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# Preparation of gentamicin dioctyl sulfosuccinate loaded poly(trimethylene carbonate) matrices intended for the treatment of orthopaedic infections

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**Abstract.**
**BACKGROUND:** Infection is a common problem in trauma and orthopaedic surgery. Antibiotic-loaded biomaterials are used locally to clear infections as an adjunct to systemic antibiotics. Gentamicin-sulphate (GEN-SULPH) is commonly used in antibiotic-loaded biomaterials, although it displays high water solubility resulting in quick diffusion from the carrier.
**OBJECTIVE:** Preparation of a lipophilic derivative of gentamicin to reduce solubility and obtain a slower release. Subsequently, entrapment of this lipophilic gentamicin within poly(trimethylene carbonate) (PTMC) matrices.
**METHODS:** Hydrophobic ion-pairing was used to prepare lipophilic gentamicin (GEN-AOT). The susceptibility of *Staphylococcus aureus* NCTC 12973 and *Staphylococcus epidermidis* 103.1 for GEN-AOT was tested and the viability of fibroblasts upon exposure to GEN-AOT was assessed. GEN-AOT was then loaded into PTMC films.
**RESULTS:** GEN-AOT was successfully prepared as confirmed by FTIR-spectroscopy. GEN-AOT was bactericidal for *S. epidermidis* and *S. aureus* at 0.5 μM and 8.5 μM, respectively. At 1.1 μM GEN-AOT no reduction in fibroblast viability was observed. At 11 μM the reduction was ∼50%. PTMC discs loaded with GEN-AOT were prepared by compression molding.
**CONCLUSIONS:** Lipophilic GEN-AOT was at least as potent as GEN-SULPH. For *S. epidermidis* it was even more potent than GEN-SULPH. More than 50% fibroblast cell viability was maintained at bactericidal concentration for both bacterial strains.

Keywords: Orthopaedic infection, local infection treatment, gentamicin, antibiotic modification, poly(trimethylene carbonate), drug delivery

## 1. Introduction

The incidence of infection seriously threatens modern-day advances in the field of orthopaedics and traumatology. Most infections related to implanted fracture fixation devices and orthopaedic implants are caused by Staphylococci [4, 15]. Bacteria present in a biofilm on the surface of an implant, necrotic bone or living tissue can enter a state of reduced growth and become less susceptible to killing by growth-dependent antimicrobials [11]. Treatment of these infections is therefore difficult, and usually involves

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systemic administration of antibiotics over an extended time of approximately 6 weeks. Antibiotic-loaded biomaterials are used as an adjunct treatment for clearing these infections. Two types of antibiotic-loaded biomaterials are used in the clinic: Non-degradable poly(methyl methacrylate) (PMMA) blocks and beads, which need to be removed after the treatment has finished [7] (these PMMA based materials have a poor antibiotic release profile [6]), and degradable biomaterials such as calcium phosphate cements [12] or collagen fleece [5]. The latter may be advantageous as they do not need removal. However, usually antibiotic release is not simultaneous with degradation [9, 13]. Often this leads to a suboptimal antibiotic release profile and the presence of a biomaterial without antibiotic action.

Many antibiotic-loaded biomaterials used in the clinic are loaded with gentamicin [3]. However, the sulphate salt of gentamicin, gentamicin-sulphate (GEN-SULPH) is highly soluble, which results in fast diffusion from the carrier [13]. A less soluble salt, gentamicin crobefate, has been combined with collagen fleece, extending the release kinetics, but simultaneous resorption of the carrier and antibiotic release was not achieved [9].

Poly(trimethylene carbonate) (PTMC) as opposed to other biodegradable polymers synthesized by ring-opening polymerization (ROP), degrades by enzymatic surface erosion [1, 16]. Other biodegradable polymers, like poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(caprolactone) (PCL) and their copolymers degrade by bulk erosion into acidic degradation products. So, PTMC has the advantage of degrading in a more controlled fashion which could be beneficial for the release profile of antibiotics from the material. PTMC based carriers will gradually reduce their thickness when degrading, so the diffusion pathway for antibiotics entrapped closer to the core of the carrier will be become shorter, leading to a more gradual release as compared to release from a bulk degrading matrix. Furthermore, the degradation products of PTMC are non-acidic (predominantly cyclic TMC monomer, cyclic and linear oligomers of TMC and 1,3-propanediol) [10], so these will not interfere with aminoglycoside activity and bone healing, which are impaired at low pH.

Therefore, it was hypothesized that a delivery system based on PTMC, and entrapping a lipophilic gentamicin complex, could be advantageous for a sustained antibiotic release and would be able to achieve this in the absence of negative stimuli that impair antibiotic action and bone healing.

Hence, a lipophilic complex of gentamicin was prepared by hydrophobic ion-pairing with dioctyl sodium sulfosuccinate (tradename Aerosol OT (AOT)) and characterized by means of Fourier Transform Infrared Spectroscopy (FTIR). Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) for relevant bacterial species, as well as cell viability upon exposure, were assessed for this lipophilic gentamicin. High molecular weight linear PTMC was prepared by ROP and characterized by means of gel permeation chromatography (GPC). Finally, PTMC films loaded with GEN-SULPH and GEN-AOT were prepared by compression molding, demonstrating the ability to produce PTMC membranes homogeneously loaded with lipophilic gentamicin for a sustained and controlled antibiotic release.

## 2. Materials and methods

### *2.1. Materials*

TMC monomer was obtained from Huizhou Foryou Medical Devices (China). Stannous octoate ($Sn(Oct)_2$), dioctyl sodium sulfosuccinate, gentamicin sulphate salt and sodium chloride were purchased from Sigma-Aldrich (US). Sodium acetate, potassium chloride and calcium chloride dehydrate were

purchased from Fluka (CH). Dimethyl sulfoxide (DMSO) (≥99.5%) was purchased from Carl Roth (D). *Staphylococcus Aureus (S. aureus)* NCTC 12973 was purchased from the National Collection of Type Cultures (NCTC). *Staphylococcus epidermidis (S. epidermidis)* 103.1 was obtained as clinical isolate from a patient with an infected plate osteosynthesis at the BGU Murnau, Germany. Tryptone soy agar (casein soy bean digest agar, CM0131) and Mueller Hinton (MH) broth (CM0405, LOT# 970220) were purchased from OXOID Ltd. (UK). Cation adjusted Mueller Hinton (CAMH) broth was prepared by adding $Ca^{2+}$ and $Mg^{2+}$ to the MH broth to obtain a final cation content of 20–25 mg/L $Ca^{2+}$ and 10–12.5 mg/L $Mg^{2+}$. The cation containing solutions were sterile filtered before addition to the broth. Dulbecco's modified Eagles medium (DMEM), L-glutamine, sodium pyruvate, foetal calf serum (FCS) and Trypsin/Ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco Life Technologies (CH). Tyrode buffer saline solution (TBSS) was purchased from Fluka (CH). Cell titer blue® was purchased from Promega (US).

## 2.2. Methods

*Preparation of lipophilic gentamicin-dioctyl sulfosuccinate (GEN-AOT):* AOT was used for hydrophobic ion pairing (HIP) with GEN-SULPH. Equal volumes of GEN-SULPH in buffer (10 mM sodium acetate, KCl and $CaCl_2$ (pH 5) (0.40 w/v%)) and AOT in $CH_2Cl_2$ (1.25 w/v%) were mixed by vigorous stirring for 3 h and left for 0.5 h to separate the 2 phases. GEN-AOT was isolated from $CH_2Cl_2$ by evaporation of the solvent. Both structures for GEN-SULPH and GEN-AOT are depicted in Fig. 1.

*Characterization of gentamicin-dioctyl sulfosuccinate (GEN-AOT):* The successful hydrophobic ion pairing of GEN-SULPH with AOT was confirmed by Fourier Transform Infrared Spectroscopy (FTIR) on a Bruker Tensor 27 spectrophotometer equipped with a single reflection diamond attenuated total reflection (ATR) accessory. Spectra (single measurements with 128 repeated scans per IR spectrum) were recorded for both starting compounds, GEN-SULPH and AOT, and for the GEN-AOT isolated from the $CH_2Cl_2$ layer after the HIP process. Additional to the previous stated compounds, also the spectrum for a physical mixture of GEN-SULPH and AOT was recorded.

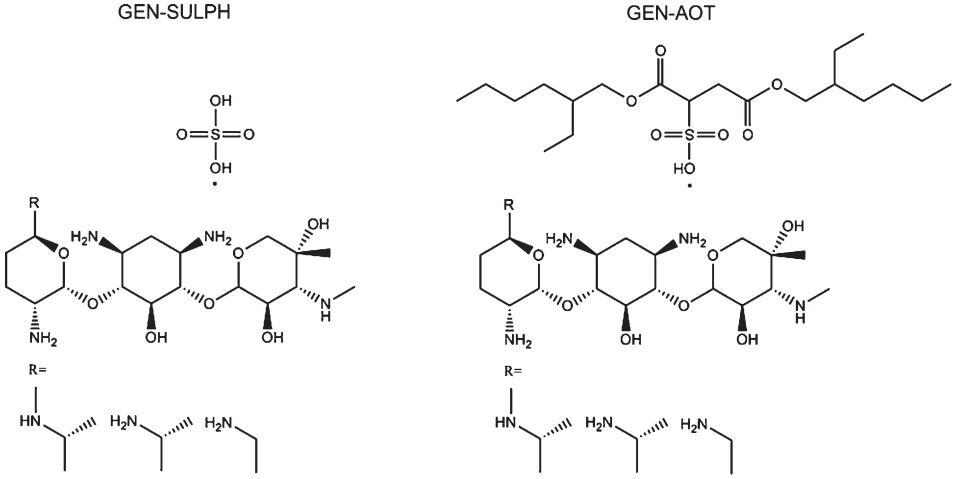


Fig. 1. Structure of GEN-SULPH and GEN-AOT.

*Antimicrobial susceptibility testing:* 2 bacterial species were selected for susceptibility testing of GEN-AOT. The used species were: *S. aureus* NCTC 12973 (*SA*) and *S. epidermidis* 103.1 (*SE*). Antibiotic solutions were prepared in CAMH broth by doubling dilution in a concentration range from 271 μM – 0.06 μM. The minimal inhibitory concentrations (MIC), the concentration for which bacterial growth is inhibited and the minimal bactericidal concentrations (MBC), the concentration for which bacteria are killed, were determined according CLSI guidelines M7-A9 (2012) [2]. All concentrations of the antibiotic solutions were measured in triplicate, as well as the positive control (no antibiotic + inoculum), DMSO control (10 v/v% DMSO in broth + inoculum) and the sterility control (no antibiotic + no inoculum). For the susceptibility testing, GEN-SULPH and AOT were solubilized and diluted directly in CAMH broth. In order to solubilize GEN-AOT and to prepare the dilution range, GEN-AOT was first dissolved in DMSO at 10× the final concentration, after which 10 μl of this solution was added to 90 μl CAMH broth in the wells of a 96-wells plate. Solubilisation of GEN-AOT in 10 v/v% DMSO did not impair viability of the bacterial strains (data not shown). Although, an end concentration of 5 v/v% DMSO in the wells did not inhibit bacterial growth, it cannot be excluded that there is a synergistic effect between GEN-AOT and DMSO [14]. Finally, 100 μl inoculum ($5 \times 10^5$ CFU/ml) of the respective bacterial strain was added to every well, to reach a final volume of 200 μl/ well.

*Viability testing for fibroblast cell line:* The hTERT fibroblast BJ-1 cell line was used for viability testing upon exposure to the antibiotics following the basic rules as prescribed in ISO-10993-5 [8]. Viability was assessed after 24 h and 72 h of exposure of the cells to the antibiotics. An antibiotic concentration range from 0.53 μM – 2.12 mM was prepared for viability testing. After 24 h and 72 h respectively, cell media containing the antibiotics was removed from the wells of the 96-wells plate and the cells were incubated for 2.5 h with cell titer blue after which the fluorescence intensity (FI) was measured ($\lambda_{excitation} = 560$ nm, $\lambda_{emission} = 590$ nm). FI was measured with a Perkin Elmer Victor 3 1420-012 multi-label microplate reader. All sample concentrations, including the controls, were measured in quadruplicate. Viability was expressed as relative percentage with respect to the untreated control. Viability = $(FI_{sample} - FI_{negative\ control}) / (FI_{positive\ control} - FI_{negative\ control}) \times 100$.

*Polymer synthesis and characterization:* High molecular weight (HMW) PTMC was synthesized by ROP of trimethylene carbonate in the presence stannous octoate ($Sn(Oct)_2$) ($2 \times 10^{-4}$ mol $Sn(Oct)_2$ per mol TMC) as catalyst (Fig. 2). No initiator was added to obtain a high molecular weight polymer. Polymerization was performed in the melt in dry vacuum sealed silanized glass ampoules for 48 h at 130°C. Number average molecular weight ($M_n$) and intrinsic viscosity (IV) were determined by gel permeation chromatography (GPC, Viscotek (US)). Chloroform was used as eluent at a flow rate of 1.0 ml/min. The GPC system was equipped with ViscoGEL I-guard-0478, ViscoGEL I-MBHMW-3078 and ViscoGEL I-MBLMW-3078 columns in series and a TDA 302 triple detector array with refractometer-, viscometer-, and light scattering detectors to determine the absolute molecular weight.

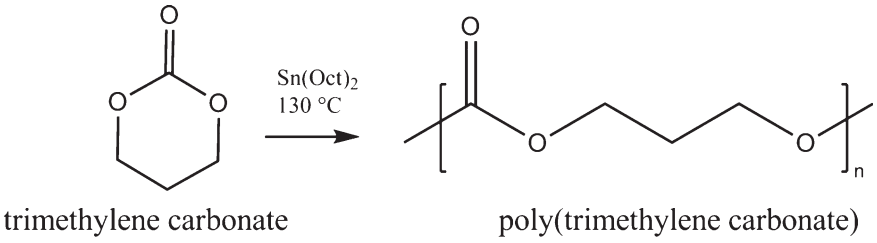


Fig. 2. Ring opening polymerization of trimethylene carbonate.

*Preparation of GEN-AOT loaded PTMC discs:* PTMC was dissolved in $CH_2Cl_2$ at a concentration of 4 w/v%. Then either no antibiotic or 10 w/w% GEN-SULPH or 10 w/w% GEN-AOT was added to this solution. GEN-SULPH was dispersed through the PTMC solution by ultrasonication for 5 min. Since GEN-AOT is soluble in the PTMC solution it was not sonicated. Subsequently, the GEN-SULPH or GEN-AOT containing PTMC solutions were stirred for 24 h. The GEN-SULPH containing solutions were then precipitated in diethyl ether and the GEN-AOT containing solutions were poured in petri-dishes after which the $CH_2Cl_2$ was allowed to evaporate for 1 week. Finally, PTMC, PTMC with 10 w/w% GEN-SULPH and PTMC with 10 w/w% GEN-AOT were compression moulded in stainless steel molds at 70°C and 300kN force with a laboratory press (Fonteijne THB008, The Netherlands). The antibiotic-containing polymer films were cooled to room temperature using cold water. 500 μm thick films were obtained, and ø 8 mm discs were punched out of these films.

*Characterization of GEN-AOT loaded PTMC discs:* Differential Scanning Calorimetry was performed on a Pyris DSC-1 Perkin-Elmer instrument calibrated with indium. PTMC, PTMC-GEN-SULPH and PTMC-GEN-AOT samples were weighed in hermetically closed aluminium pans. Measurements performed in triplicate, were carried out under a dry nitrogen atmosphere at a flow rate of 20 mL/min. Each measurement was run heating the sample from −30°C to 100°C. The sample was held for 1 min at 100°C and cooled to the initial temperature. Heating and cooling ramps were run at a rate of 5°C/min.

Optical microscopy was performed to assess the antibiotic distribution throughout the GEN-SULPH and GEN-AOT loaded PTMC films. Images of the films were captured using a Zeiss CCD camera (Axiocam HRc, Jena, Germany) attached to an optical microscope in diffuse illumination mode with a macrolens (Leica, Macrofluo, Wetzlar, Germany) using Zeiss Axiovision Vs40 (v4.6.3.0) image acquisition software. Gray values (GVs) of circular areas of the specimens with 3200 μm radius were recorded and analysed. Average GVs and standard deviation were calculated for the PTMC, PTMC-GEN-SULPH and PTMC-GEN-AOT films for this area.

## 3. Results

The composition of the product of the HIP process of GEN-SULPH and AOT was determined by FTIR. The FTIR spectrum of GEN-SULPH, AOT and the product of the HIP (GEN-AOT) can be found in Fig. 3. An absorption band at 1734 $cm^{-1}$ (Fig. 3) in the FTIR spectrum can be both observed for AOT and GEN-AOT. This band can be attributed to the stretching of the *C*=O groups of the ester of the AOT molecule. The absorption band between 1620 $cm^{-1}$ and 1610 $cm^{-1}$ (Fig. 3) present in both GEN-SULPH and GEN-AOT can be attributed to the primary amine groups present on the gentamicin molecule (Fig. 3). Vibrational bands attributed solely to GEN-SULPH and AOT can be identified in the spectrum of GEN-AOT. As GEN-SULPH is not soluble in $CH_2Cl_2$, these features suggest the successful ion-paired GEN-AOT complex formation. Furthermore, the absorption wavenumber for the stretching vibration for the amine groups present in the gentamicin-sulphate complex is located at 1620 $cm^{-1}$. The same stretching vibration in the physical mixture of GEN-SULPH and AOT was observed. For the amine groups present in the GEN-AOT complex this vibrational band is located at 1608 $cm^{-1}$.

The susceptibility of *SA* and *SE* for GEN-AOT and GEN-SULPH as represented in MIC and MBC values can be found in Table 1.

As can be seen from Table 1, GEN-AOT was at least as effective as GEN-SULPH in inhibiting and killing both Staphylococcal species. Towards *S. epidermidis* 103.1 it is more bactericidal than GEN-SULPH. AOT itself has no antimicrobial effect as can be seen from the high MIC and MBC values.

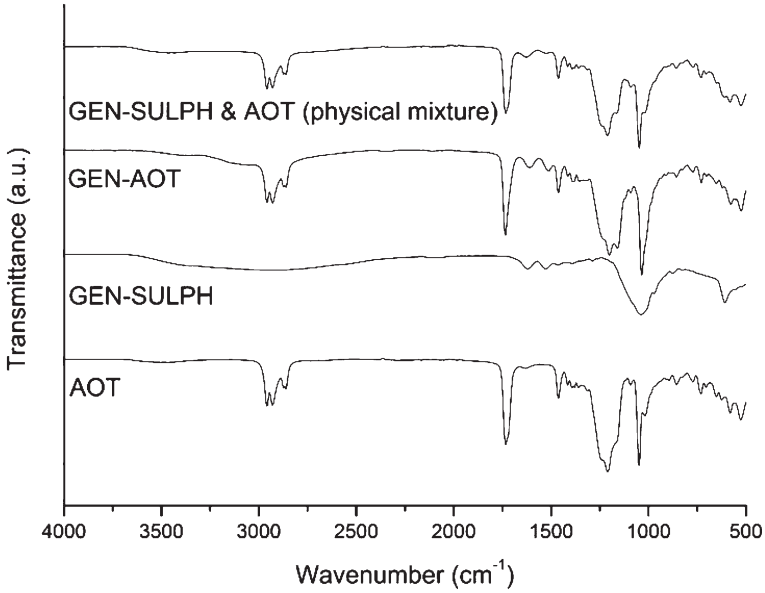


Fig. 3. FTIR spectra of GEN-SULPH, AOT, physical mixture of GEN-SULPH and AOT and GEN-AOT.

Table 1
Susceptibility of *S. aureus* and *S. epidermidis* for GEN-SULPH, GEN-AOT, and AOT

| | Gram-positive | | | |
|---|---|---|---|---|
| | *S. aureus* NCTC 12973 | | *S. epidermidis* 103.1 | |
| | MIC (μM) | MBC (μM) | MIC (μM) | MBC (μM) |
| GEN-SULPH | 2.1 | 8.5 | 0.5 | 4.2 |
| GEN-AOT | 2.1 | 8.5 | 0.5 | 0.5 |
| AOT | 544 | 544 | 544 | >544 |

The cell viability data reported in Fig. 4 indicated that GEN-SULPH did not impair fibroblast viability. Even at high concentration of $2.1 \times 10^3$ μM GEN-SULPH, 75% fibroblast cell viability was observed. For GEN-AOT, concentrations up to 1.1 μM did not negatively influence fibroblast cell viability. At 11 μM GEN-AOT fibroblast cell viability was reduced to ~50%. For free AOT, a sharp decrease in viability is seen when the concentration in the medium is increased from $11 \times 10^2$ μM to $21 \times 10^2$ μM. This decrease in fibroblast cell viability appears at a higher concentration than for GEN-AOT.

HMW PTMC with $M_n = 272$ kg/mol and IV = 2.3 was obtained, which was used for preparation of the antibiotic loaded PTMC discs.

PTMC discs, PTMC discs with GEN-SULPH and PTMC discs with GEN-AOT prepared by compression molding were prepared (Fig. 5). Both the PTMC discs and PTMC discs with GEN-AOT were transparent while the PTMC discs containing GEN-SULPH were opaque indicating a dispersion of the salt in the polymer matrix.

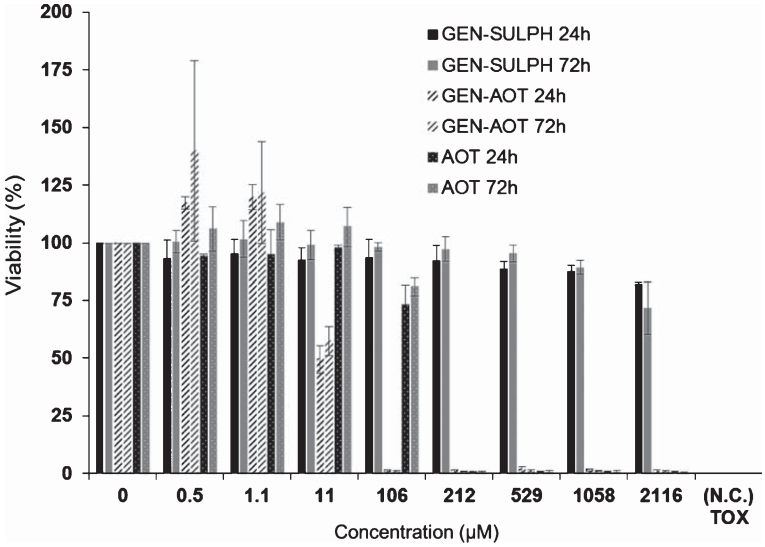


Fig. 4. hTERT fibroblast cell viability upon 24 h and 72 h of exposure to GEN-SULPH and GEN-AOT ($n = 4 \pm$ s.d., and N.C. is the negative control).


Fig. 5. ø 8 mm PTMC discs (left 4), PTMC discs with GEN-SULPH (middle 4) and PTMC discs with GEN-AOT (right 4) prepared by compression molding.

Table 2
Glass transition temperature ($T_g$) for PTMC, PTMC-GEN-SULPH and PTMC-GEN-AOT samples

| Specimen | Peak ($T_g$) | |
|---|---|---|
| | Heating ramp (T(°C)) | Cooling ramp (T(°C) |
| Pure PTMC | −14.2 | −16.1 |
| PTMC + GEN-SULPH | −12.5 | −13.7 |
| PTMC + GEN-AOT | −12.4 | −14.5 |

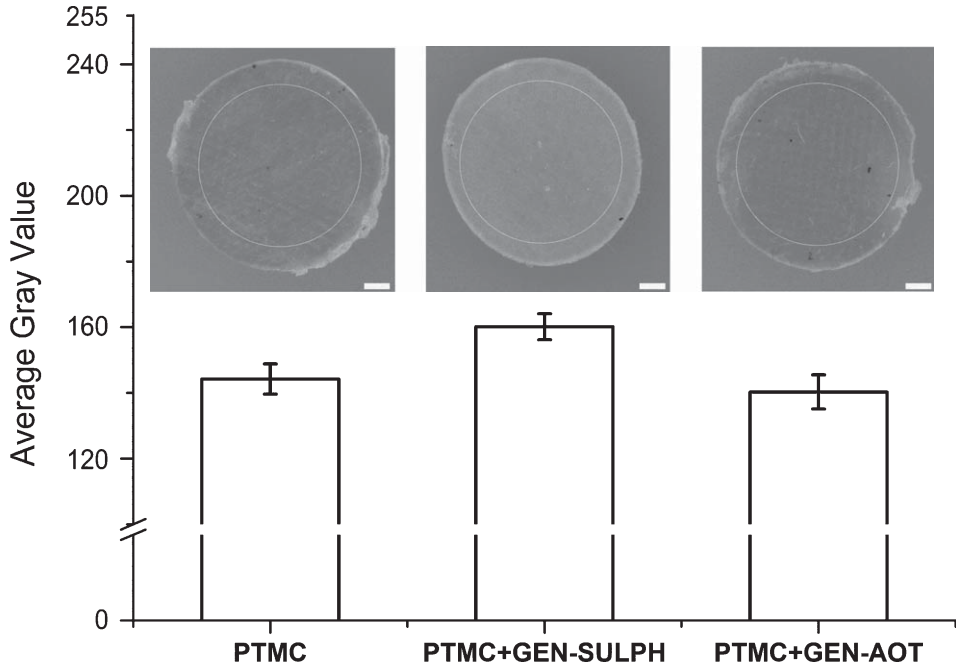


Fig. 6. Gray values obtained for PTMC discs, PTMC discs with GEN-SULPH and PTMC discs with GEN-AOT by optical microscopy in diffuse illumination mode.

The glass transition temperature ($T_g$) for the PTMC, PTMC-GEN-SULPH and PTMC-GEN-AOT discs can be found in Table 2. The GEN-SULPH and GEN-AOT loaded PTMC samples show a slight decrease of the glass transition temperature, which could be explained by the presence of a relatively high concentration of gentamicin (10 w/w%). This decrease is due to a possible enhanced interaction between the PTMC chains, established in the presence of the gentamicin.

The homogeneous dispersion of GEN-SULPH and GEN-AOT through the PTMC matrix was assessed by optical microscopy. Average gray values were recorded for all the specimens (Fig. 6). A significant higher gray value (more reflection due to scattering of larger antibiotic particles) was observed for the PTMC-GEN-SULPH specimens. For all of the films, the standard deviation of the GVs was low, indicating a homogenous dispersion/dissolution of the antibiotic through the films.

## 4. Discussion and conclusion

GEN-SULPH and GEN-AOT inhibit *S. aureus* and *S. epidermidis* and kill *S. aureus* at the same molar concentration. GEN-AOT kills *S. epidermidis* even at lower molar concentration than GEN-SULPH. This indicates that the ion pairing with dioctyl sodium sulfosuccinate does not affect the potency of the antibiotic. Even more, in the case of *S. epidermidis* GEN-AOT seems to be more effective than the sulfate salt of gentamicin; potentially it is more effective in destabilizing the bacterial membrane. Besides the susceptibility of relevant bacterial species for the hydrophobic modified gentamicin, its toxicity towards eukaryotic cells should be taken into account. The modified gentamicin should kill relevant bacterial species, but at the same time it should not impair cell viability. GEN-SULPH has limited effect on fibroblast

viability. Only at very high concentration ($2.1 \times 10^3$ μM) viability is reduced to ∼75% compared to the untreated control. AOT has an effect on fibroblast cell viability when the concentration is increased from $11 \times 10^2$ μM to $21 \times 10^2$ μM. In the presence of 11 μM GEN-AOT, cell viability was reduced to ∼50%. Reduction of fibroblast cell viability is observed at lower concentration for GEN-AOT than for AOT. Possibly, the effect of AOT on cell viability is combined with the lipophilic character of GEN-AOT which makes it possible for the GEN-AOT to cross the cell membrane and have a stronger effect on cell viability than AOT alone. However, GEN-AOT killed *S. epidermidis* at a molar concentration of 0.5 μM and killed *S. aureus* at 8.5 μM, which is below the concentration for which cell viability was reduced with 50%. HMW PTMC was successfully synthesized and was used for the preparation of GEN-SULPH and GEN-AOT loaded discs. In the PTMC the GEN-AOT was well solubilized, while the GEN-SULPH was dispersed through the polymeric matrix.

### *4.1. Outlook*

The release of GEN-SULPH and GEN-AOT from these PTMC discs will be investigated by applying by performing a serial plate transfer test (SPTT) with the previous stated relevant bacterial species. The release of GEN-SULPH and GEN-AOT from these PTMC discs will be tracked by measuring the zone of inhibition (ZOI) after o/n incubation at 37°C.

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