Development, Characterization, and Anti-Microbial Efficacy of Hydroxyapatite-Chlorhexidine Coatings Produced by Surface-Induced Mineralization

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Abstract: The surface-induced mineralization (SIM) technique was used to produce hydroxyapatite (HAP) coatings on external fixation pins with the antimicrobial agent, chlorhexidine, incorporated within the coating. The SIM process involved surface modification of the substrate with organic functional groups followed by immersion in aqueous supersaturated calcium phosphate solutions. X-ray diffraction spectra confirmed that hydroxyapatite coatings were formed. Chlorhexidine was incorporated into the coating by placing the substrate into various chlorhexidine solutions in between mineralization cycles. Total uptake was measured by dissolution of the coating into a 0.1 M nitric acid solution and measuring the chlorhexidine concentration using UV spectroscopy at 251 nm. Release rates were measured by submersion of coated substrates into saline solutions and measuring chlorhexidine UV absorbency at 231 nm as a function of time. Results show an initial rapid release followed by a period of slower sustained release. The anti-microbial efficacy of the HAP-chlorhexidine coatings was evaluated *in vitro* using a *staphylococcus aureus* cell culture. Initial results show a large "inhibition zone" formed around the chlorhexidine/HAP coating vs. coatings with HAP only. This preliminary work clearly demonstrates that SIM HAP coatings have great potential to locally deliver antimicrobial agents such as chlorhexidine at implantation sites, which may greatly reduce the incidence of pin tract infection that occurs in external fixation. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res (Appl Biomater) 53: 400–407, 2000

Keywords: surface-induced mineralization; chlorhexidine; hydroxyapatite; orthopedic; external fixation pin; antimicrobial

INTRODUCTION

External fixation is a widely used method of stabilizing long bone fractures in trauma patients as well as the treatment of non-union bone defects. Typically, the non-unions are stabilized via external rods and/or plates that are fixated to the bone via external fixation pins. These pins may remain in place for 6 months to a year depending upon the rate of healing, etc. The goal of external fixation is to provide fracture stabilization and allow for soft tissue healing.

Unfortunately, a significant problem with external fixation is that the pins can become infected. This results in pin loosening and loss of stabilization. Infected external fixation pins also make later definitive stabilization procedures much more risky because of the high rate of osteomyelitis.[1] Pin tract infection rates up to 50% using external skeletal fixation are well documented in the literature.[2] Bacteria that cause pin tract infections may arrive at the pin tracts by several routes. They can be introduced at the time of pin placement, they can seed the site hematogenously, or they can invade the site from the skin after the pin is placed. *Staphylococcus aureus* is the predominant organism cultured from infected pin tracts.

A popular and usually effective strategy for combating pin tract infection is the use of systemic antibiotic prophylaxis.[3-4] However, more recently, attention has focused on the localized use of therapeutic agents to fight infection. Potential

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TABLE I. Calcium Phosphate Minerals Used as Biomaterials and in Drug Delivery

Mineral	Formula	CaP/ ratio
Dicalcium phosphate dihydrate	$CaHPO_4 \cdot 2H_2O$	1.00
Octacalcium phosphate	$Ca_8H_2(PO_4)_6 \cdot 5H_2O$	1.33
Tricalcium phosphate	$Ca_3(PO_4)_2$	1.50
Hydroxyapatite	$Ca_5(PO_4)_3OH$	1.67
Tetracalcium phosphate	$Ca_4P_2O_4$	2.00

advantages of local delivery may include a decrease in the side-effects of systemic treatments and the possibility of higher dosages near the affected site thereby improving efficacy and reducing the treatment duration.

Currently, several strategies have been used to locally deliver antibiotics clinically. These include the use of biodegradable polymers,[5-7] the attachment of the therapeutic agents directly to the surfaces of metal implants,[8] and dip or spin coating the therapeutic directly onto the implant surface. While these coating methods offer new approaches for delivering therapeutic agents locally, the coating materials themselves do not form strong bonds with bone and, therefore, do not contribute to the stabilization of the pin.

In order to create a therapeutic coating that has a dual beneficial effect — osteoconductive combined with localized therapy, researchers have recently focused on materials such as calcium phosphate minerals that possess osteoconductive properties and the ability to locally deliver of therapeutic agents.[9-10] There is substantial evidence reported in the literature that shows that calcium phosphate coatings are effective in improving the bone bonding to orthopedic implants and external fixation pins.[11-12] This has resulted in improved performance of implants[13] and improved fixation of external fixator pins in both animal models and human clinical trials.[14] In addition, hydroxyapatite ceramics have been used as a delivery system for chemicals,[15] antibiotics,[16-17] and anticancer drugs.[18] Table I lists the various calcium phosphate minerals that have been used in orthopedic applications or for drug delivery (either as bulk materials or as coatings).

The goal of this work was to demonstrate the idea of using calcium phosphate coatings as delivery vehicles for antimicrobial agents to help combat pin tract infection. We have focused on using an aqueous-based surface-induced mineralization process (SIM) to produce a hydroxyapatite (HAP) coated pin that has the potential beneficial properties of improving fixation while delivering an anti-infective compound, chlorhexidine (CHX). Utilizing chlorhexidine as the anti-infective compound has numerous advantages. Chlorhexidine has long been used as a skin antiseptic due to its broad antibacterial effects. It is active against a wide range of gram-positive and gram-negative organisms. Bacterial resistance to chlorhexidine is extremely rare, which makes long-term prophylactic use less dangerous. Chlorhexidine has also been shown to decrease infection rates when coated onto intramedullary devices in an animal model.[9] In addition, chlorhexidine is currently FDA approved for coatings on intravenous catheters, and these catheters have been shown to be effective at decreasing line-related infection in human trials.[19]

MATERIALS AND METHODS

Surface Induced Mineralization

The surface induced mineralization (SIM) process was used to coat external fixator pins with a uniform layer of hydroxyapatite. A schematic of the SIM process is shown in Figure 1.

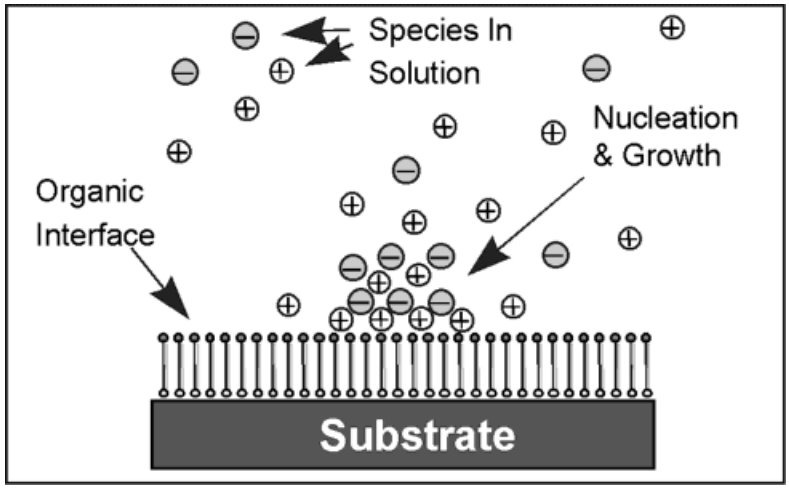

Figure 1. Schematic drawing of surface induced mineralization process. The substrate is first modified with silane coupling molecules followed by immersion in supersaturated calcium phosphate solutions. Heterogeneous nucleation and growth occurs on the templated interface.

Substrates used in this study included stainless steel external fixator pins (Synthesis), silicon wafers (polished on one side with a uniform oxide layer of 100 Å) and Ti-6Al-4V metal alloy (Goodfellow). Both the SiO_2 and Ti-alloy substrates were cut into approximately 1.25×2.5 cm coupons. The pins were used as received. Surface modification of the substrates was accomplished using self-assembled monolayers (SAMs) as the template for inducing mineralization. The SAMs where attached to the substrates attached as follows. The samples were cleaned by ultra sonication for 2–3 min in chloroform (Aldrich) in order to remove any organic material. Residual trace amounts of organic contamination were then removed by exposure of the substrates to an air plasma, produced in radio frequency glow discharge chamber**,** for 10 min. Hydroxylation of the surface was performed by placing the samples into a 0.1 M KOH solution for 2–3 min. The resulting hydroxides were then protonated by immersing the samples in 0.1 M HNO_3 solution for 10 min. The substrates were then thoroughly washed with deionized, reverse osmosis water (Millipore, MilliQ System) and blown dry with a stream of dry nitrogen gas (liquid nitrogen blow-off).

SAM formation was accomplished by placing the wafers in a 0.5 wt% vinyl terminated silane ($Cl_3Si(CH_2)_9CHCH_2$): cyclohexane solution for 30 min with gentle stirring. The samples were then rinsed in 2-propanol (Aldrich) in order to remove any residual silane followed by sonication in chloroform for 5 min.

The vinyl terminus group of the alkylsilane was subsequently modified to sulfonic acid by exposure of the derivatized substrates to SO_3 gas in a reaction vessel for 1 min. Following sulfonation, the samples were sonicated for 10 min in deionized water, and blown dry with nitrogen gas. Samples were then stored under a nitrogen atmosphere.

SAM formation on the surface and conversion of the vinyl terminus to sulfonic acid was characterized at each step by X-ray photoelectron spectroscopy (XPS), contact angle measurements, and ellipsometry. XPS and contact wetting angle measurements confirmed the near-quantitative conversion (100%) of the vinyl SAM to the sulfonic acid.

Supersaturated calcium phosphate mineralization solutions were prepared using reagent grade chemicals (Fisher Scientific) and deionized, reverse osmosis (Millipore) CO_2-free water, filtered (0.22 μm Millipore filters) before use. The filters were prewashed to remove any residual wetting agents or surfactants. Calcium and phosphorus concentrations of stock solutions were determined by inductively coupled ion plasma spectroscopy (ICP).

Calcium phosphate mineralization experiments were carried out in sealable glass containers. Supersaturated solutions were prepared by the slow addition of a calcium chloride solution to a solution containing potassium phosphate monohydrate and potassium phosphate dihydrate. Total calcium ion concentration in the supersaturated solution was 5.00×10^{-3} M. The solution Ca/P ratio was 1.67, and the pH was 6.5. Immediately following solution preparation, a rack containing the SO_3-SAM substrates was placed into the supersaturated solution, the vessel was sealed, maintained at room temperature, and gently stirred. Because homogeneous precipitation usually occurs in the supersaturated solution after about 90 min, the samples were removed after 60 min (considered 1 cycle). During this time approximately 1 μm of hydroxyapatite coating was deposited. Coatings of approximately 7 μm were prepared by successive cycles in fresh calcium phosphate solutions. After the desired thickness was obtained, the substrates were rinsed with deionized water and blown dry with N_2 gas. Samples were then analyzed by field emission scanning electron microscopy (LEO 982), X-ray diffraction (Phillips), and Fourier transform infra-red spectroscopy (Nicolet 860).

Chlorhexidine Incorporation into HAP Coatings

To incorporate chlorhexidine into the coatings, the samples were first coated with one cycle of HAP and then dipped into 2 wt% chlorhexidine digluconate (Sigma) solution (in water) and then dipped into a solution containing 0.05M KH_2PO_4 and 0.05 M Na_2HPO_4. The substrates were then returned to a fresh mineralization solution that contained chlorhexidine digluconate (4.0 mM). After mineralization, the samples were then rinsed with deionized water and blown dry with nitrogen. This process was repeated at least 4 times until the coating was of the desired thickness (6–10 μm).

Lipid Overlayer

Lipid overlayers were added to coatings prepared above by placing the substrates into a solution that contained 25 mg/mL L-α-phosphatidylcholine lipid (Sigma) and 75 mg/mL chlorhexidine digluconate in methanol for 24 h. The chlorhexidine was added to the solution to create a saturated solution so that chlorhexidine would not release from the coating due to solution undersaturation with respect to chlorhexidine. Samples were then rinsed with deionized water and blown dry with a stream of nitrogen gas**.** Lipid overlayers were used to retard the release of chlorhexidine from the coatings.

Chlorhexidine Uptake and Release

Chlorhexidine uptake and release was measured by UV spectroscopy. Calibration curves were measured by assaying solutions with known chlorhexidine concentrations either in 0.1 M nitric acid (for total uptake) or 0.15 M NaCl (for release experiments) solutions. Two different absorption wavelengths were used (231 and 251 nm) to assay the chlorhexidine. Since nitric acid produced an interference at the main peak of 231 nm, a secondary absorption peak at 251 was used to quantify chlorhexidine solution concentrations.

Bacterial Inhibition Testing

Three separate surface preparations were used in the *in vitro* bacteriological experiments. The preparations were: (1) hydroxyapatite only; (2) hydroxyapatite and chlorhexidine; and (3) hydroxyapatite and chlorhexidine with a lipid overlayer. The pins were cut into 1.5 cm pieces and three pieces of each

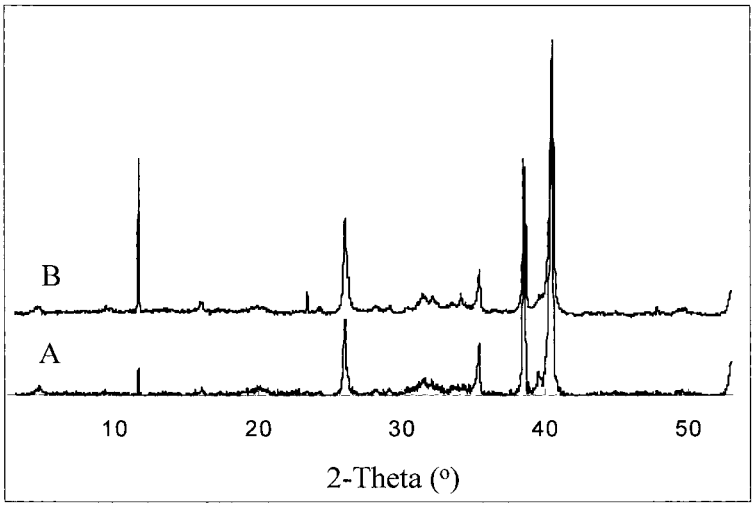

Figure 2. X-ray diffraction spectra of (A) HAP coatings and (B) HAP/chlorhexidine coatings precipitated onto flat substrates.

pin were then placed on separate plates of Meuller–Hinton media inoculated with *Staphylococcus aureus* ATCC 29213. A sixth plate was used to compare one piece from each of the five separate pins. The pin pieces were left on the media for 48 h. The "inhibition zone" that formed around the implants was measured in millimeters.

RESULTS

Coating Composition and Morphology

To ensure that the incorporation of the chlorhexidine into the coating did not significantly alter the coating chemistry or structure, X-ray diffraction (XRD), scanning electron microscopy (SEM), and Fourier transform infra red spectroscopy (FTIR) were performed. Figure 2(A) shows a typical XRD spectra for the calcium phosphate coatings without any chlorhexidine. Results show that the coating is composed entirely of hydroxyapatite ($Ca_5(PO_4)_3OH$), which is not surprising, because the solution conditions used for the deposition had a Ca/P ratio of 1.67 (similar to HAP) and were supersaturated with respect to hydroxyapatite. Similar XRD results were seen after the incorporation of the chlorhexidine into the HAP coatings [Fig. 2(B)]. There was no indication that the incorporation of the antimicrobial agent resulted in either the formation of other calcium phosphate phases or the transformation of the initial HAP layer into other materials.

Attenuated total internal reflectance FTIR spectroscopy, using a ZnSe crystal, was used to verify that the chlorhexidine compound was incorporated into the coating. In addition, the presence of the lipid overlayer was also confirmed by FTIR. Figure 3 shows the FTIR spectra obtained for (1) a hydroxyapatite coating (3A), (2) a HAP/chlorhexidine coating (3B) and a HAP/chlorhexidine/lipid coating (3C). The FTIR spectrum for the HAP coating exhibited typical phosphate absorption bands around 1050 cm^{-1}. As seen in the spectrum of the HAP/chlorhexidine coating, the chlorhexidine formed a phosphate complex that had strong absorbance peaks between 1000–1150 cm^{-1}. In addition, absorbance peaks that are indicative of the C—C (1450–1512 cm^{-1}) and C=N (1600–1670 cm^{-1}) bonds, present in the chlorhexidine molecule, appear in the spectrum. Confirmation of the lipid layer present on the HAP/chlorhexidine coating was also established using ATR-FTIR. There was no apparent alteration of the mineral chemistry or desorption of the chlorhexidine compound, as compared with Figure 3(B), but lipid presence on the coating was confirmed by the appearance of C—H stretching and bending peaks between 2800–3000 cm^{-1}.

The morphology of the coatings was evaluated using scanning electron microscopy (SEM). This technique was used to assess whether the incorporation methods altered the coating morphology, topography, uniformity, or physical structure. Figure 4 shows a typical morphology for the HAP coating without chlorhexidine. All coatings were continuous across the substrate and had a uniform thickness. As can be seen, the coatings were composed of crystalline leaflets that have nucleated and grown on the substrate surface. This morphology is in stark contrast to coatings that are produced via vapor deposition routes (Fig. 5), in which the coating morphologies appear largely amorphous; the formation of individual crystallite formation is not evident.

Chlorhexidine incorporation into the coating shows a very similar morphology to coatings with chlorhexidine (Fig. 6). There was no indication that the incorporation of chlorhexidine caused either the dissolution of the existing HAP layer or the formation of any other mineral phase. In addition, the size of the individual leaflets was similar to those found in the HAP coatings, indicating that the crystal growth rates were not significantly inhibited. Figure 7 shows the hydroxyapatite/chlorhexidine coating after the release experiments (48 h). As shown in figure, the HAP coating exhibited only a

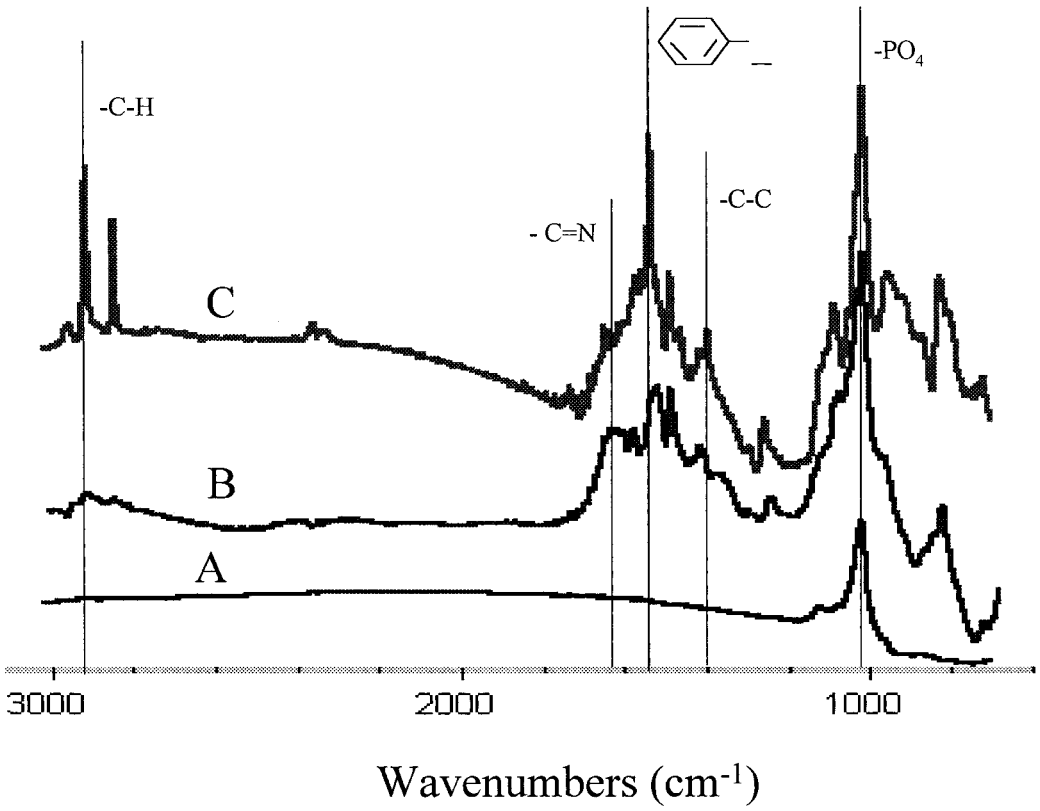

Figure 3. Attenuated total internal reflectance FTIR spectra of various coatings precipitated via SIM methods: (A) HAP only, (B) HAP/chlorhexidine coating, and (C) HAP/chlorhexidine/lipid coatings.

small fraction of dissolution as observed by the roughened crystal edges. These results indicate that the coatings topography was not significantly altered by the incorporation or the subsequent release of the chlorhexidine, and that the small amount of coating dissolution that does occur was uniform across the surface rather in the form of discrete dissolution pits.

Chlorhexidine Uptake and Release

For the chlorhexidine containing coating to be effective, it is necessary to produce a coating that contains a significant amount of chlorhexidine per unit area. To approach this problem, a deposition method was developed that involved the precipitation, from an aqueous solution, of chlorhexidine phosphate material onto an initial calcium phosphate layer**.** The formation of a chlorhexidine phosphate precipitate was

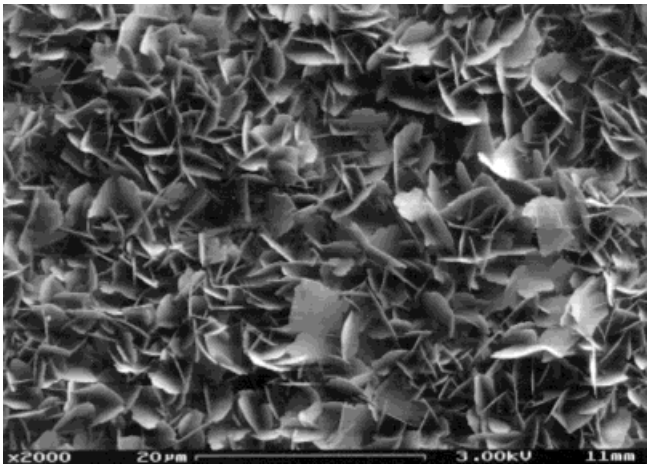

Figure 4. Scanning electron micrograph of a hydroxyapatite nucleated and grown from aqueous solutions via surface induced mineralization.

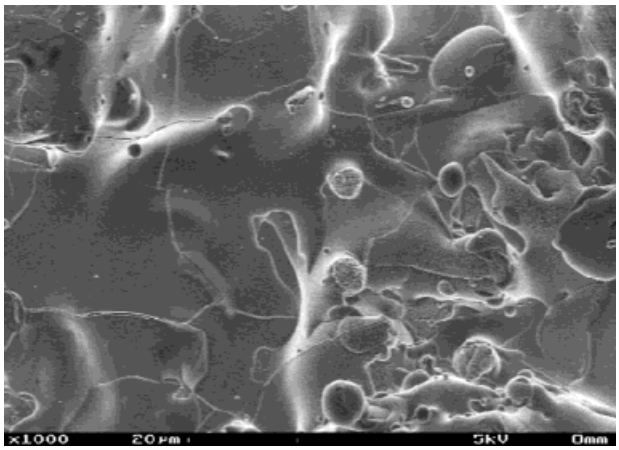

Figure 5. Scanning electron micrograph of a calcium phosphate coating produced via plasma spraying routes.

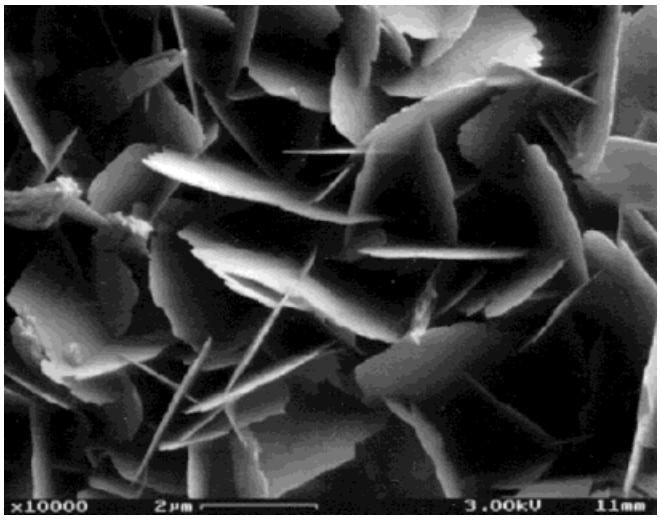

Figure 6. Scanning electron micrograph of a hydroxyapatite coating after the incorporation of chlorhexidine.

discovered serendipitously when we attempted to dissolve chlorhexidine digluconate directly into the mineralizing solution. Using this to our advantage, a method was developed that involved forming an initial calcium phosphate layer, followed by dipping the substrate briefly into a chlorhexidine digluconate solution. This was immediately followed by dipping into a phosphate solution, whereby the chlorhexidine phosphate precipitated at once on the HAP layer. While the incorporation of the chlorhexidine into the coatings was verified by FTIR, quantitative measurements on chlorhexidine uptake were measured by dissolving the coating into 0.1M nitric acid and assaying the chlorhexidine concentration by UV spectroscopy. Using this method, the total chlorhexidine uptake was determined to be from 0.10–0.17 mg cm^{-2}.

Once methods were established that produced HAP coatings that contained chlorhexidine, release profiles were investigated. Figure 8 shows a plot of the release kinetics of chlorhexidine into saline solutions as a function of time. As seen in the figure, the HAP/CHX coating exhibited a rapid initial release of chlorhexidine, which was followed by a period of slow release. In fact, almost 80% of the total chlorhexidine was released within the first several hours. Since external fixation pins may remain in place for several months, it was felt that it was necessary to extend the total release period and retard the initial release rate.

The approach taken to retard the release kinetics was to use a lipid overlayer onto the coated sample. The theory behind doing this was to place a biocompatible layer over the coating that would slowly dissolve and allow the release of the chlorhexidine. The release of the chlorhexidine would be slowed due to the presence and subsequent removal of the lipid layer. As seen in Figure 8, the lipid layer did retard the initial release rate. Whereas without lipid most of the chlorhexidine was release in the first few hours, the presence of the lipid slowed to release to over 24 h.

Bacterial Inhibition

We have preformed preliminary *in vitro* bacteriological experiments on several of the coatings produced thus far in the work. Three surface treatments on stainless steel pins were evaluated: (1) HAP only, (2) HAP/CHX, and (3) HAP/CHX/lipid. Results were based on the size of the "inhibition zone" or area around the pin on the media where the antimicrobial effect of the chlorhexidine can be seen. The inhibition zone diameters were measured in millimeters and are shown in Table II. Figure 9 shows the comparison of inhibition zone diameter for all three samples. The hydroxyapatite coating consistently showed no antimicrobial effect, with the HAP/CHX having good efficacy and HAP/CHX/lipid showing the best efficacy *in vitro*.

DISCUSSION

The goal of this work was to produce and evaluate the efficacy of antimicrobial hydroxyapatite/chlorhexidine coatings on external fixator pins using the surface induced mineralization process. Our initial results indicate that chlorhexidine was incorporated into the calcium phosphate coatings and that these coatings demonstrated significant antimicrobial efficacy *in vitro*.

The surface induced mineralization process was used to coat external fixator pin with a uniform layer of calcium phosphate mineral.[20] This process is based on the observation that, in nature, living organisms use macromolecular templates, which facilitate the nucleation and growth of mineral phases from aqueous solutions.[21-22] Rather than using biological macromolecules, the SIM process attaches simple functional groups on the underlying substrate. Once the substrate has been chemically modified, it is then placed into an aqueous supersaturated calcium phosphate solution, where solution ionic concentrations, pH, and temperature are maintained under conditions of solution supersaturated with re-

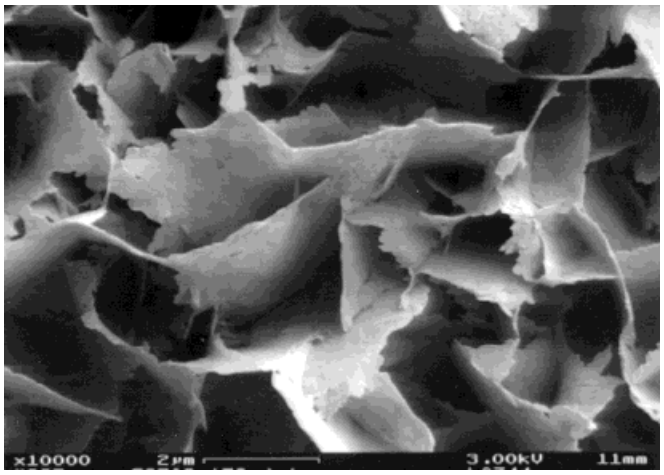

Figure 7. Scanning electron micrograph of a hydroxyapatite/chlorhexidine coating after soaking for 48 h in saline solution.

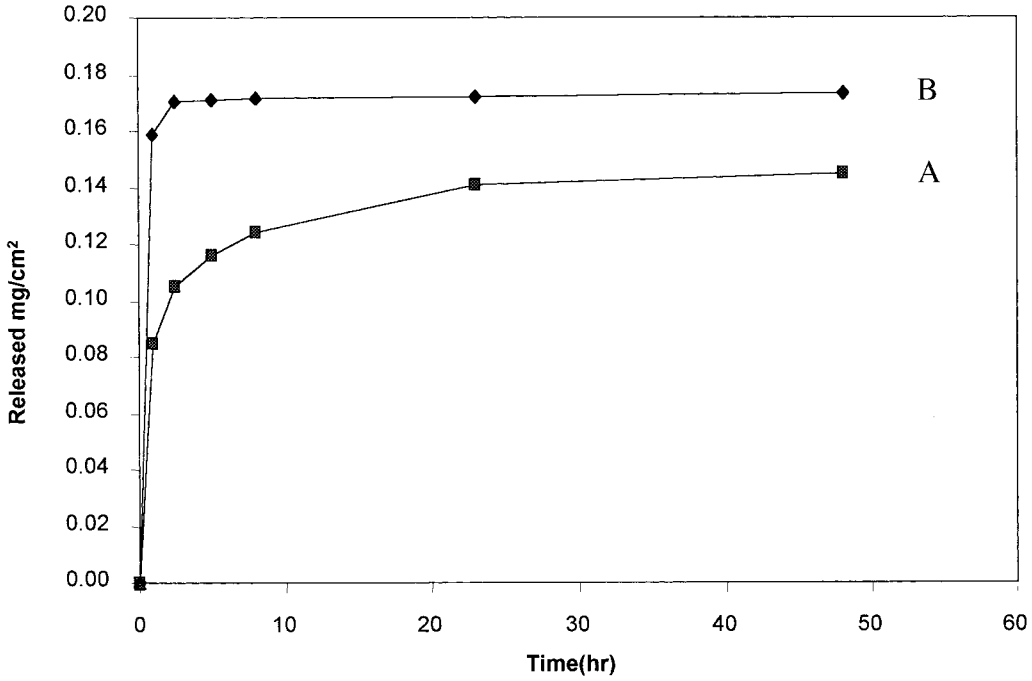

Figure 8. Comparison of the cumulative release of chlorhexidine from HAP coatings (A) with and (B) without a lipid overlayer.

spect to the desired mineral phase. Because the process is aqueous based, the incorporation of therapeutic agents, such as chlorhexidine, during the mineral deposition step is easily accomplished.

The antimicrobial agent, chlorhexidine, was incorporated into the coatings via the formation of a chlorhexidine phosphate precipitate into the hydroxyapatite coating. Conformation that the precipitate was a phosphate containing species and not due to other species present in the mineralizing solution (such as Ca^{2+}, Na^{+}, Cl^{-}) was established by ATR-FTIR.

Chlorhexidine release from the coatings exhibited a very rapid initial release period (1 h) followed by a longer and much slower rate of release. In all cases, close to 80% of the total chlorhexidine present in the coatings was released within the first several hours. This rapid release profile has been observed by other researchers studying calcium phosphate materials for drug delivery.[23-24]

While the mechanism of chlorhexidine uptake is clearly determined by the formation of a chlorhexidine phosphate precipitate layer, the rapid release must be due, in part, to a desorption or partial dissolution of the chlorhexidine phosphate layer. This was also supported by SEM micrographs of the coatings after the release studies, which show that very little of the HAP coating has undergone dissolution and, therefore, chlorhexidine release cannot be solely attributed to coating dissolution. While these coatings exhibited reasonable antimicrobial activity and represented an excellent beginning, longer and slower release periods were desired.

The use of lipid overlayers to retard drug release has been reported.[10,23] The theory of using lipid is that these coatings form a hydrophobic barrier on the implant surface thereby slowing the release of the therapeutic agent. In this work, the

TABLE II. Bacterial Inhibition Zones Formed around Coated Pins, In Vitro

Coating	Piece 1 (mm)	Piece 2 (mm)	Piece 3 (mm)	Mean (mm)
HAP only	0.0	0.0	0.0	0.0
HAP/chlorhexidine	14.0	13.0	14.0	13.7
HAP/chlorhexidine/lipid	26.0	20.0	23.0	23.0

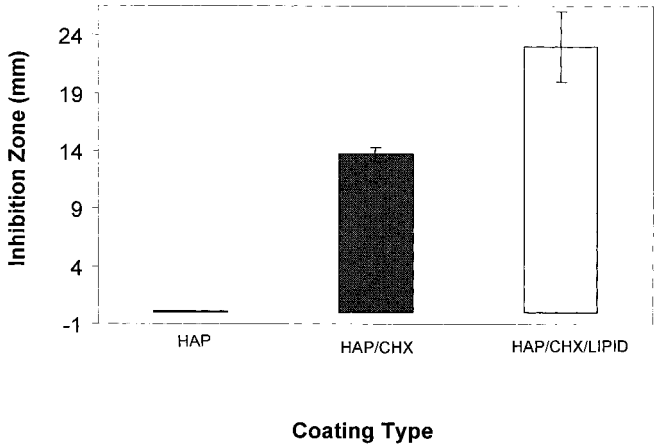

Figure 9. Comparison of inhibition zone diameters (in millimeters) formed around pins coated (n = 3) with (A) HAP coatings, (B) HAP/chlorhexidine, and (C) HAP/chlorhexidine/lipid.

lipid L-α-phosphatidylcholine, extracted from egg yolk was utilized. Our initial work with this lipid used a lyophilized compound, but it was later determined that slower release rates were obtained using the un-lyophilized compound. It was also found that it was important to have chlorhexidine in the lipid solution to prevent the desorption of the material from the coating. Utilizing this technique resulted in a reduction in the initial release rate and expanded the release region to over 24 h.

The antimicrobial efficacy of the coatings was significant varied depending upon coating type. HAP coatings alone displayed no antimicrobial effect, whereas the HAP/CHX coatings showed antimicrobial efficacy. The use of the lipid overlay also increased the antimicrobial efficacy by two-fold over the coating without a lipid coating. These results show that coatings of HAP/CHX/lipid exhibit excellent promise as carriers for the local delivery of chlorhexidine and other therapeutic agents.

This method represents a unique approach to producing a therapeutic coating that reduces external fixator pin tract infection while increasing the pin stability. Elimination of pin infection as well as enhancing the pin/bone interfacial strength should decrease the necessity of premature pin removal as well as pin loosening. The goal of this work was to investigate the feasibility of using hydroxyapatite coatings as a vehicle for locally delivering antimicrobial agents to the affected site. This strategy provides duel benefits in that the hydroxyapatite coating would allow bone bonding to the pin, thus enhancing stabilization, while the localized release of chlorhexidine would reduce the likelihood of infection.

With these promising *in vitro* results, we next plan to evaluate the HAP/CHX/lipid pin in an *in vivo* model. In addition, we plan to (1) investigate a dose response relationship between the chlorhexidine concentrations in the coating antimicrobial; (2) investigate the type of release mechanism that has the most beneficial effect on infection resistance (burst release vs. sustained release); and finally (3) evaluate the mechanical integrity of the coatings.

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