Ph.D. Thesis

SECOND GENERATION POLYMETHYL-METHACRYLATE-SORBITOL CAPSULES – DEVELOPMENT AND ELUTION STUDIES OF A LOCAL ANTIBIOTIC DELIVERY VEHICLE

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ABBREVIATIONS

ANOVA: analysis of variance, **BPB:** bromophenol blue, **HPLC:** high performance liquid chromatography, **LADV:** local antibiotic delivery vehicle, **MIC:** minimal inhibitory concentration, **PMMA:** polymethyl-methacrylate, **SEM:** scanning electron microscopy, **w/w %:** weight percent

1. INTRODUCTION

Osteomyelitis is a difficult-to treat infection of the bone and bone marrow caused by bacteria originated from trauma, implant use, surgery, by direct colonization from a proximal infection or through systemic circulation, which tends to progress unless adequate therapy is applied. The conventional treatment of osteomyelitis includes a combination of surgical and antimicrobial principles. Surgical approaches require radical debridement of all infected tissue whereas antimicrobial therapy involves high dose of prolonged (4-6 weeks) parenteral antibiotic administration. Such antibiotic therapy is expensive and prone to complications and toxic side effects. To overcome adverse effects local antibiotic therapy is an alternative. By acting directly at the site of the infection the local drug delivery vehicles (LADV) can ensure high drug concentration locally.

Using LADV to treat local infections is preferable over systemic therapy, as the main benefit of these devices is to obtain locally high antibiotic concentration at the site of the infection without increasing systemic toxicity. Hence, these devices can either replace or supplement the parenteral antibiotic administration, as the bioavailability of the systemic drugs is uncertain at the affected area due to poor vascular supply.

In 1970, Buchholz and Engelbrecht reported in their pioneering work on the use of antibiotic-impregnated polymethylmethacrylate (PMMA). Since then, the antibiotic laden PMMA represents the standard in prevention and/or treatment of deep bone infections.

However, the release properties of substances from acrylic bone cement are suboptimal. Previous studies concluded that antibiotic-laden PMMA shows biphasic release kinetic with rapid initial burst being followed by sustained low rate of release. Furthermore due to the hydrophobicity of PMMA, <10% of the trapped drug is released from such composite materials. To increase the accessible antibiotic content, PMMA have been complemented with various water soluble fillers, for example carbohydrates (dextran, sucrose), amino acids (glycine), polyols (erythrol, xylitol) or additional antibiotics. Moreover, a number of commercially used antimicrobial drugs cannot withstand the high temperature (70-110°C) during polymerization. Another limitation is that antibiotics in aqueous solutions cannot be used due to mixing and hardening problems.

2. OBJECTIVES

Our aim was to develop a LADV that provides controlled local therapy for chronic osteomyelitis, capable of using wide-range of drugs with relatively steady time-dependent release kinetics over a protracted period. We have created a PMMA-filler (sorbitol) based capsule system with different wall thickness and filler content (as an improvement of that of Börzsei et al.). We assumed that the two properties are important determinants in the formation of elution channels. Theoretically, therefore, we should be able to control the capsule wall permeability by optimising these properties.

The permeability of the capsules was preliminarily monitored by measuring the release of bromophenol blue (BPB) as a marker molecule throughout a clinically relevant period of time (42 days).

To clarify the properties responsible for wall permeability the surface morphology of the capsules was characterized by scanning electron microscopy (SEM).

According to the release profiles of the BPB we selected the most effective combinations of wall thickness and sorbitol content for further investigations. We also inteded to assess the in vitro antibiotic release profiles of the preselected capsules for various clinically relevant antibiotics (gentamicin, tobramycin, amikacin, clindamycin).

3. MATERIALS AND METHODS

3. 1. Preparation of PMMA-sorbitol capsules

Sixteen groups of capsules were prepared with different wall thickness and filler content. The chosen filler was sorbitol, an anticariogenic food sweetener that is inexpensive, water-soluble and is metabolized either slowly or not at all by bacteria.

We have prepared 4 types of Surgical Simplex P PMMA Bone Cement mixtures (Stryker-Howmedica-Osteonics, Rutherford, USA) containing 40, 50, 60 and 70 weight percent (w/w %) of sorbitol powder.

First, the sorbitol was ground and passed through a filter with a pore size of 160 μm, and then homogeneously dry-mixed with PMMA polymer powder. After adding the liquid monomer to the powder mix, cylindrical blocks were made.

The technique used by Börzsei et al. was further improved by using a milling machine instead of a press to prepare the capsules. Therefore capsules were carved from these blocks with wall thickness of 0.3, 0.4, 0.5 and 0.6 mm.

3. 2. Determination of capsule wall permeability

BPB (Sigma-Aldrich, Steinheim, Germany) was used as a diffusion indicator preceding antibiotic elution studies. The capsules were loaded with 40 μl of 1 mg/ml BPB solution and placed into 1 ml of physiological saline solution for 42 days.

At different time intervals (on days 1-6 and subsequently 9, 12, 15, 19, 23, 27, 32, 37 and 42) the amount of BPB filtered trough the capsule wall was measured by spectrophotometric light absorption at 592 nm.

3. 3. Analysis of surface morphology by Scanning Electron Microscopy

To clarify the properties responsible for wall permeability the surface morphology of the capsules was characterized by scanning electron microscopy (SEM). Pictures were recorded at same magnification.

The percent ratios of the developed pores were calculated by using Image J image processing software (plugin: contour plotter analyzer).

3. 4. Antibiotics and determination of their minimum inhibitory concentrations

According to the BPB release profiles four types of PMMA-sorbitol capsules (60 w/w%, 0.5 mm; 60 w/w%, 0.6 mm; 70 w/w%, 0.5 mm; 70 w/w%, 0.6 mm) were selected and tested for the following antibiotics: gentamicin (in form of gentamicin sulphate, (Sigma-Aldrich, Steinheim, Germany)), tobramycin (Brulamycin, TEVA Pharmaceuticals, Debrecen, Hungary), amikacin (Likacin, Lisapharma S.p.A, Erba, Italy) and clindamycin (Dalacin C, Pfizer Kft, Budapest, Hungary).

Commercially available PMMA beads (Septopal, Biomet Europe, Darmstadt, Germany) containing 4.5 mg gentamicin (in the form of gentamicin sulphate, mass of 7.5 mg) were used as controls.

After ethylene oxide sterilization capsules (n=8) were loaded and placed into 1 ml of physiological saline solution for 42 days. On days 1-6 and subsequently on days 9, 12, 15, 19, 23, 27, 32, 37 and 42 the capsules were returned to fresh saline. The saline being removed was stored at -80°C until being analyzed.

The minimum inhibitory concentration (MIC) values were determined by the tube dilution method on *Staphylococcus aureus HNCMB 112002* bacterial strain.

3. 5. Gentamicin, tobramycin and amikain release from PMMA-sorbitol capsules determined by agar plate diffusion assay

The amount of antibiotics filtered trough the capsule wall was measured by microbiologic agar plate diffusion assay using Mueller-Hinton agar seeded with *S. aureus HNCMB 112002.*

The antibiotic concentrations of the samples were determined and expressed as μg/ml by measuring and comparing the inhibition zone size to those of known concentration standards.

3. 6. Clindamycin release from PMMA-sorbitol capsules determined by HPLC

The amounts of antibiotic filtered through the capsules wall were determined by high performance liquid chromatography (HPLC) using an Ultimate 3000 HPLC system (Dionex, Sunnyvale, CA, USA).

Prior to analysis the samples were diluted to 10-50 times. The separation was carried out on a reverse phase C18 column (150x4.6 mm, 5 μm, Supelco Discovery, Sigma-Aldrich Kft, Budapest) at 25°C. The mobile phase was a mixture (1:1, v/v) of

acetonitrile and phosphate buffer. The flow rate was set at 1.0 mL/min, the injection volume was 10 μL and the chromatograms were detected at 205 nm.

For quantification standard solutions of clindamycin (1.5-40 μg/mL) were used.

3. 7. Statistical analysis

All statistical analysis was performed using SPSS 15.0. (SPSS Inc, Chicago, USA). The effects of capsule wall thickness and sorbitol content for the release of BPB were analyzed using ANOVA test for each sampling time ($p<0.05$).

ANOVA was followed by "post hoc" analyses of paired groups of capsules on day 6, 15, and 32 of incubation. Since 16 groups of capsules were compared at three different time points 16 x 15 x 3 = 720 analyses were performed. We identically color coded the groups of capsules, which were similar with regards their BPB release properties ($p>0.05$).

The relation between the release properties and the capsule surface morphologies was also assessed. Comparison of the surface area of pores, as determined by SEM was plotted and analyzed against several aspects.

The data are presented throughout as mean ± standard deviation (SD). Comparisons were performed using Student's t-test and statistically significant differences were defined at $p<0.05$.

4. RESULTS

4. 1. Wall permeability of capsules

The BPB release slowed down significantly with increasing wall thickness. The cumulative curves representing 40-70 w/w % sorbitol concentrations gradually flatten with increasing wall thickness. At the 70 w/w % sorbitol level, full equilibration was seen by day 6 with the 0.3 mm wall thickness. Full equilibration was postponed in time when the capsule walls were thicker: 19, 23 and 42 days, respectively.

As expected, the same tendencies were seen in capsules with lower sorbitol contents. Comparison of the effect of sorbitol content on BPB release at a given capsule wall thickness showed that less and less BPB is measured in the incubation medium when the sorbitol content decreases. Consequently, only about half the total BPB load is released in the thinnest, 0.3 mm – 40 w/w % sorbitol capsules by the end of experiment.

ANOVA analysis of the released BPB as the function of wall thickness or filler content gave highly significant differences at any time-point ($p<0.001$). As expected, the combined effect of these 2 factors had a significant cumulative impact on the release profiles ($p<0.001$).

Pair-wise, post hoc analyses also confirmed our hypothesis; considering only the wall thickness, the thinner is the capsule wall at given filler concentration, the faster the release group to which it belonged. Likewise, the higher the sorbitol content of the capsule at any given wall thickness, the faster the release of BPB.

4. 2. Surface morphology of capsules by SEM

In SEM pictures recorded at day 42, circular dark voids (pores) were present.

We found that the larger the area of surface pores, the sooner the initial burst occurs. An exponential curve provided the best fit to our data (R^2=0.868, p<0.001).

The relationship between the surface porosity and the time needed to release half of the total load gave a negative exponential correlation (R^2=0.945, p<0.001).

The relation between the surface porosity and the daily maximum release of BPB gave a strong exponential positive correlation (R^2=0.939, p<0.001), as expected.

The surface porosity versus time passed between the 25 and 75% total BPB released plot also confirmed strong negative exponential relationship between the variables (R^2=0.945, p<0.001).

4. 3. Biological activities and MIC values of the antibiotics

The bacterial inhibition assays revealed that the antibiotics retained their biological activity over 42 days.

MIC of gentamicin was 0.2 μg/ml, MIC of tobramycin was 0.2 μg/ml and MIC of amikacin was 0.8 μg/ml on *S. aureus*.

4. 4. Antibiotic release from PMMA-sorbitol capsules

First we determined the gentamicin release from PMMA-sorbitol capsules and Septopal beads loaded with equal amount of gentamicin (4.5 mg).

The beads showed typical biphasic kinetics with high initial burst on the first day followed by rapid decline. Over 50% of the total release occurred within 5 days, and the cumulative release curve plateaued after 10 days. Only 50% of the incorporated antibiotic was eluted from the beads by the end of the experiment.

In contrast, the antibiotic laden PMMA-sorbitol capsules released 70-100% of their total load depending on the wall thickness and sorbitol content. The capsules with 60 w/w % sorbitol and 0.6 wall thickness showed the lowest cumulative release, whereas the highest release by the end of the experiment was achieved with the capsules with 70 w/w % of sorbitol and 0.5 mm wall thickness. The latter ones had already emptied nearly 100% of their total load by day 27.

The release profiles of the capsules were determined by the type of the capsules as well. The release kinetics of the capsules with 70 w/w % of sorbitol and 0.5 mm of wall thickness compared best to Septopal, but showed quicker release and maximum elution. The release was fastest during the first 19 day period and continued at lower rate after. Between the days 2 and 23 these capsules demonstrated significantly higher antibiotic release than the control beads.

Other capsules with lower sorbitol contents and/or 0.6 mm wall thickness showed modest gentamicin release during the first days of the experiment. However, after the first phase of the opening of elution channels, sustained effective gentamicin release was seen throughout the rest of the observation period. Consequently, capsules with 60 w/w %

sorbitol and capsules with 70 w/w % sorbitol and 0.6 mm wall thickness released significantly more gentamicin compared with Septopal after day 9. These capsules appeared to deliver low levels of gentamicin during the first days, but gradually began to release antibiotic thereafter, giving effective sustained release throughout the 42 day.

Capsules with 60 w/w % sorbitol and 0.6 mm wall thickness released the highest levels of gentamicin throughout the second half of the experiment. Between days 23 and 42, over 800 times the MIC has been released.

We also assessed the tobramycin release profiles from the capsules. In comparing the cumulative release characteristics of tobramycin-loaded capsules with gentamicin-loaded capsules, they demonstrated similar cumulative release characteristics where identical capsule constructs were used.

Biphasic kinetic was observed also for 70 w/w % sorbitol with 0.5 mm capsules as the antibiotic release rate was high throughout the first days, but rapidly decreased with no additional release being detectable after day 27.

Capsules containing less sorbitol and/or having a thicker wall also demonstrated similar release properties. Even during the last 5 days of the experiment, the capsules released 350-660 x MIC equivalent tobramycin.

As with the results of the gentamicin release experiments, the 60 w/w % sorbitol and 0.6 mm wall thickness capsules showed the highest additional tobramycin release during the second part of the observation period.

The elution curves of amikacin were again similar to those of gentamicin and tobramycin release.

In case of clindamycin loaded capsules the drug release depended also on wall thickness and filler content. The highest initial burst was seen in 70 w/w %, 0.5 mm capsules and the capsules released 100% of the loaded clindamycin by day 42. These capsules represented the fastest release characteristic as high outburst was seen during the first days of the experiment followed by low release later.

The 70 w/w %, 0.6 mm and 60 w/w %, 0.5 mm capsules had slower release throughout the experiment without showing major difference from each other. At the beginning of the experiment the antibiotic release was modest compared to 70 w/w %, 0.5 mm capsules, nevertheless, after the opening of elution channels, ascendent release was seen throughout the rest of the observation period.

The 60 w/w %, 0.6 mm capsules showed the lowest cumulative release, however.

5. SUMMARY

The data obtained demonstrate that LADV with various controlled release kinetics can be prepared by setting capsule wall thickness and filler content. A rapid high initial release was associated with high filler content and thin capsule wall. If the filler content was low and the capsule wall thick, relatively even but slow release kinetics were observed.

In the case of the sorbitol-filled capsules, formation of elution channels starts immediately, but effective canalization is prolonged where the capsule wall is thick and/or the filler concentration is low. The more elution channels that are open, the quicker is the equilibration process, but the acceleration is dampened by the fact that in the meantime the capsule BPB concentration decreases. This allows the creation of a LADV with relatively constant and efficient discharge of its load.

Furthermore, we also aimed to determine whether the capsules have the potential to release antibiotic content in a similar manner, ensuring constant high drug levels over several weeks.

As expected, antibiotic release by all capsules occurred as a function of sorbitol content and wall thickness. Moreover the release profiles of the same type of capsules were similar to each other, and independent of the type and loading dose of the antibiotics.

All 70 w/w % sorbitol and 0.5 mm wall thickness capsules showed an initial burst effect (similar to Septopal); however, the release rate was significantly higher and more efficient compared to beads.

Other capsules showed delayed release characteristics. Due to a thicker wall and lower sorbitol content, the permeabilization of 60 w/w % sorbitol and 0.6 mm wall thickness capsules proceeded more slowly.

Therefore, if a longer (e.g. 6-week) effective antibiotic treatment is needed, we recommend a combination of capsules to complement one another. (It is also easy to create a separator divided capsule, where the two halves have different wall thickness and/or sorbitol content or create a capsule chain analogous with the bead chains.) This way, after the 70 w/w % sorbitol and 0.5 mm wall thickness compartments emptied their load the 60 w/w % sorbitol and 0.6 mm wall thickness compartments could still gave a high rate of antibiotic release for the rest of the treatment period. Notably, the additional release from the PMMA-sorbitol capsules could remain at a significantly greater level than the MIC, which offers great possibility for rapid bacterial clearing.

Therefore, besides the wide range of drugs that could be used, the amount of drug release and the duration meet the criteria of an almost ideal LADV.

Consequently, after identification of the infective microorganism and determination of the in vitro antibiotic susceptibility, it would be feasible to introduce the most appropriate antibiotic into the previously prepared and sterilized PMMA-sorbitol capsules.

We consider that, after radical debridement, inserting the combination of 70 w/w %:0.5 mm and 60 w/w %:0.6 mm capsules directly into the site of the infection could provide ideal and relative even local drug concentration, giving the best possibility of killing or inhibiting the growth of bacteria.

We conclude that the capsules can provide effective local drug delivery in a predictable manner for various length of time depending on their filler content and wall thickness.

By using a combination of capsules (or a divided capsule) with quick and protracted release characteristics, the desired aim of local antibiotic therapy can be achieved.

In this way these capsules may decrease the risk of developing antibiotic resistance, as the prolonged, unwanted subtherapeutic level can be avoided.

Theoretically any kind of antimicrobial agents can be applied through this delivery system (based on the identification of the microorganism and its in vitro antibiotic susceptibility). Other drugs, such as anticancer agents, could also be employed to treat bone metastasis.

Another advantage of our method is the inexpensive preparation of the capsules themselves.

Thus PMMA-sorbitol capsules are likely to be more reliable drug delivery vehicles compared to antibiotic impregnated PMMA bone cements**.**

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