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Antibiotic-Releasing Microspheres Prevent Mesh Infection in Vivo

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Abstract

Background—Infection remains a dreaded complication after implantation of surgical prosthetics, particularly after hernia repair with synthetic mesh. We previously demonstrated the ability of a newly developed polymer to provide controlled release of an antibiotic in a linear fashion over 45 days. We subsequently showed that coating mesh with the drug-releasing polymer prevented a *Staphylococcus aureus* (SA) infection in vivo. In order to broaden the applicability of this technology, the polymer was synthesized as isolated “microspheres” and loaded with vancomycin (VM) before conducting a non-inferiority analysis.

Materials and Methods—Seventy-three mice underwent creation of a dorsal subcutaneous pocket that was inoculated with 10⁴ CFU of green fluorescent protein (GFP)-labeled SA (10⁵ CFU/ml). Multifilament polyester mesh (7*7mm) was placed into the pocket and the skin was

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Author contributions: Sean Zuckerman, Horst A. von Recum, and Julius N. Korley developed the polymer, synthesized it as microspheres, and experimentally determined its characteristics. They helped with analysis and interpretation of data and they edited the manuscript. Kevin T. Grafmiller, Clayton Petro, Lijia Liu, and Michael J. Rosen helped conceive, design, and conduct the study and analyzed and interpreted the data. They drafted and edited the manuscript. All authors gave approval for submission of this manuscript.

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closed. Mesh was either placed alone (n=16), coated with VM-loaded polymer (n=20), placed next to VM-loaded microspheres (n=20) or unloaded microspheres (n=10), or flushed with VM solution (n=7). Quantitative tissue/mesh cultures were performed at 2 and 4-weeks. Mice with open wounds and explanted mesh were excluded.

Results—Twenty-two of twenty-three (96%) tissue-mesh samples from mesh alone or empty microspheres were positive for GFP-labeled SA at two and four-weeks. Six of seven (86%) samples from the VM flush group were positive for GFP SA at 4 weeks. Thirty-eight of thirty-eight (100%) VM-loaded pCD-coated mesh or VM-loaded microspheres were negative for GFP SA at two and four weeks.

Conclusion—Slow affinity based drug-releasing polymers in the form of microspheres are able to adequately clear a bacterial burden of SA and prevent mesh infection.

Keywords

Microsphere; Hernia; Mesh; Infection; Cyclodextrin; Antibiotic releasing

1 - INTRODUCTION

The use of synthetic mesh in ventral hernia repair allows the surgeon to achieve a more durable repair that is associated with a lower recurrence rate compared to traditional auto-tissue repair[1]. However, mesh-related complications such as acute or delayed prosthetic infection has become a concerning clinical problem. Current prevention and non-surgical treatments for mesh infection are limited, and infection of the mesh often necessitates surgical removal to avoid serious complications, such as enteric fistula[2]. A multisite cohort study of patients undergoing incisional hernia repair with permanent mesh prosthesis at 16 Veterans Affairs hospitals from 1998 to 2002 showed that 5.1% of the 1,071 mesh repairs were complicated by subsequent mesh explantation at a median of 7.3 months (interquartile range 1.4 –22.2)[3]. Infection was the most common reason for mesh explantation(69%)[3].

Mesh infection can arise weeks after implantation, but the main prophylactic options available to prevent mesh infection, including pulse lavage, perioperative systemic antibiotics, and antibiotic impregnated mesh and sutures, are all short acting. Additionally, pulse lavage with or without antibiotics lacks evidence to support its efficacy[4,5] and perioperative systemic antibiotics can produce serious side effects, especially with prolonged administration[6], limiting their use in long-term prophylaxis.

We previously demonstrated the ability of a newly developed polymer to provide controlled release of antibiotic in a linear fashion over 45 days[7,8]. The von Recum lab at Case Western Reserve University found that crosslinked cyclodextrin polymers (pCD) loaded with antibiotic release drug in a longer, more linear fashion than chemically similar dextran gels[7,8]. Cyclodextrin (CD) is a ring of 6, 7, or 8 glucose molecules that forms a pocket where drugs can bind and is commonly used in pharmaceuticals to increase solubility of poorly soluble drugs. The molecular interaction between the CD pocket and drug is the rate-limiting step in release that determines the amount of free drug able to diffuse out of the polymer. Once a drug is out of the CD pocket it is able to diffuse freely throughout the

material and may either diffuse out of the polymer or stick into another CD pocket. This type of release mechanism has been termed affinity-based release[9]. The pCD is capable of extended antimicrobial activity for more than 7 months even following commercial sterilization[10]. Given the extended time window following surgery during which mesh infections can arise, we believe the extended antibiotic delivery demonstrated by this pCD platform represents a novel approach to prevent mesh infections. We previously demonstrated that pCD-coated polyester mesh loaded with vancomycin (VM) prevented subcutaneous *Staphylococcus aureus* (SA) infection *in vivo*[11]. Despite the great potential of this polymer, its clinical applicability is limited by the fact that each different polymer coated product requires individual FDA approval[12]. Therefore, a drug delivery mechanism independent of the prosthesis would be preferable and more broadly applicable to preventing infection of any device. We reformulated the polymer as microspheres that could be administered independently from the prosthetic device, loaded the microspheres with VM and, conducted a non-inferiority analysis. We designed this study to determine if microsphere polymer complexes are technically practical and efficacious for use against prosthetic infection and to compare antibiotic-loaded microspheres against antibiotic-loaded, polymer-coated mesh with respect to their ability to prevent prosthetic infection.

2 – MATERIALS AND METHODS

2.1 - Polymer Materials

b-Cyclodextrin prepolymer (CD) was purchased from CycloLab, Budapest, Hungary. Ethylene glycol diglycidyl ether (EGDGE, $M_n = 174$) was purchased from Polysciences, Inc., Warrington, PA. All other reagents were purchased from Fisher Scientific (Pittsburgh, PA) at reagent grade.

2.2 - Mesh and Polymer Coating

A 25% w/w CD solution in dimethylformamide was prepared under stirring. Hexamethylene diisocyanate (HDI) was added to the above solution to become glucose:HDI molar ratio of 1: 0.32. Known volumes of pCD-HDI solution as prepared above were uniformly coated onto standard pieces (0.7 cm x 0.7 cm) of a three-dimensional market approved polyester mesh (Parietex TET) (Covidien, Mansfield, MA) in a glass dish, and crosslinked at room temperature 3 days. The polymer coated samples, hereafter called pCD-coated mesh, were carefully removed from the glass and were immersed in solvent, solvent/water 50/50, followed by Milli-Q water for one day each. The samples were then dried at room temperature in a dust free environment. pCD-coated meshes used for drug loading and animal studies were prepared using this methodology.

2.3 - Microspheres

The pCD microspheres were synthesized via single water-in-oil emulsification. Briefly, epichlorohydrin crosslinked β CD prepolymer (CycloLab R&D; Hungary) was dried overnight at $\sim 70^\circ\text{C}$ prior to solubilization in 0.2 M KOH to make a 25% w/v solution by vortexing. After the polymer was fully dissolved, EGDGE ($M_n \sim 174$) was added dropwise and vortexed on high for 2 min. The CD/EGDGE mixture was added to 50 ml light paraffin oil (Fisher Scientific) and homogenized for 5 min at 13,000 RPM (Brinkman Kinematica

Polytron PT3000 homogenizer equipped with a PT-DA 3012/2 TS blade). The polymer emulsion was then added to 100 ml light paraffin oil stirred at 400 RPM by an overhead stirrer (IKA-Werke Eurostar Euro-St PCV P4S1) to allow crosslinking.

After 48 h the stirrer was stopped and the oil was filtered off using a coarse frit glass filter. The crosslinked pCD microspheres were washed successively with excess hexanes, excess acetone, and finished with excess Milli-Q grade water. Microspheres were allowed to dry at room temperature for 2 d.

2.4 - Drug Loading Determination

2.4.1 - Coated mesh—VM was chosen because it is a clinically relevant antibiotic and ease of loading into our polymer. Analysis of drug loading was performed as detailed previously [11]. Briefly, dry pCD-coated mesh samples were loaded in aqueous solution of VM (5 wt%) for 3 d at room temperature, dried, extensively extracted into water, and VM analyzed spectrophotometrically at 282 nm.

2.4.2 – Microspheres—Microspheres were immersed in VM (5 wt % in water) room temperature for 3 d. The drug-loaded microspheres were then briefly washed 3 times in pure water to remove surface-adsorbed drug and dried at room temperature for 2 d followed by vacuum drying. VM present in the samples was determined spectrophotometrically at 282 nm after extensive (>30d) extraction in water.

2.5 - Animals

All animal experimental protocols were approved by the Institutional Committee for Use and Care of Laboratory Animals (IACUC), Case Western Reserve University, Cleveland, OH. Female mice aged 6–8 wk (strain: C57BL/6J; The Jackson Laboratory, Bar Harbor, ME) were used. We used females instead of males to maintain consistency with our previous study[11]. Mice were maintained on a light/dark, 12/12 h cycle at 22°C, and received food and water *ad libitum*.

2.6 - Bacterial Inoculum Preparation

The same clinical strain (Seattle 1945) of SA transformed with a green fluorescent protein (GFP)-labeled plasmid to produce SA 1945GFPuvr was used in this study as previously[11]. Briefly, the strain was recovered from –80°C, inoculated in brain heart infusion, diluted 1:50 and cultured 2.5 h to 10^8 CFU/mL via optical density. The bacteria was then diluted to 10^5 CFU/mL in sterile saline, and the wound was inoculated with 100ul 10^5 CFU/ml GFP-labeled SA for a final inoculum of 10^4 CFU/mesh

2.7 - Mesh

Commercially available polyester mesh (Parietex TET) (Covidien, Mansfield, MA) was tailored into standard pieces (7mm* 7mm) prior to implantation into the mice's dorsal wound. For the mesh plus microsphere groups, either VM-loaded microspheres or empty (i.e. unloaded) microspheres were placed on top of and adjacent to the mesh. For the polymer-coated mesh group, a piece of VM-loaded, pCD-coated mesh was implanted into the subcutaneous pocket on the animal. Control groups received uncoated mesh.

2.8 - Surgical model

After hair clipping and sterile preparation, a dorsal subcutaneous pocket was created in each mouse. A 7mm by 7mm piece of sterile multifilament polyester mesh was implanted into the wound for each group: mesh alone (n=16), VM-loaded pCD-coated mesh (n=20), VM-loaded microspheres (n=20), empty microspheres (n=10), or flushed with VM solution (n=7). All wounds were closed with sterile clippers prior to inoculation with 10^4 CFU of GFP-labeled SA (100 μ l of 10^5 CFU/ml) into the wounds. VM flush was introduced into the surgical wound in a dose of 0.7 mg (100 mL of a 7 mg/L solution) following wound closure. All study groups were followed for 2 and 4 weeks before euthanasia for analysis. The two exceptions were the empty microspheres group that was only sacrificed at two weeks and the VM flush group that was only sacrificed at 4 weeks.

2.9 - Study Groups

Our study groups included: (1) control – mesh alone (n=16), (2) VM-loaded pCD-coated mesh (n=20), (3) VM-loaded microspheres (n=20); (4) empty microspheres (n=10), and (5) VM flush (n=7). Quantitative tissue/mesh cultures were done at 2 and 4-weeks postoperatively. A portion of the animals from the mesh alone group (n=10), the VM-loaded pCD-coated mesh group (n=10), and the VM-loaded microsphere group (n=10) were followed for 2 weeks prior to euthanasia. The rest were followed for 4 weeks prior to euthanasia. At necropsy, mesh with surrounding tissue was excised under sterile conditions and weighed. Each specimen was then placed in 1000 μ L of sterile 0.9% normal saline and homogenized. Mice with open wounds and explanted mesh were excluded.

2.10 - Microbiologic Examination

Quantitative microbiologic culture was performed as detailed previously [11]. Briefly, serial 10-fold dilutions of the mesh and tissue homogenate were plated in duplicate, cultured for 72 h and counted. For plates showing no growth, the minimum number of detectable colonies (300 CFU/g) was used in statistical analyses. Cultured bacteria was checked for green fluorescence, and standard microbiological methods used to identify those not displaying green fluorescence.

2.11 - Statistical Analysis

Data were analyzed using STATA (ver. 10; College Station, TX, USA) as detailed previously [11]. Median and interquartile ranges were obtained for CFU/g counts. The minimum number of colonies detectable (300 CFU/g) was used for samples not showing bacterial growth. A P value < 0.05 was considered significant. Post-hoc analysis was performed when Kruskal-Wallis rank test was found to be significant.

3 - RESULTS

3.1 - Drug Loading Efficiency

Percent drug loading was calculated as: % drug loading = $W_{\text{drug}} / (W_{\text{sample}} + W_{\text{drug}}) \times 100$ where W_{drug} and W_{sample} are the weight of drug and sample, respectively. pCD-coated mesh

loaded $11.8 \pm 0.6\%$ VM, which is consistent with previously published work using this HDI-crosslinked pCD [7]. Microspheres loaded $9.1 \pm 0.7\%$ VM.

3.2 - Study groups

All animals survived throughout the duration of the study. One animal in the mesh alone control group (n=1/10), two animals in the VM-loaded microspheres (n=2/10), and two animals in the empty microspheres group (n=2/10) were excluded due to open wounds and explanted mesh. All excluded animals were in groups scheduled for sacrifice at 2 weeks.

3.3 - Microbiology Results

GFP SA was isolated from tissue-mesh homogenates of inoculated animals from the mesh alone, empty microsphere, and VM flush groups. Additionally, low numbers of GFP negative, coagulase negative *Staphylococci* were isolated from a few samples. These GFP negative *Staphylococci* were likely derived from skin contamination at the time of surgical necropsy and thus were excluded from analysis.

Median bacterial counts from homogenates at different survival time points were as follows. At 2 weeks: the empty microspheres group had the highest median GFP SA growth (n=8; 1.4×10^8 CFU/g), followed by the mesh alone group (n=9; 8.6×10^6 CFU/g). Both the VM-loaded pCD-coated mesh group (n=10; sterile cultures) and the VM-loaded microspheres group (n=8; sterile cultures) achieved complete clearance of GFP SA. The difference in median GFP SA bacterial counts at 2 weeks was statistically significant ($P < 0.05$; Table 1). On post-hoc analysis of 2-week data, the mesh alone group and the empty microspheres group both had higher GFP SA bacterial counts than the VM-loaded pCD-coated mesh group and the VM-loaded microspheres group ($P < 0.05$). No statistical difference was found at 2 weeks on post-hoc analysis between the mesh alone group and the empty microspheres group ($P > 0.05$). At four weeks: the mesh alone group had the highest median GFP SA growth (n=6; 4.9×10^2 CFU/g), followed by the VM flush group (n=7; 4.2×10^2 CFU/g). Both the VM-loaded pCD-coated mesh group (n=10; sterile cultures) and the VM-loaded microspheres group (n=8; sterile cultures) had no GFP SA growth at 4 weeks. The difference in median GFP SA bacterial counts at 4 weeks was statistically significant ($P < 0.05$; Table 2). On post-hoc analysis of 4-week data the mesh alone control group and the VM flush group had higher GFP SA bacterial counts than both the VM-loaded pCD-coated mesh group and the VM-loaded microspheres group ($P < 0.05$). No statistical difference was found at 4 weeks on post-hoc analysis between the mesh alone group and the VM flush group ($P > 0.05$).

Comparisons were also made between 2 week and 4 week results for the VM-loaded pCD-coated mesh group and the VM-loaded microspheres group. There was no difference in bacterial growth recovered at 2 and 4 weeks for these two groups ($P > 0.05$).

4 - DISCUSSION

Mesh infection remains a significant challenge facing hernia surgeons and lacks a gold-standard method for prevention and treatment. SA has been found to be one of the pathogens most commonly involved in mesh infection [13]. We previously created an affinity-based

pCD drug delivery platform that demonstrated the capacity to release drug in a linear fashion, which allowed therapeutically active levels of drug to be maintained for a longer duration than with traditional drug impregnated polymers that release drugs via diffusion in a logarithmic fashion[7,8]. Our previous study demonstrated that polyester mesh coated with this affinity based, drug delivering pCD was able to effectively prevent a SA mesh infection with efficacy demonstrated at 2 and 4 weeks[11]. We have now demonstrated that this pCD polymer can be synthesized as microspheres and easily placed adjacent to implanted mesh. Compared to the pCD-coated mesh, the microspheres have an equivalent ability to prevent SA mesh infection at 2 and 4 weeks.

Despite the demonstrated efficacy of the pCD coating, using the polymer as a coating for prosthetics limits its clinical applicability because each different prosthetic coated with polymer requires individual FDA approval, which significantly affects physician choice and preference [12]. Our solution to this problem was to develop a drug delivery mechanism that would allow the antibiotic delivering polymer to be administered adjacent to the mesh or prosthetic, but not as part of the mesh or prosthetic, without interfering with the surgical repair. We synthesized the antibiotic delivering polymer as isolated microspheres that could be administered separately from the prosthetic and conducted a non-inferiority analysis. Altering the polymer from a coating to microspheres did not impair the ability of the polymer to prevent mesh infection in vivo in a mouse model at 2 and 4 weeks following mesh implantation.

Compared to other polymers, our pCD offers a more linear drug release profile, has demonstrated long-term antimicrobial activity beyond 7 months and in vivo efficacy out to 28 days, and has the potential to provide full wound area coverage. Logarithmic release of antibiotic produces a spike in drug concentration that quickly drops below therapeutic levels, whereas linear release can maintain active drug concentrations longer, which is ideal when treating hernia infections that can arise weeks after mesh implantation. Most currently available polymers do not possess ideal linear release patterns. Guillaume et al. 2012 developed an ofloxacin and rifampicin impregnated biodegradable polymer that could be airbrushed onto mesh; however, despite triple coating the mesh to improve antibiotic release, 25% of the rifampicin and 40% of the ofloxacin were released in the first 5 hours [14]. The triple coated mesh effectively prevented infection throughout the entire experiment, but the study was concluded after only 72 hours [14]. Laurent et al. 2011 studied a ciprofloxacin impregnated polymer mesh coating over 24 hours and found a logarithmic decrease in the zone of inhibition for *S. aureus*, *S. epidermidis*, and *E. coli*, with the zone of inhibition completely disappearing by 24 hours for *S. epidermidis* [15]. Letouzey et al. 2011 examined an amoxicillin and ofloxacin polymer over a longer period of 10 days, but found a rapid, logarithmic release of both antibiotics over the first 2 days followed by low level release dropping to zero by day 8 ½ [16]. Antibacterial efficacy was only demonstrated out to 72 hours [16].

The polymer with performance most similar to ours is a VM impregnated coating studied by Fernandez-Gutierrez et al. 2013 that demonstrated an antimicrobial effect for at least 14 days in an agar diffusion test and prevented infection in a rabbit model out to 30 days [17]. The polymer was shown to produce near-linear release of antibiotic over 40 days, similar to our

pCD with demonstrated near-linear release over 45 days [7,8,17]. This polymer and our pCD both possess ideal release profiles and have demonstrated long-term efficacy in animal models. Unlike our pCD, the polymer studied by Fernandez-Gutierrez et al. 2013 has not demonstrated antimicrobial activity out to 7 months, and it has not been successfully synthesized as microspheres, limiting the polymer's versatility [10,17].

Antibacterial products with demonstrated efficacy that have received FDA approval include the Xenmatrix AB graft and the AIGIS_{Rx} ST polymer coated mesh [18,19]. Both of these products release minocycline and rifampin and have only been studied out to 7 days post-implantation [18,19]. The Xenmatrix AB graft initially releases antibiotic rapidly with approximately 50% of rifampin and 40% of minocycline eluted in the first 24 hours, followed by a more linear release up to day 7 [18]. It has been shown to prevent graft infection in a rabbit model for 7 days [18]. The AIGIS_{Rx} mesh releases 80% of antibiotics in the first 24 hours[20], and when used as an envelope to surround an implantable battery pack, it prevents infection for 7 days [17]. Other FDA approved, drug releasing products such as Vicryl suture and DualMesh Plus have failed to consistently demonstrate their antimicrobial efficacy [21,22,23]. Compared to our pCD, current FDA approved antibiotic releasing products have less desirable drug release profiles and have not demonstrated long-term antimicrobial activity or long-term efficacy in an animal model.

Finally, the ability of our polymer to be synthesized as microspheres that distribute throughout the wound site means that the antibacterial effect of our polymer is not limited to the immediate proximity of the prosthetic. Our microspheres can disperse throughout the wound to provide full area antibacterial coverage, unlike polymers used as prosthetic coatings.

In addition to ideal drug release properties, our polymer possesses additional chemical properties that make this new technology adaptable, safe, and convenient. First, the polymer can be chemically adapted to accommodate the loading and delivery of a diverse range of antimicrobials[8]. Second, the polymer is composed of cyclodextrin and ethylene glycol, both of which safely degrade into biocompatible components that are physiologically eradicated[8]. Third, the polymer releases antibiotic directly at the desired site of action decreasing the risk of systemic side effects. Fourth, the microspheres are convenient to use compared to other common surgical adjuncts, such as hemostatic agents. The microspheres can be prepared ahead of time, unlike products such as Flowseal that must be prepared less than 8 hours before use, and there is no need to wash away excess product to avoid adverse events, which is required when using Flowseal or Arista [24,25].

5 - CONCLUSION

pCD microspheres were easily placed adjacent to prosthetic mesh in a small animal subcutaneous model. These pCD microspheres loaded with VM were able to prevent an SA prosthetic mesh infection as effectively as the previously validated pCD-coated mesh loaded with antibiotic. pCD microspheres capable of affinity based drug delivery are a promising technology that allows local drug delivery adjacent to prosthetic surgical devices. Large

animal studies and reintroduction of bacteria at staged time intervals will help elucidate the full potential of this delivery platform.

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Figure 1. Scanning electron microscope image of microspheres

Image taken with accelerating voltage of 5kV. Microsphere size equals $256 \pm 74 \mu\text{m}$.

Table 1
Two week median GFP positive SA bacterial growth from tissue and polyester mesh homogenates following various treatment regimens

Mesh alone and empty microspheres groups had significantly higher GFP SA counts than VM-loaded pCD-coated mesh and VM-loaded microspheres groups ($P < 0.05$). No statistical difference was found between mesh alone and empty microspheres ($P > 0.05$) or between VM-loaded pCD-coated mesh and VM-loaded microspheres ($P > 0.05$).

Study groups	GFP+Animals	Median(CFU/g)	IQR(CFU/g)	P value
Control – mesh alone (n=9)	9	8.6×10^6	1.2×10^8	$P < 0.05$
VM-loaded pCD-coated mesh (n=10)	0	0	0	
VM-loaded microspheres (n=8)	0	0	0	
Empty microspheres (n=8)	8	1.4×10^8	4.2×10^8	

Abbreviations: VM = vancomycin; SA = *Staphylococcus aureus*; GFP = green fluorescent protein; pCD = crosslinked cyclodextrin polymer; CFU = colony forming units; IQR = interquartile range.

Table 2
Four week median GFP positive SA bacterial growth from tissue and polyester mesh
homogenates following various treatment regimens

Mesh alone and VM flush groups had significantly higher GFP SA counts than VM-loaded pCD-coated mesh and VM-loaded microspheres groups ($P < 0.05$). No statistical difference was found between Mesh alone and empty microspheres groups ($P > 0.05$) or between VM-loaded pCD-coated mesh and VM-loaded microspheres groups ($P > 0.05$).

Study groups	GFP+Animals	Median(CFU/g)	IQR(CFU/g)	P value
Control - Mesh alone(n=6)	5	4.9×10^2	1.1×10^3	$P < 0.05$
VM-loaded pCD-coated mesh (n=10)	0	0	0	
VM-loaded microspheres (n=8)	0	0	0	
VM flush (n=7)	6	4.2×10^2	2.0×10^3	

Abbreviations: VM = vancomycin; SA = *Staphylococcus aureus*; GFP = green fluorescent protein; pCD = crosslinked cyclodextrin polymer; CFU = colony forming units; IQR = interquartile range.