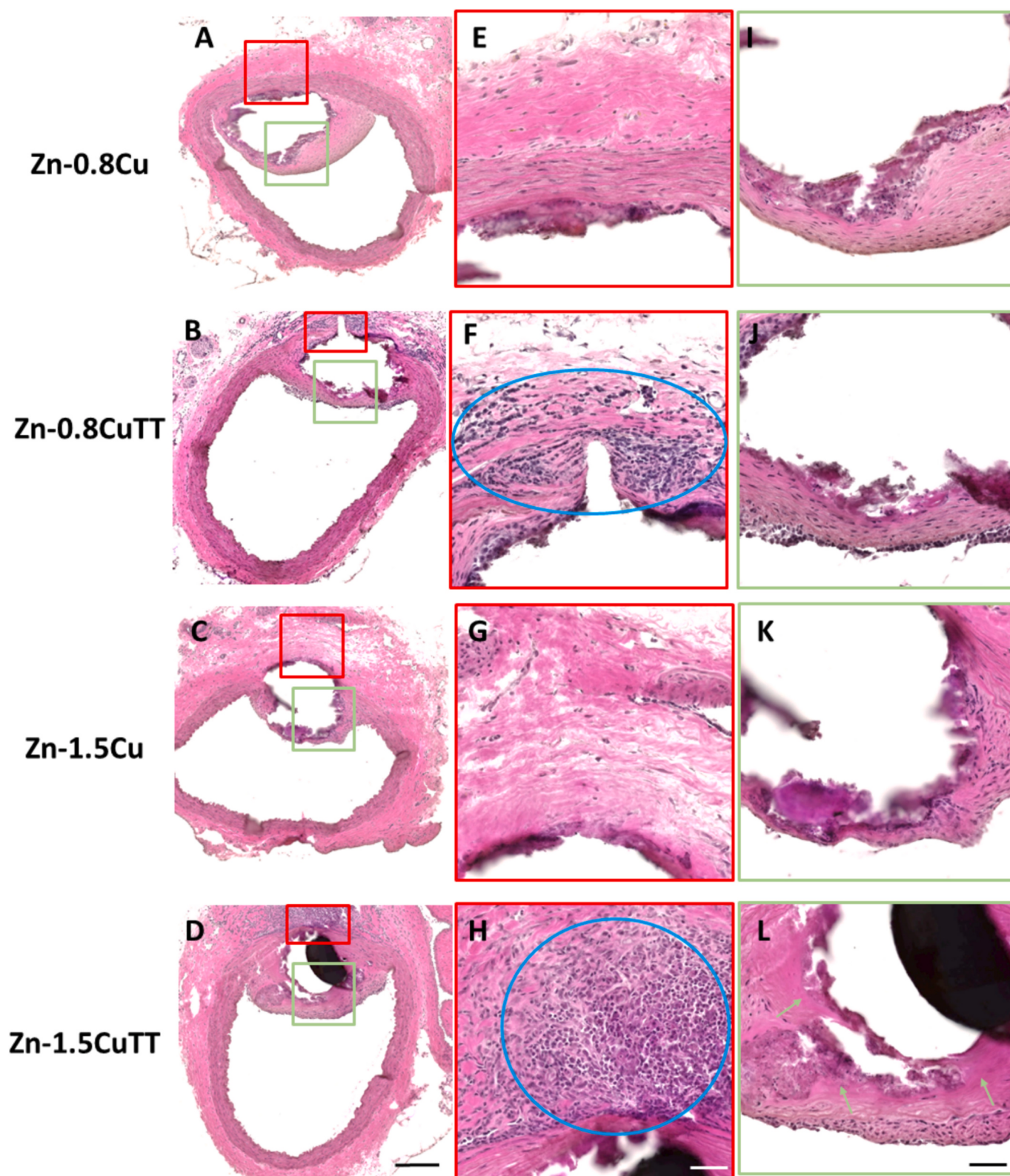


Fig. 8. A–D) Representative H&E images for each alloy type, red and green squares correspond to high magnification images, scale bar in D is equal to 200 μ m. E–H) High magnification images of arterial tissue on the mural side of the implant for each alloy type. Blue circles in F and H show areas of inflammation. Tissue defect in B and F is artifact of cryosectioning against hard metal implant. Scale bar in H is equal to 50 μ m. I–L) High magnification images of neointimal tissue for each alloy. Green arrows in L show areas of possible necrosis. Scale bar in L is equal to 50 μ m. “TT” stands for thermal treatment.

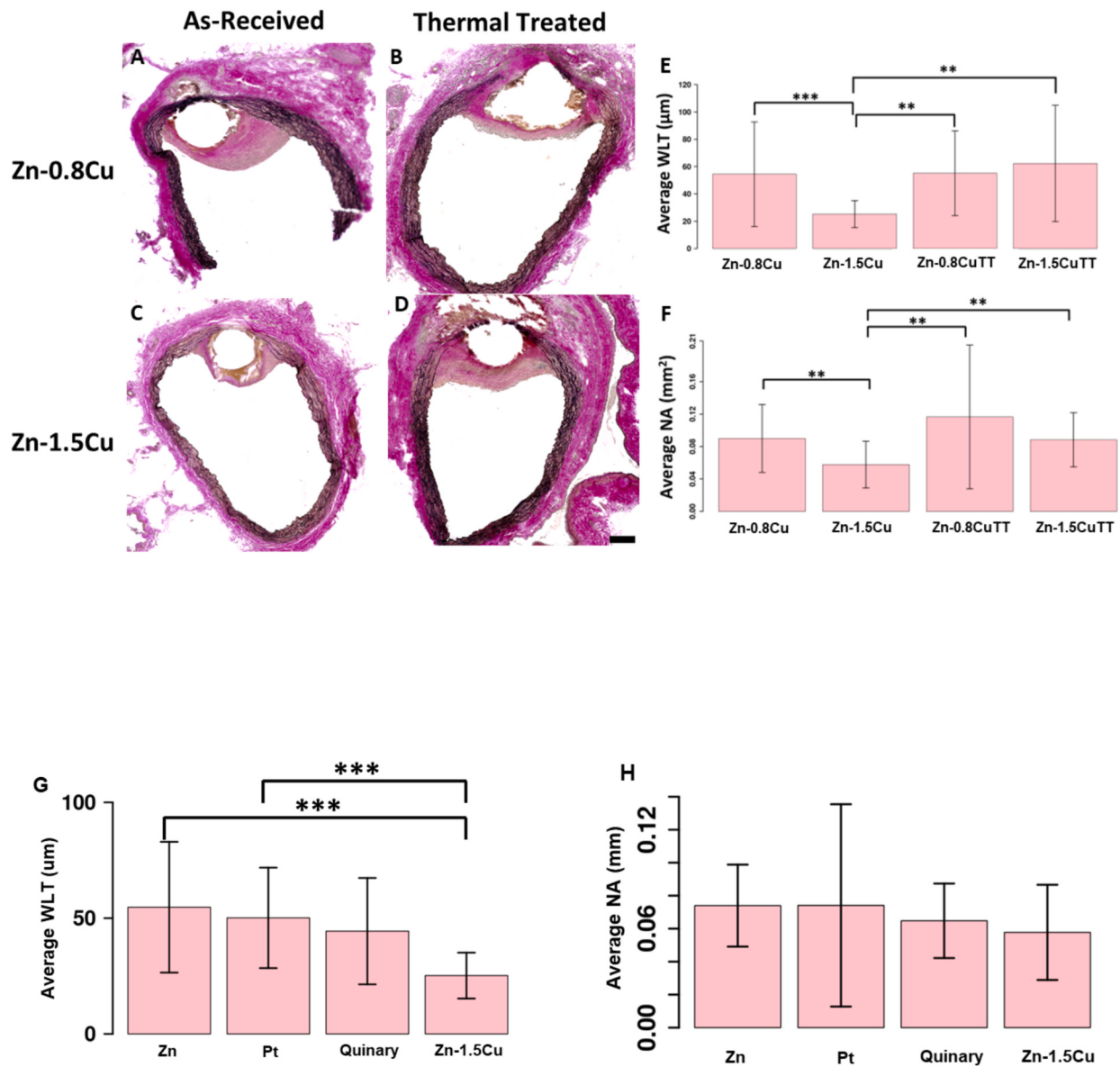


Fig. 9. A–D) Representative VVG images for the as-received and thermal treated alloys. Scale bar in D equals 200 μm . E) Graph of average wire-to-lumen thickness. Asterisks represent significance found by Kruskal-Wallis (WLT ** p -value < 0.01, *** p -value < 0.001). F) Graph of average base neointimal length for each alloy. Asterisks represent significance found by Kruskal-Wallis (NA ** p -values < 0.01). G) Graph of average wire-to-lumen thickness for zinc and platinum controls, previously reported quinary alloy and Zn-1.5Cu. Asterisks represent significance found by Kruskal-Wallis (*** p -value < 0.001). H) Graph of average wire-to-lumen thickness for zinc and platinum controls, previously reported quinary alloy and Zn-1.5Cu. No significance was found. (Zn-0.8Cu n = 12, Zn-0.8CuTT n = 12, Zn-1.5Cu n = 10, Zn-1.5CuTT n = 12). “TT” stands for thermal treatment.

3.2. In vitro corrosion

Wire samples were subjected to *in vitro* corrosion testing for 10 and 24 days, with the data summarized in Table 4. At 10 days, the 1.5 wt% Cu concentration alloy system exhibited greater average corrosion resistance than the 0.8 wt% Cu concentration or pure Zn. After 24 days of submersion under fluid, the resistance to corrosive activity was generally good or even improved, except for Zn-1.5CuTT. Overall, good corrosion resistance at 10 days was confirmed qualitatively with micro-CT (Fig. 5), where most of the wire surfaces lacked apparent signs of corrosion (Fig. 5E).

Pitting corrosion was the main corrosion mode for both alloys, with or without thermal treatment (Fig. 5A–D). Corroded wires were inspected under the microCT system, and the most corroded location along the wire was scanned. Although all alloys were degraded by pitting, the thermally treated alloys experienced more extensive pitting

corrosion than their as-received counterparts.

Solution collected from *in vitro* corrosion over 10 or 24 days was tested for the concentration of zinc and copper ions using ICP. The results are summarized in Table 5. There were no significant differences between the concentration of copper ions in the solution for the different alloys for both time points. The near-zero concentration of copper ions suggests that the biological response differences reported in the subsequent figures are mainly related to variations in material corrosion between the materials that impact the local Zn concentration.

3.3. Nitric oxide release

NO analysis demonstrated that pure copper generates the greatest amount of NO over 2 h compared to high-purity Zn and Pt reference wires and Zn–Cu alloys (Fig. 6). The bumps within the data for a material indicate non-uniform pitting corrosion activity, which increases

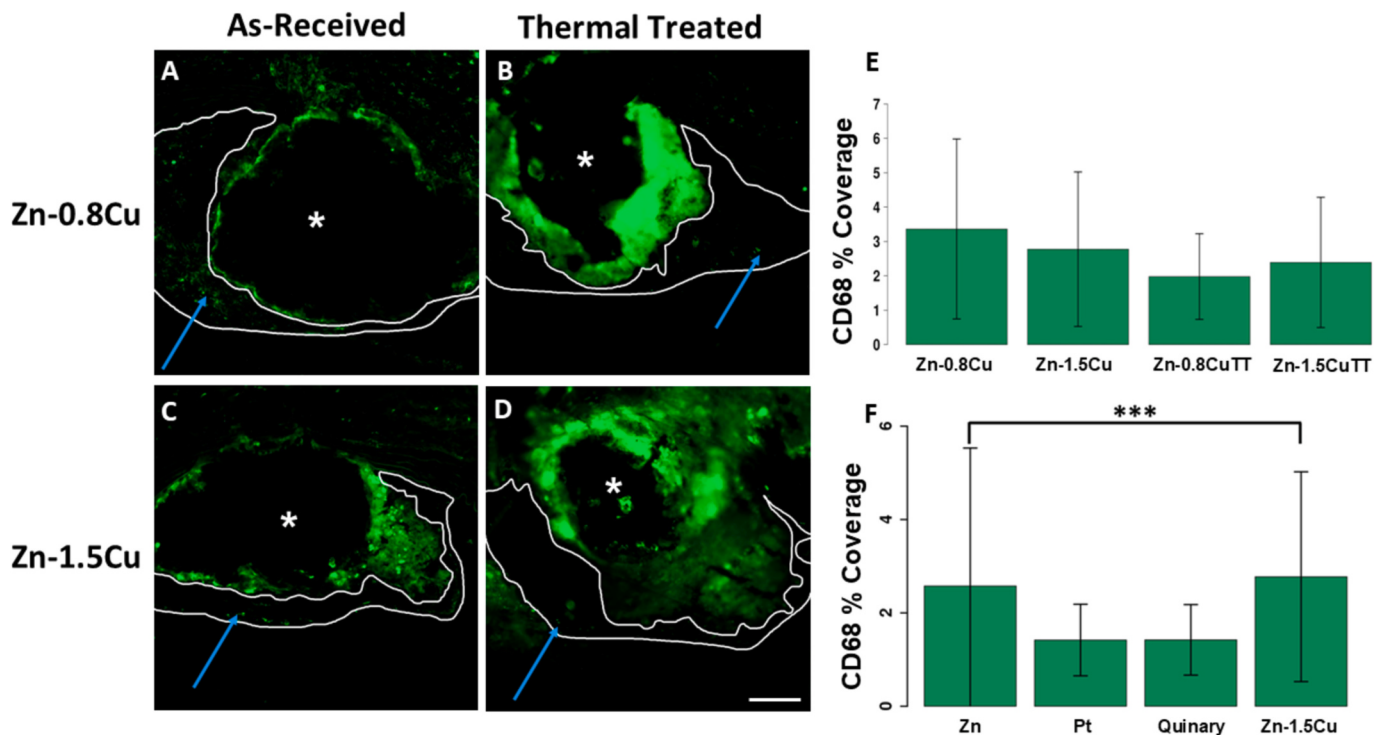


Fig. 10. CD68 immunofluorescent labeling was used to quantify macrophage signal present in the neointimal tissue. A–D) Representative CD68 images for each alloy. White outline is of neointimal tissue determined through DAPI labeling. Blue arrow identifies an example of positive signal. Scale bar in D is equal to 200 μ m. E) Graph of average % CD68 coverage for alloys. No statistical significance was found (Zn-0.8Cu $n = 12$, Zn-0.8CuTT $n = 12$, Zn-1.5Cu $n = 10$, Zn-1.5CuTT $n = 12$). F) Graph of average % CD68 coverage for Zn and platinum controls, previously reported quinary alloy [13] and Zn-1.5Cu. Asterisks display statistical significance (***) (p -value < 0.001). “TT” stands for thermal treatment.

NO catalyzed metal ion release in a burst fashion. There is a bump in NO release from pure Zn because ionic Zn is also a catalyst for NO generation [14]. But bumps are generally more frequent and extensive in the alloyed metal because ionic copper is a far more robust NO-generating catalyst than ionic Zn. The low NO generation from the Zn–Cu alloys demonstrates that a 0.8 wt% or 1.5 wt% copper inclusion in the Zn alloy generates a low average level of NO release.

3.4. *In vitro* thrombogenicity

To investigate thrombogenicity of the metals, an *in vitro* biochemical and colorimetric assay was used to determine contact pathway activation in human plasma (Fig. 7). All materials generated significantly lower FXIIa compared to the CoCr clinical control ($p < 0.05$). Additionally, higher quantities of FXIIa were produced in the pure Zn condition compared to the Zn-0.8Cu condition ($p = 0.019$). The fibrin generation assay was performed in human platelet-poor plasma (PPP), as well. There was no significant difference found in fibrin generation rate between materials. Zn-0.8Cu had a significantly delayed fibrin take-off time compared to pure Zn ($p = 0.014$), whereas Zn-1.5Cu did not ($p = 0.22$). All materials were significantly faster in activation time than the plasma alone ($p \leq 0.05$), where a faster time indicates greater thrombogenicity.

3.5. *In vivo* studies on biodegradation and biocompatibility

3.5.1. Hematoxylin and eosin staining/cell morphology

H&E staining was used to visualize cell morphology and general tissue response. Limited neointimal tissue growth is evident for all material and processing conditions (Fig. 8). The thermal treated alloys provoked some areas of moderate inflammation in the arterial wall and mild inflammation in the neointimal cap that was generally absent from the non-processed counterparts. Zn-1.5CuTT presented with some

regions of necrotic neointimal tissue, as depicted by the acellular regions in the neointima (Fig. 8D, L).

3.5.2. Morphometrics

Neointimal area (NA) and thickness at apex (WLT) measurements were conducted from the VVG images (Fig. 9). For both metrics, Zn-1.5Cu (WLT = 25 ± 10 μ m, NA = 0.06 ± 0.03 mm) was significantly less than Zn-0.8Cu (WLT = 54 ± 38 μ m, NA = 0.09 ± 0.04 mm). Furthermore, for both metrics, Zn-1.5Cu measurements were 6 times smaller for WLT and 1.5 times smaller for NA than its thermally treated counterpart Zn-1.5CuTT (WLT = 145 ± 43 μ m, NA = 0.09 ± 0.03 mm). The increase in morphometrics detected for the thermal treated alloys may be attributed to the increased inflammation that was seen in Fig. 8. Moreover, the worsened morphometric measurement for Zn-1.5CuTT could also be attributed to highly localized zinc toxicity. This was evident as areas of necrosis in Fig. 8D & L and is suggestive of a non-uniform pitting corrosion mode for that material/processing condition. When the best-performing Zn–Cu binary alloy (Zn-1.5Cu) was compared to reference materials, we found a similar neointimal area and an improved wire-to-lumen thickness (WLT).

3.5.3. CD68

Low levels of inflammatory infiltration into the neointima were detected based on CD68 labeling for macrophages (Fig. 10). There were no significant differences between the alloy and treatment groups regarding CD68 coverage. The results demonstrate that the different copper concentrations and the thermal treatment do not significantly impact the inflammatory response within the neointimal tissue. However, when the best-performing Zn–Cu binary alloy (in terms of morphometrics) was compared to reference materials (Fig. 10F), it was found to have generated a slightly worsened inflammatory response relative to pure Zn (2.8 ± 2.3 % vs 2.6 ± 3.0 %).

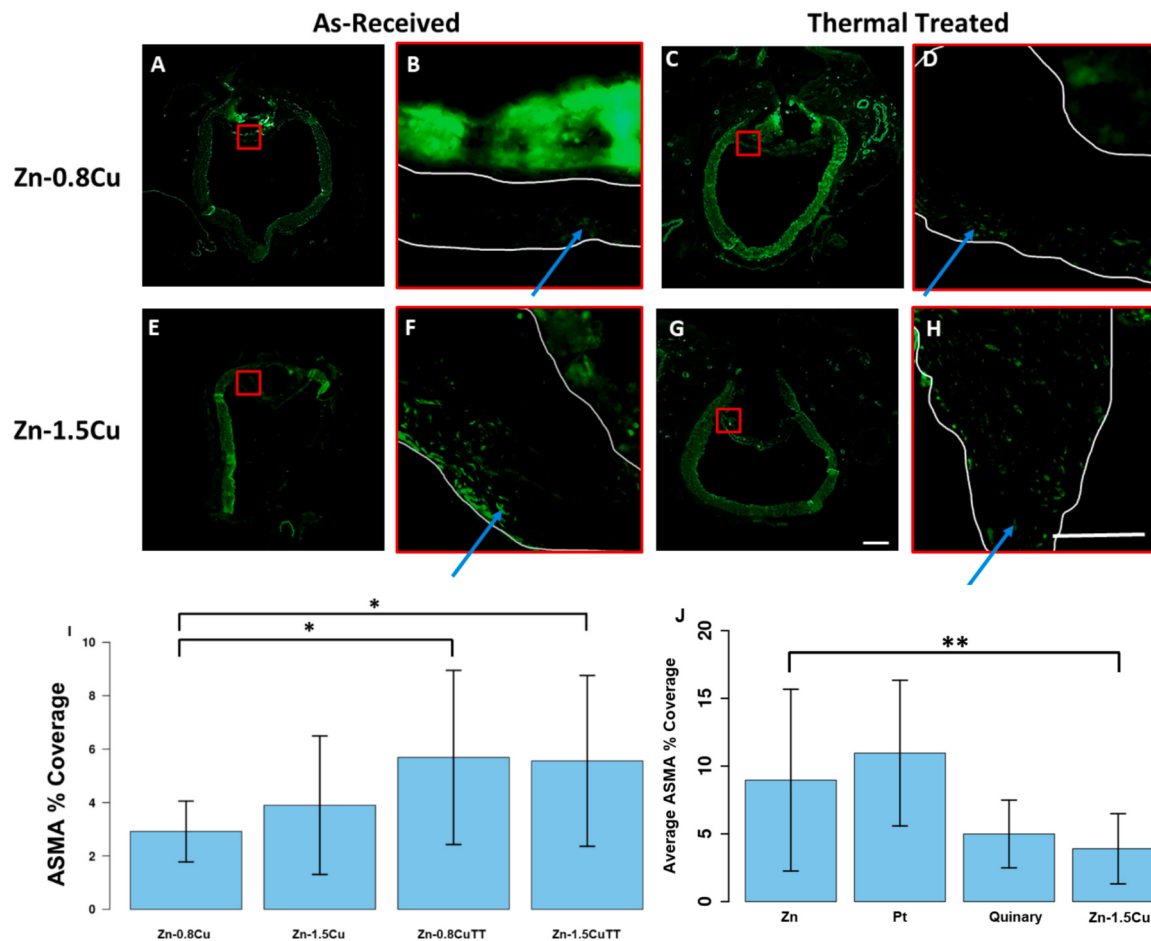


Fig. 11. α -SMA immunofluorescent labeling was used to quantify levels of smooth muscle cells in the neointimal tissue. A–H) Representative α -SMA images for each alloy. Blue arrows identify an example of positive signal. Scale bar in G is equal to 200 μ m. High magnification images show the area of highest signal detected within the neointima for each material. Scale bar in H is equal to 20 μ m. I) Graph of average % α -SMA signal for alloys. Statistical significance is shown by asterisks. * p -value < 0.05 J) Graph of average % α -SMA signal for zinc and platinum controls, previously reported quinary alloy [13] and Zn-1.5Cu. Statistical significance is shown by the asterisks (** p -value < 0.01). (Zn-0.8Cu n = 12, ZnCu1.6 n = 10). “TT” stands for thermal treatment.

3.5.4. α -SMA

α -SMA signal tended to localize along the luminal side of the neointima for each of the material types (Fig. 11A–H). This is consistent with prior reports that ionic Zn byproducts of corrosion promote the apoptosis of smooth muscle cells [26]. There was a substantial increase in neointimal smooth muscle cell coverage for the thermally treated materials (Fig. 11I) (5.7 ± 3.3 % and 5.6 ± 3.2 %). This corresponds to the worsened neointimal morphometrics. There was a significant reduction in α -SMA signal coverage for the Zn-1.5Cu material relative to the pure Zn reference (Fig. 11J) (3.9 ± 2.6 % vs 9.0 ± 6.7 %).

3.5.5. In Vivo Corrosion

In vivo corroded wires all displayed a Zn oxide layer (Fig. 12), consistent with the known corrosion products of Zn–Cu alloys [18]. Phosphate was also observed, typical for biodegradable metal implants [9,10]. The Zn-1.5Cu and Zn-1.5CuTT alloy experienced more uniform surface corrosion than the Zn-0.8Cu and Zn-0.8CuTT alloys. Moreover, there is copper enrichment at the interface between the non-degraded wire and the corrosion product for the 1.5 wt% composition (Fig. 12G and H). The Zn-0.8Cu alloy exhibited the most evident signs of intergranular corrosion (Fig. 12A).

4. Discussion

Here, we report on the biological impact of degrading Zn–Cu binary

alloys with 0.8 and 1.5 wt% additions of copper. Copper is included because it is considered a promising grain refiner in Zn metals and has become a common alloying component of advanced Zn alloy systems [27–29]. However, the biological effects of the copper component have yet to be investigated. In addition to impacting the metal’s structural and corrosion properties, elements alloyed into biodegradable metals become bioavailable as metal ions during the bulk metal degradation process. Many metal ions participate in biological activities and can dramatically alter protein and cellular functions [30–32]. Although the mechanical strength of the present Zn–Cu binary alloys is unsuitable for arterial stenting applications, the use of binary alloys made it possible to investigate the contribution of copper additions to the biocompatibility of Zn alloys. Two copper concentrations within the zinc matrix were selected in combination with conventional thermal treatments, which successfully improved the dissolution of the copper-rich precipitates, as intended (Table 2).

The similarity of average zinc and copper release into surrounding fluids during *in vitro* corrosion for the different materials (Table 5) strengthens the notion that localized differences in corrosion activity were responsible for the differences in biological responses. Aside from pitting corrosion that was more extensive for the thermal-treated metals, the analysis of *in vivo* samples demonstrated an intergranular bulk corrosion mechanism for Zn-0.8Cu, which appears slightly reduced for the thermal-treated counterpart (Fig. 12). Corrosion for the alloys with 1.5 wt% copper was relatively more uniform. Better resistance against

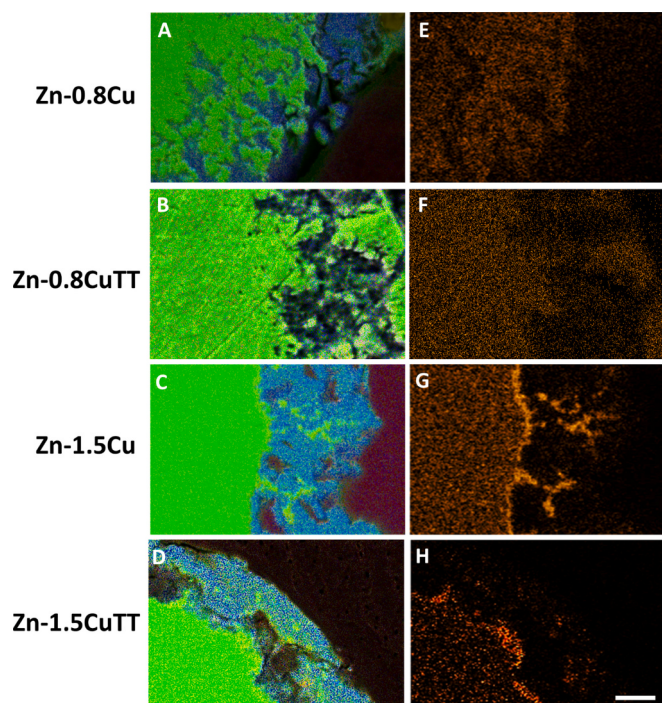


Fig. 12. SEM imaging was used to examine the degree of biodegradation and identify composition of corrosion products A–D) Representative SEM images of implanted wire polished cross sections with magnification of 1950 $\times$ showing all elements (colors: Zn — green, Cu — orange, P — yellow, C — red, O — purple). E–H) Corresponding elemental copper maps “TT” stands for thermal treatment. Scale bar in H is equal to 10 μ m.

pitting corrosion may explain why the Zn-1.5Cu material elicited a more biocompatible response than the other Zn–Cu metals and pure zinc. Although inflammation for Zn-1.5Cu relative to pure Zn slightly increased, alpha actin coverage and neointimal growth were significantly reduced. This suggests that a 1.5 wt% concentration of copper may be a suitable starting point for developing Zn alloys.

Copper (I) ions are known to strongly catalyze the release of nitric oxide (NO) from NO donors, such as S-nitrosothiols (RSNOs), as well as endogenously circulating NO donors [14]. NO, which is released endogenously in the vascular system by vascular endothelial cells, exerts strong anti-thrombotic, proliferative, and inflammatory effects [15–17]. A detectable amount of NO was generated from high purity metal wires, as-received alloy wires, and *in vitro* corroded wires (Fig. 6). The pure copper metal reference, which is known to strongly catalyze NO release, exhibited the highest level of NO release, followed by the corroded wires and then the as-received wires. The corroded materials may have produced more NO because of an increased surface area at the metal surface due to corrosion activity. Between the binary alloys, there was generally a low level of NO generation (relative to both pure copper and the zinc quinary alloy) as well as no distinct trends in NO release. Moreover, the ZnCu binary alloys were all found to reduce FXIIa activation, confirming the presence of bioactivity at the surface. Due to the importance of NO generation in the artery to generate beneficial biological effects, future work with Zn–Cu alloys should dissolve higher concentrations of copper within the Zn matrix to investigate the biological effects of increased NO release from the implant.

We found low levels of neointimal growth for both alloys and their thermal-treated counterparts. However, there was a relative increase in neointimal morphometrics for the thermally treated alloys. The increased neointimal tissue growth for the thermally treated alloys, as measured by morphometrics, may be partly attributed to the increased mural inflammation that was detected in the H&E staining (Fig. 8) and neointimal SMC presence that was detected from the alpha-actin

labeling (Fig. 11). Moreover, the worsened morphometric measurement for Zn-1.5CuTT could also be attributed to highly localized Zn toxicity. Indeed, this was evident as an area of necrosis (Fig. 8 D&L) and suggests a non-uniform pitting corrosion mode for that material/processing condition. Pitting corrosion was subsequently confirmed by micro-CT imaging as the dominant mode of corrosion for materials evaluated *in vitro* for 10 days (Fig. 5). Therefore, we speculate that more extensive pitting corrosion due to the thermal treatment led to an elevated Zn concentration at localized areas that worsened inflammation, cell viability, and neointimal hyperplasia of SMCs. The concentration of inflammation on the mural side of the implant and its relative absence in the neointima may be a consequence of the endothelium that covers the neointima tissue [33] and acts to suppress inflammation [34,35]. The lack of neointimal inflammation also demonstrates the good biocompatibility of all the metals, as we have found that alloying Zn generally worsens inflammation and neointimal morphometrics to some degree [8,12,13].

It was a surprise that thermal treatment worsened the pitting corrosion activity of Zn–Cu binary alloys. Although thermal treatment increases the solubility of the CuZn₅ secondary phases and thereby provides improved corrosion resistance, it can also increase the grain size [21], thereby increasing pitting corrosion. Since thermal treatments are generally an effective method for improving the mechanical properties of Zn alloys, additional alloying elements are needed to stabilize the grain boundaries and resist grain growth, to address the pitting corrosion susceptibility. Even without thermal treatment, the binary alloys experienced pitting corrosion. However, the thermal treatment worsened the pitting corrosion.

In addition to harming the mechanical function of a stent, pitting corrosion of biodegradable arterial implants is related to adverse biological effects. For instance, the excessive metal ion concentration that localizes near the surface of pitting sites can provoke cell toxicity and inflammation. When Zn ion concentrations surpass buffering mechanisms inside the cell, mitochondrial function is altered, which can lead to the initiation of the apoptosis cascade [36]. Moreover, supra physiological intracellular levels of copper ions can generate oxidative damage [37]. Both copper and zinc ions can be rendered relatively non-toxic by binding to metallothionein, an intracellular protein. However, copper and zinc compete for metallothionein binding sites [38]. Based on the substantially higher zinc concentrations relative to copper, as measured by ICP analysis, it is possible that Zn ions preferentially saturated the metallothionein binding sites near a pitting site. This could allow the remaining unbound intracellular zinc and copper to generate toxic effects inside local cells. Therefore, copper ions may be able to generate toxic effects inside cells even at low levels, when co-present with Zn ions. Such a mechanism may explain the necrotic regions seen for the Zn-1.5CuTT implants. Even if toxic cellular effects are not readily apparent in young, healthy animals, they may still be problematic in the aged and diseased arteries of the clinically targeted human population. For these reasons, a uniform mode of metal degradation is highly preferred.

5. Conclusion

The as-received Zn-1.5Cu implant metal exhibited the most biocompatible morphometric measurements relative to other alloys, as well as to pure Zn and Pt reference metals. There was significantly less smooth muscle cell and slightly more macrophage presence within the neointimal tissue around the Zn-1.5Cu implant relative to pure Zn. No trends were found between the alloys for nitric oxide release. The small inclusions of copper (0.8 and 1.5 wt%) were not enough to generate substantial nitric oxide. A localized pitting corrosion mode dominated for all alloys, which was worsened by the thermal treatments. The Zn-1.5Cu implant exhibited a more uniform corrosion mode of degradation relative to the other alloys, which may have contributed to the more favorable biological response.

CRediT authorship contribution statement

Lea Morath: Writing – review & editing, Writing – original draft, Visualization, Validation, Resources, Investigation, Formal analysis, Data curation. **Shebeer A. Rahim:** Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation. **Cole Baker:** Writing – review & editing, Writing – original draft, Validation, Investigation, Formal analysis. **Deirdre E.J. Anderson:** Writing – review & editing. **Monica T. Hinds:** Writing – review & editing. **Malgorzata Sikora-Jasinska:** Validation, Investigation. **Lindy Oujiri:** Writing – review & editing, Investigation. **Lisa Leyssens:** Writing – review & editing. **Greet Kerckhofs:** Writing – review & editing. **Grzegorz Pyka:** Writing – review & editing, Validation, Investigation. **Alexander A. Oliver:** Writing – review & editing. **Jaroslav W. Drelich:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. **Jeremy Goldman:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The authors are unable or have chosen not to specify which data has been used.

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