Biofilm Eradication With Biodegradable Modified-Release Antibiotic Pellets

A Potential Treatment for Glue Ear

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Objective: To develop a biodegradable, modified-release antibiotic pellet capable of eradicating biofilms as a potential novel treatment for biofilm infections.

Design: Pellets containing poly(DL-lactic-co-glycolic acid) microparticles, rifampin and clindamycin hydrochloride (3.5%, 7%, or 28% antibiotic by weight), and carrier gel (carboxymethylcellulose or poloxamer 407) were tested in vitro. Drug release was assessed using serial plate transfer testing and high-performance liquid chromatography, and pellets were tested against biofilms in an in vitro model of Staphylococcus aureus biofilm grown on silicone.

Results: Serial plate transfer testing demonstrated continuing bacterial inhibition for up to 21 days for all pellets studied. High-performance liquid chromatography showed high levels of drug release for 2 to 4 days, with greatly reduced levels subsequently; continued measurable clindamycin (but not rifampin) release for up to 21 days was achieved. Pellets made with poloxamer released higher drug levels for a longer period. Irrespective of the carrier gel used, pellets containing 7% and 28% (but not 3.5%) antibiotic eradicated biofilms successfully.

Conclusions: Antibiotic pellets can release antibiotics for up to 21 days and are able to eradicate biofilms in an in vitro model. Use of modified-release antibiotic formulations in the middle ear as a treatment for biofilms appears to be a potentially promising new therapy for otitis media with effusion.

not type with respect to growth rate and gene transcription; biofilms may cause a low-grade inflammatory reaction, which may then lead to the development of clinical symptoms of OME.7

Understanding of biofilm involvement in the etiology of OME opens potential new treatment avenues for reducing the current high rate of further surgery. However, bacterial biofilms are difficult to eradicate with conventional antibiotic therapy because they show inherently low susceptibility to treatment with antibiotics, typically requiring from 10- to 1000-times-higher antibiotic levels than those required to inhibit planktonic bacteria.7 This reduced susceptibility to antibiotics, termed recalcitrance, is distinct from antibiotic resistance occurring as a result of genetic alterations. Because biofilms are less susceptible to antibiotics, achieving sufficiently high antibiotic concentrations in the middle ear is a challenge; too low a concentration may also be responsible for the failure of oral antibiotics to confer any lasting curative effect in OME.4,8 The high recurrence rate after VT extrusion may also be explained by biofilm persistence because the VT may simply suppress mucin production or remove the effusion but leave the biofilm intact. Once the VT extrudes, the biofilm is still present and continues to exert the inflammatory response leading to effusion formation. Recurrence of OME after VT extrusion is likely to be a complex phenomenon in which adenoid disease, allergy, immune function, or any combination of these, as well as biofilms, may be important.

To achieve antibiotic levels in the middle ear high enough to eradicate biofilms, local delivery is the logical choice. Because biofilm eradication requires treatment for several weeks, a modified-release formulation would be required. This modified-release device could be inserted into the middle ear along with the first VT, release antibiotics for several weeks, and then undergo degradation without the need for surgical removal.

The aim of this study, therefore, was to explore the possibility of developing a modified-release antibiotic pellet that could be used in the middle ear as a treatment for OME.

METHODS

The pellet was based on poly(DL-lactic-co-glycolic acid) (PLGA), which already has a history of safe clinical use,9 with the addition of a carrier gel (carboxymethylcellulose [CMC] or poloxamer 407 [Pluronic F-127 (PL); Sigma-Aldrich Company Ltd]). The resulting PLGA suspension was filtered through a 40-µm filter to remove any large clumps, frozen in liquid nitrogen, and then freeze dried (ModulyoD, Thermo Electron Corporation) prior to storage at −20°C. The size of the particles was determined by laser diffraction with a particle size analyzer (Coulter LS230; Beckman Coulter). With this method, the mean particle diameter obtained was 12.31 µm (median diameter, 11.70 µm; mode, 15.37 µm).

The pellets (Figure 1) were produced by mixing UV-sterilized PLGA microparticles with a carrier gel and antibiotics and then placing the mixture into a polytetrafluoroethylene mold designed to produce pellets 3 mm long and 2.5 mm in diameter with a central 1-mm-diameter hole to allow movement of air through the pellet. The pellet dimensions were chosen so that the pellet would sit in the middle ear, held by surface tension, and could be inserted through the myringotomy.

Figure 1. Scanning electron microscopic (JSM-6060LV; JEOL Ltd) image of the surface of the antibiotic pellet (original magnification ×2000).

Effectiveness of the pellets was evaluated in 3 ways:

- Serial plate transfer testing (SPTT),15 which involves daily transfer of the antibiotic pellet to a freshly inoculated agar plate and measurement of the zone of bacterial growth inhibition occurring around the pellet as a result of antibiotic release. The zone of inhibition is proportional to the amount of drug released.
- High-performance liquid chromatography (HPLC), which allows determination of actual antibiotic concentration levels released from the pellets and allows measurement of rifampin and clindamycin separately.
- An in vitro biofilm model, which was used to assess the effect of the pellets against a biofilm in vitro.

MICROPARTICLES AND ANTIBIOTIC PELLETS

The oil/water emulsion method was used to prepare microparticles. Briefly, 1 g of PLGA was dissolved in 6.7 mL of dichloromethane (Sigma-Aldrich Company Ltd) in a glass container and added to 200 mL of 0.3% polyvinyl alcohol (Sigma-Aldrich Company Ltd). The mix was homogenized using a mixer (LSM; Silverson Machines) for 2 minutes at 9000 rpm. The emulsion, at room temperature, was then left stirring overnight (16 hours) with a 50-mm glass flea on a magnetic stirrer (Variomag Poly; Variomag) set at 300 rpm to allow the dichloromethane to evaporate. The following day, the polyvinyl alcohol/PLGA emulsion was aliquoted into 50-mL containers and centrifuged (Mistral 1000; MSE) at 3000 rpm for 5 minutes. The supernatant was discarded and the particles resuspended in deionized water and centrifuged again. The step was repeated until the samples had been centrifuged 3 times in water. The resulting PLGA suspension was filtered through a 40-µm filter to remove any large clumps, frozen in liquid nitrogen, and then freeze dried (ModulyoD, Thermo Electron Corporation) prior to storage at −20°C. The size of the particles was determined by laser diffraction with a particle size analyzer (Coulter LS230; Beckman Coulter). With this method, the mean particle diameter obtained was 12.31 µm (median diameter, 11.70 µm; mode, 15.37 µm).

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made in the eardrum before grommet insertion. The pellets were sintered at 60°C for 16 hours, taken out of the mold, and sterilized again with UV light. The pellets were stored at 4°C and used within a week of preparation.

The pellets were composed of 56-kDa PLGA 50:50 (Lake-shore Biomaterials), with a mean particle size of 12 μm, either 2% high-viscosity sodium CMC (Blanose; Aqualon/Hercules) or 20% poloxamer 407 (Pluronic F-127) (the PLGA to gel ratio was 1:0.8) and antibiotics rifampin (marketed in the United Kingdom as rifampicin; Sigma-Aldrich Company Ltd) and clindamycin (Sigma-Aldrich Company Ltd) at 3 different concentration levels: low (3.3% antibiotic as a percentage of PLGA weight; rifampin, 0.5%; clindamycin, 3%), medium (7% antibiotic as a percentage of PLGA weight; rifampin, 1% and clindamycin, 6%), and high (28% antibiotic as a percentage of PLGA weight; rifampin, 4% and clindamycin, 24%). The pellets were labeled as CMC/L, CMC/M, and CMC/H for the pellets made with CMC and low, medium, and high antibiotic concentrations and as PL/L, PL/M, and PL/H for the pellets made with poloxamer and the corresponding antibiotic levels. The different antibiotic loading levels were chosen on the basis of the published literature and preliminary experiments that indicated that antibiotic levels of around 1000 times higher than the minimum inhibitory concentration of the test S aureus strain would be required to achieve biofilm eradication; the difference between rifampin and clindamycin loading reflects the different minimum inhibitory concentrations of the test organism for the 2 antibiotics.

SERIAL PLATE TRANSFER TEST

A suspension of S aureus (a laboratory strain isolated from OME) was prepared to an optical density of 0.08 to 0.13 (McFarland 0.5 equivalent) at 490 nm measured using a spectrophotometer (Jenway Multicell Changer Spectrophotometer; Bibby Scientific Limited), and 200 μL was used to inoculate a sheep blood agar plate. The diameter of the zone of inhibition of bacterial growth around the pellet was measured with electronic calipers, with the pellet diameter itself subtracted from the overall zone. In addition to studying zones on individual days, the sum of the zones obtained on all days by a single pellet was calculated.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The pellets were immersed fully in 1 mL of phosphate-buffered saline, with phosphate-buffered saline aspirated and replaced every 2 days and the aspirated solution analyzed. High-performance liquid chromatography was performed with an HPLC system (Beckman System Gold; Beckman Coulter) using an HPLC column (Eclipse XDB-C8; Agilent Technologies) (150 mm long, with a 4.6-mm internal diameter and 5-μm silica particles with an 8-carbon side chain), with a flow rate of 1 mL/min at 40°C. The mobile phase consisted of aqueous sodium dihydrogen phosphate (15mM, pH 2.5) with 10% acetonitrile mixed with a varying amount of methanol (65% to 80%) in a gradient elution method. Detection was performed at 234 nm for rifampin and 210 nm for clindamycin. The lower detection limit for both antibiotics was 2 μg/mL; for rifampin this equates to 333 times the minimum inhibitory concentration of the test S aureus strain; for clindamycin, to 20 times.

BIOFILM MODEL

A variety of biofilm models is available; to keep to a size similar to the middle ear cavity, a new model of biofilm grown on a silicone disk was developed. Briefly, overnight cultures of S aureus (a laboratory strain isolated from OME) were sampled with an inoculation loop and suspended in tryptone soya broth (TSB) to an optical density of 0.08 to 0.13 (McFarland 0.5 equivalent) at 490 nm measured using a spectrophotometer. Silicone disks (silicone elastomer, 6 mm in diameter and 1 mm thick; Goodfellow) were placed into the suspension and incubated at 37°C, initially on an orbital shaker (Stuart orbital incubator SI30; Jencons-PLS) for 1 hour and then static for 1 hour. The disks were rinsed 3 times in phosphate-buffered saline prior to being aseptically placed into a sterile bijou bottle containing 2 mL of TSB. The bijou bottles were incubated at 37°C (Fir-labo Incubator HPE; Firlabs). The model was evaluated and validated prior to use in these experiments, with confirmation that biofilms grow on the silicone disks and develop the reduced susceptibility to antibiotics that is typical of biofilms.

The antibiotic pellet was added to 5-day-old biofilms in TSB, with the TSB being changed every 2 days during the 21 days of antibiotic exposure. After 21 days, the antibiotic pellets were removed, but the silicone disks were reincubated in fresh TSB (in the absence of any antibiotic) for an additional 5 days to allow any bacteria still alive to regrow (resuscitation experiment). The disks were then removed from TSB and exposed to trypsin for 15 minutes, the trypsin was then changed to phosphate-buffered saline, and the suspension was sonicated in a water bath for 5 minutes prior to plating.

STATISTICAL ANALYSIS

Comparison of the mean antibiotic level and inhibition zones on individual days was by 1-way analysis of variance performed using SPSS statistical software (version 19; SPSS Inc), with Tukey honestly significant difference post hoc analysis.

RESULTS

SERIAL PLATE TRANSFER TESTING

The SPTT results showed that the pellets tested continued to inhibit bacterial growth for up to 21 days (Figure 2), with fairly stable drug release from 10 days onward. Increasing antibiotic loading led to increased zones of inhibition; this effect was seen only during the first 13 days for CMC pellets but throughout 21 days of testing for poloxamer pellets. The sum of zones obtained by CMC/L pellets (284.9 mm) was significantly lower than that seen in CMC/M pellets (375.9 mm, P = .001) and CMC/H pellets (424.0 mm, P < .001), but the difference between CMC/M and CMC/H pellets was not statistically significant (P = .06). The total zone for the PL/H pellet (496.3 mm) was significantly larger than the zones obtained by PL/M pellets (391.1 mm, P < .001) and PL/L pellets (354.9 mm, P < .001), but the difference between PL/L and PL/M pellets was not statistically significant (P = .22).

Poloxamer pellets resulted in larger zones of inhibition than did CMC pellets with the same antibiotic loading. The sum of zones for low and high antibiotic loading was higher with poloxamer pellets (P = .005 and .004, respectively), but the difference was not statistically significant in medium antibiotic concentrations (P = .90).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

High-performance liquid chromatography results showed that clindamycin continued to be released until days 16,
22, and 14 from CMC/L, CMC/M, and CMC/H, respectively, and until days 22, 20, and 22 from PL/L, PL/M, and PL/H, respectively (Figure 3). However, for rifampin, the levels beyond day 4 were minimal—below the detection threshold of 2 µg/mL (data not shown). As with SPTT, increasing antibiotic loading led to greater amounts of drug released, but this effect was confined to days 2 and 4 for both clindamycin and rifampin. Poloxamer pellets with low and high antibiotic loading generally led to higher clindamycin levels than did corresponding CMC pellets, with the difference being statistically significant on days 18 and 20 (all P<.01).

**BIOMFAST ERADICATION IN VITRO**

Irrespective of the carrier gel used, the pellets made with low antibiotic concentrations failed to eradicate biofilms, but the pellets containing medium and high concentrations successfully eradicated biofilms in the in vitro model. No emergence of resistance was encountered. Pellets without antibiotics had no antibiofilm properties.

**COMMENT**

Otitis media with extrusion is a common condition, but its current treatment with VT insertion has significant shortcomings because further surgery is required in approximately one-fifth of cases. However, the recent implication of biofilms in the etiology of OME has opened new therapeutic avenues that have the potential to improve current treatment.

Therefore, we investigated a modified-release antibiotic pellet as a potential new treatment for OME. The pellets, based on PLGA, CMC, or a poloxamer and using the antibiotics rifampin and clindamycin, were tested in vitro, with drug release assessed using SPTT and HPLC and with the pellets tested against biofilms in an in vitro S aureus biofilm model. The results presented in this report indicate that the pellets containing the 2 higher antibiotic levels tested can eradicate biofilms in vitro; although SPTT demonstrated continued bacterial growth inhibition for up to 21 days, HPLC indicated that only clindamycin was being released in measurable amounts after the first few days but at relatively low levels. Nevertheless, the results indicate that such a strategy could, in principle, be a useful new treatment against OME biofilms.

**CHOICE OF MODIFIED-RELEASE DEVICE**

The application of novel drug delivery for the local treatment of biofilms has received much attention, especially in relation to implantable materials. Biodegradable polymers have also been identified as a potential method of delivering antibiotics in a modified-release manner, with particular interest in infected orthopedic implants. However, within the middle ear, some studies of using modified drug delivery to eradicate middle ear infections have also been published. Goycoolea and Muchow studied modified-release ampicillin from a poly-L-lactic acid support in cat and chinchilla animal models and found that the method was effective at eradicating infection without any ototoxic effects. While...
encouraging, this is not a true representation of human OME because animal models are either acute infections or eustachian tube obstructions and no true animal model of OME exists; the important link to biofilms also requires exploration. Another study using the same poloxamer (Pluronic F-127) to deliver vancomycin as a treatment of experimentally induced methicillin-resistant S. aureus middle ear infection\textsuperscript{20} is again encouraging but not a true representation of human OME.

Poly(DL-lactic-co-glycolic acid) forms one of the most commonly used biodegradable systems\textsuperscript{21} with a long history of safe clinical use in sutures, implants, prosthetic devices, and drug delivery systems, and it is generally considered to be very safe.\textsuperscript{22} The individual types of PLGA
have different molecular weight, different ratios of lactic to glycolic acid, and can be made as differently sized microparticles; all these characteristics affect the rate of degradation. For our purposes, 56-kDa PLGA 50:50 with a mean particle size of 12 µm was suitable. The safety, versatility, and current clinical use made PLGA an ideal starting point for developing new treatments for OME.

The PLGA microparticles adhere to each other when heated (sintered) to form a porous matrix in the desired shape. The spaces in the matrix between PLGA microparticles are filled by a gel that contains the antibiotics. The modified-release properties of the device are thus conferred by the gel containing the antibiotics as well as by the PLGA matrix. The gel contains antibiotics and releases them at a slow rate depending on water ingress and concentration gradients, whereas the PLGA matrix governs water ingress through the pores and PLGA degradation over time increases the release of the gel and drug from within the matrix. Poly(DL-lactic-co-glycolic acid) undergoing bulk erosion; consequently, at the end of its life, the device simply disintegrates into small fragments. In addition, PLGA undergoes hydrolysis to lactic and glycolic acid, both of which are metabolized in man without adverse reaction.

Carboxymethylcellulose and poloxamer are hydrogels with modified-release properties, and hydrogels have been widely studied because of these properties. Hydrogels are polymeric materials consisting of hydrophilic macromolecules cross-linked to form a 3-dimensional network and have the ability to imbibe large quantities of water without dissolving. Drug molecules can then be dispersed or dissolved within the hydrogel and its retained water and are released through diffusion.

Carboxymethylcellulose is a cellulose derivative that is widely used as a food viscosity modifier, is a component of many toothpastes, forms the outer gel lining of nasal packing or wound dressings, and has modified-release applications. Carboxymethylcellulose is available in a range of viscosities, with the high-viscosity CMC chosen in these studies leading to a more prolonged drug release rate. Although generally considered safe, one animal study of its use as ear-packing material suggested that it might affect hearing, but another found no concerns. Although CMC can be degraded by cellulase, this enzyme is not present in man, and the gel (whether CMC or poloxamer) may therefore be dissipated down the eustachian tube once the PLGA degrades.

Poloxamers, also known by their trade name Pluronics, are triblock copolymers consisting of a central hydrophobic polyoxypropylene chain flanked by 2 hydrophilic polyoxyethylene chains. Variation in the length of the polymer chain and the exact percentage of polyoxyethylene content lead to variation in the molecular weight, physical form, and drug-release properties. In addition to being used in modified drug release, poloxamers are also mucoadhesive and have thermoreversible properties, which means that they can be injected into the body as a liquid that turns into a gel at body temperature. Poloxamers are widely used in cosmetics and mouthwashes and generally considered safe, with animal experiments showing no ototoxic effects and only temporary hearing impairment likely due to conductive loss caused by gel in the middle ear. Poloxamers are not biodegradable but are rapidly excreted in the urine following systemic administration.

Overall, our results indicate that the 20% poloxamer 407 gel used in these experiments would be a better choice than 2% high-viscosity CMC because the drug is released in larger quantities for a longer period.

**DRUG-RELEASE PROFILE**

The type of a modified-release delivery device studied in this research, a matrix system with drug dispersed evenly throughout the polymer matrix, has several advantages over other types, most notably the ease with which drug delivery rate, dosing, lifetime of the device, and its size and shape can be adjusted; it is for these reasons that this formulation was chosen for biofilm eradication. However, a disadvantage is the difficulty of achieving drug release at a constant level, with the typical release profile being of a burst followed by gradually reducing levels.

The pellets studied herein are therefore typical of a matrix device because increasing drug loading of the pellets increased the amount of drug released and because of the release profile (an initial burst followed by release at much lower levels). Serial plate transfer testing and HPLC give complementary information about drug release; the former assesses both antibiotics together with the pellet sitting on a moist agar plate, and the latter measures levels of antibiotics separately with the pellet immersed in saline. Having the pellet sitting on a moist agar plate is more likely to be representative of the middle ear conditions following VT insertion; having the pellet fully immersed in saline (changed every 2 days) for HPLC experiments likely represents extreme conditions designed to draw the maximum amount of drug out of the pellet.

Our experiments using SPTT showed a much less steep fall in the amount of drug released (using zones of bacterial inhibition) than did our experiments using HPLC, with continued drug release for 3 weeks. However, HPLC showed that only clindamycin was being released at measurable levels beyond day 4; because SPTT assesses both antibiotics together, no information can be drawn about clindamycin/rifampin differentials on SPTT. Although the lower rifampin levels released may relate to lower rifampin loading into the device (chosen to reflect the differential susceptibility of the test organism to the 2 antibiotics), it is more likely that the lower levels released are the result of poorer rifampin water solubility: the hydrogel imbibes a large quantity of water containing the drug; therefore, if a drug is less water soluble, the hydrogel will be less effective as a modified-release agent.

The downside of having only 1 antibiotic is that emergence of resistance is more likely than when multiple antibiotics are used. The antibiotic concentrations released are also important, because levels below the minimum biofilm eradication concentration (MBEC) would again favor emergence of resistant strains without eradicating the biofilm itself. Assuming that the MBEC level is 1000 times higher than the level required to inhibit growth of planktonic bacteria (the minimum inhibitory concentration), then the pellets we studied can...
achieve those levels on day 2 as tested by HPLC, but not on subsequent days.

BIOFILM MODEL

Despite concerns that only 1 antibiotic rather than 2 may be released and that levels may be lower than the MBEC beyond day 2, the pellets containing the 2 higher antibiotic levels investigated nevertheless managed to eradicate Staphylococcus aureus biofilm grown in an in vitro model. The lowest concentration studied did not achieve this, but no emergence of resistance was encountered. The high initial burst of antibiotics—a useful antibiofilm strategy—clearly was sufficient for biofilm eradication despite lower antibiotic levels on subsequent days. The effectiveness of the pellet in the in vitro model also supports the idea of local drug delivery as a means of optimizing the therapeutic window, achieving at the site of infection antibiotic levels much higher than what could be obtained with systemic administration while minimizing any systemic toxic effects.

FURTHER INVESTIGATIONS

Although the success of this pellet in vitro is encouraging, much further research is needed before such a strategy could reach clinical use. At present, we have tested 2 antibiotics, rifampin and clindamycin, chosen for the initial experiments because they both work well against biofilms10,11 and are effective against S aureus (chosen as a typical biofilm organism) and because the use of 2 antibiotics minimizes resistance.12,13 Further research should incorporate other antibiotics relevant to otitis media and test them against other bacteria, since organisms other than S aureus are important in OME. In addition, better optimization of drug delivery could ensure more sustained release with antibiotic levels consistently above the MBEC. It would also be useful to explore diffusion of antibiotics through the middle ear effusion. The experiments undertaken to date were performed in vitro, but future in vivo testing may also be appropriate, both in terms of toxicity and efficacy; although there is no exact animal model of OME, recent reports have described potentially useful animal models of OME caused by a biofilm infection. The antibiofilm strategy discussed herein may also be relevant to other clinical conditions in which biofilm infection is important. Examination of the effectiveness of antibiotic pellets against OME would require a clinical trial.

CLINICAL APPLICATIONS

Although further developments are required, a novel strategy based on modified-release antibiotic pellets for the treatment of OME biofilms may someday help improve the care of patients. The pellet could be inserted into the middle ear at the time of VT insertion, with the aim of reducing the need for revision surgery. Alternatively, the pellet may be useful on its own when inserted in combination with a myringotomy, thus avoiding VT insertion and its potential complications (including those related to water exposure) altogether. A future refinement could include stimulus-responsive drug delivery that would release antibiotics in response to the presence of an active infection.

In conclusion, the understanding that biofilms play a key role in the etiopathogenesis of OME opens the possibility of better treatments aimed at reducing the need for repeated VT insertion. The modified-release antibiotic pellet studied herein can release antibiotics for up to 3 weeks and can eradicate biofilms in an in vitro model. Although further developments are necessary, the results indicate that such a strategy could, in principle, be a useful new treatment against OME biofilms.

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