PLGA-GENTAMICIN BIOCOMPOSITE MATERIALS WITH POTENTIAL ANTIMICROBIAL APPLICATIONS IN ORTHOPEDICS

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Abstract
The purpose of this study was to synthesize a PLGA (polylactic-co-glycolic acid)-gentamicin biodegradable material followed by HPLC determination of the drug’s encapsulation efficiency in the polymer. The chosen polymer, PLGA, is a biocompatible and biodegradable synthetic polymer, approved by the FDA, with adjustable mechanical properties, highly used as a controlled release drug delivery system. One novel feature of the study is the inclusion of an antibiotic in the composite material to combat the planktonic growth and biofilms formed by some of the major bacterial species involved in the aetiology of osteomyelitis. The double emulsion synthesis method was chosen, as the most suitable for encapsulating water-soluble drugs, such as gentamicin. For the quantitative assay of gentamicin, a new HPLC chromatographic method was used, consisting in an initial treatment with FMOC-Cl (9-fluorenylmethylchloroformate) to derivatize gentamicin, followed by UV detection of the derivative at λ = 265 nm. The antibacterial activity of the composite material was confirmed by the qualitative and quantitative assays performed in this study to determine the minimal inhibitory concentration and the minimal biofilm eradicating concentration.

Rezumat
Scopul acestui studiu l-a reprezentat sinteza unui material biodegradabil de tip PLGA-gentamicină, urmată de determinarea eficienței de încapsulare a medicamentului în polimer. Polimerul ales, PLGA (acidul poli lactic-co-glicolic) este un polimer sintetic biocompatibil și biodegradabil, aprobat de FDA, cu proprietăți mecanice ajustabile, extrem de utilizat ca sistem de cedare controlată a medicamentelor. Un element de noutate al studiului îl reprezintă includerea în materialul compozit a unui antibiotic activ față de creșterea planktonică și față de biofilmele formate de principalele specii bacteriene implicate în etiologia osteomielitei. Ca metodă de sinteză s-a ales metoda emulsiei duble, aceasta fiind cea mai adecvată pentru încapsularea medicamentelor hidrosolubile, precum gentamicina. Pentru determinarea cantitativă a gentamicinei s-a utilizat o metodă cromatografică HPLC nouă, care a presupus inițial derivatizarea gentamicinei cu clorură de 9-fluorenilmetilchloroformat (FMOC-Cl), urmată de detecția UV a derivatei la λ = 265 nm. Activitatea antibacteriană a compozitului a fost confirmată prin metodele cantitative utilizate pentru determinarea concentrațiilor minime inhibitoarei și de eradicare a biofilmului.

Keywords: PLGA, gentamicine, biocomposite, antimicrobial activity assay

Introduction
A considerable number of studies have reported the use of biodegradable polymers as drug carriers, which can even be inserted as bioresorbable surgical devices [17, 22]. Many new organic compounds have been synthesized and characterized [20, 21] to establish their bio-pharmaceutical applications, but of all types of biomaterials, the use of polylactic-co-glycolic acid has a huge potential as a drug transporter and as a matrix for tissue engineering [6]. Biocomposite materials are promising future candidates for biomedical applications. Biocompatible materials typically contain either a natural or synthetic polymer (polymethyl-methacrylate, poly(D,L-lactide-co-glycolide, poly(lactic-co-hydroxy-methyl glycolic acid) or a calcium phosphate (Ca10(PO4)6(OH)2 [14], Ca5(PO4)2 [11]) together with an osteoconductive drug (sodium alendronate, strontium ranelate [18]) or broad antibacterial agents (quinolones [4], aminoglycosides). The therapeutic value of some drugs including gentamicin can be improved by encapsulating them in delivery systems, for example in PLGA microspheres, which can contribute to sustained release of the drug [2].
Small molecule drugs are classified biopharmaceutically in hydrophilic or amphiphilic drugs. The first category has good water solubility and it is used for the treatment of many diseases. Clinical use may, however, be limited due to rapid clearance or adverse bioavailability, requiring repeated administration [23]. Gentamicin antibiotic was discovered in 1963 [24], remaining particularly useful in the treatment of resistant bacteria. It has extremely effective bactericidal properties against Gram-negative and some Gram-positive bacteria. Gentamicin is not metabolized, being accumulated in the extracellular space, before kidneys excretion [10]. Its use is limited by its negative effects it has, such as ototoxicity and nephro-toxicity.

Polylactic-co-glycolic acid (PLGA), a copolymer of polylactic acid (PLA) and polyglycolic acid (PGA) (Figure 1) belongs to a class of polymers approved by Food and Drug Administration, due to its exceptional biodegradability and biocompatibility. Various studies show the use of this polymer as a carrier for various types of macromolecules, such as DNA, RNA and peptides, and for drugs [13]. PLGA can encapsulate almost any size molecules.

One problem remains the quantitative assay of the drug encapsulated in the composite because it does not absorb in the UV-VIS domain. It may, however, be determined by a HPLC-DAD method after a pre-differentiation with 9-fluorenylmethyl chloroformate (FMOC-Cl). FMOC-Cl was first used as a derivatizing agent in 1983 by Einarsson et al. [5] which determined different amino acids.

**Materials and Methods**

All chemicals were of analytical grade and were purchased from Merck (Darmstadt, Germany).

**Double emulsion synthesis of PLGA- gentamicin composite**

First, an appropriate amount of drug (150 mg) was dissolved in the aqueous phase (400 µL of 0.1 M monosodium phosphate and 0.1 M disodium phosphate, pH = 6.3 with 0.5% PVA) and then this solution, A₁, was added to the oily organic phase (O), 200 mg of PLGA in 5 mL of dichloromethane (DCM). The initial water-oil emulsion was stirred vigorously for 2 - 3 minutes at 45,000 rpm with a special SilentCrusher device. Then this phase was added to the second aqueous phase (A₂), quantitatively more significant (0.4 g of PVA in 40 mL of water) under stirring. The next step was to evaporate the organic solvent under stirring for 3 hours at 1500 rpm.

The resulting suspension was frozen at -45°C then lyophilized at 0.014 mbar and -9°C for 10 hours, then at 0.014 mbar and 20°C for a further 10 hours with a lyophiliser. The resulting microparticles were kept at cold, 4-5°C.

**Synthesis of PLGA- hydroxyapatite-gentamicin biocomposite**

150 mg of gentamicin were dissolved in the aqueous phase A₁ (400 µL of 0.1 M monosodium phosphate and 0.1 M disodium phosphate, pH = 6.3 with 0.5% PVA) and then vigorously stirred for 2 - 3 minutes at 45,000 rpm with the organic phase O (200 mg PLGA in 5 mL DCM). The water-oil emulsion obtained was added to the secondary aqueous phase A₂ (0.4 g of PVA in 40 mL of water) to which 100 mg of hydroxyapatite were added.

Thereafter, the organic solvent was evaporated, followed by lyophilisation under the experimental conditions described above.

**Gentamicin determination in biocomposite by HPLC-DAD**

A Thermo Finnigan Surveyor HPLC System equipped with photodiode array detector and Thermo Finnigan Xcalibur data system was used: i) column: C₁₈ reversed phase column (Thermo Scientific) Hypersil GOLD, 250 mm x 4.6 mm I.D., particle size 5 µm; ii) mobile phase: a mixture of acetonitrile:methanol:water (85:10:5), isocratic elution; flow rate: 1 mL/min; wavelength: 265 nm.

**Calibration curve**

Stock solutions of gentamicin with concentrations range between 0 and 1 mg/mL were prepared by dissolving the drug in borate buffer (pH = 9.7). FMOC derivatization of gentamicin was achieved by dissolving FMOC-Cl in methanol (30 mM) and by adding a suitable amount (0.5 mL) over 1 mL gentamicin solution. They were left to react for 5 minutes. And then, a volume of 200 µL of this solution was diluted by 800 µL mobile phase and then injected in the HPLC system.

**Samples**

An amount of 14 mg PLGA-gentamicin composite and 9 mg PLGA-hydroxyapatite-gentamicin were left with 1 mL borate buffer (0.05 M sodium tetraborate + 0.1 M NaOH, pH = 9.7), overnight, in order to hydrolyse PLGA. A volume of 0.25 mL of the hydrolysed solution was filtered through a 0.45 µm porous membrane mixed with 0.75 mL borate buffer and 0.5 mL FMOC-Cl solution. After 5 minutes, 200 µL of this solution was diluted with 800 µL mobile phase and injected into the chromatographic system.
Antimicrobial activity assays

Microbial strains
The antimicrobial activity was tested on two Gram-positive (Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC-29212) and two Gram-negative (Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922) bacterial strains. The tested samples were represented by 10 mg/mL suspensions in dimethyl sulfoxide (DMSO) and encoded as follows: 1) PLGA-hydroxyapatite-gentamicin; 2) PLGA-gentamicin; 3) Gentamicin control; 4) DMSO negative control.

Quantitative assay of the antimicrobial properties
The binary serial micro dilution technique using 96-well microtiter plates was used to determine the minimal inhibitory concentration (MIC) values of the tested composites. The quantitative assay was performed in Muller Hinton broth for bacteria, according with CLSI, 2018.

The sterile medium was added in sterile 96-well plates and binary dilutions of each tested suspension were performed in a final volume of 100 µL. After performing the binary dilutions, 10 µL of microbial suspension adjusted to an optical density of 0.5 McFarland (1.5 x 10^8 CFU/mL) were added in each well. The MIC values were established by the macroscopic analysis of the wells content and by spectrophotometric measurement of the optical density at 600 nm using a BIOTEK SYNERGY-HTX ELISA multi-mode reader. Each experiment was performed in triplicate and repeated on at least three separate occasions.

The quantitative assessment of the influence of the tested suspensions on the microbial adherence on the inert substratum
The content of the 96-multi well plastic plates used for the MIC assay were discarded, washed three times with phosphate buffered saline (PBS) and the microbial cells adhered to the plastic walls were fixed with cold methanol for 5 minutes and stained by 1% violet crystal solution for 15 minutes. The coloured biofilm was thereafter re-suspended by 33% acetic acid solution. The optical density of the blue suspension was measured at 490 nm and the obtained values being proportional with the number of the adhered microbial cells.

Cytotoxicity assay
The cytotoxicity effect was tested on MG63 cells (ECACC 86051601) using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega G3580). For the experiments, 10^4 MG63 cells were plated in each well of 96 well plate, in DMEM:F12 medium supplemented with 10% bovine foetal serum. After 24 h, the cells were treated with different concentration of gentamicin, PLGA-gentamicin and PLGA-hydroxyapatite-gentamicin (between 1 mg/mL and 7.6 µg/mL). Treated and untreated cells were maintained for 72 h at 37ºC, 5% CO2. The toxicity was evaluated by adding CellTiter 96® AQueous One Solution and maintained the cells for 3 h at 37ºC, and quantification of absorbance at 490 nm, using the plate reader BertholdTech TriStar2S (Berthold Technologies GmbH & Co. KG, Germany). The results were presented as % of viability reported to the untreated cells and IC50 calculation.

Results and Discussion

Double emulsion synthesis of PLGA gentamicin composite
The water-oil-water emulsion method is the most appropriate method for encapsulating water-soluble drugs such as gentamicin, as opposed to simple oil-water emulsion methods, which are ideal for water-insoluble drugs [25].

Gentamicin determination in biocomposite by HPLC-DAD
Gentamicin does not have absorption in the UV-Vis domain and therefore its determination is possible after a derivatization reaction with FMOC-Cl.

FMOC-Cl reacts with the primary and secondary amino groups of gentamicin at alkaline pH and forms quite stable derivatives. Figure 2 shows how FMOC-Cl binds to a primary and a secondary amino group.

![Figure 2](image-url)

The reaction between FMOC-Cl and primary or secondary amino groups
On the HPLC chromatogram when the FMOC-amine solution was injected after the derivatization reaction, chromatographic peaks were observed at a retention time of less than 6 minutes which were attributable to the remaining FMOC-Cl reagent (excess) but also to some products resulting from the hydrolysis reaction of FMOC-Cl (Figure 3), consistent with other results presented in the literature [16].

**Figure 3.**
The hydrolysis reaction of FMOC-Cl

Chromatogram obtained for A) gentamicin standard solution of 1 mg/mL; B) determination of gentamicin from the PLGA-gentamicin microparticles
From the point of view of the amount of FMOCT-cl required to react with gentamicin, it was taken into account that each primary or secondary amino group reacted with a FMOCT-cl molecular moiety, but also that gentamicin still has in its structure other three glycosidic -OH groups which also can react at neutral pH with the derivatization agent.

The basic pH is required not only to ensure a better development for the reaction between amino groups and FMOCT as observed by Gao et al. [7] and Korös et al.[14], but also to prevent further reaction of aminoglycoside OH groups with FMOCT-cl.

As can be seen in the chromatogram of a standard solution of 1 mg/mL shown in Figure 4A, gentamicin exhibits four peaks characteristic of the four gentamicin isomers, the first at 11.2 minutes, chosen later for quantification. HPLC methods in the literature present data similar to those found in this study [3].

The obtained calibration curve has good linearity in the range of 0.05 - 1 mg/mL (r = 0.996211) as depicted in Figure 5.

\[ \text{Encapsulation} (\%) = \frac{\text{experimental gentamicin amount (mg)}}{\text{microparticle amount (mg)}} \times 100 \]

Thus we found in PLGA-gentamicin 2.85% gentamicin, and only 0.64% gentamicin in PLGA- hydroxyapatite-gentamicin biocomposite. The explanation of this small percentage of gentamicin encapsulated in the PLGA-hydroxyapatite-gentamicin microparticles results from the fact that part of the hydroxyapatite is encapsulated in PLGA in competition with gentamicin.

**Antimicrobial activity**

**Quantitative screening of the antimicrobial properties**

In the quantitative assay, the minimal inhibitory concentrations were read by wells observations [1]. In the wells containing high concentrations of compounds the culture growth was no visible, the microbial cells being killed or inhibited by the tested compounds. The results of the quantitative assay revealed that the most susceptible microorganism were *P. aeruginosa*, in which case the composites exhibited the same efficiency as the free antibiotic, as demonstrated by the equal minimal inhibitory concentration value, followed by *S. aureus* strains, when the MIC value of the composite has still remained very low (Table 1). This is particularly important, because the inclusion of gentamicin in hydroxyapatite contributes to the bone reintegration of the implant by improving the biocompatibility of orthopaedic materials, but preserving their antimicrobial activity [19].

Osteomyelitis is the inflammation of the bone marrow secondary to infection, which can progress to osteonecrosis, bone destruction and septic arthritis [15]. Epidemiological studies of the last decade have shown that the increase in the incidence and severity of acute osteomyelitis is associated with *Staphylococcus aureus*, as the most prevalent pathogen [12], an opportunistic species that can be extremely difficult to treat [9]. A postulated cause of the increased severity of osteomyelitis is the production of a toxin known as panton-valentine leukocidin (PVL) by *S. aureus* strains [26]. Many other pathogens were found to be, although less frequently involved in the aetiology of osteomyelitis, such as *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *Proteus mirabilis* and anaerobic bacteria [8].

**Table 1**

<table>
<thead>
<tr>
<th>No.</th>
<th>Bacterial strains</th>
<th>Minimal inhibitory concentration values (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLGA-hydroxyapatite-gentamicin</td>
<td>PLGA- gentamicin</td>
</tr>
<tr>
<td>1</td>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td>0.0097</td>
</tr>
<tr>
<td>2</td>
<td><em>Enterococcus faecalis</em> ATCC 29212</td>
<td>0.31</td>
</tr>
<tr>
<td>3</td>
<td><em>Pseudomonas aeruginosa</em> ATCC 27853</td>
<td>0.0097</td>
</tr>
<tr>
<td>4</td>
<td><em>Escherichia coli</em> ATCC 25922</td>
<td>0.039</td>
</tr>
</tbody>
</table>

**Figure 5.**

Calibration curve, obtained for the first characteristic peak (retention time 11.2 minutes) of the four gentamicin isomers.

**Determination of encapsulation degree**

The degree of encapsulation was calculated by plotting the mass of gentamicin recovered by HPLC in the sample (0.400 mg gentamicin in PLGA-gentamicin and 0.058 mg, respectively in PLGA-HA-gentamicin) to the microparticle mass.
The quantitative assessment of the influence of the tested suspensions on the microbial adherence on the inert substratum

Concerning the inhibitory activity of the tested suspensions against the growth of the tested bacterial strains in biofilms, the two suspensions (PLGA-hydroxyapatite-gentamicin and PLGA-gentamicin) proved to inhibit the adherence of E. faecalis strains, proving ineffective against the other monospecific biofilms (Figures 6 - 9). Therefore, we could conclude that the obtained composites are primarily useful against planktonic bacteria, but less effective against bacterial biofilms.

The composite material are quite to be biocompatible because the effects seem to be due only to gentamicin content (gentamicin IC₅₀ = 222.359, PLGA-gentamicin IC₅₀ = 272.421, respectively HA-PLGA-gentamicin IC₅₀ = 257.354) (Figure 10).

Conclusions

The synthesis of a biocompatible composite of PLGA-gentamicin and PLGA-hydroxyapatite-gentamicin, respectively, was accomplished using double emulsion synthesis as a method. For the determination of gentamicin in the samples, a new HPLC-DAD analysis method was developed involving a first step of derivatizing gentamicin with FMOC-Cl at pH 9.7. Detection was performed at 265 nm, the analysis time being relatively short. By including gentamicin and gentamicin together with hydroxyapatite in PLGA, it was obtained a material with antimicrobial properties and biocompatibility so that it could be further optimized to fight orthopaedic infections.

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References


