**Staphylococcus aureus** biofilm formation on different gentamicin-loaded polymethylmethacrylate bone cements

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**Abstract**

In this in vitro study, the formation of a **Staphylococcus aureus** biofilm on six gentamicin-loaded bone cements (CMW1, CMW3, CMW Endurance, CMW2000, Palacos, and Palamed) was determined in a modified Robbins device over a 3 days time span and related with previously (Van de Belt et al., Biomaterials 21 (2000) 1981) measured kinetics of antibiotic release by these cement brands. The influence of gentamicin release on biofilm formation was quantified by expressing the number of colony-forming units on gentamicin-loaded cement relative to the number of viable organisms on unloaded cement of the same brand. Biofilms formed on all gentamicin-loaded cements, despite the release of antibiotics, followed a consistent pattern in time with a maximum number of colony-forming units per unit cement area found between 24 and 30 h after inoculation. None of the gentamicin-loaded cements showed a reduction in biofilm formation relative to unloaded cements within 6 h after inoculation, whereas only gentamicin-loaded CMW1 and Palacos reduced biofilm formation 24 h after inoculation. Alternatively, CMW Endurance, CMW2000, and Palamed did not exhibit any initial reductions in biofilm formation, but effects started after 72, 48, and 72 h, respectively. Biofilm reduction by gentamicin-loaded CMW3 lasted the longest from 24 to 72 h. Interestingly, each cement seemed to have a different “window-of-effectiveness” with regard to reduction in biofilm formation that did not relate with the gentamicin-release kinetics. Summarising, this study demonstrates that although gentamicin loading of bone cements yields reductions in biofilm formation, **S. aureus** is able to grow on gentamicin-loaded bone cements. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** Polymethylmethacrylate; Gentamicin; Bone cement; **Staphylococcus aureus**; Biofilm formation

1. **Introduction**

Several studies have shown that local administration of antibiotics through antibiotic-loaded bone cements yields successful and cost-effective prevention of biomaterials-related infections in total joint arthroplasty, but others have questioned the clinical efficacy of antibiotic-loaded bone cements and worry about the contribution of the use of antibiotic-loaded bone cement to the development of antibiotic resistant microorganisms [1-2]. The susceptibility of staphylococci to gentamicin has dropped enormously since the introduction of gentamicin-loaded bone cements in orthopaedics. In a retrieval study, coagulase-negative staphylococci were found responsible for 88% of infections in patients with the primary arthroplasty fixed with gentamicin-loaded bone cement, while at least one of the infecting strains of staphylococci was resistant to gentamicin [3]. Recently, staphylococcal growth was reported on gentamicin-loaded cement beads in a patient with the contra-lateral hip fixed with gentamicin-loaded bone cement [4]. Also in vitro studies have shown that bacteria adhere to antibiotic-loaded acrylic bone cements and can survive in the presence of high antibiotic concentrations [5-8].

The benefit of antibiotic-loaded bone cement is the assumed high local concentrations of antibiotics that are said to be achieved. In vitro studies have demonstrated a high initial release of antibiotics from polymethylmethacrylate bone cements in the first hours, but little is known about the prolonged release, long-term effects on the development of an infectious biofilm and the actual antibiotic concentration resulting from antibiotic release out of bone cements around an implant in a living organism [9-12]. Previously, we compared the gentamicin release...
release out of six different polymethylmethacrylate bone cements, and found initial release rates varying between 8.6 and 14.1 μg/cm²/h. The initial release rates tended to increase with increasing surface roughness of the cement, while the total amount of antibiotics released within 1 week increased linearly with the porosity of the cements. Palamed showed a high initial release burst, while the total release within 1 week was highest among the cements. Palamed showed a high initial release burst, while the total amount of antibiotics released within 1 week increased linearly with the porosity of the cements. Palamed showed a high initial release burst, while the total amount of antibiotics released within 1 week was highest among the collection of six different bone cements. These differences in release kinetics may have a major effect on biofilm formation and the prevention of infection in a clinical situation.

The aim of this study is to compare the effects of gentamicin-loaded bone cements on in vitro Staphylococcus aureus biofilm formation and to relate the effects on biofilm formation with previously measured kinetics of antibiotic release of the bone cements [13].

2. Materials and methods

2.1. Cement disc preparation

Six gentamicin-loaded bone cements were used: CMW1, CMW3, CMW Endurance and CMW2000, all with 2.5 w/w% gentamicin, corresponding to 4.22 w/w% gentamicin sulphate (DePuy CMW International Ltd., Cornford road, Blackpool Lancashire FY4 4QQ, UK); Palacos (Schering-Plough, Maarssen, The Netherlands), and Palamed, both with 1.25 w/w% gentamicin corresponding to 2.04 w/w% gentamicin sulphate (Merck Biomaterial GmbH, D-64271 Darmstadt, Germany). Unloaded cements of the same brands are (commercially) available as well and were obtained from the above manufacturers. Cements were prepared by mixing the powdered methylmethacrylate with the liquid monomer in a bowl with a spatula. Manual mixing was done according to the manufacturer’s instructions and resulted in a liquid cement. The liquid cement was poured into a polystyrene mould (200 × 40 × 3.2 mm), containing holes of 6 mm diameter. This filled mould was pressed between two glass plates for 25 min. After hardening of the cement, cement discs were pulled out of the mould, and stored under dark, sterile conditions at room temperature. The total surface area of each disc was 0.87 cm² and each one weighted 100 mg.

2.2. Bacterial strains, growth condition and biofilm formation

S. aureus ATCC 12600 was grown for 24 h at 37°C in ambient air in Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK) with pH equal to 7.3. This pre-culture was used to inoculate a second culture (400 ml) which was grown overnight and used to inoculate the modified Robbins device (MRD) [14], a hollow rectangular cu

bicle (620 × 20 × 20 mm) with 10 holes in which cement discs can be plugged. The cement discs were glued onto the sample holders with silicone paste under aseptic conditions. The silicone paste did not have any antibacterial effects.

The MRD was inoculated with the overnight culture of S. aureus and left for 5 h. Hereafter, the device was flowed for 72 h with TSB growth medium at a flow rate of 63 ml/h. Of all six cements, three separate runs were performed and at each run fresh cement discs were placed in the device. Plain cement discs of one brand were placed in positions 1–5 up-stream of the flow and gentamicin-loaded cement discs of the same brand were placed down-stream in position 6–10. The temperature of the MRD was maintained at around 37°C during the experiment.

2.3. Biofilm evaluation

The cement discs were removed from the MRD, put in 2 ml of reduced transport fluid (RTF: NaCl 0.9 g/l, (NH₄)₂SO₄ 0.9 g/l, KH₂PO₄ 0.45 g/l, MgSO₄ 0.19 g/l, KH₂PO₄ 0.45 g/l, Na₂EDTA 0.37 g/l, L-cysteine HCl 0.2 g/l, pH 6.8), vortexed for 10 s and finally sonicated for 60 s for microbiological evaluation. Serial dilutions were poured onto TSB agar plates. After overnight incubation, the number of colony-forming units (CFU) on each cement disc were counted and expressed relative to the surface area of the bone cement (CFU/cm²). Subsequently, to quantify the effect of antibiotic release on biofilm formation, the number of CFU/cm² on the antibiotic releasing cement were expressed relative to the maximum number of CFU/cm² found on the corresponding unloaded cement. Data for biofilm formation were evaluated for statistical significance as described by Matthews et al. [15], using the Student t-test. After averaging the results for five gentamicin-loaded and unloaded cements discs per run, the number of study units was three for all experiments and a 95% (p < 0.05) confidence level was adapted for statistical significance [15].

3. Results

3.1. Biofilm evaluation

The number of CFU/cm² on the unloaded cements had a maximum of about 3.4 × 10⁶ CFU/cm², which was independent of the type of cement involved and was reached in all cases after 24 h. This maximum was used as the 100% level for each cement brand involved in this study.

Fig. 1 summarises the number of CFU/cm² on the six different bone cement brands as a function of time as expressed relative to the maximal number of CFU/cm² recovered from the corresponding unloaded cement. By
expressing all results relative to this maximum number, biological variations between runs were eliminated. Biofilm formation on all the six gentamicin-loaded cement brands demonstrated the same growth pattern, with an increase in biofilm formation during the first hours, a maximum around 24 and 30 h followed by a more or less gradual decrease in the number of adhering viable microorganisms. None of the gentamicin-loaded cements was able to reduce the biofilm immediately after the onset of the experiment, but each cement had a significant reducing effect on the formation of a *S. aureus* biofilm at specific points in time after the start of an experiment. Table 1 summarises the points in time for each cement at which the antibiotic releasing cement yielded a significant (*p* < 0.05) reduction in biofilm formation. CMW Endurance, CMW2000 and Palamed showed a reducing effect on biofilm formation only after 48–72 h, while CMW1, CMW3 and Palacos reduced biofilm formation.
Table 1
Statistical analysis of the effects of gentamicin-loaded bone cements over time as compared with unloaded cements of the same brand. Points in time after the start of an experiment at which antibiotic release yielded a significant (p < 0.05) reduction in *S. aureus* biofilm formation are indicated by an “*“.
Furthermore, for completeness and comparison, the initial and total gentamicin release after 1 week is presented.

<table>
<thead>
<tr>
<th>Cement brand</th>
<th>6h</th>
<th>24h</th>
<th>30h</th>
<th>48h</th>
<th>72h</th>
<th>Initial (6h) release*</th>
<th>Total (168h) release* (µg/cm²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMW1</td>
<td>—</td>
<td>√</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>13.1</td>
<td>88</td>
</tr>
<tr>
<td>CMW3</td>
<td>—</td>
<td>√</td>
<td>√</td>
<td>—</td>
<td>—</td>
<td>12.8</td>
<td>79</td>
</tr>
<tr>
<td>CMW</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>√</td>
<td>—</td>
<td>12.4</td>
<td>85</td>
</tr>
<tr>
<td>Endurance</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>√</td>
<td>9.2</td>
<td>66</td>
</tr>
<tr>
<td>Palacos</td>
<td>—</td>
<td>√</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>8.6</td>
<td>70</td>
</tr>
<tr>
<td>Palamed</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>√</td>
<td>14.1</td>
<td>140</td>
</tr>
</tbody>
</table>

*Data are valid for a similar sample geometry as employed here and are taken from Van de Belt et al. [13].

in an earlier stage. CMW3 was the only cement that significantly reduced biofilm formation during an extended period of time ranging from 24 to 72 h.

4. Discussion

This study shows the effect of different gentamicin-loaded bone cements on in vitro *S. aureus* biofilm formation. Biofilm formation on all cements follows a similar pattern in time, but the gentamicin-loaded cements demonstrate different reductions of biofilm formation, that seems unrelated with the gentamicin-release kinetics of the cements, previously measured [13].

The number of CFU/cm² in biofilms on the bone cements increases during the first 24–30 h after which a decrease sets in, irrespective of gentamicin loading. Likely, after adhesion, anchoring and growth of biofilm bacteria, parts of the matured biofilm are sloughed off in the MRD. For five out of the six cement brands, however, gentamicin release further reduces biofilm growth. From Table 1 it can be seen that biofilm reduction by gentamicin loading is only significant at specific points in time after the onset of biofilm formation, with no evident relation with the gentamicin-release kinetics. Palamed, with a high initial release burst (14.1 µg/cm²/h) only demonstrates a reducing effect on biofilm formation after 72 h, while Palacos, with the lowest initial release burst (8.6 µg/cm²/h) already showed significant early biofilm reduction at 24 h. Strangely, CMW1 showing a high initial release (13.1 µg/cm²/h), and releasing a high amount of gentamicin (88 µg) in total as well, only reduced biofilm formation significantly at 24 h. Several other studies have reported a delayed effect of antibiotic release from bone cements on biofilm formation, but biofilm formation has never been related with the antibiotic-release kinetics as in this study. Chang and Merritt [5] described that the number of microorganisms adhering to gentamicin-loaded PMMA was markedly decreased after 24 h as compared with unloaded cement due to delayed effects on bacterial viability and subsequent proliferation, while even later reductions after 96 h were reported on *Staphylococcus epidermidis* biofilm formation [7].

It must be concluded, therefore, that biofilm formation on bone cements is not only related to the gentamicin release, but may also be dependent on other properties of the cement surface, such as for instance its roughness. Palamed with its extremely high initial release burst, shows only delayed reductions, possibly associated with its rough surface [13]. Speculatively, it could be argued that the greater roughness may on the one hand, enhance antibiotic release, but on the other rugosities may prevent detachment of killed bacteria that subsequently act as a substratum for other organisms [16]. The “window-of-effectiveness” as existing for different types of gentamicin-loaded bone cements indicated in Table 1, is governed by a variety of factors, that we do not yet fully understand but that are of utmost clinical importance. Slow release of subinhibitory amounts after an initial high release burst are highly undesirable as this leads to antibiotic resistance of colonising bacteria [3,4,17–19].

In conclusion, this in vitro study shows that bacteria can adhere to gentamicin-loaded bone cements, while different cements have different “windows-of-effectiveness” on the reduction of infectious biofilms on these cements that could not be related to the gentamicin release from the cements.

References


