Antimicrobial properties of gallium(III) and iron(III) loaded polysaccharides affecting the growth of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa, in vitro*

Mark G. Best[1], Cassilda Cunha-Reis,[2] Alexey Y. Ganin,[3] Aureliana Sousa,[4] Jenna Johnston,[5] Ana L. Oliveira,[2] David G.E. Smith,[6] Humphrey H. P. Yiu*[,5], and Ian R. Cooper*[,1]

1. School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, BN2 4GJ, U.K.
2. CBQF - Centro de Biotecnologia e Química Fina Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. de Diogo Botelho 1327, 4169-005 Porto
3. School of Chemistry, Joseph Black Building, University of Glasgow, Glasgow, G12 8QQ, U.K.
4. 1i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal
5. Chemical Engineering, School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, EH14 4AS, U.K.
6. The Institute of Biological Chemistry, Biophysics and. Bioengineering (IB3), School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, EH14 4AS, U.K.

*Joint Corresponding authors:

HHPY, Tel: +44 131 451 8145. Fax: +44 131 4513129. E-mail address: h.h.yiu@hw.ac.uk

IRC, Tel: +44 1273 641189. Fax: +44 1273 642674. E-mail address: I.Cooper@brighton.ac.uk

Abstract

Antimicrobial resistance (AMR) has become a global concern as many bacterial species have developed resistance to commonly prescribed antibiotics, making them ineffective to treatments. One type of antibiotics, gallium(III) compounds, stands out as possible candidates due to their unique “Trojan horse” mechanism to tackle bacterial growth, by substituting iron(III) in the metabolic cycles of bacteria. In this study, we tested three polysaccharides (carboxymethyl cellulose CMC, alginate, and pectin) as binding and delivery agent for gallium on three bacteria (*P. aeruginosa*, *E. coli* and *S. aureus*) with a potential bioresponsive delivery mode. Two types of analysis on bacterial growth (Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)) were carried out while Iron(III) loaded polysaccharide samples were also tested for comparison. Results suggested that gallium showed an improved inhibitory activity on bacterial growth, in particular gallium(III)-loaded carboxymethyl cellulose (Ga-CMC) sample showing inhibiting effect on growth for all three tested bacteria. At the MIC for all three bacteria, Ga-CMC showed no cytotoxicity effect on Human Dermal Neonatal Fibroblasts (HDNF). Therefore, these bioresponsive gallium(III) polysaccharide compounds show significant potential to be developed as the next generation antibacterial agents with controlled release capability.

Key words:

Gallium, carbohydrates, antibacterial, antibiotics, anaerobic

1 Introduction

The increasing problem of antibiotic resistance has generated significant concerns over the ability to treat infectious disease in humans. In 2016, a high-level meeting on Antimicrobial Resistance (AMR) was held at the United Nations HQ, New York to finalise a global plan combating this world-wide crisis[1]. Notably, one research strategy tackling AMR is the search for new types of antimicrobial agents from naturally-occurring, environmentally friendly materials[2]. For example, allicin from garlic[3] and cinnamaldehyde from the leaves of the cinnamon plant[4], as well as metallic compounds (e.g. Fe- and Pt-peptides and maltolato complexes), long purported to deliver an antimicrobial effect[5, 6].

Recently, soluble gallium compounds (Ga^{3+}) have been used in several biomedical applications including the treatments of bone-associated cancers[7, 8] and infectious diseases[9, 10]. The antibacterial action of Ga^{3+} is strongly linked to sharing similar properties with Fe^{3+} ions (including electronic configuration, atomic radius and coordination chemistry). The survival of many microorganisms relies upon the availability of iron, particularly Fe^{3+}, which takes part in numerous redox driven biological processes such as respiration and DNA synthesis, and in particular their activity as iron siderophore analogues[11]. The electron transition between a 3+ state to 2+ (and vice versa) only requires low energy for Fe systems, and therefore these crucial processes readily occur. However, such redox transition requires much higher energy for Ga ($Ga^{3+} \rightarrow Ga^{+}$ while Ga^{2+} has not yet been identified) and substitution of Fe^{3+} with Ga^{3+} in protein binding sites disrupt these vital processes[12]. Given that this mode of antibacterial action is different from those of conventional, organic antibiotics, gallium could prove to be less vulnerable to resistance mechanisms such as reduced uptake due to restricted permeability of bacterial cell membranes[13]. Since the use of

gallium as a drug product (gallium-67 citrate) has already been approved by the FDA[9], Ga-bound natural products could be considered to be safe for trial use as an antimicrobial agents.

Many gallium-based materials reported for research on antibacterial activity are water-soluble species such as gallium nitrate[14-16] and other organometallic Ga-complexes (e.g. Ga-maltolate[17,18], Ga-EDTA[19], Ga-desferrioxamine[20], Ga-porphyrins[21-24] and Ga-quinolone[25]). Release of these water-soluble gallium compounds can be difficult to control. This causes problematic applications as dry environment is likely to be required, while water is difficult to avoid in many biological systems. A feasible controlled release platform will greatly improve the usability of some of these antibacterials. To address this, gallium-based bioglass materials have been developed and shown such a controlled release by varying the composition of the glass, in particular the calcium content[26-30]. However, such a control is still strongly linked to the solubility of the material, regardless of whether the bacteria are present in the media or not. To achieve a controlled release, gallium species have been incorporated onto a degradable polymer substrate. Ma *et al.* have demonstrated the preparation of poly(ether urethane), or PEU, blended with Ga-protoporphyrin or mesoprotoporphyrin complex[11]. This material have shown antibacterial activity towards both *Staphylococcus epidermidis* (Gram-positive bacterial species) and *Pseudomonas aeruginosa* (Gram-negative bacterial species), but there is limited information on Ga release due to non-bacterial solvation or degradation. Valappil *et al.* have reported the use of carbonxymethyl cellulose (CMC) as the polymer substrate with the gallium species (Ga^{3+} ions) directly bound onto the carboxylic acid groups on the polymer structure[31]. Since this crosslinked polymer is not water-soluble, the release of Ga was theorised to be a consequence triggered by the digestive action of

viable *P. aeruginosa* cells in the test matrix, initiating bioresponsive delivery of gallium. The antibacterial activity of a related material gallium alginate (Ga-Alg) has also been reported when used in conjunction with bioglass[32].

In our study, a family of bioresponsive antibacterial materials are prepared based on gallium and iron(III) crosslinked polysaccharide materials (carboxymethyl cellulose, pectin, alginate). These polysaccharides were chosen because of the carboxylic acid groups on their polymer backbone, allowing the co-ordination of Ga^{3+} and Fe^{3+} ions onto the polymers. Three representative bacterial species were used as test organisms, namely, *Escherichia coli* (NCTC 8196), *Staphylococcus aureus* (NCTC 6571) and *Pseudomonas aeruginosa* (NCIMB 10548). All three species are regarded as potentially significant species capable of causing infectious diseases and as a threat to human health due to their high rate of resistance to contemporary antimicrobials. Enzymatic degradation profiles and cytotoxicity of the metal-loaded polysaccharides were also examined to provide essential information about these materials for further development of clinical applications.

2. Materials and methods

2.1 Materials

Gallium nitrate ($Ga(NO_3)_3.H_2O$, 99.9%) was supplied by Alfa Aesar, UK. Ferric nitrate ($Fe(NO_3)_3.9H_2O$, 99%), carboxymethyl cellulose (CMC, 250 kDa, D.S.=0.9), pectin (from citrus peel, galacturonic acid ≥74.0 %), cellulase from *Aspergillus niger* (>0.8 U/mg), pectinase from *Aspergillus niger* (>1.0 U/mg), alginate lyase (≥10,000 U/g), Phosphate-buffered saline (PBS) and resazurin were all purchased from Sigma Aldrich, UK. Alginic acid sodium salt was purchased from Fisher Scientific UK. Sodium

acetate buffer (0.2 M, pH 7) was prepared from sodium hydroxide (CertiFied AR grade, Fisher Scientific, UK) and glacial acetic acid (Sigma-Aldrich, UK). Human Dermal Neonatal Fibroblasts (HDNFs) were purchased from the Coriell Institute for Medical Research, Camden, New Jersey, USA, Dulbecco's Modified Eagle Medium (DMEM) high glucose was supplied by Lonza, foetal bovine serum (FBS), penicillin/streptomycin and TrypLE Express were purchased from Gibco. Dimethyl sulfoxide (DMSO, laboratory grade) was purchased from Fisher Scientific, UK. All water used was deionised water. All chemicals and materials were used as received without further purification. All microbiological media were purchased from Fisher Scientific (UK).

2.2 Preparation of Ga^{3+} and Fe^{3+} polysaccharides

The preparation procedure of Ga^{3+}-CMC has been published previously[31]. This method was adapted for the preparation of all Ga^{3+} and Fe^{3+} polysaccharide materials used in this work. In general, 1.0 g of the polysaccharide source (carboxymethyl cellulose CMC, pectin, and alginate) was dissolved in 150 mL of deionised water in a 250 mL conical flask under magnetic stirring. A 0.1 M solution of $Ga(NO_3)_3$ or $Fe(NO_3)_3$ was added drop-wisely to the polysaccharide solution causing a gelatine precipitate to form. The amount of Ga^{3+} and Fe^{3+} added was calculated from the maximum ion exchange capacity of the polysaccharides (based on the amount of carboxylic acid group). To ensure an exchange close to 100%, an excess of metal (50%) was added. The gel was stirred overnight with the flask sealed with parafilm to minimise evaporation. Finally the gel was recovered by filtration and allowed to dry in air to form a hard solid. The dried solid was then ground to a powder for antibacterial tests and materials characterisation. All procedures were carried out at ambient temperature.

2.3 Characterization of Ga^{3+} and Fe^{3+} polysaccharides

Thermogravimetric analysis (TGA) was used to determine the inorganic content of the samples. A TA Instrument STD600 thermobalance was used for this purpose. In a typical experiment, a ca. 5 mg sample was heated up in flowing air (100 mL min^{-1}) at a heating rate of 5°C min^{-1} to 700°C where the sample was held isothermal for 60 min. The residual weight was due to inorganic oxides, Ga_2O_3 or Fe_2O_3. Details of calculation can be found in Supplementary Information. The surface morphology and the metal distribution of samples were studied by a scanning electron microscopy (SEM) and Energy Dispersive X-ray (EDX) spectroscopy. Energy-dispersive X-ray (EDX) spectroscopy was performed in conjunction with scanning electron microscopy (SEM) on a Philips XL30 ESEM with an attached Oxford Instruments x-act EDX detector. EDX spectroscopy was used to determine the elemental composition of the materials, whilst the micrographs taken provide a visual representation of the morphology of the products. About 0.5 mg of each material was placed on carbon tape; the materials were insufficiently conductive and gold sputtering was required to enhance the conductivity prior to imaging. All micrographs and EDX spectra were recorded using a beam current of 20 kV. A Thermo Scientific Nicolet iS5 spectrometer was used for FTIR spectroscopy with 16 scans for each sample at a resolution of 4 cm^{-1}.

2.4 Enzymatic degradation profile

Degradation profile of the Fe^{3+} polysaccharides materials was studied using the corresponding enzyme (e.g. pectinase for Fe^{3+} pectin). Iron(III) polysaccharides were

chosen for the study due to the colour (visible light absorbance) allowing spectrophotometric measurement. In a typical experiment, 20 mg of Fe^{3+} polysaccharide powder was suspended in sodium acetate buffer solution (5 mL, 0.2 M, pH = 7). A neutral pH was chosen to avoid acid/base-initiated digestion of the carbohydrate material. Enzyme (1 mg/mL) was then added and the suspension was incubated at 37°C. After a set time interval, the sample was cooled down using an ice bath to slow down the enzymatic action. The solution was then filtered and the supernatant was collected. The absorbance of supernatant at 450 nm was recorded using a Shimadzu UV-mini 1240 spectrometer. The % degradation values were calculated against that from a sample fully digested using conc. HCl. In general, at a certain time point, % degradation = (A_t / A_f)×100%, where A_t was the absorbance of the supernatant at time t while A_f was the absorbance of the fully digested sample. All experiments were triplicated and the absorbance data presented was the average values of the three experiments. Sodium acetate buffer was chosen to avoid precipitation of iron(III) salts (*e.g.* iron(III) phosphate from PBS or iron(III) carbonate from sodium carbonate buffer).

2.5 Evaluation of cell viability

2.5.1 Culture of Human Dermal Neonatal Fibroblasts

Human Dermal Neonatal Fibroblasts (HDNFs) were seeded at a density of 1.0×10^4 cells/cm^2 and cultured in DMEM high glucose, supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin, at 37 °C and 5% CO_2. Culture medium was replaced every 3 days until 80% confluence was reached. Then, the culture medium was removed, the cells were washed twice in sterile PBS and incubated for 5 minutes in TrypLE

Express. In order to neutralize trypsin, 5 mL of culture medium were added. After counting, the cells (passage 9) were seeded in 96 well plates (1.0×10^4 cells/well) and incubated under the same conditions for 24h.

2.5.2. Preparation of liquid extracts

Extracts from the compounds under study were prepared based on ISO standard ISO10993-5 for cytotoxicity evaluation of medical devices. DMEM high glucose, supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin, which was the medium used for cell culture, was employed as the extraction vehicle in this study. Test materials were mixed with the extraction vehicle at 50 mg/mL and incubated for 24 hours at 37 °C. Then, the mixtures were centrifuged for 10 minutes at 14000 rpm to remove the non dissolved fraction, and the supernatant was recovered. A ten-fold serial dilution was performed in order to obtain solutions up to 1/1000 of the concentration of the original extract. The possible effects of the extraction procedure on the extraction vehicle, and subsequently on cell viability, were assessed by preparing a control group in which culture medium with no test sample was subjected to all the extraction conditions.

2.5.3 Evaluation of cell viability after exposure

Possible cytotoxic effects caused by the developed formulations were extrapolated from cell viability testing in HDNFs, after 24 hours of exposure to the extracts and respective dilutions (ISO10993-5). HDNFs seeded in 96 microplates (1.0×10^4 cells/well) were incubated at 37 °C, 5% CO_2 for 24 hours, after which, culture medium was replaced with the extracts and respective dilutions. A negative control, which was used to normalize the results, was prepared by incubating the cells with their regular culture medium, and a positive control by incubating the cell with 20% DMSO in culture

medium. A control group for the extraction procedure was also prepared. In that group, cells were incubated with culture medium that did not contain any test sample but was subjected to all the extraction conditions. After 24 hours of incubation under controlled conditions, the extracts and medium from the controls were removed and cell viability was measured using a resazurin assay. Briefly, 100 µL of a solution of 10% resazurin (0.1mg/mL in PBS) were added to each well and incubated for 3 hours at 37°C. After incubation, 100 µL were collected and the fluorescence was read at 530 nm excitation and 590 nm emission. The percentage of cell viability in each condition was obtained after normalization to the negative control. Three independent replicates were performed, in which all the conditions were tested in triplicate. The results are expressed as the average ± standard deviation of the independent experiments. Statistical significance of the results was analyzed using a One Way ANOVA test, followed, by a Tukey's multiple comparisons test.

2.6 Minimum inhibitory and minimum bactericidal concentrations (MIC and MBC)

2.6.1 Culture of bacteria

Bacterial cultures were obtained from the in-house culture collection of the School of Pharmacy & Biomolecular Sciences at the University of Brighton. The cultures were streaked on nutrient agar (Oxoid, UK) and aerobically incubated for 18 hours. After that time, for each test species, three morphologically correct colonies were aseptically transferred to 10 mL Isosensitest broth (Oxoid, UK), and aerobically incubated for 18 hours in an orbital shaker (Stuart Scientific, UK).

2.6.2 Determination of MIC and MBC

These experiments were conducted according to the macrodilution protocol of Andrews[33]. Briefly, a solution of each of the test compounds was constituted using Isosensitest broth (Oxoid, UK), and serially diluted two-fold by pipetting to obtain a concentration dose range. To each test tube, an aliquot (approx. 10 µL) of overnight culture of each test bacterium was added to give a final concentration of approximately 1×10^6 CFU/mL. A positive growth control of bacteria in the Isosensitest broth plus a negative control of Isosensitest broth only was also set up for each test. Each test was performed in triplicate, and the tubes were incubated at 37°C for 20 hours.

After this period, the Minimum Inhibitory Concentration (MIC) was determined by macroscopic observation of the tubes. Those tubes without visible growth were recorded as containing sufficient concentration of the test compound to inhibit growth, and the tube with the lowest concentration of the test compound registering no visible growth was recorded as the MIC value. From each of the tubes that demonstrated no macroscopic growth, 200 µL was plated on to Isosensitest agar (each tube plated in triplicate, and each dilution series plated in triplicate), in order to determine the Minimum Bactericidal Concentration (MBC). These plates were incubated at 37°C and observed for growth at 24, 48 and 72 hours. The plates with the lowest concentration of test compound to yield no bacterial colonies were recorded as the MBC values. For growth inhibition assays in anaerobic environment, the same experiment was carried out in a Whitely A35 Anaerobic Workstation (Don Whitely Scientific) using anaerobic gas mixture (80% N_2, 10% H_2, 10% CO_2; supplied by BOC). For this, *E coli* (P4 wild-type strain) was used for both anaerobic and aerobic growth experiments.

3. Results and discussion

3.1 Characterization of Ga^{3+} and Fe^{3+} polysaccharides

All carboxymethyl cellulose (CMC), pectin and alginate formed crosslinked gels with Ga^{3+} or Fe^{3+} solution. The structure of Ga^{3+} bound CMC, pectin and alginate are proposed in Figure S1. From Table 1, TGA analysis revealed the metal content of the samples varied from 1.10 – 1.62 mmol/g (or 6.6 – 10.6 % w/w) which is close to saturation based on the number of carboxylic acid groups on the polymer. The EDX elemental mapping on the SEM micrograms (Figure 1) reveals homogeneous distribution of both Ga^{3+} and Fe^{3+} within the samples. This is consistent with the literature[31].

Table 1. Metal content of Ga^{3+}- and Fe^{3+}-polysaccharide samples from TGA.

Sample	Metal content [mmol/g]	Metal content [% w/w]
Ga^{3+}-CMC	1.52	10.6
Ga^{3+}-alginate	1.10	7.7
Ga^{3+}-pectin	1.35	9.4
Fe^{3+}-CMC	1.19	6.6
Fe^{3+}-alginate	1.62	9.0
Fe^{3+}-pectin	1.36	7.6

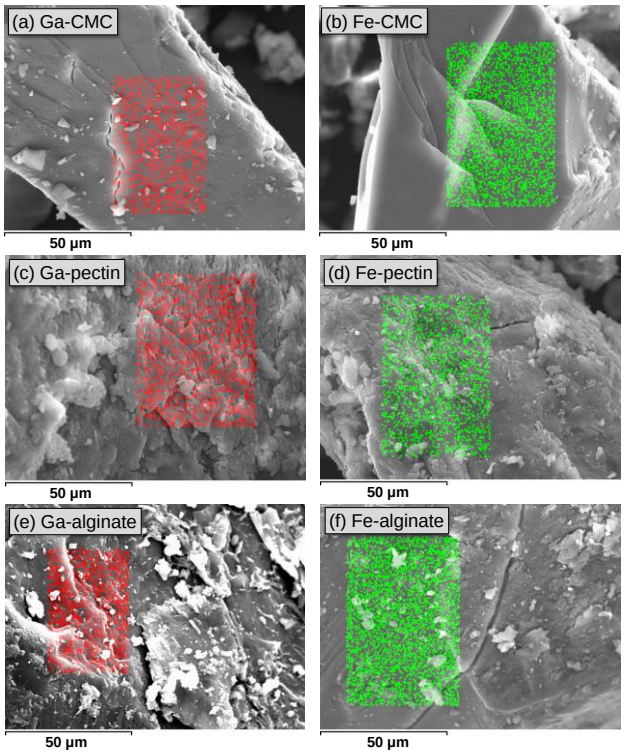

Figure 1. Scanning electron micrographs (SEM) and corresponding EDX mapping for (a) Ga-CMC, (b) Fe-CMC, (c) Ga-pectin, (d) Fe-pectin, (e) Ga-alginate, (f) Fe-alginate showing homogeneous distribution of metal ions within the samples.

The FTIR spectra of CMC samples show typical stretching signal for ν(C=O) at ~1730 cm^{-1} and ν(C-O) at ~1590 cm^{-1} corresponding to the carboxylic acid groups on the polysaccharide backbone (Figure 2). The remaining significant peaks can be assigned as follows: 3310 cm^{-1} for –OH stretching ν(OH), 2920 cm^{-1} for –CH stretching ν(C-H), 1410 cm^{-1} for $–CH_2$ symmetrical bending $\delta(CH_2)$ or C-OH bending δ(C-OH), 1310 cm^{-1} for $–CH_2$ wagging $\delta(CH_2)$ and 1030 cm^{-1} for C-O-C stretching modes ν(C-O-C). All investigated products retain most of the spectroscopic features associated with the original CMC polymeric network suggesting that the CMC structure has been unchanged upon the ion-exchanging process. The FTIR spectra of alginate and pectin samples (Figure 2b and c) show similar features, in particular the carboxylic acid

groups at ~1730 and ~1600 cm^{-1}. In terms of their structure, three model polysaccharides are highly compatible.

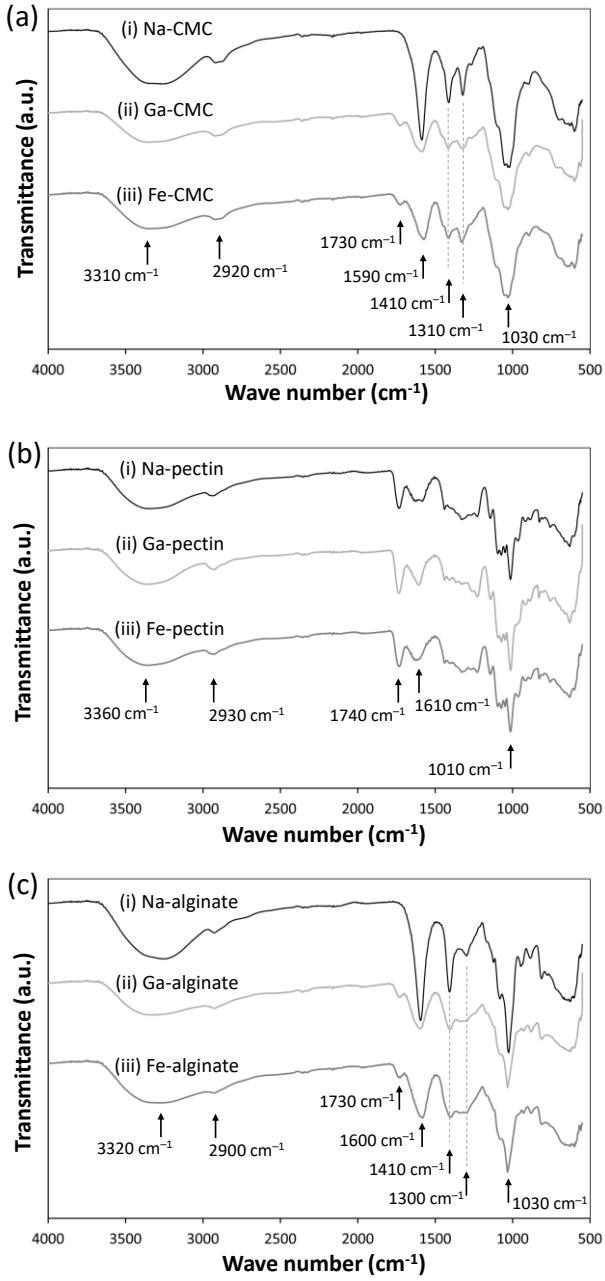

Figure 2. FTIR spectra for metal loaded polysaccharide samples (a) carboxymethylcellulose; (b) alginate and (c) pectin. The as-received Na^{+} polysaccharides are indicated with black lines (i). Ga^{3+} loaded samples are indicated with light grey lines (ii) while the dark grey lines are for Fe^{3+} loaded samples (iii). The

main features of these polymers are highlighted at a region between 1580 – 1750 cm^{-1}, where the bands associated with the carboxylate groups are located.

3.2 Enzymatic degradation profile

To develop bioresponsive, biodegradable platforms for controlled release and delivery of bioactive agents, the release profile and kinetics of the bioactives due to biodegradation of material is essential. In order to probe the metal ion release profile from Fe^{3+} polysaccharides under enzymatic action, a spectrophotometric experiment was setup. It is not straight forward to probe the release of Ga^{3+} due to its colourless nature. Study on Fe^{3+} samples instead could provide some information for the degradation profile of Ga^{3+} release from the polysaccharide samples because of the similarity of their structures. Figure 3 showed the degradation profile of Fe-CMC and Fe-pectin in presence of cellulase (hydrolyzing of the 1,4-β-D-glycosidic linkages in cellulose)[34] and pectinase (hydrolyzing (1→4)-α-D-galacturonan methyl ester and α-1,4-glycosidic linkages)[35]. Alginate lyase (hydrolyzing the (1-4)-β-D-mannuronate linkages)[36] was shown to have low activity towards Fe-alginate, only < 15% degradation after 4 days, possibly due to the strong crosslinking from Fe^{3+} ions. Degradation of Fe-CMC (Figure 3a) showed a linear profile over 24 h and reached around 80% degradation. In contrast, an almost complete degradation for Fe-pectin was achieved in 8 h (Figure 3b). Although the enzyme concentration used in both experiment was roughly the same (1 mg/mL or 1 U/mL), it is difficult to directly compare between these enzymes since they have different activity profile towards their substrates.

The non-enzymatic degradation profiles of Fe-CMC and Fe-pectin were also shown in Figure 3. In absence of cellulase, Fe-CMC did not release any Fe^{3+} ions while Fe-pectin showed some release (up to ~15%). This is due to possible ion exchange with Na^{+} ions from buffer. No such ion exchange was observed from Fe-CMC if the crosslinking due to Fe^{3+} is stronger. This could also contribute to the slower degradation rate under an enzymatic action. Results from this set of experiments indicate that Fe-CMC could be more suitable for long-term release of Fe^{3+} than Fe-pectin and this could be the same case for Ga^{3+} loaded polysaccharides.

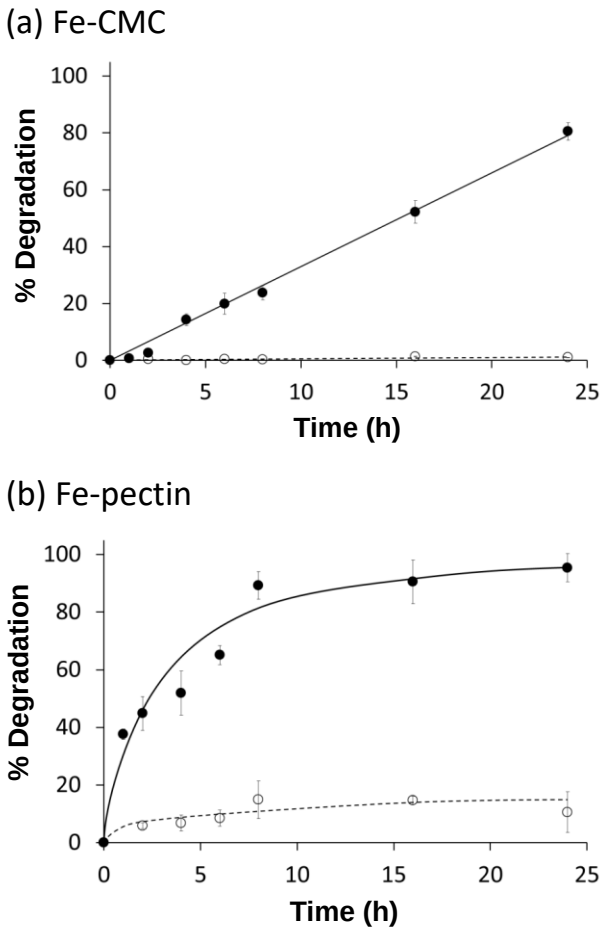

Figure 3. Degradation profile for (a) Fe^{3+}-CMC in 1mg/mL cellulase and (b) Fe^{3+}-pectin in 1 mg/mL pectinase. The dashed lines are control experiments in buffer with no enzyme.

3.3 Cell viability after exposure to the compounds

In this study, the cell viability of HDNFs after exposure to extracts of both metal loaded and the base materials was used as an indicator of the potential cytotoxic effects of the developed compounds. The Resazurin assay chosen for this study allows evaluation of the metabolic activity of cells as an indicator of cell viability and it is widely used to extrapolate the toxicity of natural polymer materials (e.g. silk and alginate). The three chosen polysaccharide materials (pectin, Na-CMC and alginate sodium salt) have been generally accepted as safe for human administration. Alginate is generally regarded as having good biocompatibility, evidenced by over 30 years of use as a hydrogel material for cell growth, tissue engineering[38] and wound healing[39]. Carboxymethyl cellulose is commonly used in food and pharmaceutical industries[40], and has also been exploited for preparing hydrogels for tissue engineering[41] while pectin showed a wide range of biomedical applications[42] However, cytotoxic effects have been reported for gallium, depending on both the gallium moiety and the anionic component[43] and iron, in a tissue and dose dependent manner[44]. Therefore, it was essential to investigate if the inclusion of metal components in the formulations conferred these compounds a cytotoxic character, by analysing the effect of their extracts on cell viability. Figure 4 shows that the extraction procedure had no interference with the ability of the culture medium to provide the adequate conditions for cell growth. Compared to the negative control, the base polysaccharide materials caused a reduction in cell viability that ranged from approximately 60% in the case of Na-CMC ($P \leq 0.0001$), 42% for pectin ($P \leq 0.05$) and 31% for Na-alginate. However, no statistical significance was found between Na-alginate and the negative control ($P \geq 0.05$), and since the obtained viability is around 70%, this compound showed no

cytotoxic effects towards HDNFs. Ga-based compounds showed a similar pattern. Cell viability downturned around 65% in cells incubated with Ga-pectin extracts ($P \leq 0.0001$), 50% with Ga-alginate ($P \leq 0.001$) and 34% with Ga-CMC. Again, Ga-CMC showed no statistical difference in comparison with the control ($P \geq 0.05$), and the viability value bordering 70% indicates no cytotoxic effects. Fe-based samples displayed a very different outcome. Whereas no viability decrease was detected in the samples incubated with Fe-CMC samples ($P \geq 0.05$), a major decline (84%) was observed when the cells were incubated with Fe-pectin ($P \leq 0.0001$) and Fe-alginate ($P \leq 0.0001$), in which case there was 0% of cell survival. These results show that at 50 mg/mL, Fe-pectin and Fe-alginate present a very strong toxic effect on HDNFs. Despite the statistical significance that was found in some cases by comparing each formulation used at 50 mg/mL with the control group, the same was not always verified for apparently different viability values, when multi-comparisons were performed using all the sample groups. The detailed results of a Tukey's multi-comparisons test performed to assess the differences between the samples at 50 mg/mL and the negative control can be consulted on Table S1. From 5 mg/mL and below, the viability of HNDFs, when compared to the control group, was not compromised by exposure to any of the extracts, ($P \geq 0.05$). These results show that the cytotoxic effects of Ga and Fe-based formulations are concentration dependent. Concentrations as high as 50 mg/mL pose considerable limitations to cell survival and growth.

There are several routes by which cell toxicity can be mediated, namely the catalysis of highly reactive oxygen radicals formation in iron mediated toxicity[45], and enzymatic reactions that inhibit deoxyribonucleotide synthesis, partially responsible for the antineoplastic activity of gallium[46]. However, in this study, it is likely that the viability drops found using the highest concentration may have a strong contribution from the

rheological properties of the base polysaccharide used to develop the final formulations, other just than from Ga and Fe. At high concentrations, these materials form gels[37], and in this case, caused the gelation of the culture media (see Figure S2). Gelation reduces the supply of nutrients and oxygen to the cells, leading to death, and that was particularly noticed on Na-CMC samples. Decreasing the concentration to 5 mg/mL, not only reduced the amount of Ga and Fe available for cellular uptake, but also drastically decreased the viscosity of the culture media, resulting in a non toxic effect for all the tested formulations. Fe^{3+} ions are required for biological function, and usually considered as biocompatible, yet, when present at high levels, they can disrupt iron homeostasis and become cytotoxic[47]. On the other hand, Ga-based materials have now been exploited in many biomedical uses, including use as a tracer in nuclear medicine, as a treatment for various cancers[48] and for non-Hodgkin's lymphoma[49]. Therefore, materials capable to deliver Ga such as these demonstrated here should be considered for these therapies. As with most pharmacological agents, the safe applicability of these compounds will rest on the delicate balance between the concentration that is necessary to produce a therapeutic effect, and the toxic effects induced at that concentration. In the particular case of this study, given the obtained cytotoxicity profiles, the safe use of the developed compounds will rely on the minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) found for target bacteria. The results obtained here show that if MIC and MBC ≤ 5 mg/mL these compounds can be safely used as antimicrobial agents without inducing toxicity in human primary fibroblasts of dermal origin, HDNFs.

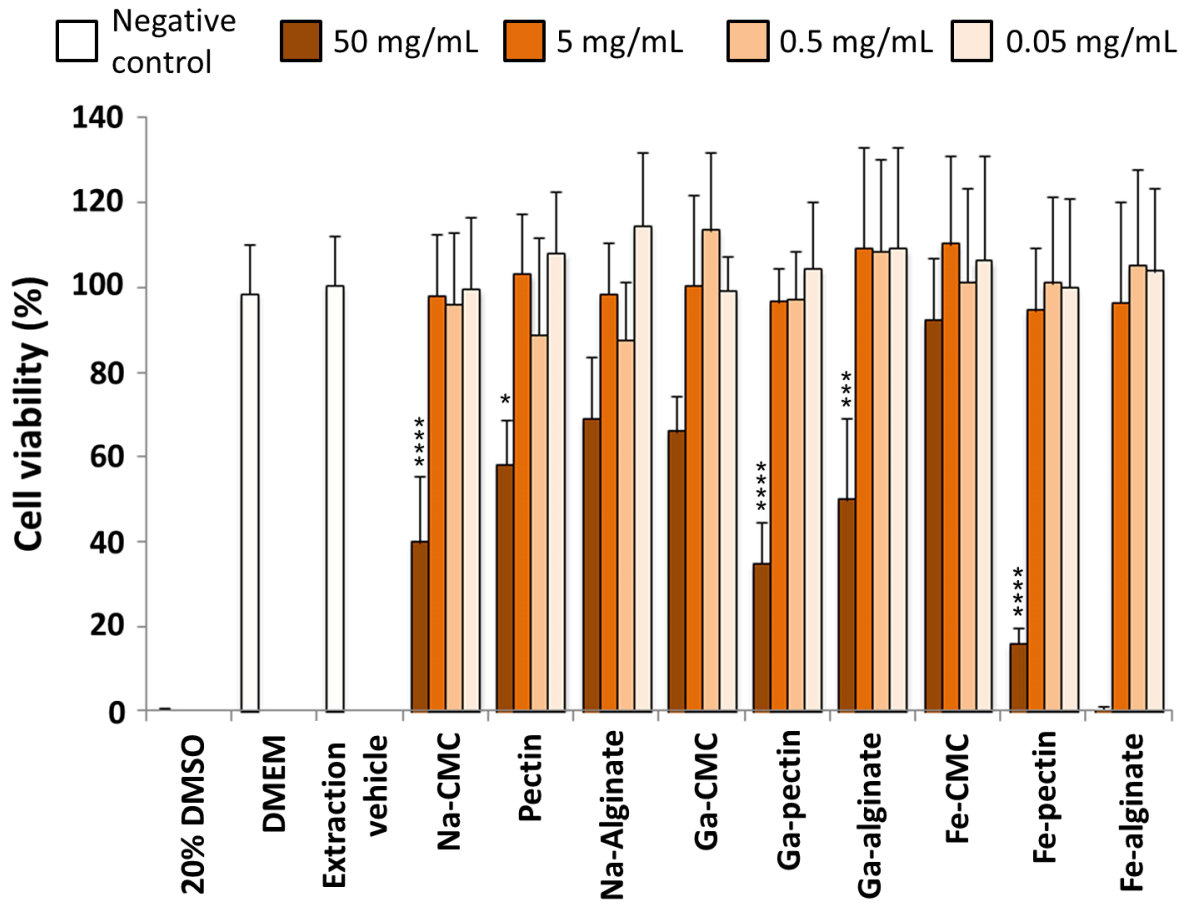

Figure 4. Viability profiles of HNDFs after 24 h exposure to the extracts of test compounds. Results are averaged from triplicate samples of three independent replicates. Statistical significance was established using a One-way ANOVA test, followed by Tukey's multiple comparisons test. * indicates significant differences ($P \leq 0.05$), ** indicates very significant differences ($P \leq 0.01$), *** ($P \leq 0.001$) and **** $P \leq 0.0001$) represent extremely significant difference, when compared to the negative control.

3.4 Minimum Inhibitory and Minimum Bactericidal Concentrations (MIC and MBC)

Results indicate that each test organism presents a unique susceptibility profile, when compared against compounds without the attached metal of interest (Table 2). In general, Na-CMC showed no observable degree of inhibition on bacterial growth even at a high concentration (> 10 mg/mL) while pectin and Na-alginate exhibited no effect on all three bacteria. Ga-CMC showed significant inhibitory effects on all three tested bacteria but Ga-alginate had no effect. Ga-pectin showed good inhibitory effect only on *P. aeruginosa*, but not on other test species. Among the Fe samples, both Fe-CMC and Fe-pectin showed some inhibitory effect on *S. aureus* growth but Fe-alginate had minimal demonstrable effect on all three bacteria. When compared among the three polysaccharide base materials, alginate samples showed the least inhibitory effect on all three bacteria. This can be linked to the slowest degradation of the samples as tested in Section 3.3. Ga-CMC seemed to be the most promising candidate for developing as an antibacterial agent.

Table 2. Minimum Inhibitory and Bactericidal Concentrations (MIC and MBC, respectively) for each of the test organisms, against the test metal compounds bound to polysaccharide, and the base polysaccharides as a control (X indicates no macroscopic inhibition of growth). All experiments were triplicated (n=3).

Bacteria		***Pseudomonas aeruginosa***		***Escherichia coli***		***Staphylococcus aureus***	
Sample tested		MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Non-reactive sample	Na-CMC	>10	>10	>10	>10	>10	>10
	Na-alginate	X	X	X	X	X	X
	Pectin	X	X	X	X	X	X
Gallium sample	Ga-CMC	0.625	2.5	2.5	>10	1.25	2.5
	Ga-alginate	X	X	X	X	X	X
	Ga-pectin	0.625	0.625	10	10	10	10

Iron(III) sample	Fe-CMC	10	10	>10	10	2.5	>10
	Fe-alginate	X	X	X	X	10	10
	Fe-pectin	5	5	10	10	1.25	1.25

These results, in combination with the viability study (Figure 4), indicate that Ga-CMC, Ga-pectin, and Fe-Pectin are very promising antimicrobial agents with no apparent toxicity to primary human dermal fibroblasts at MIC and MIB (≤ 5mg/mL) for *P. aeruginosa.* For *S.aureus*, the results were very similar, however, MBC using Fe-CMC was >10 mg/mL, and this study cannot guarantee that concentration will be safe for human cells. Regarding *E. coli*, only the MIC for CMC was lower than 5 mg/mL. The viability study has shown that for sample concentrations ≥ 50 mg/mL a marked toxic effect is observed in HDNFs, and for concentrations ≤ 5 mg/mL no toxic effects are observed. However, there is one order of magnitude between these two values, and it would be useful to study a narrower concentration range, in order to obtain the LC50 and to investigate the safety of these compounds at 10 mg/mL.

3.5 Action of Ga-CMC on bacteria in anaerobic conditions

Because of the physiological relevance of oxygen levels, a separate experiment was carried out to investigate the effect of reduced oxygen level in atmosphere on the MIC of Ga-CMC. It was reported that the oxygen levels in infection usually are much lower than ambient atmosphere[50]. For example, it is about 5% O_2 equivalent in perfused tissues while a near anaerobic condition is found in sites of inflammatory damage. On balance, Ga-CMC is the most promising materials testing in the MIC/MBC assay. *E. coli* is facultatively anaerobic and is resilient in low O_2 environment, hence it is an ideal model organism for this purpose. The wild-type *E. coli* strain P4 was used for this experiment as we have previously carried out thorough investigation of its sensitivities

to Ga as chloride and nitrate salts under aerobic and anaerobic conditions.[51] The MIC for Ga-CMC under anaerobic conditions was found to be 10 mg/mL, compared with 0.625 mg/mL in aerobic conditions. In a separate study on gallium salts (gallium nitrate and gallium citrate) with the same P4 strain carried out in our laboratory, similar results were also observed. Under aerobic conditions, the MIC values are 0.04 and 0.08 mg/mL for nitrate and citrate respectively. Both MIC values elevated to 0.08 and 0.32 mg/mL when the experiment was carried out in anaerobic conditions. Such an elevated MIC (up to x 20 times) is consistent with data reported for other Ga compounds on *E coli* (our data[51] and data reported from literature[52]), as well as other bacterial species[53,54]. Under reduced oxygen levels, *E coli* substantially alters gene expression and metabolism. These adaptations are reflected in the higher concentration of antimicrobial agent that is required to inhibit its growth.

Although the gallium compounds evaluated in this study seem to have potential to be developed as bioresponsive antibacterial agents, which is in urgent need globally, further research is required to unfold the mystery of their interaction with bacterial cells, both in aerobic and anaerobic environment, and the key mechanisms in growth inhibitory. Another area of interest is the delivery and the controlled release of gallium. The use of polysaccharides allows gradual release of gallium, which is difficult to achieve using soluble compounds such as gallium nitrate and maltolate but use of other ligands should also be investigated. Biodegradable polymers such as polysaccharides can also deliver, or co-deliver, other antibacterial agents, hence widening their applications. Using these platforms for delivering positively charged antibacterial agents such as tobramycin, polymyxin B, colistin and nisin is an attractive prospect. In addition to antibacterial properties, gallium compounds have also shown

other medical uses including anticancer properties. This makes biomedical research on gallium compounds very attractive.

The WHO have indicated that the proportion of *E. coli* and *S. aureus* reported as being resistant to commonly used agents is increasing. A key concern for *E. coli* is resistance to fluoroquinolones and third or later generation cephalosporins as well as carbapenems, whilst *S. aureus* resistance to beta-lactams is also of prime importance as these antibiotics are routinely used in human treatments[55]. Our data suggests that the bacteria responded to each sample in a species-specific manner. There is potential for these samples to be developed against specific target species, and therefore against specific infections. This could be an advantage when considering the need to deliver site- and species-specific therapy to a patient whilst minimising the potential for resistance to develop amongst co-existing flora. It also suggests that the growth response will be species dependant, and not simply effective against all Gram negative or Gram positive species.

4. Conclusions

Ga-CMC has demonstrated antibacterial activity against the three test bacterial species: *E. coli, S. aureus,* and *P. aeruginosa*, and revealed to be the most promising formulation among all tested samples. The data presented in this work indicate that these compounds present an attractive alternative for the development of antimicrobial actives, in a species-dependent manner, using polysaccharides to deliver an antibacterial activity. Cytotoxicity work helps to further screen the suitability of each compound for clinical applications. By testing antibacterial active doses in a sensitive

cell marker, using commonly available *in vitro* assays, those that are tolerated by mammalian cells can be selected as candidates for future development.

We also conducted a study on the enzymatic degradation kinetics of these metal loaded polysaccharide samples, which have the potential to be developed as bioresponsive delivery agents of gallium and other antibacterial metal species. Our new approach using anaerobic assay also examination of antibacterial activity under more realistic physiological conditions. The results in this investigation direct the conclusion to gallium loaded materials as the preferred material for further investigation as a mammalian cell-viable antibacterial agent.

Supporting Information Available

The following files are available free of charge at XXX, including illustration of proposed Ga^{3+} binding sites for carboxymethyl cellulose, pectin, and alginate, fluorescent microscope images of cells during viability tests, and statistical analysis of cytotoxicity data.

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