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Tetracycline delivery from fibrin controls peritoneal infection without measurable systemic antibiotic

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The addition of antibiotics to an adhesive haemostat results in an ideal system for the treatment of a localized infectious disease. Fibrin sealant (FS) is a biocompatible, resorbable, adherent haemostat that can deliver antibiotics. Previous use of fibrin to deliver antibiotics resulted in rapid release and limited bioactivity. We have reported previously that poorly soluble antibiotics significantly retard release from FS, resulting in extended delivery *in vitro*, and overcome antibiotic-resistant infection. We now report that localized antibiotic delivery from FS controls peritoneal infection without measurable systemic antibiotic. Rats and mice were implanted with preformed FS discs containing tetracycline free-base to evaluate control of peritoneal sepsis and to measure serum tetracycline levels. Infection was initiated with *Staphylococcus aureus*. Morbidity and mortality were evaluated for 14 days. Serum was isolated from jugular vein blood with subsequent evaluation for antimicrobial activity. Mice prophylactically treated with FS-tetracycline (FS-TET) 500 mg/kg 2 days before infection cleared the *S. aureus* infection, resulting in 100% survival. Mice treated with FS-TET 500 mg/kg 7 days before infection survived. Mice treated with FS-TET 1750 mg/kg 35 days before infection also survived. Rats treated with FS-TET 500 mg/kg had undetectable serum tetracycline levels, whereas *in vitro* release of tetracycline from FS-TET pellets in rat serum was readily detected. We conclude that fibrin is an excellent vehicle for extended delivery of low solubility tetracycline. Tetracycline delivered from FS is an appropriate chemotherapy for *S. aureus* peritonitis. FS-TET controls localized infection without a measurable concentration of systemic tetracycline.

Introduction

Infection control is of prime importance in treatment of diseases and injuries. Currently available antibiotics, delivered topically or systemically, are effective against most infections. However, remote sites with limited vascularity require high antibiotic doses or prolonged treatment, which increases the risk of toxicity or induction of bacterial resistance. Therefore, *in situ* treatments have been investigated, albeit with limited success. Fibrin was one of the first *in situ* delivery vehicles evaluated for infection control.[1] Fibrin has several unique characteristics that make it an ideal candidate as a delivery matrix for pharmaceuticals and biologics in humans.[2] Composed of a natural biopolymer, it is readily resorbable. Its inherent adhesive properties allow agents trapped in its three-dimensional matrix to be localized where the fibrin is deposited, and its haemostatic/sealing properties prevent rapid elution.

Fibrin is formed by the enzymatic conversion of fibrinogen by thrombin and the subsequent cross-linkage by activated Factor XIII. Human fibrin components (fibrinogen, thrombin and Factor XIII) can be isolated from fractionated human plasma.[3] The components can then be injected or sprayed through a dual-chambered catheter for *in vivo* delivery, or combined *in vitro* (so as to be moulded into capsules) and implanted. Biological or chemotherapeutic agents can be added to the fibrin for *in situ* delivery. Cross-linked fibrin monomers create 1–10 μm pores[4]

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through which trapped compounds are released. The release of trapped compounds is governed by a diffusion–dissolution mechanism, whereby the product within the fibrin matrix dissolves and is also released during fibrinolysis.[2]

Fibrin supplemented with antibiotics has been used to treat experimental osteomyelitis,[5,6] repair experimental bone defects (e.g. cortical drill hole, heterologous cancellous transplants and osteomies),[7] facilitate orthopaedic wound healing,[8] treat endocarditis,[9,10] repair rectovaginal and complex fistulas[11] and treat experimental keratitis.[12] However, for these types of 'difficult to treat' infections, the duration of antibiotic release was inadequate. Most ($\geqslant$85%) of each antibiotic tested was released in the first 72 h.[1,13–15] Release of antibiotics over this relatively short time period most likely resulted from the rapid diffusion of the small, ionic molecules[15,16] that were designed for maximum absorption during oral and parenteral delivery. Because of this lack of long-term bioactivity and the fear of viral transmission from fibrin (a human blood product), antibiotic delivery from fibrin was abandoned in favour of polymethylmethacrylate beads,[17] sponge collagen,[18] liposomes[19] and plaster of Paris beads.[20] Unfortunately, release kinetics similar to those obtained with fibrin have also been reported for each of these matrices when tested with typical clinical formulations of antibiotics. Additionally, and in most cases, their poor resorbability necessitated a second surgery to remove them, thus exposing patients to potential re-infections and increased morbidity.

We have reported previously on the *in vitro* activities of low solubility antibiotics (<1 mg/mL) delivered from a virally inactivated fibrin product. In contrast to commonly used antibiotics, use of less soluble antibiotics significantly retards their release from fibrin resulting in extended delivery (42 days) *in vitro*.[21] With this technique, high local concentrations of antibiotics can be sustained for weeks and thereby overcome some bacterial resistance mechanisms.[22] In a new series of experiments, we now extend and augment the previous data to report on the use of fibrin to deliver antibiotics for long-term infection control *in vivo* and confirm that the efficacious release of antibiotic from fibrin sealant (FS) results in insignificant systemic exposure to the antibiotic.

Materials and methods

Preparation of fibrin discs

Fibrin components (FS) were generously provided by the American Red Cross (Rockville, MD, USA) as separate vials of lyophilized fibrinogen containing Factor XIII [topical fibrinogen concentrate (TFC)] and lyophilized thrombin. FS discs were prepared by mixing 157 mg TFC, 15.7 mg thrombin and 0.68 mL of 40 mM $CaCl_2$ in a 20 $\times$ 3 mm plastic mould to produce a sheet of fibrin from which discs were obtained using a sterile biopsy punch (Acu-Punch; Acuderm, Inc., Fort Lauderdale, FL, USA). Tetracycline free-base (Sigma Chemical Co., St Louis, MO, USA) was added to the fibrinogen and thrombin just before hydration with $CaCl_2$ solution. Control discs contained no tetracycline.

Fibrin disc imaging

FS discs (with or without tetracycline) were air dried for 28 h over Drierite (W. A. Hammond Drierite Co., Ltd, Xenia, OH, USA), dehydrated in ethanol and at the critical point under CO_2 flux, covered with a 500 Å layer of gold. Discs were viewed with a Stereoscan-100 scanning electron microscope (SEM) (Cambridge, MA, USA) at 15 kV to image tetracycline within the FS.

In vivo studies

In vivo studies were performed in accordance with ethical standards established by the Institutional Animal Care and Use Committees of Kent State University and Akron General Medical Center. Male BALB/c mice (20 $\pm$ 2 g) were obtained from Taconic Farms (Germantown, NY, USA) and female Sprague–Dawley rats (120 $\pm$ 5 g) were obtained from Harlan (Indianapolis, IN, USA). Animals were acclimatized for 5 days before use and provided with unlimited access to food and water in light- and temperature-controlled rooms. For each experiment, animals were weighed and randomized into experimental or control groups.

Sepsis survival with dose escalation. BALB/c mice were anaesthetized with ketamine hydrochloride 40 mg/kg (Ketaset, Fort Dodge Laboratories, Fort Dodge, IA, USA) and xylazine 7.5 mg/kg (Xyla-Ject, Phoenix Pharmaceutics Inc., St Joseph, MO, USA), administered im. FS discs (with and without tetracycline) were prepared as detailed above just before surgery. The mice (six per group) were shaved over the abdominal surface and the site cleaned with Providine (Barre-National Inc., Baltimore, MD, USA). Under aseptic conditions, a 5 mm incision was made through the skin and the peritoneal wall, through which FS-tetracycline (FS-TET) discs (tetracycline 50, 125, 250 or 500 mg/kg) were implanted. The peritoneal incisions were closed with absorbable sutures (4–0 VICRYL; ETHICON Co., Ltd, Somerville, NJ, USA), and the skin closed with stainless steel clips (AUTOCLIP; Clay Adams Brand, Becton Dickinson, Sparks, MD, USA). Mice were rested for 2 days and subsequently infected with 10^8 cfu of *Staphylococcus aureus* ATCC 49976 (American Type Culture Collection, Manassas, VA, USA), by ip injection (an LD_{90} dose had been determined previously). Control mice received fibrin discs containing no tetracycline. Mice were evaluated daily for sepsis-associated morbidity and mortality. Survival at 14 days post-infection was reported for each group.

Sepsis survival with extended tetracycline release. In other experiments to determine the *in vivo* duration of bio-

available tetracycline delivered from FS, mice (five per group) were rested for up to 35 days after implantation of discs containing tetracycline 500, 1250 or 1750 mg/kg. The mice were subsequently infected with 10^8 cfu of *S. aureus* ATCC 49976. Survival data (percentages) were transformed to arc sin square root to make direct comparisons, and analysed by one-way ANOVA with differences between groups determined by Dunnett's test.

Serum levels after tetracycline release. To determine whether tetracycline released from FS in the peritoneum entered the systemic circulation, FS-TET discs were implanted intraperitoneally into Sprague–Dawley rats and serum samples were evaluated for bioactive tetracycline over 30 days. Briefly, groups of six rats were surgically implanted with FS or FS-TET discs (500 mg/kg) ip (discs prepared as described above) while anaesthetized with Metofane (Abbott Laboratories, Abbott Park, IL, USA). Under aseptic conditions, a 10 mm incision was made through the skin and the peritoneal wall, through which FS-TET discs (500 mg/kg) were implanted. The peritoneal incisions were closed with absorbable sutures (4–0 VICRYL), and the skin closed with stainless steel clips (AUTOCLIP). Peripheral blood (0.5 mL) was collected using tuberculin syringes (Monoject, 1 cc Syringes; Sherwood Medical, St Louis, MO, USA) from alternating, contralateral jugular veins while rats were anaesthetized with Metofane. Collections were made on alternate days for 2 weeks followed by once a week until week four. Serum was obtained from whole blood via centrifugation at 3000**g** for 10 min at 4°C in serum separator tubes (Microtainer, Becton Dickinson, Franklin Lakes, NJ, USA) and frozen at −70°C until assayed. To control for potential freezing and serum protein binding effects, serum samples from untreated rats were doped with 2 mg/L tetracycline and stored with the other samples.

In vitro tetracycline release study

FS-TET discs (50 mg tetracycline per disc) were also evaluated *in vitro* for tetracycline release after incubation in normal rat serum to confirm release of bioactive tetracycline from FS discs. FS-TET discs were prepared as described above and placed individually into wells of 24 well plates (VWRSP, Pittsburgh, PA, USA). Freshly obtained Sprague–Dawley rat serum was added as 1 mL to each well. Plates were incubated at 37°C with 5% CO_2 for 9 days with serum exchanged daily and frozen at −70°C until assayed.

Tetracycline concentration assessment

In order to measure biologically active tetracycline that might have left the peritoneum and entered the peripheral circulation, a tetracycline bioassay was used. While not having the sensitivity of other detection methods, the tetracycline bioassay permits the detection of active drug recovered from *in vitro* and *in vivo* experiments. Bioactive tetracycline was measured in the serum samples collected from the *in vivo* and *in vitro* release studies according to the agar well method of Bennett *et al*.[23] as modified by Tan *et al*.[24] Briefly, duplicate tetracycline standards (1–5 mg/L in normal rat serum) or experimental serum samples were added as 125 μL to wells punched from nutrient agar seeded with *Bacillus subtilis* spore suspension (Difco, Detroit, MI, USA). Plates were incubated at 30°C for 18–20 h before measuring zones of growth inhibition. Zones were measured to the nearest 0.1 mm with a caliper. Tetracycline standards were used to construct a standard curve from which tetracycline concentrations contained in experimental serum samples were extrapolated.

Results

Fibrin disc imaging

Scanning electron micrographs of native (i.e. not glutaraldehyde fixed) fibrin discs are presented in Figure 1. The homogeneous, reticular network of fibrin was readily visible and revealed irregularly sized pores ranging from 5.6 to 27.8 μm in diameter. Tetracycline crystals appeared to be trapped by the FS matrix as solid particles extending from within the fibrin. Control discs demonstrated a smooth, flat appearance resulting from collapse of the unfixed, dehydrated fibrin protein (Figure 1).

In vivo sepsis survival studies

Both of the sepsis studies in mice indicate that morbidity and mortality were inversely dependent on the dose of tetracycline. The dose escalation study resulted in 100% prophylaxis at 500 mg/kg (Figure 2). Furthermore, tetracycline 500 mg/kg protected 100% of the mice for at least 1 week (Figure 3). Additionally, tetracycline 1250 mg/kg protected 100% of the mice for at least 2 weeks and 90% of the mice for at least 5 weeks (Figure 3). Figure 3 also shows that tetracycline 1750 mg/kg protected 100% of the mice for at least 5 weeks ($P \leqslant 0.01$ as compared with control animals).

Tetracycline release studies (in vivo and in vitro)

Serum samples were evaluated in the tetracycline bioassay compared with known standards. Tetracycline concentrations were then extrapolated from the standard curve ($r^2 = 0.93$). Results of the tetracycline bioassay demonstrated that FS-TET discs released measurable amounts of tetracycline into rat serum *in vitro* over 9 days (Figure 4). In fact, 17.6 ± 3.3 of the 50 mg tetracycline contained in the discs was recovered in the 9 days of *in vitro* delivery into rat serum. In contrast to the *in vitro* serum samples, sera from rats implanted with FS-TET did not contain measurable tetracycline by bioassay (Table). These results were not a

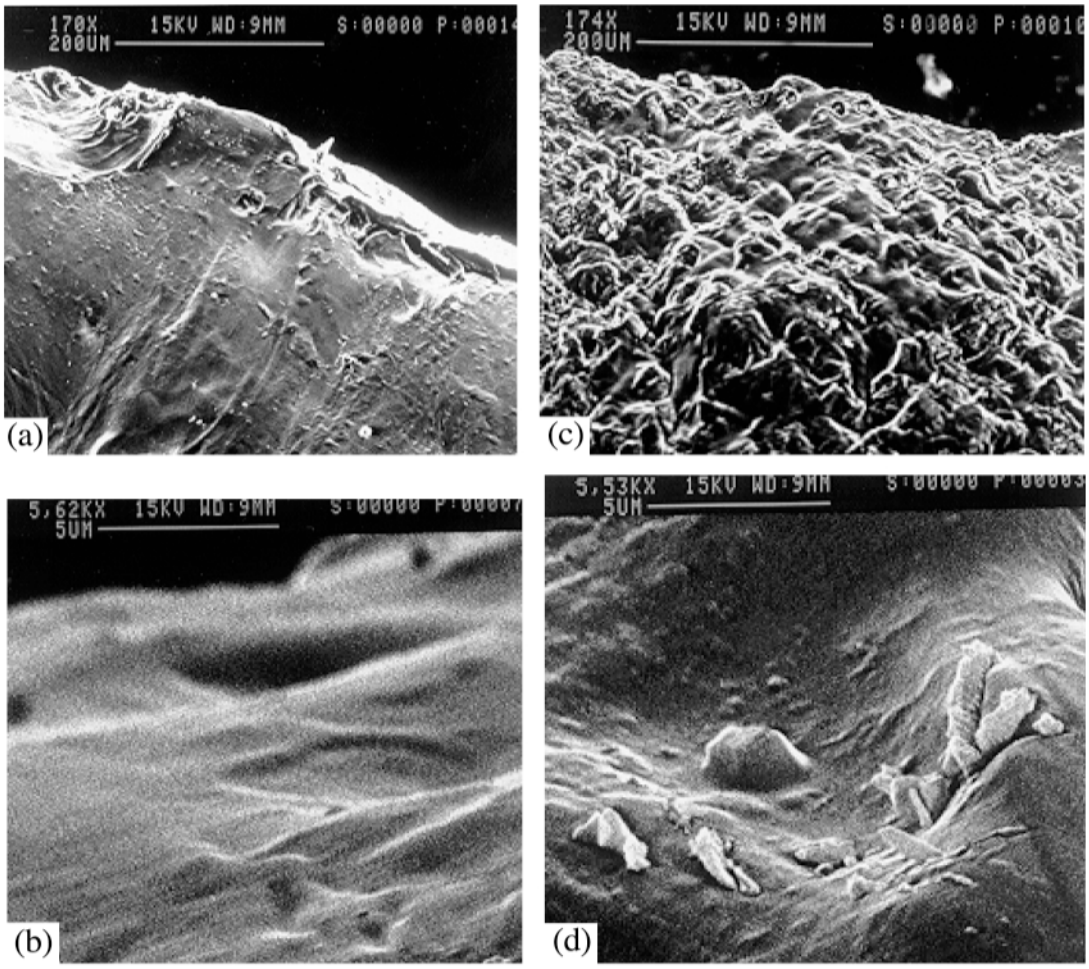

Figure 1. (a and b) Scanning electron photomicrographs of native (unfixed) FS disc [bar is 200 μm in (a) and 5 μm in (b)]. (c and d) Native FS disc containing 25 mg tetracycline free-base [bar is 200 μm in (c) and 5 μm in (d)]. Note the resulting ridges and valleys as the fibrin forms about the tetracycline crystals. Pore sizes range from 5 to 28 μm.

result of loss of tetracycline activity due to freezing or to serum protein binding, as determined by doped serum controls (Table).

Discussion

Effective antibiotic chemotherapy necessitates antibiotic concentrations at or above the MIC within the tissue or space harbouring the infectious microorganisms. Traditional antimicrobial chemotherapy approaches this goal by using the oral or parenteral administration of highly soluble compounds, thus allowing the systemic circulation to deliver efficacious doses of antibiotic. This therapy requires sufficiently concentrated and repeated dosing of antibiotic so as to allow for appropriate, local drug concentrations after dilution into the systemic blood volume. However, poorly vascularized tissues and spaces are underserved by this antibiotic delivery method. Furthermore, the characteristic properties of each drug (to sufficiently penetrate tissues) limits the number of therapeutic options. One alternative technique for control of localized infections of relatively avascular or poorly penetrated tissues is antibiotic delivery from a biocompatible, non-toxic material typically administered during invasive tissue management (e.g. wound debridement or culture acquisition) or as part of minimally invasive procedures (endoscopy, for example). Several relatively inert materials (e.g. polymethylmethacrylate beads,[17] sponge collagen,[18] liposomes[19] and plaster of Paris beads[20]) have been examined, along with the more natural protein polymers like fibrin, for their ability to deliver antibiotics.

Used in various forms as an absorbable haemostat, fibrin has been readily mixed with antibiotics as a means to control infection of open wounds. Thus fibrin was one of the first delivery vehicles evaluated for infection control *in situ*.[1] The mechanical and biochemical characteristics of fibrin made it an ideal candidate as a drug delivery vehicle, in that it is (i) composed of a natural biopolymer, (ii) adherent, (iii) non-toxic, (iv) biocompatible and (v) resorb-

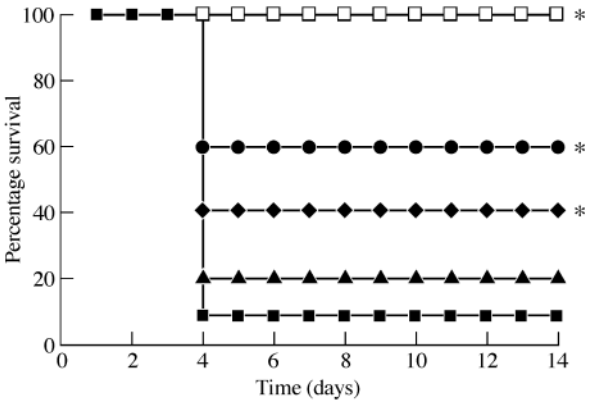

Figure 2. Dose-dependent survival of *S. aureus*-infected mice after prophylactic treatment using tetracycline free-base delivered from FS. Mice were surgically implanted with 6 × 3 mm FS discs containing tetracycline 50 (▲), 125 (◆), 250 (●) or 500 (□) mg/kg into the peritoneum 2 days before intraperitoneal infection. Control mice (■) were implanted with FS discs without tetracycline before infection. Morbidity and mortality were inversely dose dependent. $*P \leqslant 0.01$ by Dunnett's test as compared with the FS control group.

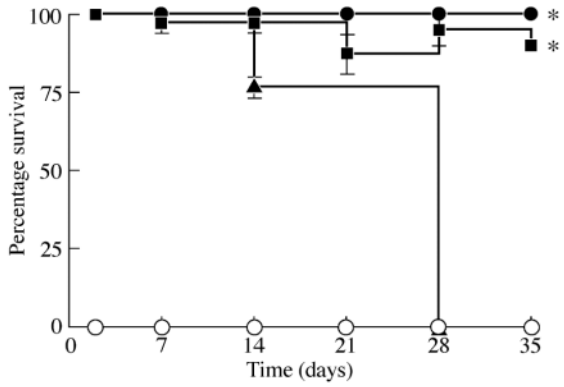

Figure 3. Survival of *S. aureus*-infected mice after prophylactic treatment using tetracycline free-base delivered from FS. Mice were surgically implanted with 6 × 3 mm FS discs containing tetracycline 500 (▲), 1250 (■) or 1750 (●) mg/kg into the peritoneum 2–35 days before intraperitoneal infection. Control mice (○) were implanted with FS discs without tetracycline before infection. Morbidity and mortality were inversely dose dependent. $*P \leqslant 0.01$ by Dunnett's test compared with the FS control group.

able. However, the practice of drug delivery from fibrin was discontinued in the USA when found to be ineffective and a potential source of pathogen transmission.[3] Today, human plasma products are treated to neutralize and/or remove microorganisms.[3] The modern use of fibrin to mediate haemostasis and seal vascular leakage was developed in Vienna in 1972 when the cryoprecipitate of plasma (mostly fibrinogen) was mixed with a bovine thrombin solution.[8] Thus the term 'fibrin sealant' now refers to a fibrin product whose primary purpose is haemostasis and prevention of fluid loss. Nonetheless, FS has been reported by several authors to deliver antibiotics.[5–10] However, the use of most clinical antibiotics (i.e. highly soluble forms) has resulted in their rapid release (38–99%) from fibrin within 24 h[25] and poor clinical efficacy. In contrast, we have reported previously on the inclusion of poorly soluble antibiotics within FS to produce a sustained-release delivery vehicle to treat infectious disease.[2,21,22] Those previous data indicated that the cross-linked fibrin network trapped heterogeneously sized crystals of poorly soluble antibiotic that were slowly released when dissolved into the local environment. Furthermore, when poorly soluble antibiotics ($\leqslant$1 mg/ml) were loaded above their solubility limit, they were released from the fibrin at concentrations exceeding the MIC for >42 days *in vitro*[21] and were of sufficient local concentration to control peritonitis initiated by a multidrug-resistant *S. aureus*.[22]

Tetracycline was chosen for the current studies because it was readily obtained in a poorly soluble formulation and its use as a chemotherapeutic agent is well understood (even if not for treating peritonitis). We have studied several antibiotics delivered by this method (e.g. erythromycins, tetracyclines and penicillins) and conclude that relative insolubility is the determining factor for long-term release kinetics.[21,22] In addition, since this study proposes the local use of antibiotics in higher than normal concentrations, it could be more effective against resistant strains of bacteria.[22] The use of tetracycline to treat peritonitis represents an uncommon therapy that tests our hypothesis while preventing antibiotic resistance to conventional chemotherapeutics routinely used to control this infectious disease. Although tetracycline is not often used to control *S. aureus in vivo*, we report complete efficacy using the mouse peritonitis model. Escalation of the antibiotic identified tetracycline 500 mg/kg as the lowest dosage providing 100% protection from the *S. aureus* infection. Furthermore, a single implanted disc of tetracycline

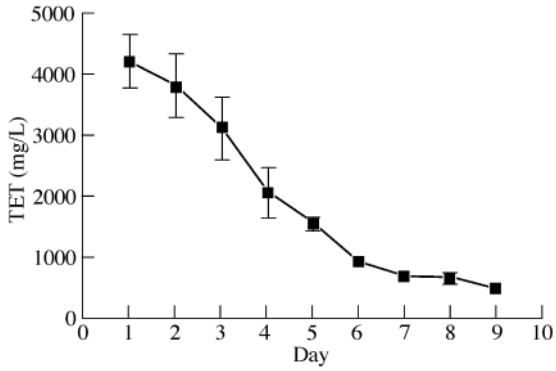

Figure 4. Determination of tetracycline (TET) concentrations eluted from FS discs incubated in normal rat serum. Discs (n = 3) were doped with 50 mg of tetracycline hydrochloride just before incubation.

Table. Summary of tetracycline concentrations determined by bioassay

Sample	Zone (mm)[a]	S.D.	TET (mg/L)
Standard 1	10.4	0.7	1.0
Standard 2	15.5	0.6	2.0
Standard 3	19.1	0.6	4.0
Standard 4	20.6	0.6	5.0
Rat serum	NMZ		<1.0
Doped rat serum	15.4	0.2	2.2
Serum from FS-treated rats	NMZ		<1.0
Serum from FS-TET-treated rats	NMZ		<1.0

Abbreviations: FS, fibrin sealant; NMZ, no measurable zone; S.D., standard deviation of the mean; TET, tetracycline.
[a]Mean zone of inhibition measured in millimetres by the agar diffusion bioassay using *B. subtilis* spore suspension, values determined from replicate samples; $n = 20$ for standard curve, $n = 20$ for normal rat serum, $n = 5$ for doped rat serum, $n = 11$ for FS-treated rats and $n = 11$ for FS-TET-treated rats (500 mg/kg ip).

500 mg/kg provided 100% protection to infected mice 7 days post-tetracycline delivery. A more substantial dose of tetracycline (1750 mg/kg) delivered from FS implanted 35 days before infection with *S. aureus* also resulted in 100% survival of mice. These data indicate that tetracycline is effectively released from the fibrin for extended periods of time. Testing of other poorly soluble antibiotics may provide an appropriate listing of specific drugs by indication and efficacy.

Our SEM analysis of the tetracycline crystals trapped by the fibrin as it polymerized indicates that the cross-linked fibrin network forms irregularly sized pores ranging from 5.6 to 27.8 μm in diameter about the tetracycline and through which trapped tetracycline is released into the local environment as the crystals dissolve. These results are consistent with, and extend, results of previous SEM studies that identified regular pore formation (1–10 μm) resulting from the fibrin polymerization.[4,13] Furthermore, they provide a physical explanation for both the retention of the antibiotic and its sustained release.

Further testing was then carried out to alleviate concerns of systemic side effects. Analysis of serum obtained from the systemic circulation of rats with FS-TET implants indicates that tetracycline is not likely to cross into the systemic circulation in bioactive amounts when slowly released locally into the peritoneum. Furthermore, *in vitro* release of tetracycline from FS into serum indicates that the tetracycline is bioactive and not altered by serum protein binding or freezing. Together, these data strongly suggest that the antibiotic concentration released from FS locally is sufficient to manage a lethal *S. aureus* peritonitis, but not sufficient to spill over into the systemic circulation.

We conclude that FS is an excellent vehicle for extended delivery of low solubility tetracycline for infection control. This study extends and augments our previous reports that slow release of the tetracycline can occur over weeks depending on the loading dose held by the FS. Additionally, doses of tetracycline delivered into the peritoneum that result in 100% protection of mice from a lethal peritonitis do not result in measurable, bioactive tetracycline concentrations in the systemic circulation. Other properties that make FS (a natural biopolymer) an ideal delivery vehicle include its adhesiveness, biocompatibility, resorbability and haemostatic capability. FS impregnated with antibiotic would thus be an ideal candidate for treatment of difficult infections such as osteomyelitis, endocarditis, keratitis, otitis, or rectovaginal and complex fistulas. This ability to release antibiotics in appropriate concentrations, for clinically useful durations, so as to manage *in vivo* infectious disease locally without systemic side effects could also reduce the risk of inducing bacterial resistance.

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