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The impact of storage conditions upon gentamicin coated antimicrobial implants

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A systematic approach was developed to investigate the stability of gentamicin sulfate (GS) and GS/poly(lactic-co-glycolic acid) (PLGA) coatings on hydroxyapatite surfaces. The influence of environmental factors (light, humidity, oxidation and heat) upon degradation of the drug in the coatings was investigated using liquid chromatography with evaporative light scattering detection and mass spectrometry. GS coated rods were found to be stable across the range of environments assessed, with only an oxidizing atmosphere resulting in significant changes to the gentamicin composition. In contrast, rods coated with GS/PLGA were more sensitive to storage conditions with compositional changes being detected after storage at 60 °C, 75% relative humidity or exposure to light. The effect of γ-irradiation on the coated rods was also investigated and found to have no significant effect. Finally, liquid chromatography–mass spectrometry analysis revealed that known gentamines C1, C1a, and C2 were the major degradants formed. Forced degradation of gentamicin coatings did not produce any unexpected degradants or impurities.

Original Article

The impact of storage conditions upon gentamicin coated antimicrobial implants

1. Introduction

Over the past decade, scientists have been working at the frontier between materials science and pharmaceutics to develop new orthopedic devices with functionalized antibacterial coatings [1–5]. This interest has been driven by the ongoing problem of periprosthetic joint infection in 0.3–9% of patients following total replacement of the hip, knee or ankle [6]. Patients with implanted devices are particularly susceptible to infection due to compromised host defense at the implant/tissue interface [1,7]. Furthermore, implant adherent bacteria typically exist as a biofilm, which protects the former from the host immune system and antibacterial agents [8,9]. Treatment of such bacterial biofilm usually requires surgical removal of the implant, followed by replacement in one or two stages depending on the nature of the infection [6]. Therefore, it becomes necessary to explore alternative procedures for preventing implant associated infections in the first instance.

One such method is the application of antimicrobial coatings to the biomedical implant. Such coatings can disrupt the adhesion of bacteria to the implant surface, and bolster the host’s immune system in the interface region between implant and tissue [1]. Gentamicin is the antibiotic most widely exploited in antibiotic coatings of implants due to its relatively broad antibacterial spectrum and thermal stability [1,3,4]. Other antimicrobials which have also been used in implant coatings include cephalexin [4], carbenicillin [4], amoxicillin [4], cefamandol [4], tobramycin [4], vancomycin [4,10,11], chlorhexidine [12], chloroxylenol [13], silver [14], copper [15], and zinc [16].

Gentamicin is an aminoglycoside antibiotic consisting of four major components (C1, C1a, C2, C2a) and a minor component C2b which are produced by fermentation of Micromonospora purpurea or Micromonospora echinospora (Fig. 1) [17,18]. The ratio of the individual congeners is dependent on the origin of the sample and accepted limits to these ratios are specified in the United States and European Pharmacopoeias [19,20]. During the fermentation process a number of other minor impurities (e.g. sisomicin, and JI-20B) and degradation products (2-deoxystreptamine, and garamine) can also be formed (Fig. 1) [21]. Bulk gentamicin mixtures as an active pharmaceutical ingredient (API) are typically characterized by high performance liquid chromatography (HPLC) with the detection of...
the major components facilitated by UV detection of their ortho-phtaldehyde derivatives [22,23], pulsed electrochemical detection [24–26], charged aerosol detection [27] or evaporative light scattering detection (ELSD) [24,28,29]. Liquid chromatography–mass spectrometry (LC–MS) has also been employed to establish impurity profiles of gentamicin mixtures [30–32].

A particular formulation has been developed and investigated in previous studies [33–36], with the objective of clinical use in cementless hip prostheses. In vitro testing of grit blasted and porous titanium coupons coated with 1.0 mg gentamicin per cm² and a poly(lactic-co-glycolic acid) (PLGA) overcoat has shown them to be as efficacious as a commercially available antibiotic-loaded bone cement for preventing infection [33,34]. Subsequent in vivo studies have demonstrated both the antibacterial efficacy of this coating formulation and that it supports the integration of bone tissue into the implant surface through its relatively fast in vivo dissolution [35,36]. As part of this convergent technology development it is necessary to investigate the stability of the active drug coating under common sterilization and storage conditions used for medical implants.

The degradation of gentamicin as a pure API or in solution is well established [37]. However, alternative degradation pathways might be possible on interaction of gentamicin with the surface of a medical device. Friess and Schlapp [38] have reported the short-term stability of gentamicin loaded PLGA microparticles and collagen/PLGA composites sterilized by ethylene oxide treatment as well as β- and γ-irradiation. Ethylene oxide sterilization resulted in chemical changes to the gentamicin drug substance, while storage of irradiated samples at 40 °C and 75% relative humidity (RH) resulted in degradation of the polymer support. To our knowledge there have been no reported studies for the degradative behavior of gentamicin on titanium or hydroxyapatite device surfaces.

The goal of this study was to evaluate the degradation pathways of gentamicin coatings on hydroxyapatite implant devices under common storage conditions. While gentamicin is known to be a heat stable antibiotic when stored under standard conditions as an API, incorporation on the surface of a material may lead to different stability levels and degradation pathways. Accordingly, a range of temperatures were studied to establish the thermal stability/lability of gentamicin on surfaces. The impact of sterilization by γ-irradiation of the drug/device combination products was also investigated. To facilitate these studies a simple yet robust method was developed for the extraction of the drug coating from the combination devices. Extracts of the drug coating were analyzed by HPLC with ELSD to determine the ratio of the major components in the gentamicin coating. The extracts were also analyzed for common gentamicin degradants and impurities using LC–MS. In the present study gentamicin sulfate (GS) coated hydroxyapatite rods were used as a convenient model system for larger implant devices.

2. Experimental

2.1. Reagents and chemicals

GS was obtained from Lek (Lubljana, Slovenia). HPLC grade water, acetonitrile and methanol were from Sigma-Aldrich (St Louis, MO, USA). Trifluoroacetic acid (99%) (TFA) was from Sigma-Aldrich. Nitrogen gas (purity: 99.995%) was supplied by BOC (Guildford, UK). GS and GS/PLGA coated hydroxyapatite rods were obtained from DePuy Synthes (Cork, Ireland), produced via a process equivalent to previous published work on this coating [35,36]. The rods were received in sealed aluminum foil pouch packaging.

2.2. Instrumentation and chromatographic conditions

2.2.1. LC–ELSD chromatographic conditions

The LC-ELSD apparatus consisted of an 1120 Compact LC system, a 385-ELSD evaporative light scattering detector and ChemStation B.04.03 software (all from Agilent Technologies, Santa Clara, CA, USA) for data acquisition. Nitrogen (purity 99.995%) was used as the evaporation gas at a flow rate of 1.6 L/min. ELSD was operated with the nebulizer and evaporator temperatures of 40 °C.

The LC-ELSD method was adapted from those previously reported by Agilent Technologies [39] and Clarot et al. [28]. An
Atlantis T3 C18 column (150 mm × 4.6 mm, 5 μm, Waters, Milford, MA, USA), maintained at 25 °C in the 1120 Compact LC column heater, was used for chromatography. The mobile phase was an isotropic mixture of an aqueous solution of trifluoroacetic acid (0.2 mol/L, pH 1.5) and methanol in a ratio of 92:8 (v/v). The mobile phase flow rate was 1 ml/min.

All GS standard solutions were prepared in water. The injection volume was 10 μL. GS standards were prepared at concentrations of 0.1–1 mg/mL and used to calibrate the detector response.

2.2.2. LC–MS chromatographic conditions

The LC–MS apparatus consisted of a 2695 Separations Module, a 2996 Photodiode Array Detector, an LCT Premier KD160 mass spectrometer and MassLynx 4.1 software (all from Waters, Milford, MA, USA) for data acquisition. Chromatographic column and conditions employed for LC–MS studies were the same as given above for the LC–ELSD data generation.

2.2.3. 1H nuclear magnetic resonance (NMR) spectroscopy analysis

1H NMR spectra were recorded in D2O on a Bruker Avance NMR spectrometer operating at 600 MHz at room temperature (20 °C).

2.3. Isolation of gentamicin sulfate components

Pure samples of the major components of GS were isolated using the LC–ELSD. An initial analysis of GS performed using the LC–ELSD method reported above with an injection volume of 50 μL, provided retention times for the C1,2, C2, C2a, and C1 components. The ELSD detector was subsequently disconnected and fractions of the eluent were collected from the detector inlet at the previously determined time intervals (C1, 5.0–6.0 min, C2, 6.9–7.9 min, C2a, 9.0–9.9 min, C1, 11.0–12.0 min). Fifteen injections of a 0.25 mg/mL GS solution were processed. Evaporation to dryness of the pooled fractions of samples of gentamicin C1,2, C2, C2a, and C1, in good purity, as determined by LC–ELSD, NMR spectroscopy and high resolution mass spectrometry (HRMS).

2.4. Chemical stability

The stability of GS coated rods under a range of storage conditions was studied over 30 days. The variations of storage conditions investigated are shown in Table 1.

(i) Thermal: samples were stored at a low temperature (4 °C) or high temperature (60 °C) in sealed vessels to establish the thermal stability of the gentamicin on the rods.

(ii) Sensitivity to oxidation: oxidizing storage conditions were simulated by suspension of samples over a solution of 30% m/m hydrogen peroxide, while in contrast to simulate storage in a non-oxidizing environment samples were stored under nitrogen gas at 20 °C. Both were shielded from light.

(iii) Humidity: humidity variations were simulated by sample storage at low humidity (20% RH, suspension over saturated potassium acetate solution) or high humidity (75% RH, suspension over saturated sodium chloride solution) in sealed containers at 20 °C.

(iv) Light: low light storage conditions were simulated by storage of the samples in open foil pouches at 20 °C while high light conditions were achieved by storage of the samples under a fluorescent light.

(v) Irradiation: GS coated rods were subjected to sterilization by γ-irradiation (carried out by DePuy Synthes) at low (25 kGy) and high (40 kGy) doses.

On completion of the storage period, the organic material was extracted from the coated hydroxyapatite rods by immersion in a solution of TFA/acetonitrile/water (0.3/3/97, v/v/v) [200 mL]. The vessel was sonicated for 1 h at room temperature. The resulting solution was filtered and evaporated to dryness. Portions of the residues (10 mg) were dissolved in water (1 mL) and analyzed by LC–ELSD and LC–MS. LC–ELSD results were recorded as % (m/m) based on the assumption that structurally similar gentamicin congeners have the same ELSD response factors [42] (Table S1). LC–MS results were recorded as peak area ratios relative to the gentamicin C1 peak area.

As summarized in Table 1, the samples of GS coated rods were originally received in sealed foil pouches under a protective atmosphere. For the light, humidity and oxidation stability tests the pouches were opened to enable exposure of the coating to the appropriate test conditions. Accordingly, exposure to open atmosphere in addition to the specific stability tests was incorporated in the study.

3. Results and discussion

3.1. Analysis of bulk gentamicin sulfate

The European Pharmacopoeia defines acceptable limits for the composition of GS (Table 2) [19]. The bulk GS used for preparing coated devices in this study was examined using LC–ELSD to determine its composition before the application of the device coating (Fig. 2). The composition of the bulk material was also determined separately using 1H NMR spectroscopy. The anomeric protons of the four gentamicin constituents could be separately assigned by resolution of the signal at 5.9 ppm. Integration of this signal revealed the ratio of gentamicin components [43]. The LC-
ELSD and $^1$H NMR spectroscopy determinations for our GS were in agreement, and were within the European Pharmacopoeia limits. This also verified that the major components of GS displayed an equivalent response in ELSD detection.

### 3.2. Isolation of gentamicin sulfate components

The individual gentamicin C1, C1a, C2 and C2a congeners were isolated using HPLC to provide pure standards for use in our LC–ELSD and LC–MS analyses (Fig. S1).

### 3.3. Chemical analysis of gentamicin coated hydroxyapatite devices

In order to study the gentamicin coating on medical devices it was desirable to have a method for the extraction of the coating into a solution. The extraction of the gentamicin coating could be effectively achieved by sonication of the coated rods in a solution of trifluoroacetic acid, acetonitrile and water in a ratio of 0.3:3:97 (v/v/v) at room temperature. Subsequent analysis of the concentrated extract by LC–ELSD revealed a gentamicin composition of 41% C1, 8% C1a, 43% C2 and 8% C2a. While this composition was marginally outside of the European Pharmacopoeia specification for gentamicin drug product, it was considered sufficiently close to that of the bulk GS to indicate that the extraction method was efficient and non-destructive.

### 3.4. Stability of the gentamicin sulfate coating studied by LC–ELSD

One of the core objectives of this study was to assess the chemical stability of gentamicin coatings on hydroxyapatite devices and determine if there were environmental factors impacting on shelf life. For the purpose of this pilot study, GS coated hydroxyapatite rods supplied by DePuy Synthes were subjected to a 30 day forced degradation study to determine the influence of temperature, humidity, atmospheric oxidation and light exposure. For these stability studies, it was important that the sealed foil pouches in which the rods were supplied were opened for the stability tests (other than thermal and irradiation studies) to evaluate effect of exposure to stress factor as compared to the packaged products. Also the hydroxyapatite rods coated with both GS and PLGA were subjected to the same forced degradation studies.

#### 3.4.1. Thermal stability

Gentamicin is well noted for being a heat stable antibiotic, retaining its activity even after autoclaving [44,45]. It is this stability under autoclave conditions that makes it a favored antibiotic for use in antimicrobial coated devices [1,3,4]. In the present study, storage of the rods in sealed pouches at temperatures of 4 °C and 60 °C had no noticeable effect on the chemical composition of the coating consisting of just GS (Fig. 3A). When the rods coated with both GS and PLGA were stored at 60 °C in sealed pouches, the resulting gentamicin composition showed a small but significant reduction in the level of C2 (43% down to 36%) relative to the other congeners. This result indicates that the thermal stability of GS may be reduced in coatings containing PLGA.

#### 3.4.2. Stability to humidity

The International Pharmacopoeia describes GS as being hygroscopic and susceptible to gradual degradation on exposure to a humid atmosphere with the decomposition being faster at higher temperatures [46]. Storage of the GS and GS/PLGA coated rods in the opened foil packaging at a low RH of 20% resulted in no change to the congener ratios of gentamicin in the coating. However, storage of both types of coated rods at 75% RH resulted in small decreases in the level of gentamicin C2 relative to the other congeners (Fig. 3B). The presence of PLGA in the coating had little effect on the stability of gentamicin under high humidity. The changes observed in this study were relatively small but it should be noted that the high RH test was conducted at room temperature over a limited period of 30 days. A combination of high RH and high temperature might be expected to exert more of an effect on the gentamicin coating over longer time periods.

#### 3.4.3. Stability to atmospheric oxidation

Oxidative processes are known to occur in the biosynthetic interconversion of gentamicin and its several known impurities

![Fig. 2. LC–ELSD chromatogram of gentamicin sulfate [39].](image-url)
Fig. 3. The effect of 30-day storage conditions on the composition of the GS coating determined by HPLC–ELSD. Control: control extraction, GR: GS coated rod, GPR: GS and PLGA coated rod, LT: stored at 4°C, HT: stored at 60°C, LH: stored under 20% RH, HH: stored under 75% RH, LL: stored in dark, HL: stored under light, LO: stored under nitrogen atmosphere, HO: stored over 30% H₂O₂.

Fig. 4. The effect of 30-day storage conditions on relative level of common impurities in the GS coating determined by LC–MS. Control: control extraction, GR: GS coated rod, GPR: GS and PLGA coated rod, LT: stored at 4°C, HT: stored at 60°C, LH: stored under 20% RH, HH: stored under 75% RH, LL: stored in dark, HL: stored under light, LO: stored under nitrogen atmosphere, HO: stored over 30% H₂O₂.
such as JI-20B [47]. It was therefore expected that an oxidizing atmosphere might lead to degradation of gentamicin. Also the amino and hydroxyl functional groups in GS are reported as being susceptible to chemical oxidation [37]. Furthermore, the glycoside linkages of several aminoglycosides are known to be cleaved under oxidizing conditions [48]. In the present study, no changes were observed in the composition of GS and GS/PLGA coatings stored in open pouches under an inert nitrogen atmosphere. Storage of the coated rods in open pouches in an oxidizing atmosphere, over a solution of hydrogen peroxide (30%, m/m), resulted in measurable changes to the gentamicin composition of GS and GS/PLGA coatings. Small increases in the relative level of gentamicin C2 were observed, while gentamicin C1 was noticeably decreased from 41% to 34% in both the GS and GS/PLGA coatings (Fig. 3C).

3.4.4. Stability to light exposure

Hydroxyapatite rods coated with either GS or GS/PLGA were stored in the dark and under a fluorescent light for 30 days in an open atmosphere in both cases. After this period of storage, no changes were observed in the gentamicin composition of rods stored in the dark (Fig. 3D). The GS coated rod also appeared to be stable to storage under the fluorescent lamp with no measurable change in the gentamicin congener ratios measured by LC–ELSD, while the GS/PLGA coated rod did undergo changes when stored under the lamp. Under the high light exposure conditions, the C2 level was significantly reduced to 31% in the GS/PLGA coating. This indicated that even though gentamicin appeared to be stable to light, the presence of other components (such as PLGA) in antimicrobial coatings could lead to significant photodegradation.

3.5. Analysis of common impurities by LC–MS

The extracts obtained from the stability studies were also subjected to LC–MS analysis to screen for common gentamicin impurities and degradants. The characteristic ions for many of these gentamicin impurities have been previously reported [30–32]. Total ion count chromatograms showed the presence of the five gentamicin C components. The presence of key degradants and impurities in the extracts were determined by ion extraction method.

Garamine fragmentation ions were detected in all samples as multiple peaks due to fragmentation of the major gentamicin congeners inside the mass spectrometer. However, m/z 322 peak was also observed at 2.59 min, which was not observed with any heavier ions, suggesting that this peak represented garamine degradants formed prior to LC–MS analysis. Measurement of this peak in the extracts revealed garamine levels of 1%–6% with the maximum level being observed in samples stored under a highly oxidizing atmosphere (5%–6%). No garamine was detected in the control extract taken from a rod not subjected to forced degradation studies.

The extracts were expected to contain gentamines in significant levels if severe degradation of the corresponding gentamicin C components had occurred under the tested environmental conditions. Gentamine C1, arising from degradation of gentamicin C1, was observed at levels of 2%–5% in most extracts. However, there was noticeably more gentamine C1 (19%) observed in samples subjected to highly oxidizing conditions. Low levels of humidity also led to an increase in gentamine C1 levels (7%–9%). The C1a and C2 gentamines showed a similar increase in levels for samples that experienced a highly oxidizing atmosphere.

G-418, sisomicin and JI-20B are common in batches of GS and are related to gentamicin through biotransformative processes [47]. Sisomicin and the antibiotics JI-20B and G–418 were observed at levels below 1% in all the samples (Fig. 4).

The storage conditions employed in this study generally had little effect on the composition and degradation of the gentamicin coated rods; the exception being the highly oxidizing environment (Fig. 4). After 30 days the only major degradants observed were gentamines arising from the cleavage of the A-ring from the B–C gentamicin ring system. All the LC–MS peaks observed in this study could be attributed to previously reported gentamicin impurities [30–32]. Thus, there was no evidence that gentamicin underwent substantive unique degradation pathways when applied as a device coating at readily detectable levels .

3.6. Stability to γ-irradiation

γ-Irradiation is a common method for the sterilization of medical devices and has previously been employed for gentamicin containing PLGA composite particles [38]. Friess and Schlapp have reported that free radicals, detected by electron spin resonance spectroscopy, were formed in gentamicin loaded particles sterilized by exposure to 28.9 kGy of γ-irradiation. These free radicals did not persist beyond four weeks in gentamicin and had no detectable effect on the 1H NMR spectrum of gentamicin. [38] In our study, we employed 25 kGy and 40 kGy dosages of γ-irradiation to sterilize GS and GS/PLGA coated hydroxyapatite rods. LC–ELSD and LC–MS analysis of extracts after low and high dose γ-irradiation did not display any significant changes compared to extracts from unsterilized rods (Fig. 5). These results confirmed that γ-irradiation is a suitable sterilization method for use with gentamicin coated medical implants.

As stated, LC–ELSD analysis revealed that the change in composition of the gentamicin congeners (gentamicin C1, C1a, C2 and C2a) was very small across the range of conditions studied. The largest variation seen in rods stored under high humidity and
oxidation. The proportion of gentamicin C2 changed by 5% (Figs. 3B and 3C). A comparison of the change in composition of the minor impurities using LC–MS revealed that the biggest change was seen in the amounts of gentamine C1 formed after storage (Fig. 6). Under conditions of low humidity both GS coated rods and those coated with GS and PLGA showed a significant increase in levels of gentamine C1 detected. This effect was even more pronounced when rods subjected to highly oxidizing conditions were examined. In addition, an increase in the other gentamine impurities (gentamines C1a and C2) was also evident.

4. Conclusions

GS and GS/PLGA coated hydroxyapatite rods were subjected to high and low temperature, humidity, oxidation and light exposure environments over a period of 30 days. LC–ELSD and LC–MS analyses of extracts from GS coated rods indicated that they were stable under storage at 60 °C and also to light exposure. High humidity had a minimal effect on the composition of GS. In contrast, rods coated with both GS and PLGA were more sensitive to storage conditions with compositional changes being detected after storage at 60 °C, 75% RH or exposure to light. Storage of both GS and GS/PLGA coated rods in an oxidizing atmosphere resulted in significant changes to the gentamicin composition but no unexpected impurities were detected by LC–MS. The major degradants detected were the gentamines arising from degradation of the corresponding gentamicin C components. Finally, the effect of γ-irradiation on the gentamicin composition of GS and GS/PLGA coated rods was also investigated and found to have no significant effect. Based on this preliminary study, investigation of long-term storage of the GS and GS/PLGA coated rods under appropriately controlled conditions (namely protected from light, avoiding high temperature and exposure to air) is warranted.

In conclusion, a systematic approach has been developed to investigate the chemical stability of drug coatings applied to medical implant devices. This methodology was applied to the study of hydroxyapatite rods coated with gentamicin to determine the degradative behavior of gentamicin on a device surface.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jpha.2016.05.002.

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