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Scaffold-based Anti-infection Strategies in Bone Repair

Christopher T. Johnson[1,2,3] and **Andrés J. García**[1,4,*]

[1]Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA

[2]Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, GA

[3]School of Medicine, Emory University, Atlanta, GA

[4]Woodruff School of Mechanical Engineering, Georgia Institute of Technology, Atlanta, GA

Abstract

Bone fractures and non-union defects often require surgical intervention where biomaterials are used to correct the defect, and approximately 10% of these procedures are compromised by bacterial infection. Currently, treatment options are limited to sustained, high doses of antibiotics and surgical debridement of affected tissue, leaving a significant, unmet need for the development of therapies to combat device-associated biofilm and infections. Engineering implants to prevent infection is a desirable material characteristic. Tissue engineered scaffolds for bone repair provide a means to both regenerate bone and serve as a base for adding antimicrobial agents. Incorporating anti-infection properties into regenerative medicine therapies could improve clinical outcomes and reduce the morbidity and mortality associated with biomaterial implant-associated infections. This review focuses on current animal models and technologies available to assess bone repair in the context of infection, antimicrobial agents to fight infection, the current state of antimicrobial scaffolds, and future directions in the field.

Key Terms

Biomaterial; Scaffold; Bone Repair; Regeneration; Infection

Implant-associated infection is a significant clinical problem[23]. Bacterial colonization of implants is associated with surgical sites, central line access points, ventilators, surgical drains and shunts, urinary and central venous catheters, and others. Current strategies used to prevent such infections include, but are not limited to, antibiotic therapy, healthcare-provider hygiene, environmental controls such as isolation or negative pressure rooms, surface coatings and modifications, sterilization, and the use of sterile technique during procedures. Nearly all types of bacteria and fungi are capable of infecting implanted devices[23]. Some of the most common pathogens include *Staphylococcus aureus*, *Staphylococcus epidermis*, *Pseudomonas aeruginosa*, *Propionibacterium acnes*, beta hemolytic *Streptococcus*, *Proteus mirabilis*, and *Escherichia coli*[37,77]. The development of

*Corresponding author. Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA, USA. Tel.: +1 404 894 9384; fax: +1 404 385 1397. andres.garcia@me.gatech.edu.

biomaterials with antimicrobial properties to prevent device-associated infection is a rapidly expanding field.

DEVICE-ASSOCIATED INFECTIONS IN BONE RECONSTRUCTION

In the field of orthopedics alone, 2–5% of all procedures involving implants are complicated by infection[23]. This number can be as high as 30% when open fractures are present[93]. Significant morbidity and even death are associated with implant-related infections, with outcomes often leading to complete implant removal, surgical debridement of the affected tissue, and long-term antibiotic therapy[55,56]. Device-associated infections not only occur from direct implantation of bacteria, but also develop post-operatively following hematogenous bacteremia, or direct spreading from a nearby infection site[55,56]. Further complicating treatment is the emergence of antibiotic-resistant bacteria[34]. Choosing the correct antibiotic for initial treatment is directly correlated with successful infection management and becomes more difficult in the case of nosocomial infections, due to the inherent resistance that these organisms possess[75]. The above circumstances motivate the development of implantable materials with antibacterial properties to significantly improve surgical outcomes and reduced patient morbidity and mortality. Engineered scaffolds for regenerative medicine applications provide a framework for tissue repair as well as a substrate for the inclusion of antimicrobial properties.

BIOFILM AND NONUNION DEFECTS

Device-associated infection is characterized by bacterial adhesion, colonization, and biofilm development, which is outlined in Figure 1[9,94]. The most common organisms associated with orthopedic implant infections include the gram positive strains *Staphylococcus epidermis*, *Staphylococcus aureus*, and *Propionibacterium acnes*, as well as the gram negative *Pseudomonas aeruginosa*[94]. Osteomyelitis is inflammation of the bone, which can be due to biofilm formation, causing increased bone resorption and reactive bone formation[55,56]. These biofilms are composed of secreted bacterial components, such as protein, lipid, lipopolysaccharide, and DNA[9], forming a matrix around the bacteria that provides protection from antibiotic therapy and immune defenses[21,57]. Bacteria in a biofilm have higher mutation rates[30], and can display increased virulence[73] than if growing planktonically, and when exposed to antibiotics, mutation rates increase further, allowing for accelerated development of a drug-resistant phenotype[64]. Moreover, incomplete resolution following therapy leads to highly resistant cells, or persistors, that then replenish the biofilm[58]. These characteristics paired with availability of only semi-effective treatment options leave a significant, unmet need for the development of therapies to combat device-associated biofilm and infection[9,63].

Non-union bone defects are fracture injuries that cannot heal without intervention. Currently, standard medical therapies include the use of bone auto- and allo-grafts, or delivering high doses of therapeutic protein, such as bone morphogenetic protein 2 (BMP-2), to facilitate healing. However, there are unacceptably high failure and complication rates associated with these interventions[25], which are significantly increased when an infection develops[47,60,66]. Advances in biomaterials and regenerative therapies

have led to the development of engineered scaffolds capable of correcting non-union defects without the need for bone grafting procedures[27]. These strategies for bone repair often rely on biomaterial-based scaffolds to bridge the defect. This provides a convenient framework to introduce antibacterial agents to prevent and treat infection after surgical intervention.

Engineering regenerative medicine implants to overcome bacterial contamination is a critical and emerging area of biomaterials research. These technologies require rigorous *in vitro* and *in vivo* evaluation, bringing together the fields of microbiology and biomaterials engineering. Significant progress has been made in the design of infection-resistant surfaces, as recently reviewed by Campoccia *et al.*[14,15]. Therefore this review will focus on relevant animal models and techniques to assess antimicrobial tissue scaffolds in the context of bone repair, potential therapeutic additives to fight infection, and the current and future of scaffolds with infection resistant properties to promote bone repair.

ANIMAL MODELS TO ASSESS INFECTION AND BONE REPAIR

Successful evaluation of antimicrobial scaffolds for bone regeneration requires the development of reliable and robust infection models. This proves to be a very challenging task, as pathogenic bacteria are required to induce the infection without causing overly adverse harm to the host. Furthermore, the model should provide a sustained infection over a prolonged period of time to have increased relevance to human health. Several animal models to assess fracture therapy exist, and appropriate model selection was discussed in depth by Mills and Simpson[69]. Bone regeneration is most frequently evaluated using critical-sized, segmental defect models. Critical-sized segmental defects are bone injuries that do not spontaneously heal, allowing for assessment of bone regeneration due to the therapy, such as implantation of a scaffold.

Few validated models exist to evaluate bone regeneration in infected defects. These models introduce infection to bone repair models. Table 1 summarizes validated models developed to characterize the efficacy of antimicrobial bone repair scaffolds. The most common repair model extends the rat femoral segmental defect[102] to include pathogenic bacteria[17–20,103]. Femoral segmental defects have been widely used in regenerative medicine studies, and allow for the evaluation of a long, weight-bearing bone that will not spontaneously heal. This procedure requires bone fixation hardware and effectively tests the reparative capacity of regenerative scaffolds. To introduce infection, two distinct inoculation techniques exist. In one, a segment of the femur is excised, the bone is stabilized, bacteria is introduced, and the surgical site is closed. Once enough time has elapsed for the infection to become established, reoperation occurs and the infected tissue is debrided and a sterile regenerative scaffold is placed[17–20]. This method has been used to evaluate osteogenic protein-1[17] as well as systemic antibiotic therapy paired with recombinant human BMP-2[18] and recombinant human osteogenic protein-1[19] in the presence of infection. This technique provides clinical relevance, as it mimics how implant infections are treated, but two surgical procedures may increase variability and add more stress to the animal. The second approach requires a single procedure, where the defect is created and stabilized and the implant is placed. Following implant placement, the pathogen is injected into the implant (or the implant is inoculated prior to implantation), simulating intraoperative contamination[103].

This technique is advantageous because it only requires a single procedure, which may reduce variability associated with surgery. This model was later adapted to realize a 50% overall infection rate in order to reduce the chances of observing false-negative (type II error) *in vivo* results[74]. An infected femoral segmental defect model in the rabbit has also been reported where infection was induced 48 hours after bone excision and defect stabilization by a percutaneous injection of a bacterial suspension[88]. These models provide an economical way to assess bone-healing strategies, but are complicated by requiring defect fixation with plates and wires. Stabilization can pose a problem when assessing the antimicrobial abilities of regenerative scaffolds if the stabilization pins become infected and cause failure[17].

Self-stabilizing segmental defects could be a means to avoid complications associated with infected stabilization hardware. Self-stabilization is achieved by removing a segment of a non-weight bearing bone, such as the radius. This allows for the study of regenerative implants in critically sized defects of long bones that will not self-heal, but may not be as clinically relevant since many orthopedic procedures require fixation of long bones. Bi *et al.* developed a lapine radial segmental defect infection model to assess localized antibiotic release compared to systemic therapy[8]. In this model, a defect was created and a bacterial suspension was placed in the wound. After 30 minutes, the area was washed, the implant was placed and the wound was closed. This model only requires a single procedure and also simulates intraoperative contamination. Although several different animal models have been developed to assess bone repair, to our knowledge a validated murine model has not yet been published, even though murine models have been used extensively throughout the osteomyelitis literature[78].

The advent of *in vivo* imaging systems has significantly improved the analysis of biomaterial-associated infections[87]. Genetic engineering of bioluminescence genes into clinically relevant bacterial strains allows for *in vivo* monitoring of infection. Commercially available gram positive (Xen29 *S. aureus*) and gram negative (Xen5 *P. aeruginosa*) strains contain a stable luminescence reporter, and can be tracked over time *in vivo*, providing the assessment of infection progression[48], and treatment efficacy[49]. However, limitations do exist. For example, the luminescence signal detected is not a direct marker of the number of bacteria, but of the metabolic activity of the colony[32,48,49]. The population of bacteria making up a biofilm is composed of both rapidly dividing and quiescent cells. This heterogeneity may be a possible explanation for the large variability between bacterial counts and bioluminescent signal. The use of bioluminescent bacteria has been successfully established *in vivo* in the context of osteomyelitis[36,42], suggesting that this technology could be adaptable to monitoring scaffold-associated infections in bone repair. Nevertheless, genetic modification of bacteria through bioluminescent gene insertion could reduce the virulence of the clinically isolated strains, which could complicate the evaluation of infection resistant materials.

In addition to bioluminescent bacteria, several *in vivo* probes utilizing fluorescent, magnetic, and radioactive tracers have been developed. Near infrared (near-IR) imaging probes that specifically identify bacteria have received heightened interest as a viable alternative to luminescent bacteria. Discrimination between infection and inflammation is the key

challenge associated with their development[31]. Eggleston and Panizzi provide an extensive review on this topic[31]. Our lab has recently developed near-IR probes that specifically discriminate between infection and inflammation through targeting the products produced by the inflammatory response[91]. Reactive oxygen species (ROS) are characteristic of the body's response to biomaterials implants, whereas large quantities of nitric oxide (NO) are produced by macrophages and neutrophils in a direct response to bacteria. Dual administration of ROS- and NO-selective probes allows for the simultaneous *in vivo* observation of infection and inflammation with high specificity[91]. Furthermore, we have shown these fluorescent probes exhibit increased sensitivity compared to bioluminescent strains. Fluorescent probes also have a dose dependent response to the number of bacteria regardless of metabolic activity, in a strain independent manner[28]. Other strategies to achieve specificity include utilizing antimicrobial peptides that have been labeled with radioactive isotopes and paired with clinically available imaging systems, such as SPECT (single photon emission computed tomography)[12], and labeling the antibiotic vancomycin with a near-IR fluorophore to identify gram positive infections[96]. The technologies described above provide real-time, *in vivo* means to monitor infection initiation, progression, and resolution, and could provide an indispensable tool in the development of infection-resistant scaffolds.

Although significant effort has been made to develop finely tuned animal models for the assessment of a materials antibacterial properties as described above, ethical concerns do exist surrounding these methods. This is especially relevant when evaluating infection resistant properties of scaffolds after a sterile implantation, which is the most clinically realistic scenario. These types of studies require large animal numbers to adequately power the analysis due to the relatively low rates of spontaneous infection developing (less than 7%) and that both the control and treatment groups will require large animal numbers to resolve a difference[54]. Concerns also exist surrounding animal welfare. Many infection models are highly variable and it can be challenging defining a sub-lethal bacterial dose that does not cause animal suffering. This is particularly difficult, as simply increasing the bacterial dose could result in sepsis and termination before the desired experimental end point.

ANTIMICROBIAL AGENTS TO FIGHT INFECTION

Several different strategies exist to combat bacterial infection. Table 2 provides a list of major antimicrobial strategies [2,24,39,40,67]. Brief overviews of the major antibacterial classes, including the advantages and limitations of each follow.

Clinically, antibiotics are the most common agent used to clear bacterial infections. They are widely used throughout clinical medicine as treatment and prophylaxis. However, over the past decades, the emergence of antibiotic-resistant bacteria, such as methicillin resistant *Staphylococcus aureus* (MRSA), have become more common[24]. Sub-inhibitory aminoglycoside antibiotic treatment can induce biofilm formation[41]. The development of biofilm can potentiate the emergence of resistant cells, further complicating the infection[89]. Biofilm requires higher doses and longer trials of therapy to eradicate infection, thereby prolonging the patient's exposure to drug side effects. Moreover, it has been shown that

bactericidal antibiotics are toxic to mammalian cells, causing mitochondrial dysfunction[50]. However, the benefits of treatment far outweigh the risks, and until viable alternatives are available, antibiotics will remain the standard of care. For a comprehensive review of antibiotic therapy including drug mechanisms, specificities, and the development of resistance, refer to Davies and Davies[24].

Silver is a broad-spectrum antimicrobial agent used in research and clinically. Silver exerts bactericidal activity on both gram positives and gram negatives through several mechanisms. Silver ions enter the bacterium and generate ROS capable of damaging DNA, they interact with membrane proteins affecting their function, and alter membrane permeability leading to cell death[67]. It is believed that silver resistance is widespread, but not realized since it is not widely tested for. A Chicago hospital revealed that over 10% of enteric bacteria exhibit silver resistance[86], and overuse could potentiate the problem. Furthermore, the bactericidal mechanisms of silver ions are not specific to bacterial cells, and also disrupt mammalian cell function placing significant concern on toxicity[5,10]. However, it has been reported that silver can be effective against antibiotic-resistant bacterial strains, and even induce susceptibility towards antibiotics that were ineffective in the absence of silver[71]. Silver can also be adapted to reduce bacterial adherence to orthopedic implants by killing adherent pathogens[95]. For a more detailed discussion, the reader is referred to Marambio-Jones and Hoek[67]. Clinically, silver has translated to several applications, including wound dressings, creams, urinary catheters and endotracheal tubes. However, little if any data has demonstrated efficacy. An analysis of 2066 patients enrolled in several clinical trials failed to show any benefits to silver-doped wound dressings[90]. Silver-coated endotracheal tubes[53] have exhibited modest efficacy in preventing bacterial colonization, whereas silver-coated urinary catheters have shown mixed results[6].

Host defense peptides or antimicrobial peptides (AMPs) have activity against bacteria, viruses, and fungi[45]. Defensins, cathelicidins and histatins are AMPs produced by many mammalian cells[26]. AMPs are amphiphilic peptides characterized by a several cationic and hydrophobic residues and exhibit broad-spectrum activity against both gram positive and gram negative bacteria[26,40,45]. The cationic residues associate with the negatively charged bacterial membrane. The hydrophobic and hydrophilic residues cause membrane penetration, leading to instability, pore formation, osmotic changes, and bacterial lysis[45]. As with all antimicrobial strategies, the development of resistance is a concern. This could be especially problematic since AMPs are part of the natural host response to pathogens and resistance could make simple infections dangerous[2,72]. Another drawback is the observation that AMPs are not stable over long periods of time in an *in vivo* environment. However, AMPs are easily engineered, and several synthetic peptides have been developed in an attempt to overcome these shortcomings[11]. It has been well documented that AMPs possess immunomodulatory activity in addition to being antipathogenic[26,40,45]. AMPs modulate both the innate and adaptive immune responses to control infection and stimulate regenerative processes[40]. These attributes make AMPs an enticing candidate for antimicrobial regenerative scaffolds. However, there are no reports of human safety or efficacy trials for AMPs.

Bacteriophage therapy has gained renewed interest with the increased prevalence of antibiotic resistance[62]. Bacteriophages are viruses that specifically infect bacteria. The phage binds to a membrane receptor, introducing phage DNA into the cell. This DNA is replicated and translated by the host bacterium, leading to phage replication, progeny assembly, bacterial lysis, release of progeny, and phage propagation to surviving bacteria. Following eradication of the infecting organism, phage replication ceases, allowing for resolution of the affected tissue. Bacteriophage DNA can also code for lysins, lytic enzymes that destroy the bacterial cell wall[84], as well as polysaccharide depolymerases, enzymes that break down the biofilm matrix created by bacteria[44,61]. This allows bacteriophages to disperse biofilm as well as eradicate infection. In addition, synergism between phage therapy and antibiotics has been demonstrated[81]. Host bacterial strains can develop resistance to phage infection, which can be reduced using several different phages at once[35]. There are also concerns surrounding the immunogenicity of *in vivo* phage administration, even though adverse events have not been reported in the literature[4,70]. Currently, the safety of bacteriophage therapy administered orally[13] and cutaneously[79] has been evaluated in humans in phase I clinical trials. Preliminary results of the first controlled trial to evaluate bacteriophage efficacy in chronic otitis to treat antibiotic-resistant *P. aeruginosa* have been positive, demonstrating therapeutic value in humans[99].

ANTIMICROBIAL SCAFFOLDS FOR BONE REPAIR

Recently, tissue engineered scaffolds for bone repair have started to include antimicrobial agents to prevent or fight infection. These scaffolds provide a substrate for sustained, localized drug release, tunable degradation properties to promote tissue integration, and support for cell delivery. Rigorous evaluation of the antimicrobial efficacy exhibited by these scaffolds has proven difficult, and requires expertise in both microbiology techniques as well as biomaterials engineering. Antimicrobial scaffolds are required to be toxic to bacterial cells, while promoting local tissue regeneration and minimizing the adverse inflammatory events. Figure 2 is a schematic diagram illustrating how engineering antimicrobial properties into scaffolds for bone repair can improve outcomes associated with bacterial infection. Bacterial contamination is introduced into a model used to evaluate regenerative implants. If the contaminate is not cleared by the immune system or an infection-resistant scaffold, the infection becomes established, which may lead to the development of osteomyelitis. Infection fighting scaffolds can be implanted into defects with ongoing infection or osteomyelitis to remove the existing pathogen and facilitate repair. Further development of these technologies will allow for bone repair to occur in both sterile and contaminated conditions.

Antibiotic-releasing scaffolds

Antibiotic delivery scaffolds are the most well developed area in the literature. In orthopedics, antibiotic-loaded fillers and bone cements have been used clinically for a number of years. Zilberman and Elsner published an extensive review on antibiotic-releasing materials[104]. We will focus on advances in tissue engineering scaffolds that incorporate antibiotics.

Scaffolds provide an ideal substrate to deliver long-term bactericidal doses of antibiotics to the injury site. This is accomplished by modifying drug release characteristics through encapsulation within degradable matrices. Antibiotic releasing matrices have been used as coatings for orthopedic implants prone to infection. Sol-gel thin films have been engineered to provide sustained release of vancomycin and protect against implant associated infection of titanium rods. The addition of the thin film minimized bacterial adherence to the implant, and protected against the development of osteomyelitis *in vivo*[1]. A similar has also been applied to stainless steel K-wires[7].

Antimicrobial activity can also be engineered into scaffolds for tissue regeneration. Poly (L-lactic acid) (PLLA) nanofiber scaffolds were synthesized with poly (lactic-co-glycolic acid) (PLGA) nanospheres to provide extended release of the antibiotic doxycycline[33]. These scaffolds provided sustained antimicrobial activity against *S. aureus* and *E. coli* over 42 days in bacterial culture, demonstrating an approach to provide extended, localized antibiotic release, which would reduce the systemic side effects associated with antibiotic therapy. This is especially important when treating osteomyelitis, which typically involves extended courses of high dose antibiotics. Poly(caprolactone) (PCL) scaffolds were synthesized by electrospinning PCL using 10% and 20% (w/w) rifampin[80]. These scaffolds exhibited extended rifampin release over eight hours and were bactericidal towards *S. epidermis* and *P. aeruginosa in vitro*. Shi *et al.* demonstrated the addition of lecithin can increase the encapsulation efficiency of gentamicin and protein into PLGA microsphere-based scaffolds[85]. After an initial burst release, gentamicin release occurred for 60 days, and protein was released for 18 days. The material was active against *E. coli* while still supporting mesenchymal stem cell (MSC) viability, proliferation, and mineralization. These observations suggest that this scaffold is a viable candidate for delivering protein therapeutics as well as antibiotics, and supporting bone formation for the treatment of infected bone defects. The encapsulation of growth factors has also been paired with antibiotic encapsulation. Calcium sulfate scaffolds with chitosan microspheres containing vancomycin, recombinant human BMP-2 (rhBMP-2), or both were developed and assessed *in vitro* for bactericidal activity and regenerative properties[29]. It was shown that these scaffolds are bactericidal against *S. aureus* for 18 days and release rhBMP-2 over 6 weeks, causing increased alkaline phosphatase (a marker of osteoblast differentiation) expression. These investigators found that an optimal balance between antibiotic and growth factor release is required for optimal osteoblastic differentiation, as high antibiotic concentrations can lead to inhibition of osteoblastic differentiation. However, these techniques have not yet been extended to *in vivo* models.

As mentioned above, the current standard of care for critically sized bone defects is bone grafting. Infection is one of the most significant side effects associated with grafting procedures. Bi *et al.* engineered bone xenografts (grafts from a different animal species) composed of antigen-free calf cancellous bone combined with calf cortical bone extract and bovine BMP impregnated with clindamycin to treat critically sized defects contaminated with *S. aureus*[8]. This scaffold was evaluated *in vivo* within a rabbit radial segmental defect. After the graft was implanted, 1×10^6 colony forming units (CFU) of *S. aureus* were administered to the injury. All animals in the clindamycin-impregnated graft group healed

completely. Defect repair was observed in the clindamycin-graft group including recanalization of the medullary cavity. Systemic clindamycin therapy resulted in either non-union, or delayed union after the 16 week period, whereas the non-treatment control developed osteomyelitis characterized by reactive bone formation and resorption. This study shows that local, sustained delivery of antibiotics can overcome an infection, while still providing regenerative properties.

The bone graft substitute calcium sulfate has also been combined with antibiotics and assessed *in vivo*. The antibiotic moxifloxacin has been evaluated with commercially available Stimulan®, a synthetic semihydrate form of calcium sulfate[51]. In this study, osteomyelitis was induced in a rabbit tibia by injection of 2×10^7 CFU of a clinical osteomyelitis isolate of MRSA into the intramedullary cavity. After the infection was allowed to develop for three weeks, the rabbits underwent surgical debridement of all necrotic bone tissue and implant placement. The results showed a significant reduction in viable bacteria throughout the six week observation period. *In vitro* assessment of the delivery system showed sustained moxifloxacin release over 35 days. However, this study did not evaluate whether the regenerative properties of the moxifoxacin doped Stimulan® are still intact in the presence of sustained antibiotic release. Xie *et al.* compared bioactive borate glass to the clinically used calcium sulfate as a carrier for vacomycin to treat MRSA-induced osteomyelitis in rabbits[101]. Bioactive borate glass provided sustained vancomycin release over 28 days *in vitro* and improved mechanical properties compared to calcium sulfate. The scaffold was assessed *in vivo* using a rabbit model for osteomyelitis. After three weeks of infection, surgical debridement was performed and scaffolds were placed within the defect. Both the vancomycin-loaded calcium sulfate and vancomycin-loaded borate glass significantly reduced the number of bacteria, improved the radiographic score and improved the histopathologic score at the end of the eight-week observation period. This study further illustrates that scaffolds serve as an effective mechanism to provide sustained antibiotic therapy to eradicate osteomyelitis.

Polyurethane scaffolds have also received interest as a substrate to deliver antibiotics and growth factors for bone repair. Polyurethane scaffolds designed for prolonged release of vancomycin were compared to the clinically used vancomycin-loaded PMMA- beads[59]. Extended vancomycin release from polyurethane scaffolds could be controlled by changing the solubility of vancomycin. *In vivo* evaluation of the vancomycin-loaded polyurethane scaffolds in a contaminated rat femoral segmental defect reduced viable bacterial counts as well as the clinical standard of vancomycin-loaded PMMA beads. Importantly, nearly 10 times less antibiotic was loaded to the polyurethane scaffolds. Dual delivery of vancomycin and the growth factor BMP-2 to a *S. aureus* infected rat femoral segmental defect using a biodegradable polyurethane scaffold demonstrated increased bone formation as determined by microCT and histological analysis[38]. The addition of vancomycin to the scaffold reduced the clinical signs of infection while not affecting bone regeneration. Together, these studies illustrate that extended release of vancomycin can eradicate infection and the addition of BMP-2 can enhance regeneration in contaminated defects.

Clearly there has been significant progress in towards the development of antibiotic releasing materials for bone repair. However, as mentioned before, biofilm can offer

protection to the microorganisms against antibiotic therapy, leading to the development of resistance. In a study evaluating the efficacy of gentamicin-loaded bone cement against the well-known biofilm former *S. epidermis* in rats, it was shown that even though the number of bacteria was reduced, there was a significant increase in the number of gentamicin-resistant bacteria[92]. In another study evaluating vancomycin-releasing polyurethane scaffolds in an infected rat segmental defect, significantly fewer bacteria were recovered at two weeks[99]. Nonetheless, this was only a roughly three-fold reduction, leaving over 1×10^5 CFU/gram of bone tissue, demonstrating a significant limitation in effectively treating infections.

Silver-presenting scaffolds

Silver can be easily incorporated into materials through various manufacturing techniques such as reduction or the addition of silver nanoparticles. This ease of incorporation combined with silver's broad spectrum antimicrobial activity has led to the development of several silver-containing antimicrobial scaffolds. Several designs have demonstrated *in vitro* efficacy, but success *in vivo* has been limited.

Naturally derived tissue engineered scaffolds have been used for a multitude of applications, include bone repair. These materials can be modified to present silver and exhibit infection resistance. Collagen scaffolds were fabricated to include silver nanoparticles coated with poly(ethylene glycol) (PEG) and Triton X-100 [65]. The scaffolds had increased elasticity and antimicrobial effects against both gram positives (*B. cereus* and *S. aureus*) and gram negatives (*E. coli* and *P. mirabilis*). Silver nanoparticles have also been incorporated into type I collagen scaffolds synthesized using UV initiation of a non-toxic, water-soluble benzoin to facilitate polymerization[3]. The collagen scaffolds served to stabilize the nanoparticles and supported fibroblast and keratinocyte viability at silver concentrations less than or equal to 100 μM. Bactericidal activity (*E. coli*, *B. megaterium* and *S. epidermis*) was determined using a modified minimum inhibitory concentration assay. These studies show that collagen-based scaffolds that include silver nanoparticles can prevent bacterial growth *in vitro*, while also supporting mammalian cell viability. Further development of these technologies and evaluation in *in vivo* models is necessary to establish the feasibility of silver nanoparticle-containing collagen scaffolds for infection prevention and bone repair. Bioactive glass containing silver has been incorporated into extracellular matrix-derived hydrogels to exhibit sustained antimicrobial effects and bone regenerative properites[16]. These materials show sustained silver ion release over 25 days and is bactericidal against *E. coli* and *E. faecalis*. The composite hydrogels support dental pulp cell viability, making them a plausible candidate for tooth or bone regeneration. Silver ions have been added to composite chitosan/nano-hydroxyapatite scaffolds to add antimicrobial properties[83]. The chitosan/nano-hydroxyapatite scaffolds were immersed in silver nitrate, allowing for an ion-exchange and reduction to occur between the scaffold and silver. The scaffolds support osteoprogenitor and osteosarcoma cell viability and demonstrate antimicrobial effects against both gram positive and gram negative bacteria (*S. aureus* and *E. coli*).

PLGA has been of particular interest for bone repair due to its biocompatibility, degradable properties, and being used in FDA-approved devices. Silver was incorporated into tricalcium

phosphate (TCP) nanocomposite, mixed with PLGA and then electrospun to form a fibrous scaffold. These scaffolds provided sustained silver release at bactericidal levels *in vitro* against *E. coli*, a frequent contaminator of dental implants. The scaffolds were equally as effective as the clinical standard of tetracycline-soaked cotton swabs. However, upon media exchange in the assay, the silver scaffolds maintained antimicrobial ability due to sustained release characteristics. This study demonstrates the importance of sustained antimicrobial release, and that scaffolds for tissue engineering provide a convenient avenue to accomplish this. Zheng *et al.* reported a promising antimicrobial regenerative scaffold[103]. In this study, microporous PLGA scaffolds were fabricated to contain silver nanoparticles. Interestingly, 1.0% silver containing grafts supported increased osteoblastic differentiation and increased alkaline phosphatase activity compared to the 2.0% silver grafts *in vitro*. These scaffolds were evaluated using a rat femoral segmental defect. After implantation, 1×10^8 CFUs of vancomycin-resistant MRSA was injected into the implant. Radiographic and histological analysis showed that the 2.0% silver implants completely eliminated infection and supported defect bridging, whereas the 1.0% silver implants only reduced the number of bacteria present, but supported some bone regeneration. Control scaffolds that did not contain silver were grossly infected, demonstrating bone resorption and reactive bone formation, indicative of osteomyelitis. The *in vitro* analysis paired with the *in vivo* data show that although high concentrations of silver can inhibit osteoblast differentiation, it is more important to eliminate the contaminating bacteria to facilitate bone formation. This is a clear demonstration that developing implants capable of resisting infection while providing functional cues to facilitate bone repair is possible.

As an alternative to silver, copper ions loaded into microporous bioactive glass scaffolds reduce bacterial growth and support MSC viability and differentiation[100]. These scaffolds significantly reduced *E. coli* growth, and promoted human MSC differentiation towards osteoblasts in a dose dependent manner. Vascular endothelial growth factor levels were also elevated, suggesting the scaffold could promote vascularization.

Antimicrobial Peptides, Bacteriophage, and Other Antimicrobial Strategies

Interest has been building surrounding technologies that take advantage of alternative antimicrobial therapies. These alternatives to silver and antibiotics could expand the arsenal against infection, while also reducing the chances of bacteria developing resistance to our most efficacious treatments. Scaffolds provide a means to extend the activity of these agents by providing sustained release characteristics. Antimicrobial peptides have been introduced into scaffolds designed for orthopedic regeneration. Poly(caprolactone) (PCL)-chitosan nanofiber scaffolds were synthesized and PEG-microgels containing the cationic antimicrobial peptide L5 were electrostatically associated with the nanofibers[98]. These novel scaffolds demonstrated antimicrobial activity against *S. epidermis*, and maintained L5 stability and activity. The scaffolds supported osteoblast adhesion, spreading, and proliferation.

Stainless steel K wires used in orthopedic procedures coated with a hydroxypropylmethlycellulose (HPMC) hydrogel containing bacteriophage, the antibiotic linezolid, or both were developed to prevent MRSA infection[52]. The coated wires showed

sustained phage and linezolid release over several days, as well as inhibiting MRSA adherence in a dose dependent manner. The bacteriophage and linezolid group exhibited the greatest efficacy toward inhibiting MRSA attachment and growth, suggesting synergism exists between the co-delivery of antibiotics and bacteriophage. This claim was further supported by analysis of recovered MRSA after treatment showing reduced mutation rates in the dual treatment group suggesting lower drug resistance. This *in vitro* evaluation of scaffolds presenting bacteriophage and antibiotics suggests the treatment could be extended to an *in vivo* environment to prevent infection associated with stainless steel implants. Bacteriophage has also been evaluated in a regenerative context. In one study, the *E. coli* bacteriophage λ was loaded into microporous hydroxyapatite or beta-tricalcium phosphate scaffolds with various porosities by passive adsorption[68]. Bactericidal activity against *E. coli* K12 was observed *in vitro*, demonstrating the prophylactic potential bacteriophage loaded materials could provide.

Polyelectrolyte scaffolds assembled by electrostatic interactions of chitosan gamma-polyglutamic acid and carboxy-methylcellulose were developed for treating dental bone defects. These scaffolds supported pre-osteoblast cell adhesion and viability *in vitro*, and antimicrobial activity against *S. aureus* and *E. coli*. Scaffold biocompatibility was assessed by extracting the second pre-molars of beagle dogs and replacing them with the material. The scaffolds were explanted after 10 weeks and histology revealed no adverse foreign body reaction.

Neutrophils and macrophages produce peroxide and other free radicals to kill invading pathogens. This mechanism was extended to electrospun polycaprolactone (PCL) scaffolds with different concentrations of calcium peroxide to exhibit antimicrobial activity by releasing a significant initial burst of peroxide[97]. This short-term antimicrobial response was effective in controlling *E. coli* and *S. epidermis in vitro*, illustrating broad applicability. The nanowires supported osteoblast viability for four days of culture despite the cells being exposed to toxic peroxide levels for the first 24 hours. This novel method of direct peroxide generation from a PLC scaffold shows that burst release from materials can be toxic to bacteria but still provide a means to promote bone growth.

Berberine is a natural antimicrobial agent that exhibits activity against several different organisms and is non-toxic to mammalian cells. For these reasons, Huang *et al.* incorporated it into a chitosan coating on a nano-hydroxyapatite/polyamide66 scaffold developed for bone regeneration[43]. These scaffolds provided a continuous release of berberine over 150 hours and were bactericidal to *S. aureus*. Furthermore, the scaffolds supported MG63 cell adhesion, proliferation, and spreading, supporting that berberine is nontoxic. However, this material has not been evaluated *in vivo*. These data provided preliminary evidence that berberine may be suitable for *in vivo* evaluation to provide antimicrobial and regenerative properties in a bone repair setting.

Preventing biofilm formation may be another way to protect against chronic osteomyelitis. Sanchez *et al.* demonstrated biofilm dispersal agents reduce infection *in vivo*[82]. A polyurethane scaffold containing D-amino acids was contaminated with *S. aureus* and implanted into a rat femoral segmental defect. The treated scaffolds significantly reduced the

number of contaminating bacteria, showing that preventing biofilm formation can improve post-operative outcomes, by preventing the biofilm from shielding the bacteria from endogenous antimicrobial defenses.

CONCLUSIONS AND OUTLOOK

Preventing infection in the presence of biomaterials implants is a major unmet need and will significantly improve patient outcomes. Currently, implant infection leads to removal, and significant medical costs from reoperations and extended antibiotic therapy. Moreover, after an initial infection, patients are at a much higher risk for relapse, further complicating management and causing increased patient morbidity. As medicine advances, we have become more and more reliant on implantable devices to more effectively correct patient problems, which increase the risk of implant-associated infections[23]. Demand exists for the prevention of orthopedic implant infections due to the frequency of their occurrence, as well the challenges associated with combating osteomyelitis. Despite improvements in intraoperative techniques and the invention of antibiotic-doped cements and fillers, infection continues to be a significant issue associated with non-union defects. Furthermore, the increased prevalence of antibiotic resistant bacteria raises concern over widespread use of antibiotic presenting materials. This suggests alternative antimicrobials such as silver, antimicrobial peptides or bacteriophage could help to preserve the efficacy of our most potent weapons against infection. These alternative strategies to fight infection offer exciting opportunities to introduce new properties into scaffolds. For example, the rapid expansion, but self-limiting characteristics of a bacteriophage infection provide a way to engineer materials that respond only when a pathogen is present. Scaffolds can shield the phage from the host response, while providing activity only in the presence of offending bacteria. Antimicrobial peptides can enhance the body's defenses against pathogens, and even promote wound healing. Scaffolds can serve a means to extend the stability of these peptides and enhance their utility.

In addition to extending the stability of antimicrobial agents, scaffolds provide a highly controlled means to release therapeutics. Modulation of scaffold degradation typically correlates with therapeutic drug release. Traditionally, bone repair is driven by a scaffold degradation leading to therapeutic release. The drug release recruits cells and further promotes scaffold degradation, leading to tissue healing. This process is outlined in Figure 3. Degradable scaffolds are also advantageous from an *in vivo* infection resolution point of view. Implanted biomaterials are prone to infection after implantation by transient bacteremia causing colonization and direct bacterial spreading from infection sites[55,56]. Degradable scaffolds provide the benefit of controlled therapeutic release while facilitating integration into the native tissue.

Degradable scaffolds to treat infection and regenerate bone have been primarily investigated in the bioactive glass literature[76]. These studies are mostly centered on extending the release of antibiotics to provide continuous antimicrobial activity[22,46,101]. However, a significant gap exists in understanding how the degradation properties of scaffolds influence antimicrobial efficacy *in vivo*. Future studies could focus on optimizing scaffold degradation properties to efficiently eliminate pathogens and guide the bone repair process. These

studies can then be extended to characterize and understand how engineering extended release of antimicrobial therapies affects drug activity though drug scaffold interactions. Modifications of scaffolds to provide continuous release may negatively impact the efficacy of the loaded therapeutic.

The next generation of antimicrobial scaffolds for bone repair will optimally balance antimicrobial delivery with regenerative therapeutics. This could be achieved by tuning material properties such as porosity, charge, degradation speed, density, antimicrobial agent, growth factors, and the bulk material. Understanding how material design choices prevent bacterial contamination, biofilm development, eradication of existing osteomyelitis, while simultaneously regenerating bone, will lead to optimized scaffold designs.

In order for these new technologies to translate into the clinic, several challenges need to be overcome. The development of robust, controlled, and reproducible animal models of infected scaffolds is a critical need for the success of this fast emerging field. Animal models that utilize bioluminescent bacteria allow for real time monitoring of infection progression without animal sacrifice, which addresses some of the ethical concerns of biomaterial infection research. Reproducible, controlled infections that accurately simulate clinical scenarios are required to effectively evaluate experimental materials to prevent infection and facilitate bone regeneration.

Scaffolds provide an ideal substrate for designing regenerative therapies due to the exquisite engineering control we have over them. They provide a platform for controlled drug release, a substrate for therapeutic cell delivery, tunable degradation characteristics that facilitate replacement by regenerating tissue, reduced immunogenicity, and response to the surrounding environment. It is clear that progress is being made towards the development of infection-resistant bone repair implants. However, the *in vivo* validation of these technologies is still in its infancy. The advancement of *in vivo* imaging techniques, paired with robust bone repair models will facilitate the translation from the bench to the bedside.

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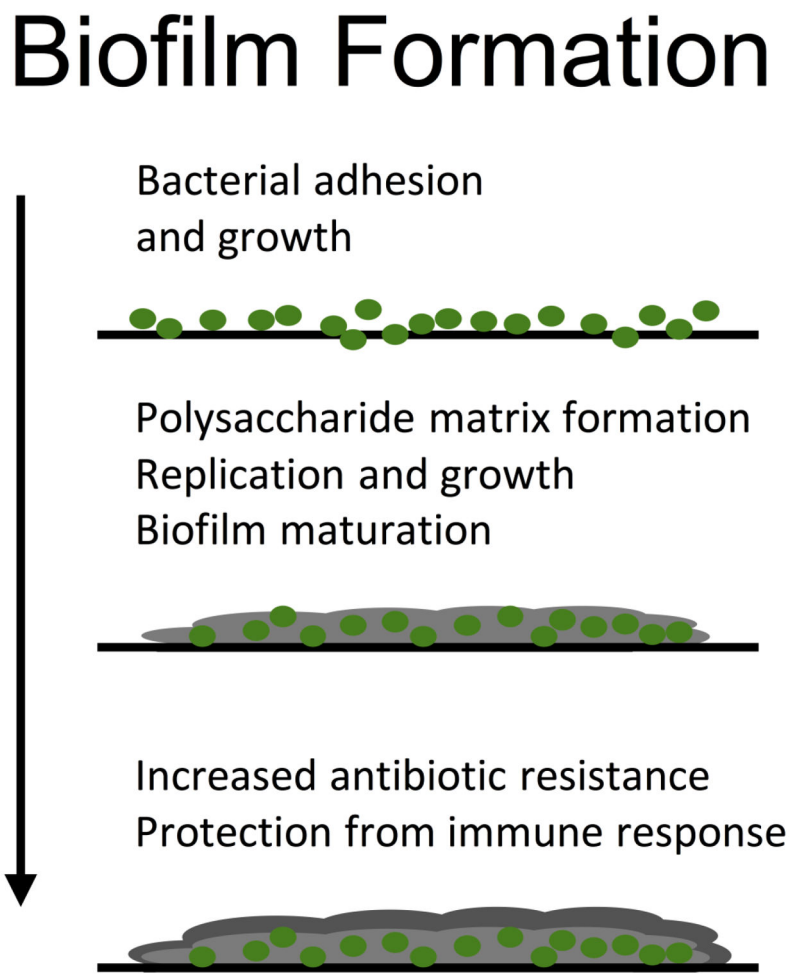

Figure 1. Bacterial adhesion and biofilm development

Biofilm formation begins by bacteria adhering and growing on a surface. As the pathogen continues to replicate a polysaccharide matrix is deposited. This matrix protects the pathogen from the host immune system and increases the development of antibiotic resistance.

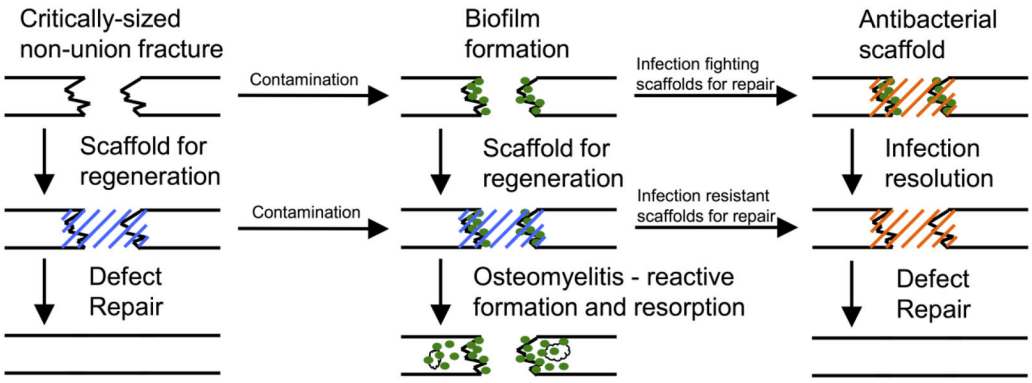

Figure 2. Critically-sized non-union bone defects are used to assess the therapeutic efficacy of regenerative scaffolds

Contamination of these defects can be introduced before or after the scaffold is placed to establish the infection. Absence of antimicrobial agents will lead to the development of osteomyelitis, which is characterized by bone resorption and reactive bone formation. Infection resistant scaffolds are designed to prevent initial bacterial colonization whereas infection fighting scaffolds can be used to resolve an established biofilm and promote defect repair.

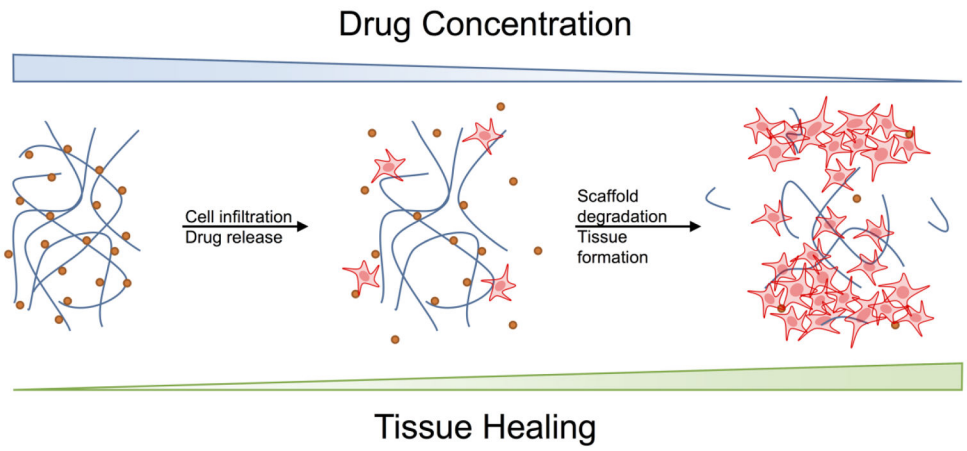

Figure 3. Scaffold based drug delivery for tissue repair

Current regenerative medicine strategies focus on delivering therapeutics to drive cell recruitment and tissue repair. As cells are recruited the scaffold degrades, releasing therapeutics, and promoting integration. Next generation biomaterials will include abilities to prevent or eliminate pathogens and provide regenerative cues.

Table 1

Infection-based segmental defect models

Ref.	Animal	Procedure	Advantages	Disadvantages
17–20,103	Rat	Femoral segmental defect, debridement following 2 weeks later	Evaluates long weight bearing bone, widely validated, not self healing	Failure if infection extends to fixation pins, two surgical procedures required
74,103	Rat	Femoral segmental defect, contaminated scaffold	Evaluates long weight bearing bone, widely validated, not self healing, single operation	Failure if infection extends to fixation pins, complicated procedure
88	Rabbit	Femoral segmental defect	Evaluates long weight bearing bone, not self healing	Failure if infection extends to fixation pins
8	Rabbit	Radial segmental defect	No fixation device required, evaluates long bone, not self healing, single procedure, simulates intraoperative contamination	Non-weight bearing bone

Table 2

Review articles detailing various antimicrobial strategies.

Ref.	Antimicrobial	Topics Covered
24	Antibiotics	Mechanisms of action, and how resistance has emerged
67	Silver	Antimicrobial properties of silver nanomaterials and effects on human health and the environment
2	Host Defense Peptides	Host defense peptides as therapeutics for antibiotic resistant infections
40	Host Defense Peptides	Immunomodulatory aspects of host defense peptides
39	Bacteriophage	Bacteriophages and how they can be used to treat infection