Continuous-release or burst-release of the antimicrobial peptide human lactoferrin 1-11 (hLF1-11) from calcium phosphate bone substitutes

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Objectives: In order to identify possible drug delivery systems against resistant bone infection, we determined the release of the antimicrobial peptide (AMP) human lactoferrin 1-11 (hLF1-11) from commercially available bone substitutes.

Methods: We combined six calcium phosphate cements and six granule-types with 5 mg/g hLF1-11 and measured its availability and release in vitro from cements (7 days) and granules (3 days). The integrity and antimicrobial activity of the hLF1-11 that was released during the first 24 h were measured, using mass spectrometry, and a killing assay on methicillin-resistant Staphylococcus aureus (MRSA).

Results: Most of the cements showed burst release followed by low-level continuous release, whereas the coated granules showed high burst release for 24 h. After release the peptide was active (in nine of 12 materials) and intact.

Conclusions: Different release profiles may be obtained by choosing the appropriate carrier, which supports the feasibility of biodegradable carriers releasing AMPs against resistant infections.

Keywords: bone infections, human lactoferrin, biodegradable, carriers

Introduction
Antimicrobial resistance will probably complicate future treatment of bone infection, treatment of which requires local and systemic antibiotics and often several surgical interventions. The non-degradable polymethylmethacrylate beads which are used currently as antibiotic carriers require operative removal and may induce resistant bacteria.1

We aimed to address this increasing problem by combining an antimicrobial peptide (AMP) of human origin (hLF1-11) with biodegradable carriers—which obviates the need for operative removal—and analysing its availability and release. These carriers, which consist of (a combination of) calcium-phosphate ceramics, e.g. tricalcium phosphate, Ca$_3$(PO$_4$)$_2$, are slowly replaced by ingrowing bone after implantation.2 AMPs form a novel class of antimicrobial agents of natural origin that have been identified in virtually all forms of life as part of the antimicrobial defence system. These positively charged peptides kill by forming pores in the negatively charged bacterial cell-membrane and targeting intracellular organelles, without development of resistance.3 Moreover, they seem to have an immuno-modulating effect, killing microorganisms at lower concentrations in vivo (ng/mL) than in vitro (µg/mL).4,5

Materials and methods
Peptide
hLF1-11-peptide (GRRRRSVQWCA, 1375 Da) was manufactured by solid-phase peptide synthesis using Fmoc (9-fluorenyl-methoxycarbonyl) chemistry as described previously.6 Reanalysis of peak fractions by reversed phase HPLC resulted in one major peak revealing at least 90% purity. The authenticity was confirmed by electrospray-ionization quadrupole-time-of-flight mass-spectrometry (Q-TOF MS, Micromass Inc., Manchester, UK). Thermal stability in solution, adhesion to polystyrene and solubility were tested as described previously.7

Release experiment
After mixing cement powder and liquid containing 5 mg hLF1-11 per gram of powder (liquid/powder ratio according to the manufacturer), cylindrical specimens hardened overnight at 37°C in 6 × 5 mm moulds. The cements were: Biobon (Biomet Merck Biomaterials, Darmstadt, Germany), Calcibon (Biomet Merck Biomaterials), Biofil, (experimental, DePuy CMW, Blackpool, UK), Bonesource (Stryker-Leibinger, Freiburg, Germany), Chronos Inject, (experimental, Mathys, Bettlach, Switzerland) and Norian SRS (Mathys).

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Granules were immersed in 1.0 mL of dH2O containing 5 mg of hLF1-11 per gram of material and lyophilized. After removal of the granules, the residual hLF1-11 in the vessel was measured. The granules were: Bonesave (Stryker-Leibinger), Biosorb (Science for Biomaterials, Lourdes, France), Allogran-R (Orthos, Bristol, UK), Vitoss (Orthovita Malvern, PA, USA), Cerasorb (Curasan, Kleinostheim, Germany) and Bicalphos (Medtronic, Memphis, TN, USA).

Specimens were immersed in 500 µL of dH2O and kept in sealed polystyrene 48-well plates (Costar) at room temperature on a shaking device (180 rpm). The water was replaced at regular intervals: 30, 90 and 180 min on day 1 and then 24 hourly for 7 days (cements) or 3 days (granules) and stored at −20°C.

After production and after release, three specimens per group were finely ground and suspended in 5 mL of dH2O containing 1 M NaCl and the part that was left in the carrier. From the cements, less hLF1-11 was extractable (2.65±0.34 mg/g) than had been incorporated (5 mg/g). For the granules, the amount of available peptide varied (1.5–4.6 mg/g), the rest was recovered from the vessel after lyophilization.

The amount of hLF1-11 available immediately after production (biologically available) and after the release experiment (extracted after release) was determined by fine grinding and addition of 1 M NaCl. The values represent the mean ± S.D. from three experiments, totalling at least 12 samples per carrier-material. Sterile cultures were considered 99% killing, the detection limit.

Test results confirmed stability at 37°C in water for 26 weeks, solubility up to 150 mg/mL and no adhesion to polystyrene. Sample weight showed only a small variation (<0.01 g in samples of 0.150 g). Table 1 shows the amount of hLF1-11 that was initially available for dissociation, the quantity that was actually released from the carrier, and the part that was left in the carrier. From the cements, less hLF1-11 was extractable (2.65–3.43 mg/g) than had been incorporated (5 mg/g). For the granules, the amount of available peptide varied (1.5–4.6 mg/g), the rest was recovered from the vessel after lyophilization.

All cements displayed a sustained release profile for several days, with Biobon, Biofil and Chronos releasing significantly more hLF1-11 than the three other cements (Figure 1). All granules had burst release profiles in the first day only; Bicalphos, Bonesave and Vitoss released significantly more peptide than the other three granule types (not shown).

After release, a 1375 Da peak on MS-QTOF of the correct amino acid sequence confirmed the integrity of hLF1-11 (control samples had no 1375 Da peak). The antimicrobial activity was >90% in nine of 12 carriers (Table 1).

### Discussion

Low systemic toxicity and high local drug concentrations are the main advantages of local antibiotic delivery systems. These can be used both for treatment of chronic bone infection, requiring prolonged high antibiotic concentrations and for prevention, in which a short duration local antibiotic peak concentration may suffice.8

Using methods described by Kühn, adapted for biodegradable specimens, we determined the available hLF1-11 in samples before and after release.9 Not all hLF1-11 added to cement was available for extraction, even after fine grinding and adding high salt concentra-

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**Table 1. hLF1-11 released by the carriers and antimicrobial activity against MRSA**

<table>
<thead>
<tr>
<th>Carrier</th>
<th>hLF1-11 (mg/g) released</th>
<th>biologically available</th>
<th>extracted after release</th>
<th>Killing (%)</th>
<th>hLF1-11 (log cfu) control (log cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cements</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biobon</td>
<td>0.57±0.10</td>
<td>3.25±0.18</td>
<td>2.47±0.07</td>
<td>98</td>
<td>4.1</td>
</tr>
<tr>
<td>Biofil</td>
<td>0.57±0.19</td>
<td>2.70±0.13</td>
<td>1.96±1.08</td>
<td>72</td>
<td>6.3</td>
</tr>
<tr>
<td>Bonesource</td>
<td>0.10±0.05</td>
<td>2.69±0.06</td>
<td>2.65±0.45</td>
<td>97</td>
<td>4.4</td>
</tr>
<tr>
<td>Calcibon</td>
<td>0.22±0.02</td>
<td>2.89±0.27</td>
<td>2.25±0.45</td>
<td>88</td>
<td>5.1</td>
</tr>
<tr>
<td>Chronos</td>
<td>1.69±0.30</td>
<td>3.43±0.07</td>
<td>2.19±0.24</td>
<td>92</td>
<td>4.8</td>
</tr>
<tr>
<td>Norian</td>
<td>0.12±0.10</td>
<td>2.65±0.12</td>
<td>2.66±0.30</td>
<td>83</td>
<td>6.2</td>
</tr>
<tr>
<td><strong>Granules</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allogran</td>
<td>2.52±0.38</td>
<td>2.64±0.57</td>
<td>0.00</td>
<td>99</td>
<td>3.8</td>
</tr>
<tr>
<td>Bicalphos</td>
<td>4.25±0.30</td>
<td>4.31±0.60</td>
<td>0.07</td>
<td>99</td>
<td>3.8</td>
</tr>
<tr>
<td>Biosorb</td>
<td>2.53±0.68</td>
<td>2.63±0.84</td>
<td>0.00</td>
<td>99</td>
<td>3.8</td>
</tr>
<tr>
<td>Bonesave</td>
<td>1.58±0.21</td>
<td>1.83±0.40</td>
<td>0.00</td>
<td>99</td>
<td>3.8</td>
</tr>
<tr>
<td>Cerasorb</td>
<td>1.50±0.20</td>
<td>1.52±0.18</td>
<td>0.11</td>
<td>99</td>
<td>3.8</td>
</tr>
<tr>
<td>Vitoss</td>
<td>4.43±1.46</td>
<td>4.61±1.00</td>
<td>0.00</td>
<td>99</td>
<td>3.8</td>
</tr>
</tbody>
</table>

*The mean ± S.D. from three experiments, totalling at least 12 samples per carrier-material. Sterile cultures were considered 99% killing, the detection limit.*

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Release of hLF1-11 from calcium phosphate


