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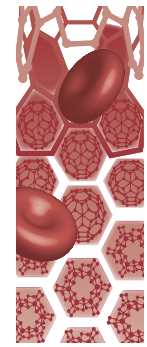

Calcium phosphate nanocoatings and nanocomposites, part 2: thin films for slow drug delivery and osteomyelitis

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During the last two decades although many calcium phosphate based nanomaterials have been proposed for both drug delivery, and bone regeneration, their coating applications have been somehow slow due to the problems related to their complicated synthesis methods. In order to control the efficiency of local drug delivery of a biomaterial the critical pore sizes as well as good control of the chemical composition is pertinent. A variety of calcium phosphate based nanocoated composite drug delivery systems are currently being investigated. This review aims to give an update into the advancements of calcium phosphate nanocoatings and thin film nanolaminates. In particular recent research on PLA/hydroxyapatite composite thin films and coatings into the slow drug delivery for the possible treatment of osteomyelitis is covered.

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During the last hundred years due to its similarity to hard tissue, the material of choice for the enhancement of bioactivity has been calcium phosphates [1–6]. A number of biodegradable nanocoated biomaterials based on calcium phosphates are currently being examined for applications for slow drug delivery and dispersion of pharmaceuticals and minerals to the targeted area. In these new devices, which comprises a polymeric or ceramic matrix and porous inorganic particulate matter, factors such as the chemical composition as well as the critical pore size are the main factors that influence the dissolution rates. Additive particulate sizes which can be incorporated to these films can be from a few nanometers up to microns dependent on the required thickness and the function of the films. They are usually designed to contain nano and meso pores for drug loading.

Classical approach of the systemic drug delivery has in the past generated a number of problems that can possibly be solved by local or targeted delivery. These include influence of the dissolving drugs to the whole body rather than pin point local delivery. Moreover, aside from reduction of toxicity to healthy cells within the whole environment, targeting these drugs directly to required locations have the possibility of improving drug efficacy and efficiency, resulting in a significant cost savings for the health care system.

During the last decade a number of research groups have been describing the production of novel nanocoatings and thin film nanolaminates with hydroxyapatite (HAp) and other calcium phosphates for clinical applications. The nano to micro particles of oxide or mixed ceramics, and calcium phosphates are the inorganic components and natural or synthetic polymers such as collagen, chitosan and biodegradable polymers such as polylactic acid (PLA) are the matrix materials in these new composites.

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These nanocomposites can be synthesized by mixing the inorganic particles physically through heat or by solvent introduction into an already existing polymeric matrix material. Evaporation of the solvent aids the formation of the nanocomposite or the thin films.

The development of tissue engineering in the past has been related directly to the types of scaffolding materials used. At present, a number of synthetic bone graft biomaterials such as calcium phosphates are available as options to autogenous bone for augmentation, repair or substitution [3]. New advancements associated with calcium phosphate scaffolds and their improvements in microstructure and surface properties have created new opportunities for bone regenerative technologies. These recent developments also make the calcium phosphate scaffolds to be thought of as being at least biologically constructive instead of as only osteoconductive scaffolds specifically with the addition of biologic materials such as bone morphogenic proteins and stem cells [7–9].

Evidence has shown that porous calcium phosphate has a direct influence on the proliferation and differentiation of human mesenchymal stem cells (MSCs). Tissue engineering along with new bioactive molecules enhanced the possible applications of calcium phosphate as scaffolds able to guide the behavior of these cells and the efficiency of bone regeneration as well as being carriers of these cells [7,9].

In this review, we aim to introduce the recent developments of nanocoatings and thin films and composites containing calcium phosphate-based nanoparticles currently being investigated for the delivery of pharmaceutical substances and their use in medicine and specifically for the treatment of osteomyelitis.

Surface modifications & liposomes for drug delivery applications

In bioceramics, the critical pore size and interconnectivity can be altered in order to control the ease of delivery and dispersion of a material to the targeted area. Targeting usually achieved with appropriate functionalizing of the surfaces. The pores may range from a few nanometers to micron sizes dependent on the pharmaceuticals used or areas intended to be delivered such as long bones. Delivery systems based on calcium phosphate which is similar at least chemically to the hard tissues, have the potential to increase drug efficacy while at the same time minimizing toxicity to nondiseased cells. Nano drug-delivery systems, embedded within a matrix or not, also have the exceptional attribute of being capable of delivering and controlling dissolution with high precision due to their high surface areas. It is not surprising that the number of research papers covering drug, gene and mineral delivery of nanoparticles, nanocoatings and composites published during the last decade is very high [9–32].

The appropriate dissolution rates and their control within the human body is the main concern for drug carriers containing nanoparticles and nano thin films [33]. The use of calcium phosphate as a delivery system also broadens its effectiveness as a result of their capacity to locally deliver minerals as well as calcium and phosphate, other active ions and biogenic materials such as bone morphogenetic proteins (BMPs) and MSCs if required to be used in the successful treatment of bone diseases. In addition the surface modification approach it can also be used to achieve enhancements of stability and long range solubility control of nanocoatings and thin films in aqueous media, as well introduction of new material properties and functions. In principle through the use of a wide range of biological, chemical and/or physical surface modifications methods, the surfaces of nanostructured materials such as nanocoatings can be altered and functionalized to assist us in slow drug delivery. In the quest for the surface modifications of nanostructured materials, approaches such as macro micro and nanocoating have emerged as the leading strategies resulting in better functionalization of the surfaces of materials and for better osseointegration in the long term.

The biological modification of surfaces of nanocoatings is at times essential for the functionality of the devices. Biospecific molecules can be incorporated into the nanocoatings or thin films by using physical or chemical methods, thus presenting biospecific sites for the further immobilization of ligands specific to these molecules. The immobilizations of specific ligands such as antibody–antigen and receptor–ligand can be carried out using biologically specific reactions [34]. Current research work in these areas is very promising.

It is well known that different biomedical applications require different functions and properties of materials. As a result, techniques available to modify nanostructured materials or thin films can vary in order to meet the demands of various biomedical systems. In spite of the advantages offered by nanocoatings and nanoparticle containing composite thin films, such as their small surface pore sizes and loading efficiency, a number of issues such as control of the appropriate drug release rates, restricted their use clinical applications.

The targeting ability and efficacy of any drug delivery system are sometimes hindered by the rapid dissolution of the carrier system within the human body. A good example is their side effects in chemotherapy drug delivery for the cancer patients. The long circulation time within the blood is the primary concern for drug carriers of both local and systemic delivery. For

this reason, a number of investigations have been carried out to examine ways in which 'long-circulating-time' carriers can be designed and engineered. Among these, the surface modification of thin films and nanocoatings with a variety of polymeric macromolecules or nonionic surfactant has been demonstrated to be the most effective for maintaining the presence of drug delivery particles in the blood for prolonged periods [35].

The use of surface modification is used in gene therapy in an effort to obtain controlled delivery of small interfering RNA and plasmid DNA (pDNA) particularly in an acidic pH environment [25–28]. The use of cationic liposomes as transfection vectors has become an ideal choice and most widely employed in the transfer of pDNA due to their weak immunogenicity and low toxicity [24]. A study by Zhou *et al.* [25] has suggested that coating calcium phosphate with liposomes could provide consistently efficient and satisfactory delivery of pDNA. Using mammalian cell culture, their findings showed the application of a lipid coating resulted in a tenfold increase in the transfection of pDNA compared with uncoated calcium phosphate [25].

Considered as one of the most clinically recognized thin film nanoscale systems, liposomes consist of a single layer or multiple concentric lipid bilayers that encapsulate an aqueous compartment are currently utilized in the delivery of antifungal drugs, vaccines and genes [25,36–41]. The exceptional clinical profile of liposome coatings in comparison to other delivery systems is based on their reduced toxicity, biodegradability and capacity for size, and surface manipulations [42]. An improvement in the biocompatibility of liposomes as well as an increase in nanoparticle hydrophilicity and stability in plasma can be achieved through the encapsulation of nanomaterials such as calcium phosphate within liposomes (Figure 1). In Lewis work multilayered liposomes were produced with the incorporation of nano-HAp and other minerals such as strontium, magnesium and zinc [42]. Figure 1 shows a transmission electron microscopy (TEM) image of multilayered liposomes containing nano-HAp particles produced in a calcifying buffer. The figure was obtained in FEI Morgagni 268D transmission electron microscope (Eindhoven, The Netherlands) at 80 kV [42]. The work showed an excellent encapsulation that can help to control drug delivery rates in medical applications such as chemotherapy drug delivery for oncology patients. This observed ease of coating and release delay ability is one of the strong reasons, calcium phosphate based nanoparticle containing thin films and liposome coatings are ideal candidates for drug delivery and bone regeneration systems [42–45]. In addition, combinatory therapy–modalities can be accomplished by utilizing the ability of liposome coatings to carry hydrophobic and hydrophilic moieties as well as their capacity to incorporate therapeutic and diagnostic agents into a single liposome-delivery system [42].

Huang *et al.* [39] have suggested that the nucleation process for new bone formation could be improved by the presence of negatively charged liposome coatings. In their experiments carried out in miniature swine, artificial bony defects on one side where implanted with liposome-coated tri calcium phosphate while defects on the other side served as controls. They reported that at three weeks postimplantation, dense connective tissues surrounded the implant material and new bone formation was visible after 6 weeks.

Using a different strategy, Wang *et al.* explored the possibility of producing collagen-calcium phosphate scaffolds with the incorporation of liposome thin films for the controlled release of bioactive molecules in bone regeneration and repair [40]. They suggested that bisphosphonate (BP) functionalized liposome encapsulation could be isolated within mineral-containing scaffolds can be better drug delivery system that localize their drug cargo to the directed area. The liposome encapsulation used consisted of cholesterol, distearoylphosphodioline, distearoylphosphoethanolamine-poly(ethylene glycol). Based on their observations, the encapsulation of BP within liposomes displayed a strong affinity to the scaffolds and the drugs entrapped within the BP-liposomes showed a slower release rate from the scaffolds as compared with drugs that were un-encapsulated or encapsulated in polyethylene glycol-liposomes. In a similar study by Chou *et al.* it was shown that this liposome coating thin films reduced the drug release rates in both BP and antibiotic additions [22,46].

A study by Xu *et al.* [36] explored the possibility of synthesizing a multifunctional thin film drug carrier with sustained drug release capability provided by the inner core liposome and osteoconductivity for bone cells supported by the HAp outer layer. The liposomes were produced from 1,2-dimyristoyl-sn-glycero-3-phosphate and 1,2-dimyristoyl-sn-glycero-3-phosphocholine, which is then loaded with the lipophilic drug indomethacin. The release profile of indomethacin was measured at two different pH levels (4 and 7.4). As expected by coating the liposome with HAp, they reported a reduction in release rate of indomethacin in comparison to uncoated liposomes. They also reported that without these coatings, the rate of drug release occurred more rapidly at pH 7.4 rather than at pH 4.

It has been reported that the management of possible postoperative infections from bone grafts and prostheses as well as the treatment of bone diseases

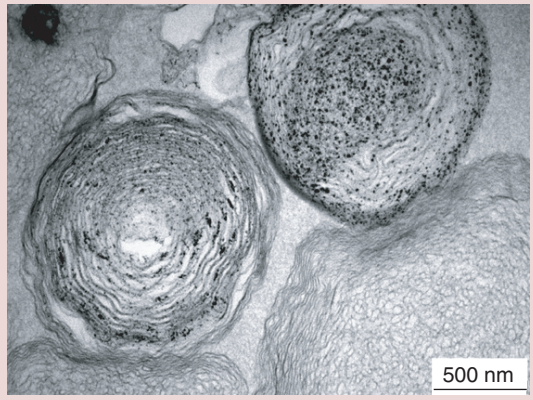

Figure 1. Transmission electron microscopy image of multilamellar lipsomes containing nano hydroxyapatite particles and minerals in a calcifying buffer (scale 500 nm).
Reproduced with permission from [42].

such as bone metastases will benefit greatly if there is a delivery system which has a high affinity toward bone tissues thereby maximizing its therapeutic effect on bone-related diseases [36,39]. Using this approach, Anada *et al.* [37] attempted to develop a calcium phosphate-binding liposome coating for a bone targeting drug delivery system by synthesizing an amphipathic molecule bearing a BP head group to recognize and bind to HAp. Liposomes loaded with the drug doxorubicin adsorbed onto the surfaces of HAp were observed to significantly reduce the number of viable human osteosarcoma MG63 cells. Based on these observations, they suggested that the system can be excellent coated carriers for anticancer drugs as they specifically target bone tissue [36].

Infection & osteomyelitis

It is widely accepted in the medical community that wound contamination as well as postoperative infections following implantation or during surgical intervention in orthopedics and maxillofacial surgery can result in serious clinical problems and could jeopardize the osseointegration process. For these reasons, antibiotics either administrated orally or intravenously are often provided as prophylactics.

By far the most frequent complications related to the use of implantable medical devices such as orthopedic or dental prostheses and endotracheal tubes are bacterial infections. *Pseudonomas aeruginosa* is regarded as an opportunistic pathogen causing indwelling device-related infections especially catheters. *P. aeruginosa* infection is leading cause of morbidity and mortality in cystic fibrosis patients. On the other hand *Staphylococcus aureus* infection causes serious infectious complications such as severe sepsis, septic-thrombosis and/or severe deep-seated infections (endocarditis, osteomyelitis and other metastatic infections). One of the basic hospital and surgical intervention acquired biofilm infections are those associated with *S. aureus* and *Staphylococcus epidermidis* strains, including methicillin-resistant *S. aureus* (MRSA). A thorough and comprehensive understanding of the molecular bases of biofilm formation and their adhesion may help us to fight biofilm infections.

Osteoarticular infection is frequently caused by coagulase-negative Staphylococci as main aetiologic agents in late infections as well as Streptococci, Enterococci and anaerobes. The number of infections is estimated to be around 0.5– 2.0% of cases and it increases continuously due to the rise in the need for implants.

The European Center for Disease Prevention and Control1 (ECDPAC) reported that approximately 4,100,000 patients are estimated to acquire infections in the European Union every year [47]. The number of deaths occurring as a direct consequence of these infections is estimated to be at least 37,000 patients and these infections are thought to contribute to an additional 110,000 deaths each year. In the USA, it was estimated that approximately 1,700,000 patients acquired infections for the year 2002. It has been estimated that 5% of patients undergoing clean surgical procedures and up to 20% of patients having intra-abdominal surgical procedures develop a surgical site infection. Such infections result in 3.7 million excess hospital days and more than US$1.6–3 billion in excess hospital costs per year.

In order to mitigate this problem different strategies have been proposed on either preventing and/or controlling bacterial infections. Modification or development of thin film or nanocoated multilayer devices with surface properties that have an effect against microbial adhesion or viability seems to be a promising approach for the prevention of device-related infections. Another strategy is to modify the surface of medical devices biologically, chemically or physically to render the surface free of microbial adhesion. Multifunctional thin films or nanocoatings can facilitate this new approach.

In clinical applications an ideal implant coatings must provide surgeons with several benefits such as providing primarily anchorage with appropriate bioactivity involving osteoconduction and if possible, osteoinduction. The nanocoating or thin films used should also provide antimicrobial property to prevent implants from developing acute or postoperative infections.

As stated earlier the development of bone infection is based on the formation of a bacterial biofilm where the bacteria differentiate from planktonic into sessile forms that protect some bacterial cells that can

be released from the biofilm after antibiotic treatment has ceased.

Bacteria adhesion is a complex phenomenon affected by many factors, including properties of surface materials, some characteristics of bacteria itself and the environment where the adhesion takes, such as the presence of serum protein or bactericidal substances [48]. Some of the proposed theory and model of adhesion seem to be limited because they consider physical interaction between the surface and bacteria and neglecting biological aspects of adhesion in which specific bacterial structure responsible for adhesive activities called adhesins that control cell to cell or cell to abiotic surface adhesion. Bacteria may have different adhesives for different surfaces (different acceptor). The ionic strength and pH of the medium in which the adhesion takes place influence the charge of the cell wall and of the substrate (in terms of surface chemistry, charge and hydrophobicity) and therefore affects their interaction.

The antibiotic dosage required to act on bacteria in biofilm conditions can be many folds higher in concentration of the drug required for treatment of planktonic cells. Consequently, it is difficult to completely eradicate active infection by means of 'systemic antibiotics' which in doses active in biofilm can be toxic for a patient.

In orthopedics, osteomyelitis mainly occurs in tissues within the infected area, and consequently targeted delivery of antimicrobial agent locally is a more appropriate form of treatment. One of the most effective ways of achieving targeted delivery of antibiotics is to use a carrier device that can be placed into the body at a specific site such as coating on an implant, which will release slowly the correct antibiotic dosage. The major challenge associated with the use of antibiotic is ensuring retention of antibiotic release and activity for a prolonged period of time post operation [49].

Treatment of osteomyelitis can be with immobilization and antibiotic therapy with a number of drugs including flucloxacillin, gentamicin, tobermycin or vancomycin, and fusidic acid. Surgical drainage and removal of damaged bone, as its presence prevents healing, sequestrum may be possible but recurrence is common. Currently, commercial products in the form of pellets composed of acrylic polymers or ceramics such as calcium sulfate or bioactive glass are available as slow drug delivery devices. However, due to the problems related to their dissolution rates, shape, sizes and chemical composition, they are either nonresorbable that requires second surgery to remove or quickly resorbed by the body's natural physiological process, thus limiting their effectiveness. In addition, past investigations have shown that some antibiotics have been reported to be ototoxic and nephrotoxic at high dosage. For most controlled release systems, the loaded dosages are usually high, and therefore the systemic exposure of antibiotic in blood and urine is the major safety concern.

The concept of pathophysiology of osteomyelitis which has been widely accepted is the infected bone becomes devascularized and the resulting sequestered portions of necrotic cortical bone harbor bacteria. This sequestered, necrotic, infected bone is responsible for the chronicity of osteomyelitis. Pus and granulation tissue then surrounds the infected fragments and through increased intraosseous pressure, bacterial toxins and enzymes, further contribute to devascularization of the surrounding bone. The granulation tissue that surrounds the infected area as the infection becomes chronic is replaced by relatively avascular fibrous tissue, and stimulation to form new reactive bone referred to as involucrum takes place within the surrounding tissues and in the periosteum permeative mesenchymal cells that wraps around the sequestered, necrotic, infected bone. Antibiotics and antibodies must cross this involucrum and relatively avascular fibrous tissue to reach the microorganisms once the fibrous and bony encapsulation takes place. Consequently, the very effective effort by the body to quarantine the host from the infection also isolates the microorganism from the defenses of the host. The infection becomes chronic and cannot be eradicated when this pathological standoff occurs. This condition is the basis for intervention surgery and the excision of necrotic sequestra. Antibiotic therapy completes the treatment of chronic osteomyelitis by eradicating the microorganisms once they are no longer isolated [50].

The quest for a more effective means of delivering antibiotics without the complications related to long-term intravenous access and the toxicity of systemic antibiotics has been ongoing. Hence, the most accurate method of assessing diseases is provided by models that utilize eradication of infection as a criterion for success, the histological findings of new bone formation, inflammation, sequestration and intraosseous bacteria, combined with cultures.

In the past, ceramics as well as other materials incorporated to the thin films and bulk composites have been suggested as potential candidates to be used as biodegradable drug delivery systems. However, manipulating these materials into an appropriate shape with adequate microporosity in order to be fitted into bone defects of different size and form was found to be difficult. Recently, it has been demonstrated that marine shells, foraminifera and corals incorporated into thin films and nanocoatings with specific microspherical carriers offer desired functions

for the delivery of BP (paminodrate) and an antibiotic (gentamicin) [51–55]. This has been possible by virtue of its nano and mesoporous structure and architecture of the foraminifera shells (Figure 2A) which are difficult if not impossible to produce with our current manufacturing methods [44,45]. Foraminifera and coral in addition to their unique interconnected porous structure are made of calcium carbonates that can be easily converted to bone such like phosphate structures [44,45].

Bone repair drug delivery systems & osteomyelitis

In early 1900s, a calcium phosphate compound (tricalcium phosphate) was first successfully used in bone repair followed by the first clinical study [1]. It took more than six decades until porous calcium phosphate scaffolds were proposed for the treatment of bony defects [2]. The reason behind the research and development of calcium phosphate based biomaterials for bone repair, augmentation and substitution was due to the similarity in composition between biological and synthetic apatites [55].

For bone repair and slow drug delivery systems, the most appropriate materials are the calcium phosphate based bone substitutes [3]. They offer easy production, drug carrying capability and supply both calcium and phosphates during dissolution. They can be easily incorporated within thin films and nanocoatings [55,56]. There have been a large number of studies carried out on both experimental and commercial calcium phosphate based drug carriers during the last decade [10–34]. Due to their wide areas of applicability, the delivery of antibiotics has become a major focus in the prevention and treatment against infection during or postoperative surgical interventions.

Biodegradable composite thin films

Biodegradable polymer thin films loaded with gentamicin have been synthesized to act as a 'composite coatings' for metallic implants and fracture fixation devices in an attempt to prevent implant-associated infections [48,57–59]. Due to their tendency to uptake and release pharmaceuticals and minerals as well as the capability to degrade over time, the use of biodegradable polymer thin films is advantageous. In addition, by controlling the pore sizes and interconnectivity of these particles, the rates of drug release could be tailored to suit the treatment.

In our experimental work [53–59], hydrothermally converted coralline HAp particles containing nano and mesopores were loaded with medically active substances, which cover both the surfaces and interconnected pores of the particles that ranged from a few hundred nanometers to micron sizes (Figure 3).

The influence of HAp particles within PLA matrix on the release of gentamicin as well as its release kinetics have been previously examined [59]. It was discovered that the release kinetics of gentamicin appeared to obey "Power law Korsmeyer Peppas' model with mostly diffusional process through a number of different drug transport mechanisms. Statistical analysis revealed a very significant difference on the release of gentamicin between gentamicin-containing PLA (PLAGM) and gentamicin-containing HAp microspheres within PLA matrix (PLAHApGM).

These thin film composite coatings, such as PLA-HApGM displays slower release rates than PLA matrix

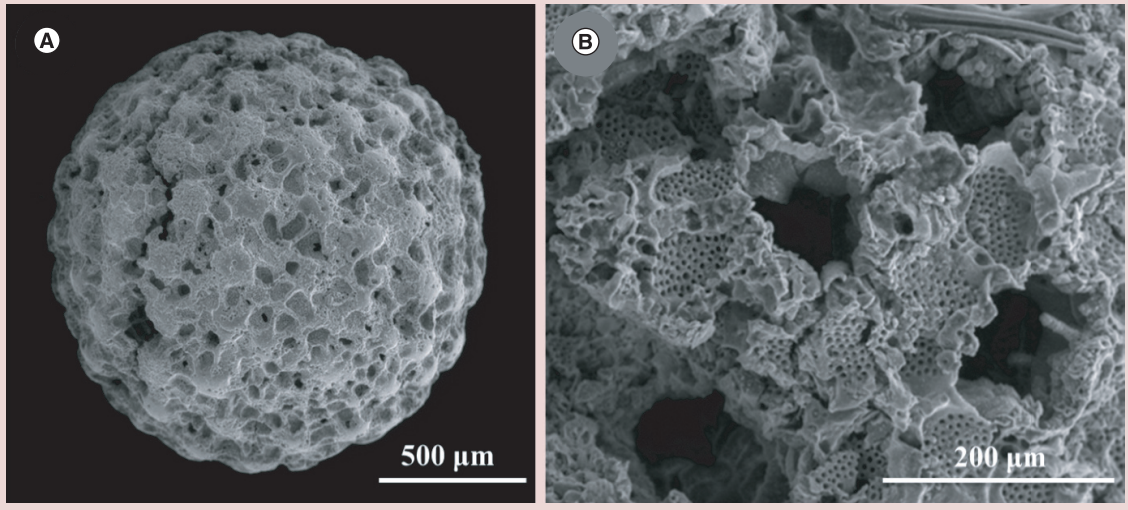

Figure 2. (A) *Foraminifera* hydroxyapatite microsphere. (B) Enlarged degraded surface of *Foraminifera* hydroxyapatite structure within simulated physiological environment.

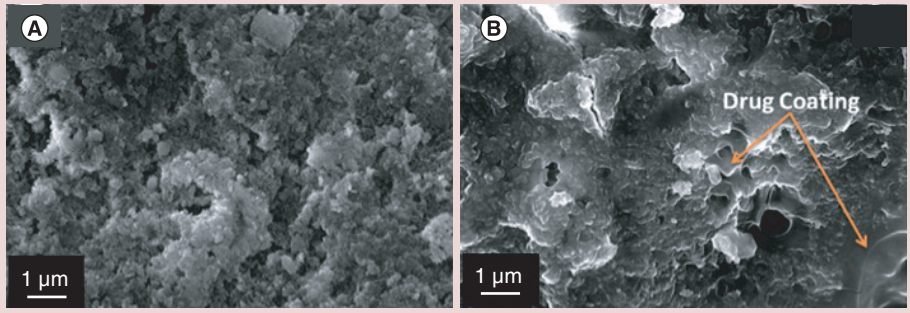

Figure 3. (A) Hydrothermal converted coralline hydroxyapatite surface. (B) Gentamicin-coated coralline hydroxyapatite surface.

alone. As stated earlier HAp and other calcium phosphates are also the source of Ca^{2+} for the regeneration and repair of diseased bone tissue. It was also reported that the release profiles, exhibited an early burst stage and then a steady state release rate with significant antimicrobial activity against *S. aureus* (SH1000) even at high concentration of bacteria. The devices also indicated significant ability to control the growth of bacterial even after 4 weeks of drug release. It was suggested that clinical release profiles can be easily tuned from drug-HAp physicochemical interactions and degradation kinetics of polymer matrix. It was concluded that the developed systems could be applied to prevent microbial adhesion to medical implant surfaces and to treat infections mainly caused by *S. aureus* in surgery.

It was reported that the degradation of the polymeric network towards the end of the initial release will 'favor' the dissolution of any drug residue. Accordingly, this phenomenon (diffusion and degradation) should take place at the conclusion of surface drug release (when dissolution of drug have created secondary porosity inside the polymeric network and resultant 'fragility' of it) and/or dependent on factors such has the surface area, dissolution rate and the molecular weight of the PLA film used.

For the coral converted to hydroxyapatite material (coralline HAp) structure contains meso- and nanopores and during the initial drug loading period gentamicin penetrates the pores of the 'HAp-coral' and coats its surfaces and its porous network. The gentamicin contained in these porosities will be released gradually. Hence, the process of polymeric matrix thin film composites containing drug carrying particulate matter can be thought to proceed in three stages that can be named as 'progressive dissolution process'.

In the first stage (stage 1), the initial release (burst) of gentamicin can be regarded as the direct dissolution of surface bound drugs in water or the physiologic environment due to the exposed outer surface area and concentration gradient. It was shown in that the initial burst took approximately 1 week for gentamicin. At this stage it can be assumed that the release is purely governed by diffusion of drugs from polymeric matrix surface [60,61].

In the second stage the dissolution is driven by the internal diffusion of drugs impregnated within the matrix possibly in the porous part of the matrix generated during preparation. For gentamicin release from PLAHApGM samples, this stage is preceded by 'lag phase' which occurs between 1 and 2 h of release. The presence of HAp loaded with gentamicin could in many ways hinder (or slow down) the release of gentamicin through these micropores. This stage is relatively much slower compared with the previous one due to the drug transporting through mainly from the surface areas and large pores of the particles. This stage proceeds with release from narrow pores of the nano and mesopores of the particles to the matrix and then to the environment. There is no degradation of particles at this stage but dissolution of the drugs only.

The third stage involves slow degradation of the polymeric matrix combined with dissolution and deterioration of the particles (Figures 4 & 2B) and slow release of the added drugs or the minerals into the environment. This is terminal release phase or stage for loaded devices. At this stage, there is pore growth due to both mass loss by polymer degradation and pore coalescence (micropores coalescing (or joining) to form larger pores). In recent dissolution studies of BPs, for the device containing HAp (PLAHApBP), the slower dissolution rates observed were reported to be due to the strong bonding of BP to apatite (HAp) as well as encapsulation within the particles. It was earlier reported that for BP containing calcium phosphate particles have strong affinity to the nanocrystalline apatites with adsorption phenomena that occurs at the surface of apatite crystals [62].

Antibacterial efficacy & biofilm formation

Published work states that poor diffusion and penetration of antibiotic through the biofilm, contribute to

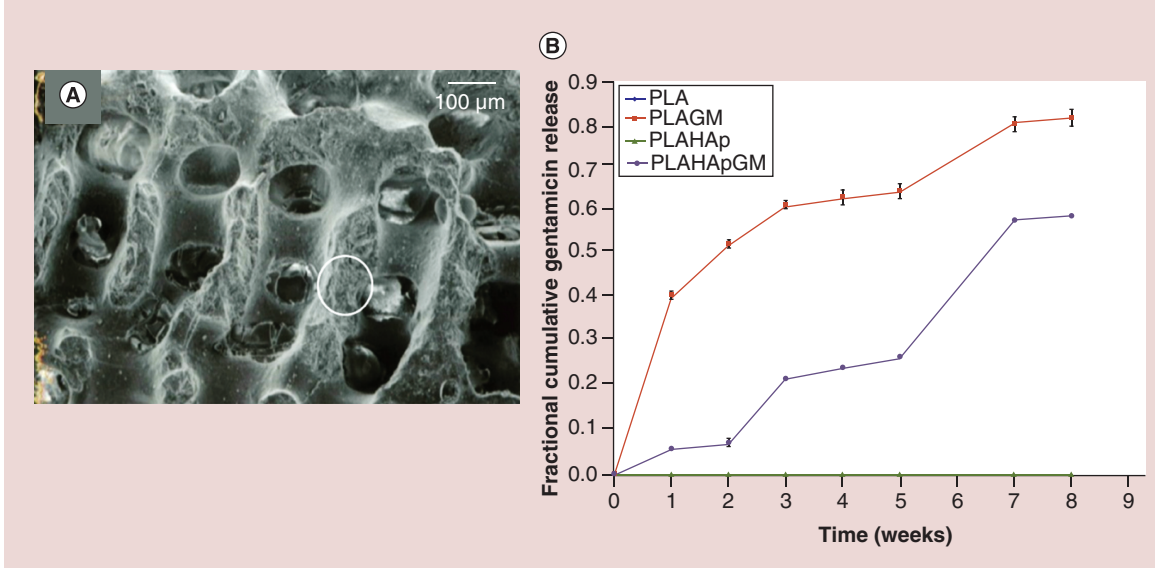

Figure 4. Gentamicin drug delivery device produced from polylactic acid-coralline hydroxyapatite thin film composite. (A) The scanning electron microscopy image of the coralline HAp surface used for drug delivery (only the struts are used as drug delivery vehicles shown in a circled area), and **(B)** drug release profile of only the gentamicin within PLA (PLAGM) and HAp coralline particles loaded with gentamicin embedded within a PLA matrix (PLHApGM) showing lower amounts of drugs released as well as a longer release rate up to 8 weeks. GM: Gentamicin; HAp: Hydroxyapatite; PLA: Polylactic acid.

the persistence of biofilm infections especially those associated with implanted devices [63–66]. A number of reasons on the microbial resistance to antimicrobial agents have been postulated. An increase in the depletion of oxygen and nutrients resulting into slow growth of bacteria, adaptive stress responses and formation of persister cells are hypothesized to constitute a multilayered defense. In recent research efforts the focus is directed toward disabling biofilm resistance, which may enhance the ability of existing antibiotics to treat infections involving biofilms [67]. It has been reported that in most cases, biofilm can be prevented aggressively by antibiotic in their early stages and can also be treated by chronic suppressive therapy. Mah *et al.* [68] suggested that the use of traditional antibiotics combined with drug that interferes with biofilm-specific resistance would be the right approach to render biofilms more susceptible to treatment.

A recent study by our group has suggested that effective drug delivery devices with adequate slow release rates can be produced using converted coralline HAp particles loaded with medically active drugs and substances embedded within a PLA thin film matrix [48,58–59]. To investigate the antibiotic delivery efficiency of the composite thin films produced and observe the biofilm formation, we carried out a number of tests.

In this current work, we prepared PLA films and film composites based on the previously published work and loaded with gentamicin. Schematic representation of the process is given in Figure 5. The films were cut into circular shape and glued on the sterilized cylindrical coupons. They were sterilized using UV for 40 min. Trytpic Soy Broth (TSB) for *S. aureus* and Mueller Hinton II BR cation adjusted media (MHB) for *P. aeruginosa* were prepared by thoroughly dissolving 30 g of TSB powder (Bacto™ TSB (BD) and 22 g of MHB powder in 1 l of polished 18 MΩ (MilliQ, Millipore, Victoria, Australia) water, respectively. The solutions were autoclaved to sterilize (121°C, liquid cycle) and stored at room temperature.

S. aureus (ATCC 25923) and *P. aeruginosa* (ATCC 15692) were used for this study and were cultured in a shaking incubator, 250 rpm at 37°C under anaerobic and aerobic conditions, respectively. For static biofilm formation, cells were grown in broth medium overnight, and diluted 1:100 into TSB and MHB media, respectively. These cell suspensions were inoculated into a 12-well plate containing triplicate of samples glued on coupons. The plate was sealed with sterile breathable film (Aeraseal; Excel Scientific, CA, USA) and statically grown at 37°C, 5% CO_2 for 24 h. Biofilm samples were washed with PBS, stained using SYTO9 Green fluorescent Nucleic Acid Stain (Life Technologies Corp, CA, USA) and fixed with 4% paraformaldehyde.

The morphological changes of the biofilms were analyzed using confocal laser scanning microscopy (CLSM; Nikon A1, Tokyo, Japan), using oil-immersion lens (70 Objective lens and numerical aperture of 1.4 with Z-series images taken in 1.0 mm slices)

with NIS Elements Confocal software. A total of eight images were acquired randomly from each specimen.

In biofilm study, four biofilm image features calculated by COMSTAT [69] were chosen to characterize biofilm development by *S. aureus* and *P. aeruginosa* on PLA thin film composites. These variables, biomass, average thickness, roughness coefficient and surface to biovolume ratio were selected for interpretation of biological and physical characteristics of biofilm [67] on these surfaces. Biomass represents the overall volume of the biofilm, and also provides an estimate of the biomass in the biofilm, average thickness provides a measure of the spatial size of the biofilm, roughness represents a measure of biofilm heterogeneity and surface to biovolume ratio provided us how large a portion of the biofilm is exposed to the nutrients flow. Recorded CLSM images were reconstructed using IMARIS imaging system (Bitplane AG, Zurich, Switzerland) for biofilm structural quantification in computer statistics software COMSTAT and presented as 3D structures.

The preliminary results obtained from 5 days experiments for *S. aureus* (SH1000), and *Pseudomonas aeruginosa* on PLA thin film samples are both strains at day 5 had grown into micro colonies, reflected by their low surface to volume ratio compared with biofilm at day 1. On the other hand, microscopy images of *S. aureus* on the films showed large and high micro-colonies on PLA, PLAHAp and PLAHApGM samples while on PLAGM high single cells and small cell clusters were observed (Figure 6). Confocal images on the composites without and with gentamicin shows the intensity of the biofilm formation (Figure 6A & C) and the effect of the antibiotics after 24 h (Figure 6B & D).

It can be suggested that cells were able to attach on the surface of PLAGM samples but the drug released from the surface suppresses the ability of bacteria to make biofilm. It could also be envisaged that there is possibility under flow-biofilm growth conditions that more drugs would be released from the surface and may suppress the attachment of bacteria on the surfaces. Being high-level antimicrobial resistant in Gram-negative, *P. aeruginosa* displays similar structure characteristics on the surface of PLA thin films.

P. aeruginosa showed a stronger tendency to form microcolonies on the surface of PLA films than *S. aureus*, which was indicated by higher roughness coefficients. The higher surface to biovolume ratio of *P. aeruginosa* is an indication of its flat growth on the surface compared with *S. aureus*. This is also consistent with the lower average thickness of the biofilm it forms on the surface. It was also observed that *S. aureus* grows faster than *P. aeruginosa* on PLA surfaces indicated by lower surface to volume ratio, higher average thickness and hence higher biomass in the biofilm.

The effect of antibiotic in the films (PLAGM and PLAHApGM) against biofilm after 24 h seemed to be minimal for *S. aureus* and no at all to *P. aeruginosa*. This is possibly due the fact that static conditions may influence low release rate of drugs from the device and also 24 h is not enough time to realize polymer degradation and significant release of antibiotic.

In summary, all of the PLA and PLA/HAp gentamicin loaded thin films under experimental conditions exhibited significant ability to prevent bacterial growth even at high concentration of bacterial. The prolonged ability to release drug from these films was tested by subjecting films for 4 weeks under the same experimental conditions. It was observed that even after releasing drugs for 4 weeks, films were still able to release enough gentamicin to prevent microbial activities.

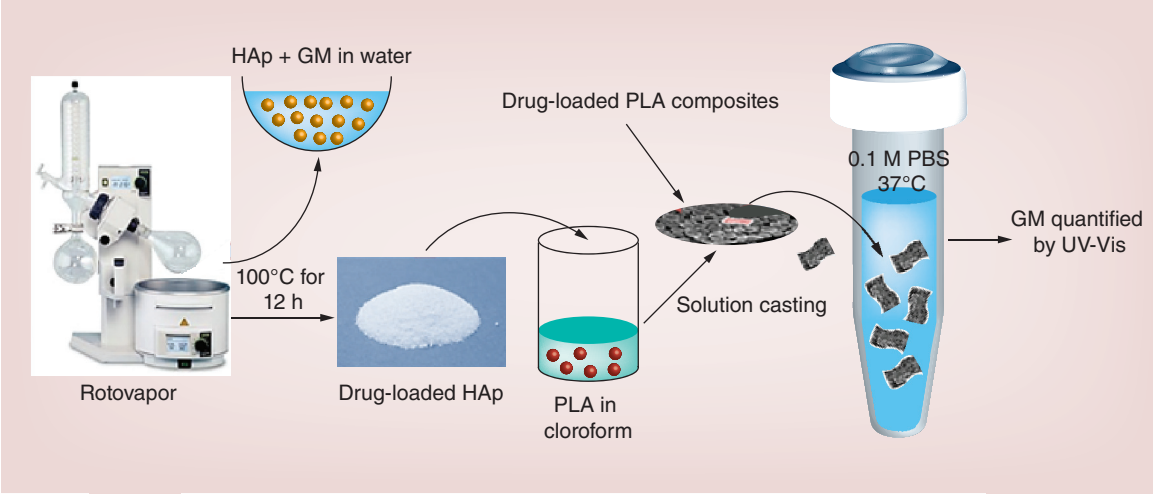

Figure 5. Schematic representation of the drug loading and release method used in this current work.
GM: Gentamicin; HAp: Hydroxyapatite; PBS: Phosphate-buffered saline; PLA: Polylactic acid.

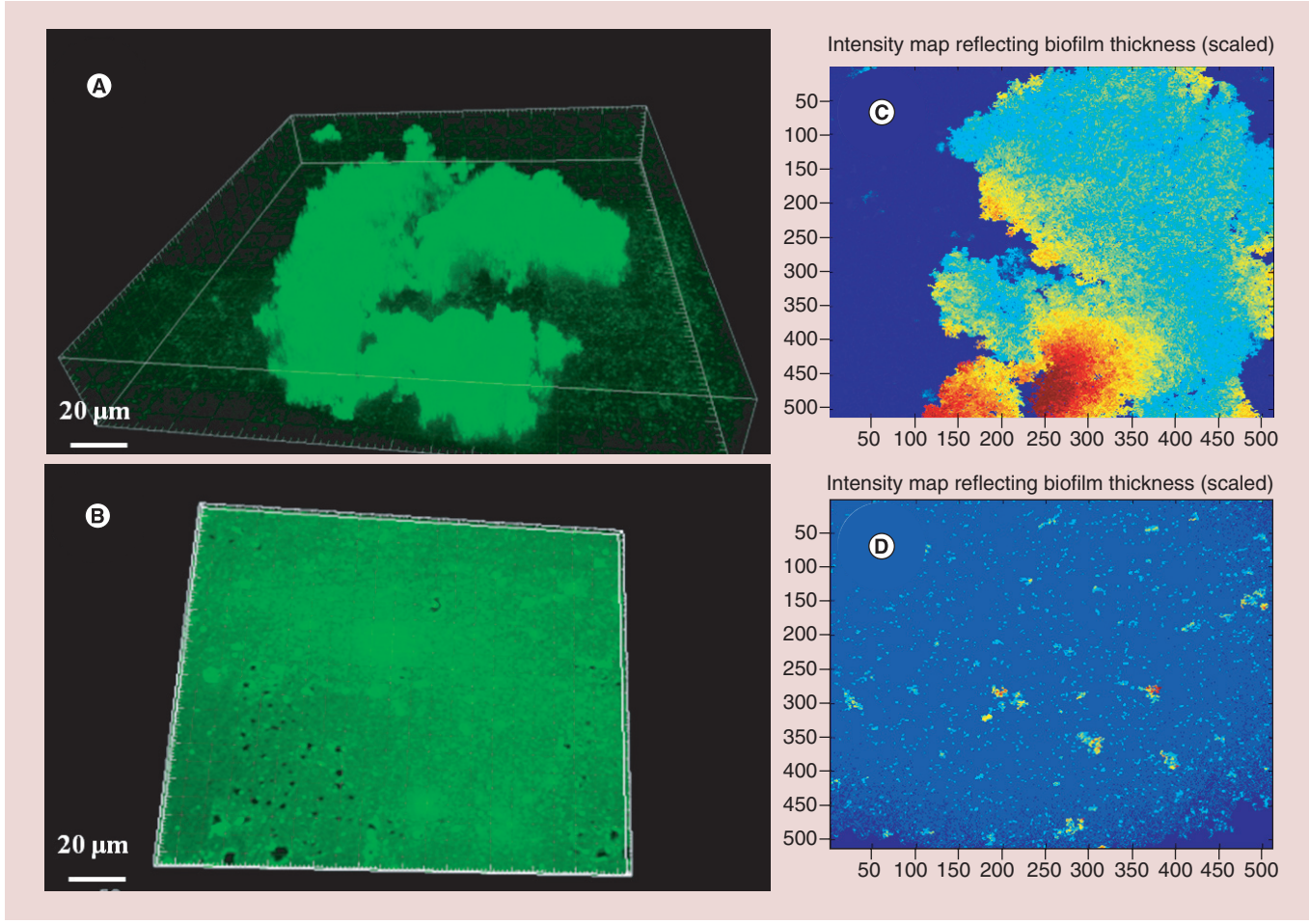

Figure 6. Confocal microscopy images showing 24 h biofilm growth of *Staphylococcus aureus*. Biofilm growth on **(A)** PLAHAp, **(B)** PLAHApGM films and their intensity map reflecting biofilm thickness in **(C & D)**, respectively, where **(B & D)** reflects the effectiveness of the gentamicin-loaded composites.
GM: Gentamicin; HAp: Hydroxyapatite; PLA: Polylactic acid.

Conclusion & future perspective

At the moment, we are gradually discovering new ways of reproducing structures that are commonly found in nature with desirable properties. The use of biological microstructures is one versatile approach for the reproduction of inorganic structures with identical features. This is achieved by using techniques in biomineral-inspired self-repairing materials chemistry. Multifunctional and multilayered nano coatings and assembly will assist us in this endeavor.

Slow drug delivery and tissue engineering are two common tools that could assist to help us to solve a number of bone related deficiencies and repair. Currently, the demand is clear for better tissue engineering scaffolds that possess more natural bioresponsive environments favorable in guiding the natural processes of regeneration. To meet this biological challenge, novel design and synthesis steps must be incorporated into the new generation scaffolds. We believe that there needs to be a new thinking in tissue scaffold systems that are responsive in which the synthesized biomatrix evolves in real-time to meet the requirements and optimize for the adaptive growth and regeneration of human tissues, while delivering the right biogenetic materials, pharmaceuticals and minerals. Consequently, the environments of the scaffolds will be further adjusted as cells proliferate and differentiate. 3D printing methods are currently used for a number of inorganic and metallic materials and stem cells and other biologic structures are incorporated to these new generation 3D materials for organ regeneration and repair.

During the last decade atomic force microscopy was used as a powerful platform in nanomedicine for studying and controlling the forces involved in cell adhesion and biofilm formation. Atomic force microscopy is well regarded in measuring the small interaction forces between a sharp probe and the surface of a sample. A relatively more recent equipment the single-cell force spectroscopy (SCFS), a single

cell can be attached on the probe in order to measure the interaction forces between the cell probe and a solid substrate or another cells. SCFS-based techniques have recently established as an important tool for understanding how microbial pathogens attach to surfaces and form biofilms [64]. This can be a turning point in our fight against biofilms and infections. SCFS assays will allow us to clarify the specific and nonspecific forces driving cell adhesion on a single-cell basis. Future work in this area will open new avenues for the development of new tools, devices, methods and multifunctional coatings capable of detecting and destroying biofilms.

Nevertheless, nanosynthesis based on biological principles of design and assembly is still in its infancy. Better understanding of biofilm formation and the cell forces is pertinent. The use of bio-inspired nanofabrication techniques including multifunctional multilayer coatings in nanoscale thicknesses for slow drug delivery and tissue repair is a unique approach. This has enormous potential to improve scaffold or lab-on-chip designs with the capability to diagnose, identify, stop biofilm formation, self-repair, micro-evolve and osteointegrate fully.

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Executive summary

Background

- Currently, researchers are focusing on the fabrication of new nanocoatings, nanomaterials, nanolaminates and thin film nanocomposites that are appropriate for applications such as drug delivery and tissue engineering.
- Innovative approaches driven by nanotechnology are now available for the production of synthetic bone-like calcium phosphate nanomaterials.

Surface modifications and liposomes for drug delivery applications

- The appropriate circulation time in addition to the dissolution rates within the human body is the main issue for drug carriers containing nanoparticles.
- The surfaces of nanostructured materials such as nanocoatings can be modified and functionalized with several types of reagents via various biological, chemical and/or physical methods.
- The surface modification approach can be used to achieve functional improvements by design, better osteointegration and stability of nanomaterials and nanocoatings in aqueous media.

Infection & osteomyelitis

- Treatment of osteomyelitis can be with immobilization and antibiotic therapy with a number of drugs including gentamicin.
- Modification or development of thin film or nanocoated multilayer devices with surface properties that have an effect against microbial adhesion or viability seems to be a promising approach for the prevention of device-related infections.
- Bacteria adhesion is a complex phenomenon affected by factors such as the properties of surface materials and characteristics of the bacteria.

Bone repair drug delivery systems & osteomyelitis

- The delivery of antibiotics has become a major focus in research for the use in the treatment of bone infections or as prevention against infection during surgical interventions.
- Although not fully satisfactory, the implantation of antibiotic-loaded PMMA microspheres and calcium sulfate based microspheres and powders into the infection site are the approach currently being used in both orthopedics and in maxillofacial surgery.
- Biodegradable calcium phosphate drug delivery system would be better suited to this endeavor as it can also introduce calcium and phosphate ions and range of minerals to assist bone growth.

Biodegradable composite thin films

- The use of biodegradable polymer thin films is advantageous in the fight against bacterial infection.
- This is due to their tendency to uptake and release pharmaceuticals and minerals as well as the capability to degrade over time.

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