Antimicrobial silver-hydroxyapatite composite coatings through two-stage electrochemical synthesis

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Abstract

A two-stage electrochemical synthesis method was developed to prepare antimicrobial silver-hydroxyapatite (Ag-HA) composite coatings. In the first stage, a titanium substrate was coated with hydroxyapatite (HA) through electrochemical crystallization from aqueous solution. In the second stage, silver nanoparticles were deposited onto the HA layer through electrochemical reduction of aqueous Ag^+ to Ag^0 . Silver nanoparticles having a narrow size distribution were found to deposit uniformly on the HA layer. The quantity and size of silver nanoparticles were shown to be controlled by the electrochemical deposition time under constant applied electrical current density. Ag-HA composite coatings were found to be bioactive based upon the promotion of the deposition of additional calcium phosphate onto the Ag-HA coating surface from simulated body fluid. Staphylococcus aureus bacteria growth was inhibited by coatings of Ag-HA, while HA coatings without silver had little effect on growth of the bacteria. The novel composite Ag-HA coatings can potentially be used in orthopedic and dental implants to simultaneously improve osseointegration and prevent post-surgical infection.

Keywords:

Hydroxyapatite, Electrochemical, Composite, Silver

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1. Introduction

Hydroxyapatite (HA) is a calcium phosphate compound that is found naturally as the mineral component of teeth and bone tissue. Orthopedic and dental implants are often coated with HA or related calcium phosphate compounds in order to speed osseointegration following surgery [1]. A number of in vivo and clinical studies have shown that HA promotes the osseointegration of load bearing implants [2–4]. As a result of its bioactivity, HA coatings have been used commercially for decades in dental and orthopedic applications. Commercial HA coatings are most commonly applied to implants using plasma spray [5], but a variety of other low temperature techniques can be used, including sol-gel synthesis [6], electrophoretic deposition [7], and electrochemical crystallization [8]. The electrochemical method allows crystalline HA to be deposited rapidly from aqueous solution under mild operating conditions. In addition, curved surfaces such as dental implant screws and orthopedic fixation pins may be evenly coated electrochemically in a single step. It was recently discovered that sub-micron thick HA coatings deposited electrochemically retain strong electrical polarization that is known to promote osseointegration [9]. The electrochemical technique therefore offers a low cost route for surface modification of implants with HA that has chemical and electrical properties that promote osseointegration. Strongly adherent, sub-micron thick HA layers can be electrochemically synthesized on the surface within a few minutes.

One of the most serious complications following implant surgery is bone infection. To reduce the incidence of bacterial film formation on implant surfaces, local delivery of antibiotics is desirable [10]. In some procedures, surgeons use polymeric bone cement as a carrier for antibiotic release. Bone cements are typically based on non-biodegradable poly(methyl methacrylate), making the cement a non-ideal material for extended drug delivery. Moreover, there is evidence that local delivery of antibiotics significantly increases the likelihood of infection with antibiotic resistant bacteria following implant revision surgery [11]. One of the major concerns with the use of antibiotics is the relatively rapid rate that bacteria can become resistant to treatment [12, 13]. Rapid clearance of antibiotic molecules from the body requires repeated doses for systemic delivery, and makes extended release through local delivery challenging.

Silver is well known for its broad spectrum antimicrobial activity, and has been gaining increasing attention as an alternative to traditional antibiotic molecules. It has high efficiency and low toxicity for long-term use [14–16]. Silver ions, silver compounds and silver particles have been used in pharmaceuticals and in medical

38 devices to replace antibiotics or other antibacterial agents [17–21]. Bacteria may
39 be killed upon surface contact with metallic silver nanoparticles, and through the
40 extended release of low concentrations of silver ions through oxidative dissolution
41 of the nanoparticles [22]. Silver nanoparticles have the advantages of long
42 term antimicrobial activity from a single application, and a low probability of a
43 microorganism developing resistance. A variety of chemical and physical meth-
44 ods have been developed to synthesize silver nanoparticles [23–26]. Among these
45 approaches, the reduction of Ag^+ to Ag^0 is attractive for the potential to rapidly
46 produce large quantities of product at low cost [27–30]. Silver ions can be reduced
47 either chemically or electrochemically, with various synthesis methods allowing
48 key nanoparticle properties such as particle size, size distribution, purity, crys-
49 tallinity and shape to be controlled. Silver nanoparticles have been formed within
50 thin ceramic coatings made through sol-gel synthesis by chemically reducing Ag^+
51 to Ag^0 [31–33].

52 Silver nanoparticles or silver atoms incorporated into HA coatings provide an-
53 timicrobial properties while maintaining the bioactivity of HA. Several methods
54 have been used to create silver-containing HA coatings, including plasma spray
55 [34], sputter coating [35], sol-gel synthesis [36], electrolytic synthesis followed
56 by ion exchange [37], and electrophoretic deposition [38]. In the present study,
57 a novel two-stage coating process is reported in which two electrochemical reac-
58 tions are carried out in sequence to form a silver-hydroxyapatite (Ag-HA) com-
59 posite thin film on a titanium substrate. The present study is motivated by the
60 recent discovery that electrochemically synthesized sub-micron thick HA coat-
61 ings retain strong quasi-permanent electrical polarization [9]. Electrical polariza-
62 tion is known to enhance biomineralization, cell adhesion, cell proliferation, and
63 osseointegration [39–41]. The first stage of the synthesis is the electrochemical
64 crystallization of HA from an aqueous solution onto a titanium substrate acting
65 as a cathode. In the second stage, silver is electrochemically reduced from silver
66 ions in aqueous solution onto the HA-coated titanium. The two stages are carried
67 out under similar operating conditions, offering a rapid low cost implant coating
68 method. The end result is a sub-micron thick HA coating decorated with silver
69 nanoparticles. Separating the process into two stages allows the HA coating prop-
70 erties to be controlled independently of the silver loading. The silver loading can
71 then be controlled in the second without altering the synthesis conditions known
72 to produce polarized HA in the first stage. The resulting Ag-HA coating has the
73 potential to act as an antimicrobial implant surface that inhibits bacterial growth
74 while simultaneously enhancing osseointegration. Previous studies have shown
75 that the loading of silver into HA must be optimized in order to provide an an-

tibacterial effect without inducing cytotoxicity, as reviewed recently [42]. Future work will examine the cytotoxicity of the Ag-HA coatings made by two-stage electrochemical deposition in order to optimize the silver loading for potential in vivo applications.

2. Experimental procedure

2.1. Materials

Tris(hydroxymethyl)aminomethane (>99.8%), $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ (>99%), NaCl (>99.0%), and K_2HPO_4 (99.99%) were purchased from Sigma-Aldrich. Aqueous HCl solution (37%) was purchased from Mallinckrodt Chemicals. Titanium (Ti, 0.89 mm thick) and platinum (Pt, 0.127 mm thick) foils were obtained from Alfa Aesar. AgNO_3 (ACS grade) was obtained from Amresco.

2.2. Electrochemical crystallization of HA

A pre-cleaned Ti plate (12.5 x 12.5 x 0.89 mm) was used as the cathode while a Pt foil (25 x 25 x 0.127 mm) was used as the anode in the electrochemical crystallization. An electrolyte solution was prepared by dissolving 138 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane, 1.3 mM CaCl_2 , 0.84 mM K_2HPO_4 in deionized water. The solution was buffered to pH=7.2 with 37% HCl. The anode and cathode were immersed into the electrolyte solution. The electrolyte solution was heated in an oil bath to maintain constant temperature. When the temperature of the electrolyte solution arrived at $\sim 95^\circ\text{C}$, a constant current density of 12.5 mA/cm^2 (relative to Pt electrode area) was applied for 2 minutes. The reaction results in selective crystallization of HA onto the titanium cathode.

2.3. Electrochemical reduction of silver

After the electrochemical deposition of HA, the electrodes were transferred to a 125 ml aqueous solution of NaCl. The solution was heated to 95°C under vigorous stirring. Then AgNO_3 having same concentration as the NaCl was added to the solution immediately before applying a DC electric field. Deposition time, was varied to investigate its effects on Ag-HA formation. The electrochemical reaction resulted in selective deposition of Ag nanoparticles onto the HA coated titanium cathode.

2.4. Characterization of the coatings

Morphology and composition of the samples were examined using a Zeiss-Leo DSM982 field emission scanning electron microscope (SEM) equipped with a Phoenix EDAX energy dispersive X-ray spectrometer (EDX). Surface composition was obtained with a Kratos AXIS Ultra DLD X-ray photoelectron spectrometer (XPS), equipped with a monochromatic Al anode X-ray gun ($K\alpha=1486.6$ eV). For crystal structure determination, four identically synthesized samples were scraped off the titanium substrate and combined to obtain enough material in the form of powder to analyze with X-ray diffraction (XRD). The powder was then analyzed with a Philips PW3020 XRD spectrometer using Cu $K\alpha$ radiation ($\lambda = 1.5418$ Å).

2.5. Mineralization from simulated body fluid

Solutions of 1.5× simulated body fluid (SBF) were prepared following the literature [43]. Ag-HA composite coatings were placed into vials containing 5 ml of 1.5× SBF at 37°C with pH of 7.25. After 24 hours, the samples were taken out of the solution, rinsed with deionized water, and placed in a desiccator to dry. The mineralization was examined using SEM of the dried samples. Electrochemically synthesized HA coating without silver deposition was used as a control sample for comparison.

2.6. Antibacterial activity

A solution of *E. coli* (BL21(DE3)) bacteria in tryptic soy broth was grown overnight with shaking at 37°C. The bacteria concentration was then diluted with tryptic soy broth until the solution's absorbance value at 490 nm was 0.01. Bacteria growth curves were produced by placing 3 HA and 3 Ag-HA samples into wells of a 24 well plate. Each well was then filled with 2 ml of bacteria suspension, as well as three control wells with just bacteria suspension. The samples were then incubated at 37°C. Bacteria growth was measured at various times (0 hr, 2 hr, 4 hr, and 8 hr) using light scattering at 490 nm (PerkinElmer EnSpire Multimode Plate Reader) by placing 1200 µL of bacteria solution into 6 wells (200 µL in each well) of a 96 well microtitre plate. The 1200 µL solution was then placed back into the solution of their respective samples after each measurement. To measure a number of live bacteria in each treatment condition, bacteria solutions at the 0-hr time point and 8-hr time point were used to spread onto tryptic soy agar plates. The agar plates were then incubated at 37°C for 12 hr. Numbers of the bacteria colonies from each plate were counted.

3. Results and Discussion

3.1. Deposition of silver nanoparticles onto HA by electrochemical reduction

In the first electrochemical reaction stage, HA was crystallized onto a commercially pure titanium substrate using a current density of 12.5 mA/cm^2 at $\sim 95^\circ\text{C}$ for 2 minutes, following the same procedure described in recent publications [9, 44]. The electrochemical synthesis of HA under these conditions produces a uniform submicron thick layer of rod-shaped HA crystals. Previous studies have demonstrated that the longest crystal axis is associated with the crystallographic c-axis, and that there is some preferential orientation of the c-axis normal to the substrate [45, 46]. The first electrochemical stage was kept constant in all experiments. In the second electrochemical reaction stage, the HA-coated titanium was transferred to a different electrolyte solution and used as the cathode for the electrochemical reduction of Ag^+ to Ag^0 .

In the second stage, the HA coated titanium was placed in a solution of 1.25 mM NaCl while heating to 95°C . When the desired temperature was obtained, 1.25 mM of AgNO_3 were added under vigorous stirring. Upon adding AgNO_3 , the precipitation of AgCl immediately occurred. The precipitation of AgCl was employed to keep the concentration of Ag^+ ions low and relatively constant. The solubility of AgCl in water at the reaction conditions results in a concentration of free Ag^+ ions of approximately 0.14 mM [47]. After adding the AgNO_3 95°C , a constant current density of 12.8 mA/cm^2 (based on the surface area of the platinum anode) was applied for 90 s in order to electrochemically reduce Ag^+ to Ag^0 at the surface of the HA coated titanium cathode. Fig. 1a shows an SEM image of the Ag-HA coating formed under these conditions. The rod-shaped HA crystals are visible, having a morphology similar to that synthesized under similar conditions in previous studies [9, 44]. Close inspection of the SEM image reveals that the HA crystals are covered by discrete nanoparticles less than 40 nm in diameter. It is assumed that the nanoparticles are metallic silver deposited onto the surface. Energy dispersive X-ray (EDX) analysis confirms the presence of silver, as seen in Table 1. All other elements detected in the EDX spectrum are associated with HA and the underlying titanium substrate.

Four samples synthesized under the same conditions as the sample shown in Fig. 1 were scraped off of the titanium substrate in order to obtain enough powder for XRD analysis of crystal structure. The XRD pattern from the collected powder is shown in Fig. 2. The pattern has a broad shoulder, indicating the presence of some amorphous material. The peaks in the XRD pattern were all matched to those of either metallic silver or HA powder according to the JCPDS standard

Figure 1: SEM image of a representative Ag-HA coating. Silver was electrochemically reduced using a constant current density of 12.8 mA/cm² applied for 90 s.

Element	Wt%	At%]
O	27.7	53.5
P	4.4	4.4
Ca	6.3	4.8
Ag	7.4	2.1
Ti	54.3	35.1

Table 1: Composition of Ag-HA coating as determined by energy dispersive X-ray spectroscopy on the sample shown in Figure 1.

178 patterns. The XRD pattern confirms the presence of metallic silver, and the ratio of
 179 peak height to width indicates that the crystal domain size is small. The Scherrer
 180 equation was used to estimate the size of silver crystals. The equation is written
 181 as:

$$D = \frac{K\lambda}{\beta \cos(\theta)} \quad (1)$$

182 where D is the mean size of the crystalline domain, K is the dimensionless shape
 183 factor, λ is the X-ray wavelength, β is the line broadening at half the maximum
 184 intensity (FWHM), and θ is the Bragg angle. The calculated silver crystal size
 185 based on the (111) peak is 28 nm. The crystal domain size is consistent with the
 186 size of the discrete spherical particles observed on the surface of HA crystals in
 187 SEM images of Ag-HA films.

188 Further confirmation of the presence of metallic silver particles was obtained
 189 using XPS of the Ag-HA coatings. The XPS spectrum is shown in Fig. 3. The
 190 peak positions were charge corrected relative to the C 1s peak of adventitious
 191 carbon on the sample surface. The peaks located at 374.0 eV and 368.0 eV corre-

Figure 2: XRD pattern of Ag-HA coating obtained by applying a constant current density of 12.8 mA/cm² for 90 s.

192 spond to Ag 3d3/2 and Ag 3d5/2, respectively. No other peaks were observed that
 193 could be attributed to other forms of silver. Since XPS is very sensitive to the el-
 194 elemental composition on the surface, the spectrum indicates that the nanoparticles
 195 observed on the HA coating are comprised of metallic silver.

196 It is believed that metallic silver is deposited on the surface due to the electro-
 197 chemical reduction of Ag⁺ through the cathodic reaction:

198 The reaction requires electrons to be transported from the cathode to silver cations
 199 adsorbed on the cathode surface. Since the cathode is almost completely covered
 200 by a layer of HA, the HA layer acts as a resistive barrier to electron transport.
 201 Cyclic voltammetry experiments on HA thin films have shown that HA becomes
 202 electrically conductive after applying a field strength of 15 kV/cm [48]. The HA
 203 layer on the cathode under these conditions is less than 1 μm in thickness [9].
 204 Therefore, a voltage drop across the HA film thickness of less than 1.5 V is suf-
 205 ficient to have significant electrical current through the HA coating. To maintain
 206 a constant current density of 12.8 mA/cm² under the conditions used for electro-
 207 chemical reduction of silver, the required potential across the electrodes was over
 208 20 V due to the relatively low ionic strength of the solution in equilibrium with

Figure 3: XPS spectrum of Ag-HA coating obtained by applying a constant current density of 12.8 mA/cm² for 90 s.

precipitated AgCl. The voltage drop across the HA layer is likely sufficient to allow electrical conduction through the coating. The presence of silver nanoparticles at the tips of rod shaped HA particles, as seen in Fig. 1a, is supporting evidence that the HA nanocrystals are electrically conductive during the synthesis so that Ag⁺ is directly reduced on the surface.

The role of NaCl during synthesis is to lower and moderate the concentration of Ag⁺ by precipitating solid AgCl. To confirm this, two control experiments were carried out. In the first experiment, the same procedure used to produce the sample shown in Fig. 1a was followed except that NaCl was not added. In the second electrochemical reaction stage, the electrolyte solution consisted of 1.25 mM of AgNO₃. An SEM image of silver nanoparticles deposited electrochemically from the 1.25 mM AgNO₃ solution is shown in Fig. 4a. With the higher Ag⁺ concentration of 1.25 mM, the silver particles that are observed are much larger. Spheroidal silver particles are visible on the surface having diameters averaging approximately 75 to 100 nm. In a second control experiment, the same procedure was used except that the Ag⁺ concentration was reduced to 0.08 mM. To maintain similar ionic strength as that of the 1.25 mM AgNO₃ solution, 1.25 mM NaNO₃ was added to 0.08 mM AgNO₃. An SEM image of the sample produced using 0.08 mM Ag⁺ is shown in Fig. 4b. Using a lower Ag⁺ concentration resulted in fine

228 silver nanoparticles decorating the surface of the HA crystals. Close inspection of
 229 the SEM image shows that the silver nanoparticles are approximately 15-20 nm
 230 in average diameter. In a recent study, an electrochemical cathodic reaction was
 231 used to coprecipitate HA and Ag [49]. It was found that Ag nanoparticles could
 232 only be deposited by coordinating free Ag^+ ions with cysteine. Forming coordina-
 233 tion complexes with Ag^+ has the effect of reducing the concentration of free silver
 234 cations available for electrochemical reduction. In the present study, precipitation
 235 of solid AgCl is a simple alternative that can be used to maintain low Ag^+ con-
 236 centration. In principle, the electrolyte solution could be used repeatedly, as the
 237 silver ions consumed by electrochemical reduction can be replenished at the ion
 238 concentration in equilibrium with solid AgCl. The control experiments indicate
 239 that low Ag^+ concentration is desirable for producing smaller nanoparticles.

Figure 4: SEM images of Ag-HA coatings obtained by electrochemical reduction of Ag^+ in 90 s under a current density of 12.8 mA/cm^2 from a solution of (a) 1.25 mM AgNO_3 and (b) $0.08 \text{ mM AgNO}_3 + 1.25 \text{ mM NaNO}_3$

240 To examine the nanoparticle growth rate, the silver reduction reaction was car-
 241 ried at a fixed current density while varying the time that the current was applied.
 242 The applied current density was held constant at 12.8 mA/cm^2 using an electrolyte
 243 solution of 1.25 mM AgNO_3 and 1.25 mM NaCl . The reaction time was varied
 244 from 90 s to 300 s, and the weight percentage of silver in the resulting samples
 245 was measured using EDX. Fig. 5a shows the silver weight percentage plotted ver-
 246 sus time. If Ag^+ is available at the surface of the cathode, then the total mass of
 247 silver metal deposited on the surface is expected to be proportional to the total
 248 number of electrons transferred from the cathode. Under constant current density,
 249 the number of electrons transferred is related to the current density multiplied by
 250 the time that current is applied. In addition to electrons, the silver reduction reac-

tion is also dependent on the availability of Ag^+ at the cathode surface. As Ag^+ ions at the cathode surface are consumed by the reduction reaction, the growth of silver nanoparticles requires the transport of Ag^+ to the cathode surface through diffusion and Coulombic attraction. If mass transfer of Ag^+ becomes limiting, then the silver metal deposition would slow down and would no longer be directly proportional to the quantity of electrons transferred. Instead, electrons would be consumed by the electrolysis of water, which is the primary competing Faradaic reaction. Over the time range investigated, the weight percent silver versus time is well represented by a linear fit, as seen in Fig. 5a. The results suggest that mass transfer of Ag^+ is not limiting the reaction in the time range investigated. As the reaction is carried out for longer time, the silver nanoparticles grow larger and more polydisperse. The SEM image in Fig. 5b shows the surface of the sample produced using the longest investigated time of 300 s. The SEM image shows numerous polydisperse silver nanoparticles, with the largest near 200 nm in diameter.

Figure 5: (a) Weight percent silver deposited versus time from 90 s to 300 s. The dashed line is a linear fit of the data. (b) SEM image of the sample produced after 300 s. Silver was electrochemically reduced using a constant current density of 12.8 mA/cm^2 .

3.2. *Biom mineralization from simulated body fluid*

Simulated body fluid (SBF) is a synthetic electrolyte solution that mimics the ionic strength, composition, and pH of human blood plasma [43]. When HA is submerged in SBF for extended time, additional calcium phosphate may be deposited from the SBF onto the HA surface. Calcium phosphate deposition from SBF offers an in vitro test of the activity of the HA surface for enhancing biomineralization. It is known that negative surface charge on HA promotes calcium

phosphate deposition, while positive surface charge retards calcium phosphate deposition [39]. Electrochemically synthesized HA thin films on titanium, similar to those in the present study, were recently shown to have strong negative surface charge as a result of quasi-permanent remnant electrical polarization acquired during synthesis [9]. To explore the in vitro activity of the Ag-HA thin films for promoting biomineralization, films were submerged in 1.5× SBF at 37°C and pH=7.25 for 24 hours. The solution used is called 1.5× SBF to reflect that the ionic strength is 150% of that found in human blood plasma [43]. The higher ionic strength of the SBF increases the rate of calcium phosphate deposition, enabling shorter experimental times to observe deposition [39].

Figure 6: SEM images of HA and Ag-HA after exposure to 1.5× SBF for 24 h. (a) and (b) are top and side views of the HA coating, (c) and (d) are top and side views of Ag-HA coating.

In the SBF experiments, two types of samples were compared. The first sample was HA produced after the first stage without depositing Ag nanoparticles. The second sample was the Ag-HA sample in which Ag nanoparticles were deposited in a second stage of 12.8 mA/cm² current applied for 90 s using an elec-

287 electrolyte solution of 1.25 mM AgNO_3 and 1.25 mM NaCl (the same conditions used
288 for the sample shown in Fig. 1a). After 24 hours, a new porous calcium phosphate
289 layer was deposited on both samples, as shown in Fig. 6. The top view images in
290 Fig. 6a and Fig. 6b show a porous morphology consistent with calcium phosphate
291 deposited from SBF onto HA under similar experimental conditions in previous
292 studies [9, 39]. The surface morphology is similar for the HA and Ag-HA sam-
293 ples. The side view images shown in Fig. 6b and Fig. 6d allow the thickness
294 of the layer deposited from SBF to be estimated. For both the HA and Ag-HA
295 samples, the layer deposited from SBF is approximately $1.2\text{ }\mu\text{m}$ thick. In the side
296 view images, the base of the coating is at the lower left, while the surface is at
297 the upper right. Close examination of the SEM images shows the rod-shaped HA
298 crystals synthesized electrochemically remain at the base of the coating. For the
299 Ag-HA coating, Ag nanoparticles are visible in the side view image (Fig. 6d). The
300 Ag nanoparticles appear as a line of bright dots in Fig. 6d located at a distance
301 approximately $1/3$ of the total thickness from the base of the coating. The SBF
302 experiments indicate that the electrochemical deposition of Ag has no measurable
303 effect on the activity of the thin films in promoting in vitro biomineralization. The
304 Ag nanoparticles appear to remain on the surface of the Ag-HA coating, where
305 they can potentially provide long term antimicrobial activity.

306 3.3. Antibacterial activity

307 Antibacterial activity was measured by culturing *E. coli* in the presence of HA
308 and Ag-HA coatings. The two types of coatings were prepared under the same
309 experimental conditions to create the HA and Ag-HA samples in the comparative
310 SBF experiments described in the previous section, except the first stage HA de-
311 position time was extended from 2 min to 5 min, and the silver deposition time
312 was extended to 6 min. The longer silver deposition time was chosen based on
313 preliminary experiments that showed higher silver loading was necessary to effec-
314 tively kill *E. coli*. The Ag-HA samples made under these conditions had a silver
315 content of approximately 6 atomic percent as measured by EDX. Each coating
316 was applied to a flat plate of titanium having a total surface area of 1.56 cm^2 .
317 Each coated titanium plate was submerged in 2 ml of bacteria suspension, and
318 bacteria growth was measured over time through light scattering at 490 nm from
319 samples of the bacteria suspension. A total of 3 HA coatings, 9 Ag-HA coatings,
320 and 3 controls were compared. The control samples consisted of the bacteria sus-
321 pension without a submerged titanium plate. Fig. 7 shows the results of bacteria
322 growth over 8 hours, with light scattering at 490 nm reported in absorbance units.
323 The control experiment shows the exponential increase in light scattering consis-

tent with the uninhibited growth of bacteria. The light scattering is slightly lower
 in the presence of the HA coating, but within the range of error, indicating the
 HA coated titanium alone did not significantly affect bacteria growth. With the
 Ag-HA coating, there was significant variation in the results. In 6 out of 9 sam-
 ples, bacteria growth was almost completely inhibited for 8 hours, as indicated
 by the low light scattering shown in Fig. 7 for the data labeled "Ag-HA". In the
 remaining 3 Ag-HA samples, no inhibition of bacteria was observed, When data
 from all 9 Ag-HA samples are included, as shown in Fig. 7 data labeled "Ag-HA
 (all samples)", the error bars are significantly greater, but still show statistically
 significant inhibition compared to the control and HA samples.

Figure 7: Growth of *E. coli* bacteria when exposed to HA and Ag-HA, as measured by light scattering. Data points indicate the mean value measured, and error bars indicate standard deviation.

To measure the number of live bacteria remaining, bacteria samples at the 0
 hr and 8 hr time points were spread onto agar plates and cultured at 37°C. Fig.
 8 shows the number of colony forming units counted after 12 hours on the agar
 plates. The 6 Ag-HA samples that were effective in inhibiting bacteria growth
 as measured by light scattering in Fig. 7 were also effective in killing nearly
 all bacteria, as indicated by the low number of colony forming units in Fig. 8.
 The HA coated titanium had no effect on the number of colony forming units as
 compared to the control. The Ag-HA samples that were most effective against
E. coli killed nearly all of the bacteria very quickly after exposure. Bacteria may

343 be killed upon direct contact with the silver nanoparticles, or through silver ions
 344 released in solution. One possible reason why a fraction of the Ag-HA samples
 345 were ineffective against the bacteria is the potential variability in bacteria contact
 346 with the surface. As the bacteria suspension was added to the well plate, some may
 347 bacteria sediment onto the surface, while others remain suspended or sediment in
 348 the well without contacting the silver surface. Another possibility is that the Ag-
 349 HA samples display variable silver ion release kinetics. Future work will analyze
 350 the concentration of silver ions released over time, as well cell culture directly
 351 on the Ag-HA surface in order to optimize silver loading for antibacterial activity
 352 and to investigate cytotoxicity.

Figure 8: Number of *E. coli* colony forming units after 12 hr growth onto agar plates at 37°C for bacteria exposed to HA and Ag-HA samples for 0 and 8 hr as compared to the control without a titanium plate. The height of the bars is the mean measured value and the error bars indicate standard deviation.

353 4. Conclusions

354 The two stage electrochemical method allows hydroxyapatite coatings to be
 355 prepared that are simultaneously bioactive and antimicrobial. Separating the HA
 356 film growth and Ag nanoparticle synthesis into different electrochemical stages
 357 allows the properties of both the HA coating and Ag nanoparticles to be easily

358 controlled independently. Using low concentrations of Ag^+ in solution, metallic
359 silver nanoparticles can be formed through electrochemical reduction on the HA
360 coating surface. The silver loading on the surface is directly proportional to the
361 time used for electrochemical reduction under constant current density. The tech-
362 nique therefore allows for low-cost and facile control of silver loading. The novel
363 Ag-HA coating is shown to inhibit bacteria growth, suggesting that the coating
364 may be used in biomedical implants to reduce the risk of infections following
365 surgery. Further work is needed to optimize silver loading for antimicrobial activ-
366 ity, and to examine potential cytotoxicity. In addition, the results indicate that the
367 nanoscale HA crystals are electrically conductive under moderate applied volt-
368 ages, potentially allowing other electrochemical reactions to be carried out on the
369 HA coating surface.

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