Controlled Time-Release Antibiotic Nanoparticles for Implants and Bone Grafting

L. Amarapalli, S.K. Thanikanti, A. Adusumilli, S. Dammalapati and P. K.-L. Fu

Governors State University, University Park, IL, USA, pfu@govst.edu

ABSTRACT

The purpose of this study is to address our invention of developing a unique nanoparticulate system that is capable of delivering antibiotics in a locally applied and extended-release manner for patients receiving implants and bone grafting. This invention involves three major components: first, encapsulate both hydrophobic antibiotics (chloramphenicol and rifampicine) and hydrophilic antibiotics (vancomycine and acyclovir) into nanoparticles. Second, incorporate antibiotic nanoparticles in a polymeric coating material (nitrocellulose, polyvinylpyrrolidone, and polyvinyl alcohol) with a volatile carrier solvent (ethyl acetate or ethanol). Third, directly apply the resulting product from component 1 and 2 to implants and any bone crafting material such as hydroxylapatite directly before surgery. When the volatile carrier solvent evaporates, the coating polymer with embedded antibiotic nanoparticles will form a thin film that is capably attached to the surface of the implant material. Local application of encapsulated antibiotics directly to the surgical sites can provide a non-oral, non-intravenous, controlled time-release treatment, which would allow continuous administration of antibiotic therapy over the prescribed time span of the individual antibiotics used. This invention will provide a novel chemotherapeutic regime for the prevention and treatment of bacterial, fungal, and viral infections often occurred in transplant patients with a more efficient effective dose.

Keywords: drug-delivery, conrolled-release, encapsulation, implant, nanoparticle, liposome, micelle

1 INTRODUCTION

Usually antibiotics are administrated orally, absorbed from the gastrointestinal tract, extensively metabolized in the liver, and then distributed throughout the body. A small therapeutic concentration (between 5 to 10 percent) can be achieved in the brain after 1.5 to 5 hours of administration of the drug.[1-3,9,10] Since it has to be systematic circulated, a much higher initial dose is needed for the effective dosage to reach the brain. However, higher initial dosages cause more severe side effects including headache, bloody diarrhea, fever, nausea, severe blisters of the skin, jaundice, etc.[4,11] These can probably be reduced by administrating the effective dosage directly to the infected area.

Nanoparticle-encapsulated antibiotics can provide an alternative method to improve antimicrobial efficacy against drug-resistant strains.[5,6] Studies have shown that drug delivery systems using nanoparticle-encapsulated antibiotics as a carrier for biomedical applications can improve efficacy in solubilizing, protecting, and targeting drugs for specified delivery.[7-9,12,13] Nanoparticles such as liposomes and micelles have been used to protect drugs within a realtively impermeable bilayer or mutlilayer environment, and to prolong release times by isolating the encapsulated drugs from systematic degrading enzymes and promoting their diffusuion across the bacterial envelope.[12-14] A combination of various sizes of micelles, inverse micelles and liposomes can be used to achieve the prolonged release of antibiotics, each having different half-life of drug release.

Normal micelles have a hydrophobic core and a hydrophillic surface allowing the encapsulation of hydrophobic molecules in an aqueous solution. Inverse (or reverse) micelles, with a hydrophillic core, are created using the microemulsion method to encapsulate hydrophillic molecules. Liposomes are colloidal lipid-bilayer vesicles ranging from a few nanometers to several micrometers.[15] Liposomes have a double membrane and can encapsulate both hyrophillic molecules(in their core) and hydrophobic molecules (in the lipid bilayer) in an aqueous solution. By using a combination of unilamellar and multilamellar liposomes as well as regular and inverse micelles containing antibiotics, sustained delivery of the antibiotic over the specified period can be achieved.

Common implant materials such as PMMA and hydroxyapatite have a high surface area which provides a substantial amount of attachment area for the nanoparticles. Nanoparticulated drug-cocktail is mixed with a polymeric coating material and is then dissolved in a carrier solvent. A thin film of nanoparticle-containing polymer is the brushed on the upper surface of the implant material which will be set within 20 minutes. When the carrier evaporates, the antibiotic-containing nanoparticles are stably attached to the surface of the implant. This is a novel chemotherapeutic regime for the prevention and treatment of bacterial, fungal, and viral infections often occurring in transplant patients can be achieved by local application of encapsulated antibiotics directly to the surgical site. It will provide a non-oral, non-intravenous, controlled time-release treatment, which would allow continuous administration of antibiotic therapy over the prescribed time span of the individual antibiotics used with a more efficient effective dose to prevent post-operative infections.

2 EXPERIMENTAL METHODS

2.1 Antibiotic Nanoparticles

Double emulsion solvent extraction technique is used to create drug delivery vehicles. PLGA (polylactic-co-glycolic acid) and 5% (w/v) polyethyleneglycol (PEG) is dissolved in about 2 ml of dichloromethane (DCM) separately. Suitable polymers generally include PEG, PLGA and copolymers having molecular weight between 1,000-5,000 daltons. About 3 ml of antibiotic stock solution in PBS is measured using a drug to polymer ratio of 1:20. Both the drug and the polymer solutions are mixed with high a speed vortex mixer to form a stable emulsion. About 100 ml of 0.2% (w/v) aqueous polyvinylchloride solution is prepared by continuous stirring in moderate heat for about 1 hr. The drug-polymer emulsion is poured in to polyvinyl alcohol (PVA) solution which leads to the double emulsification of the particles. The mixture is sonicated for about 30 minutes and the particles are collected by centrifugation (about 15 min at about 13,000 rpm). The particles are washed with deionized water twice after the supernatant is discarded and are then resuspended in water and stored under refrigeration before Transmission Electron Microscopy (TEM) imaging.

2.2 Unilamellar Liposome Formulation[water-oil-water (w/o/w) Emulsion]

The Primary oily phase was prepared by mixing 5 mL (4.94g) of α-tocopherol with 0.0019g of L-α phosphaditylcholine (500 µM) and 5 ml of α-tocopherol (4.72g) with 0.006 g of palmitic acid (500 µM). Either luminescence marker or hydrophilic drug is dissolved in water (water phase). The water phase was titrated into the primary oil phase with constant stirring under low heat. The mixture was sonicated for 1 hour, then centrifuged for 15 minutes at 13,000 rpm. The fluid was discarded. Then the aqueous phase was prepared by mixing 5 ml of α-tocopherol (4.72g) with 0.006 g of palmitic acid (500 µM) and 20 mL of de-ionized water with 4 mL of Surfynol465. Finally the w/o emulsion formed after sonication and centrifugation, is titrated with the final aqueous solution and is sonicated for one hour under high sheer to create the unilamellar liposome. Thus the final product is a water–oil–water emulsion.

2.3 Multilayer Liposome Formulation (Water-oil-water-oil (w/o/w/o Emulsion)

An organic phase is prepared by dissolving 500 mg of sodium 1,4-bis [(2-ethylhexyl) oxy]- 1,4- dioxobutane – 2 - sulfonate in 4 ml of ethyl acetate. 23 uM of fluorescin dye is dissolved in about 1 ml of water to form an aquoeus phase. The aqueous phase is titrateddropwise in to the organic phase with constant stirring. Reverse micelles are formed within this water-in-oil (w/o) emulsion. After mixing 2 ml of the organic phase is evaporated, resulting in a water-in-oil emulsion having a total volume of 3 ml. The final water phase is formed by dissolving 500 mg of sodium 1, 4 – bis [(2-ethylhexyl)oxy] -1,4 - dioxobutane-2- sulfonate in 40 ml of water. The above w/o emulsion is added dropwise into the final water phase to form a w/o/w liposome. The coating material requires an organic solvent as a carrier and hence the liposome is suspended in the organic phase to mix homogenously with the coating polymer. The final organic phase is formed by dissovling 500 mg of AOT in 10 ml of ethyl acetate. The foregoing w/o/w liposome is added to the final organic phase to produce multi-layer w/o/w/o liposomes.

2.4 Reverse Micelles (Water-oil (w/o) Emulsion)

2.2 grams of Surfynol 465 is mixed with 5 ml of vitamin E (or vitamin F) with gentle heating and continuous stirring. Once the Surfynol 465 is dissolved, water phase comprising 2 ml of water and the drug is added dropwise with constant stirring. The mixture is then sonicated for 15 min.

2.5 Nanoparticles (Oil-water-oil (o/w/o) Emulsion)

A first oil phase is formed by dissolving the hydrophobic anti-bacterial drug chloramphenicol in 5 ml of ethyl acetate. The first oil phase is then titrated into a 5 ml water phase containing 500 uM palmitic acid with constant stirring. Micelles are formed in this o/w emulsion. Stirring is continued until all of the ethyl acetate from the first oil phase is evaporated by bringing the volume down to about 5 ml which conforms to the original volume of the water phase. Nanoparticles are formed with chloramphenicol entrapped inside the micelles. The final oil phase is formed by dissolving 12 or 18% w/v of polycaprolactone (PCL) and 500 uM palmitic acid in ethyl acaetate with stirring at 50^{o} C. Rhe final oil phase is removed from the heat and the above nanoparticles are added dropwise into the final oil phase with constant stirring. Stirring is continued until the solution reaches room temperature. PCL fibers then form a thin layer surrounding the double layered nanoparticles.

2.6 Stabilizer and Thickening agent

2.5 % w/v of human collagen can be added to each of the above preparations as a stabilizer. Carbomer, a synthetic high molecular weight polymer of acrylic acid, is used as a tickening agent to increase the viscosity of the formulations.

3 RESULTS

3.1 Implants

SEM images of the surfaces of polyacrylate implant materials show different depth of grooves which are capable of embedding the liposomes and nanoparticles.

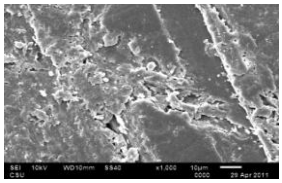

Figure 1. SEM image of Formulation A

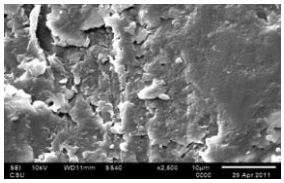

Figure 2. SEM image of Formulation B

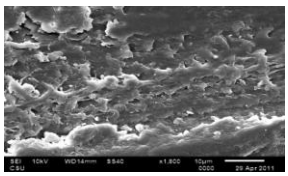

Figure 3. SEM image of Formulation C

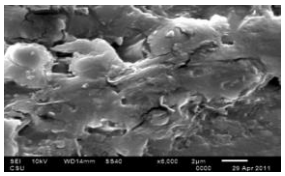

Figure 4.SEM image of Formulation D

3.2 Rifampicin Nanoparticles

TEM images of the encapsulated rifampicin nanoparticles at lower scale showed no aggregation of the nanoparticles within the matrix.

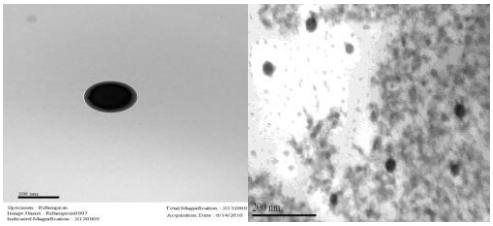

Figure 5: TEM of encapsulated Rifampicin nanoparticles. Indicated Magnification is X120000 and the Total Magnification is X132000.

3.3 Liposomes and Nanoparticles

In our study, we use the QuantomiX capsules methodology developed by WETSEM®. This technique eliminates many of the artifacts that result when preparing wet samples for Electron Microscopy. This technology enables imaging of our samples that contain oily and volatile solvent.

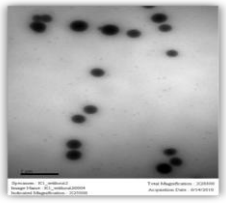

Figure 6. TEM images of unilamellar Liposomes

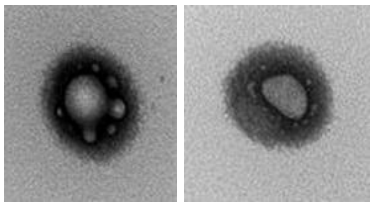

Figure 7. TEM images of multilamellar Liposome

3.4 Fluorescence Studies of Rifampicin Nanoparticles

Fluorescence spectroscopic analysis studies was conducted to confirm the encapsulation of the rifampicin in the nanoparticles. Fluorescence spectrum was recorded before and after the encapsulation of drug.

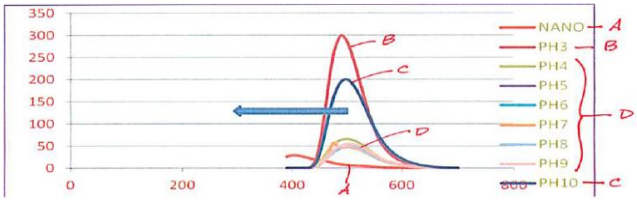

Figure 8. A. Nanoparticle B. Nanoparticle at pH 3 C. Nanoparticle at pH 10 D. Nanoparticle from pH 4 – pH 9

Fluorescent intensity dramatically decreased at the same concentration after the encapsulation. Rifampicin nanoparticles also showed a blue shift in the spectra which indicated the solvent environment had shifted from a hydrophobic environment to a more hydrophilic, polar environment. This data reveals the successful encapsulation of drug into the nanoparticles.

3.5 Fluorescence studies of Liposomes

Fluorescence study is the secondary technique which was conducted to provide an evidence for encapsulation of drug in to nanoparticles and examine the nanoparticle. The graph provieds a good evidence for the encapsulation of the drug in to the nanoparticles. Graph shows a decreased intensity of fluorescence of drug in water when compared to that of the drug in the reverse micelle which indicates that drug is encapsulated in to the reverse micelle. Liposomes have more decreased fluorescence intensity when compared to that of the reverse micelle because of the multi lamellar structure of the liposomes.

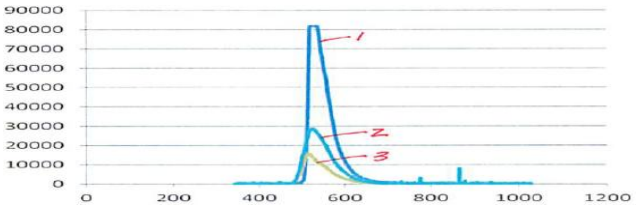

Figure 9. 1. Fluorescein dye in aqueous solution
2. Encapsulated fluorecein dye in reverse micelle
3. Encapsulated fluorescein dye in Liposome

3.6 Cell pentration studies of Liposomes

The cell penetration studies were carried out using the human dermal fibroblast cells. After 2 hours of incubation of cells with the nanoparticles, cell membranes were lysed using 5 percent N-lauryl sacrosine sodium salt solution. Fluorescent readings were compared before and after the cell lysis demonstrating the increased fluorescent intensity from the burst cells and the cell uptake of the fluorescent marker.

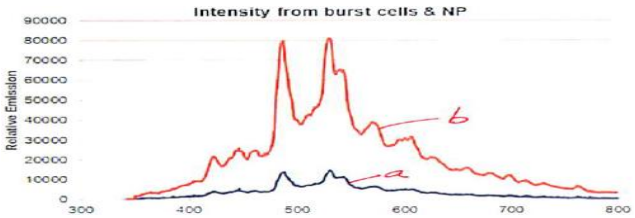

Figure 10. a. Fluorescent intensity before cell lysis b. Fluorescent intensity after cell lysis

3.6 Controlled release study

Human cerebrospinal fluid (CSF) was used to examine the controlled release kinetics. A luminescence marker was encapsulated inside reverse micelle and nanoparticles instead of the antibiotic in order to monitor the controlled release. Riboflavin was used to simulate the hydrophobic drug and fluorescein dye was used to simulate the hydrophilic drug. The nanoparticulate drug was coated on to the implants using the coating material. The final product was be submerged inside the human cerebrospinal fluid and the fluorescence was measured. The intensity of fluorescence indicates the amount of drug that is released from the coating polymer into the cerebrospinal fluid.

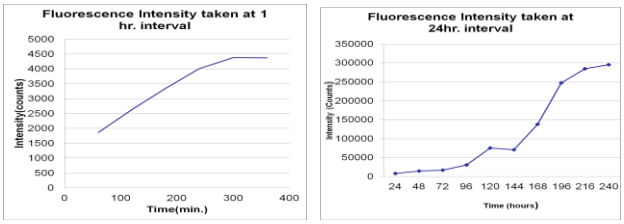

Figure 11. Controlled release study of reverse micelles. A plot of fluorescence intensity vs time.

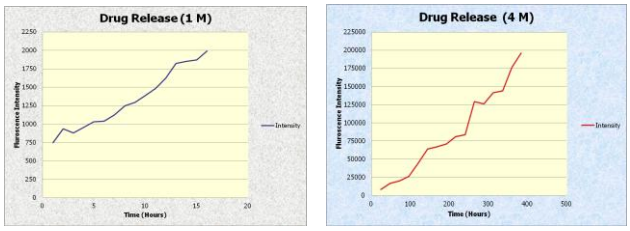

Figure 12. Controlled release study of liposomes. A plot of fluorescence intensity vs time.

4 DISCUSSION

The following study has the ability to prevent bacterial infections in patients with cranial transplantation. Preparation of antibiotics in a nanoparticulate form can be used to prevent infections with usage of less amount of drug and lesser side effects when compared to that of the antibiotics prepared for oral administration. The antibiotic drug prepared in nanoparticulate form can be directly applied to the location of surgery which reduces the first pass metabolism. SEM images of PMMA implant shows depth groves which indicates the capability of embedding the nanoparticles. Fluorescence studies show that the drug is completely encapsulated in to the prepared nanoparticles and TEM images shows the formation and size of the nanoparticles. Controlled release study results indicated that the prepared nanoparticles provided sustained release of the drug for about 15 days.

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REFERENCES

[1] Kolarz, B. N.; Trochimczuk, A. W.; Wojaczynska, M.; Drewniak, M. *Angew. Makromol Chem.* 2003 , 217 19 29
[2] Dowding, P.J.; Vincent, B. *Colloids Surf.*, A 2000, 161 259 269
[3] Kawaguchi, Y.; Itamura, Y.; Onimura, K.; Oishi, T. *J. Appl. Polym. Sci.* 2005, 96 1306 1312
[4] Owens, B.D.; Kragh Jr, J.F.; Wenke, J.C.; Macaitis, J.; Wade, C.E.; Holcomb, J.B. *J Trauma*., 2008, 64(2), 295-299.
[5] Wade, A.L.; Dye, J.L.; Mohrle, C.R.; Galarneay, M.R. *J Trauma*., 2007, 63, 836-840.
[6] Schiller, C.; Rasche, C.; Wehmoller, M.; Beckmann, F.; Eufinger, H.; Epple, M. *Biomaterials.* 2004, 1239-1247.
[7] Jesorka, A.; Orwar, O.Liposomes: Technologies and Analytical Applications *Annu. Rev. Anal. Chem.* 2008, 1, 801– 832.
[8] Stengel, G.; Simonsson, L.; Campbell, R. A.; Hook, F. *J. Phys. Chem. B* 2008, 112, 8264– 8274.
[9] Butoescu, N.; Jordan, O.; Doelker, *Eur. J. Pharm. Biopharm.* 2009, 73, 205– 218.
[10] Srinath, P.; Vyas, S. P.; Diwan, P. V. *Drug Dev. Ind. Pharm.* 2000, 26, 313– 321.
[11] Fromherz, P.; Ruppel, D. *FEBS Lett.* 1985, 179, 155–159.
[12] Parry, M. J.; Hagen, M.; Mouritsen, O. G.; Kinnunen, P.K. J.; Alakoskela, J. I. *Langmuir* **2010**, 26, 4909– 4915
[13] Kim, S. Y., Kim, H. J., Lee, K. E., Han, S. S., Sohn, Y. S. and Jeong, B. *Macromolecules* **2007**, 40, 5519– 5525
[14] Zhang, H. H., Huang, Z. Q., Sun, B. W., Guo, J. X., Wang, J. L. and Chen, Y. Q. *J. Polym. Sci., Part A: Polym. Chem.* 2008, 46, 8131– 8140.
[15] Chang, C., Wei, H., Quan, C. Y., Li, Y. Y., Liu, J., Wang, Z. C., Cheng, S. X., Zhang, X. Z. and Zhuo, R. X. *J. Polym. Sci., Part A: Polym. Chem.* 2008, 46, 3048–3057.