Development of X-ray micro-focus computed tomography to image and quantify biofilms in central venous catheter models in vitro

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Bacterial infections of central venous catheters (CVCs) cause much morbidity and mortality, and are usually diagnosed by concordant culture of blood and catheter tip. However, studies suggest that culture often fails to detect biofilm bacteria. This study optimizes X-ray micro-focus computed tomography (X-ray µCT) for the quantification and determination of distribution and heterogeneity of biofilms in in vitro CVC model systems.

Bacterial culture and scanning electron microscopy (SEM) were used to detect Staphylococcus epidermidis ATCC 35984 biofilms grown on catheters in vitro in both flow and static biofilm models. Alongside this, X-ray µCT techniques were developed in order to detect biofilms inside CVCs. Various contrast agent stains were evaluated using energy-dispersive X-ray spectroscopy (EDS) to further optimize these methods. Catheter material and biofilm were segmented using a semi-automated MATLAB script and quantified using the Avizo Fire software package. X-ray µCT was capable of distinguishing between the degree of biofilm formation across different segments of a CVC flow model. EDS screening of single- and dual-compound contrast stains identified 10 nm gold and silver nitrate as the optimum contrast agent for X-ray µCT. This

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INTRODUCTION

The majority of hospital-acquired bloodstream infections are attributed to intravenous catheters (Blot et al., 2005). Infection of indwelling central venous catheters (CVCs) occurs in 5.3 per 1000 catheter days with the mortality rate reaching as high as 35% (Kuminsky, 2007; McGee & Gould, 2003; Mermel et al., 2001). Biofilms have been shown to form within 24 h after CVC insertion and therefore play a major role in catheter-related infection (CRI) pathogenesis (Donlan, 2001; Fux et al., 2003; Götz, 2002; Raad, 1998). Biofilms are formed from bacterial cells of either mono- or multi-species which attach and multiply on either biotic or abiotic surfaces (Flemming & Wingender, 2010). The attached biofilm produces an extracellular polymeric matrix/substance (EPS) which is composed of different components and accounts for over 90% of the dry mass of the biofilm (Flemming & Wingender, 2010).

Biofilms are notoriously difficult to treat due to antibiotic tolerance (Donlan & Costerton, 2002; Hall-Stoodley et al., 2004; Mah & O’Toole, 2001). Currently, there are no biofilm-specific bloodstream markers, so any bacteria isolated using standard agar-plate culture cannot distinguish between the biofilm and planktonic phenotype. The gold standard for CRI detection is the roll-plate culture method (Curtis, 2009; Kristinsson et al., 1989; Mermel et al., 2009). However, this method often elicits false negatives, due to its inability to isolate bacteria from within the lumen, or false positives, due to host flora contamination during device removal from the skin exit site (Raad et al., 1993; Sherertz et al., 1997; Zandri et al., 2012). Frequently, bacterial biofilms are identifiable in the absence of culture-positive results (Dobbins et al., 1999; Franson et al., 1984; Hachem et al., 2009; Raad et al., 1993).

Three-dimensional architecture of the biofilm is an important parameter to assess total biofilm biomass, heterogeneity and to determine the function of external and genetic contributors to biofilm development. Currently, the majority of direct detection methods for biofilms in catheters, such as scanning electron microscopy (SEM) or confocal laser scanning microscopy (CLSM) techniques are limited by the necessity of catheter removal from the patient for accurate diagnosis and by relatively small microscopic fields so that larger scale structures and patterns may be missed. They also require optically clear or direct line of sight for imaging and the penetration depth into the biofilm is limited to a few hundred micrometres. Optical coherence tomography (OCT) provides quick, larger scale 3D imaging and can penetrate further than confocal imaging (1–2 mm), but also generally requires an optically clear material (Li et al., 2016). In this study, we explore the possibility of using X-ray micro-focus computed tomography (µCT) for biofilm imaging at voxel resolutions of 2–4 µm on in vitro CVC models.

X-ray µCT, in conjunction with various heavy metal-based contrast agents, has been conducted in animal models. This permitted non-destructive, whole-volume imaging for comparative, developmental and quantitative studies of morphology, where contrast agents were used to distinguish organs and various tissues in these animals (Metscher, 2009). This method has also been used to detect biofilms on model porous media in previous studies (Davit et al., 2011; Iltis et al., 2011). Biofilms were successfully imaged in both studies when X-ray µCT was combined with contrast agents. Davit and colleagues used a lab-based µCT scanner (SkyScan1174) with a pixel resolution of 9 µm and a medical suspension of barium sulphate (Davit et al., 2011), whereas Iltis and colleagues performed experiments using a dedicated synchrotron beamline (8.3.2 beamline, Advanced Light Source) at a pixel resolution of 4.5 µm with 10 µm hollow silver-coated microspheres as a contrast agent (Iltis et al., 2011). Stains at high concentrations (or in this case, contrast particles) may not be specific enough to allow clear demarcation between individual cells, while spatial resolution might actually be sufficient (Thurner et al., 2005).

The goal of the present study was to further develop X-ray µCT methods in order to image and quantify bacterial biofilm in vitro within CVCs as a model of a radio-opaque clinically relevant device where biofilm is characteristically difficult to study. Due to the similar X-ray absorption characteristics of biofilms and water, the first step to facilitate biofilm detection within CVCs using X-ray µCT scanning was to identify a suitable contrast agent. Energy-dispersive X-ray spectrometry (EDS) was used to estimate which stain was retained, and candidate stains were chosen based on X-ray attenuation, ease of use and user safety. After the staining protocol was finalized, the biofilm-detection sensitivity of µCT was tested by growing Staphylococcus epidermidis in CVC sections and comparing biofilm identification by culture, electron microscopy and µCT.

METHODS

Bacterial strain and in vitro central venous catheter-flow biofilm model. Cultures of S. epidermidis ATCC 35984 (maintained in 1% peptone and 10% glycerol in phosphate-buffered saline (PBS) at
pH 7.2 and stored at −80 °C) were grown in tryptic soy broth (TSB, Sigma Aldrich, UK) for 12 h at 37 °C, in 5 % CO₂.

In order to establish the efficacy of µCT as a valid method for identifying biofilm in CVCs, preliminary work was carried out on in vitro CVC-lumen-flow model, developed to allow flow on the inside of the lumen only. A 60 cm length of CVC (5 French Lifecath PICC, Vygon, Swindon, UK) was attached to a peristaltic pump using a 25 cm length of silicone tubing (size 13, Masterflex tubing, Fisher Scientific, UK). The Lifecath PICC is a radio-opaque, polyurethane CVC indicated for use in patients requiring mid- to long-term IV therapy, with outer and inner diameters of 1.7 and 0.63 mm respectively, as measured microscopically. Half-strength TSB was pumped through the catheter at a flow rate of 1.13 ml min⁻¹ in a recirculating mode with a reservoir of 100 ml. The growth medium was inoculated with 5×10⁵ colony forming units (c.f.u.) per ml of S. epidermidis. The complete system was placed in a 37 °C incubator for 5 days. The tubing was flushed twice with sterile PBS every 12 h for 5 min and the spent medium replaced with fresh sterile growth medium by replacing the medium reservoir on a daily basis and every 12 h for 5 min and the spent medium replaced with fresh sterile media assessed at 24, 48, 72, 90 and 120 h via c.f.u. counts to ensure the system was not contaminated. After 5 days, the biofilm was gently flushed with sterile PBS to remove loosely adhered biofilm and planktonic cells, and stained with osmium tetroxide and uranyl acetate for 24 h each at room temperature under the reagent conditions outlined in Table 1. The catheter was cut into 2 mm sections and two sections from the tip outlet, the middle portion and the inlet were taken for imaging with SEM and X-ray µCT. The µCT 3D data-image datasets were used to quantify the number and volume of individual biofilm aggregates and the total volume of biofilm in each 2 mm segment using MATLAB first for segmentation, followed by quantification with Avizo Fire 7 (see supplementary materials and Fig. S1, available in the online Supplementary Material).

In vitro biofilm formation. While preliminary results demonstrated the ability of µCT to identify biofilms in CVC, in-human use safety considerations with regard to the stains utilized and their lack of in vivo applicability led investigations to be carried out into alternative contrast agents. In order to screen the various candidate stains for uptake in the biofilm using EDS, biofilms were grown on sterile 10 mm diameter circular glass-microscopy coverslips (Agar Scientific, UK). Each coverslip was placed in the well of a 24-well plate (Corning, Sigma Aldrich) and

Table 1. Various contrast agents screened with energy-dispersive X-ray spectroscopy (EDS) and the accompanying materials, concentration and procedures used

<table>
<thead>
<tr>
<th>Contrast agent (and metal)</th>
<th>Concentration</th>
<th>Solution</th>
<th>Procedure</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uranyl acetate (U)</td>
<td>2 %</td>
<td>50 % ethanol</td>
<td>Mix uranyl acetate with 50 % ethanol in the fridge for 12 h (Page, 1999)</td>
<td>Oxkem, Reading, UK</td>
</tr>
<tr>
<td>Osmium tetroxide (Os)</td>
<td>4 %</td>
<td>Aqueous</td>
<td>Crush sealed powder ampoule in 0.1 M phosphate buffer (pH 7.2) (Page, 1999)</td>
<td>Oxkem, Reading, UK</td>
</tr>
<tr>
<td>Phosphomolybdic acid (Mo)</td>
<td>10 %</td>
<td>Aqueous</td>
<td>Make saturated solution then filter</td>
<td>Sigma Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Phosphotungstic acid (W)</td>
<td>3 %</td>
<td>70 % ethanol</td>
<td>Prepare stock solution of 10 % aqueous phosphotungstic acid (PTA). Mix 30 ml of stock with 70 ml absolute ethanol to make 3 % PTA in 70 % ethanol. Adapted from Metscher (2009)</td>
<td>Sigma Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Iodine (I)</td>
<td>1 % (w/v)</td>
<td>Ethanol</td>
<td>Dissolve 1 g of iodine in 100 ml of absolute ethanol (Metscher, 2009)</td>
<td>Sigma Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>10 nm Gold (Au)</td>
<td>5.7×10¹²</td>
<td>Aqueous</td>
<td>Use at full concentration</td>
<td>BBI Solutions, Cardiff, UK</td>
</tr>
<tr>
<td>60 nm Gold (Au)</td>
<td>2.6×10¹⁰</td>
<td>Aqueous</td>
<td>Use at full concentration</td>
<td>BBI Solutions, Cardiff, UK</td>
</tr>
<tr>
<td>Silver nitrate (Ag)</td>
<td>3 %</td>
<td>Aqueous</td>
<td>10 % aqueous stock solution prepared by dissolving 10 g of AgNO₃ in 100 ml deionised (DI) water. Final solution, 10 ml of acetate buffer, 3 ml of aqueous AgNO₃ and 87 ml of distilled water was mixed by shaking. Adapted from Lackie (1996)</td>
<td>Fisher Scientific, Loughborough, UK</td>
</tr>
<tr>
<td>Acetate buffer (for AgNO₃ staining)</td>
<td>Aqueous</td>
<td>Acetate buffer (4.8 ml 0.2 M acetic acid and 45.2 ml 0.2 M sodium acetate made up to 100 ml with distilled water) made by mixing 1.2 ml glacial acetic acid in 100 ml distilled water. Sodium acetate by dissolving 1.64 g sodium acetate anhydrous in 100 ml distilled water. Adjust to pH 5.6. Adapted from Lackie (1996)</td>
<td>Sigma Aldrich, Dorset, UK</td>
<td></td>
</tr>
<tr>
<td>Hydroquinone reducing solution (for AGNO₃ staining)</td>
<td>1 %</td>
<td>Aqueous</td>
<td>1 g of hydroquinone and 5 g sodium sulphite (crystals) were mixed in 100 ml of distilled water. Adapted from Lackie (1996)</td>
<td>Sigma Aldrich, Dorset, UK</td>
</tr>
</tbody>
</table>
inoculated with 1 ml of *S. epidermidis* ATCC 35984 at a concentration of 5×10^5 c.f.u. ml\(^{-1}\) in TSB and incubated at 37 °C, in 5 % CO\(_2\), with media changes performed every 24 h.

In initial experiments to evaluate metal stains as potential contrast agents, a high-vacuum SEM for EDS analysis was used and the biofilm samples were grown for 3 days. While this period was adequate to generate enough biomass to assess the differences between stain retention within the biofilm, assays to screen combinations of 10 and 60 nm gold, silver nitrate and PTA utilized an environmental SEM (ESEM) with 5 days of biofilm growth. The increase-in-growth period was performed in order to generate more biomass as well as a mature and stable biofilm to more accurately represent a longer-term biofilm infection in the CVC.

After the biofilm growth period, the coverslips were rinsed with PBS and those designated for EDS were fixed overnight in 1 ml of general fixative [3 % glutaraldehyde, 4 % formaldehyde in 0.1 M piperazine-N,N-bis-2-ethanesulfonic acid buffer (PIPES) at pH 7.2] (Page, 1999). The remaining biofilm-coated coverslips were designated for culture to enumerate the biofilm (c.f.u. and assessment of sample-to-sample variability). Both fixed and non-fixed biofilm-coated coverslips were immersed in 1.5 ml of PIPES buffer for 10 min, twice, to remove planktonic and loosely adhered cells.

**Enumeration of coverslip biofilm c.f.u.** Coverslips were immersed in 1 ml of PBS and sonicated (JPL 8050 H Professional Ultrasonic Cleaner bath, Maplin, UK) for 180 s. Following sonication, the coverslips were removed, the PBS solution serially diluted and plated using the drop-plate method (Herigstad et al., 2001) onto tryptic soya agar (TSA) plates and incubated at 37 °C, in 5 % CO\(_2\) for 24 h.

**Contrast agent evaluation with energy-dispersive X-ray spectroscopy.** Candidate µCT contrast agents were chosen on the basis of those with electron-dense metals, which had previously been used in histology or to successfully stain bacteria and biofilms for transmission electron microscopy (TEM), since these will also be expected to provide good contrast for µCT (Table 1). EDS provides a semi-quantitative measure of the relative abundance of an element associated with the surface of a sample by exploiting the distinct ionization energies of each element. This technique measures the number and the energy of X-rays produced by an element at high-speed electron bombardment. Consequently, EDS would indirectly compare and give an estimation of which of the contrast stains would most likely absorb X-rays best (X-rays are absorbed by materials at a similar energy level to the X-ray energy). By measuring the area of the curve under the peaks corresponding to a particular metal in the various contrast agents, we were able to semi-quantitatively compare the contrast agents staining the biofilm.

SEM was used to make sure that the biofilms were comparable in terms of biomass and structure. Clean, non-biofilm-coated coverslips were used as a control to assess possible background staining of the coverslip glass. After rinsing the fixed biofilms in buffer, the samples and controls were rinsed in distilled water. The samples for phosphotungstic acid (PTA) and iodine staining were first put through an ethanol dehydration series of 30, 50 and 70 % ethanol for 10 min each. As PTA was dissolved in 70 % ethanol, no higher concentration was used. Samples for iodine staining were further ethanol washed in 95 % (twice) followed by absolute (100 %) ethanol. Thereafter, the samples were immersed in either the PTA or iodine–ethanol solutions and incubated at room temperature in a fume hood overnight. The other samples that were to be stained with aequous solutions were directly immersed in the contrast agent solution after fixing, and allowed to incubate at room temperature in a fume hood overnight. The samples stained with silver nitrate were covered with aluminium foil to prevent light reaction. After staining, the silver nitrate-stained samples were drained and 1 ml of freshly prepared 1,3-dimethoxy-2-propanol and reduced solution was added and left under aluminium foil for 1 min, followed by a distilled water rinse. While colloid size was not assessed, similar methodology generates colloids in the region of 11 nm, with a range up to 65 nm (Cassar et al., 2014; Pacioni et al., 2015). The osmium tetroxide- uranyl acetate- nano gold and silver nitrate-stained samples were washed twice for 10 min with 1.5 ml 0.1 M PIPES buffer. Phosphomolybdic acid and iodine-stained samples were washed twice for 10 min with 70 and 100 % ethanol, respectively.

For dual staining, after staining with the primary contrast agent, samples were rinsed twice with distilled water, followed by immersion in the secondary stain solution and incubated for a further 12 h. The nano gold- and silver-stained rinsed biofilm and control coverslips were washed through an ethanol series of 30, 50, 70, 95 and finally 100 % ethanol for 10 min each. Phosphomolybdic acid-stained samples were rinsed with ethanol 70 %, followed by absolute (100 %) ethanol. Ethanol-rinsed coverslips were mounted on 12.5 mm aluminium SEM specimen stubs (Agar Scientific, UK) with 12 mm carbon self-adhesive discs (Agar Scientific, UK). The samples were critical-point dried (Balzers, CPD 030) before forward and EDS analysis.

**SEM and EDS.** For the initial evaluation of traditional EM stains (Fig. 1), samples were coated with carbon to increase conductivity of the biofilm surface and facilitate SEM imaging. Usually, a more conductive coating such as gold–palladium is preferred, as it increases conductivity of the sample and therefore less charging artefacts from high-speed electron bombardment occurs. However, for high-vacuum EDS, a carbon coating was used so as not to interfere with metal peaks in the EDS spectra from the contrast agents. Thereafter, the samples were placed in an SEM (FEI Quanta 200, USA), equipped with a Genesis EDS detector (EDAX, USA) at high-vacuum, 1000 W working distance, accelerating voltage 20 keV, spot size 4.5 µm, pressure 10–5 torr at ×1000 magnification, with approximately 1000 counts per second (cps) and dead time around 20 %. The cps for each metal was normalized against the total counts of all elements.

For the analysis of dual stains (Fig. 2), a more optimized system utilizing an environmental scanning electron microscope (Phillips FEI XL30 ESEM) equipped with an NSS X-ray microanalysis EDS (Noran, Thermo Scientific, USA) was used. The environmental mode allowed us to omit the coating step and thus, interference with the EDS analysis from the coating. The critical-point dried samples were placed in the SEM in environmental ‘wet’ mode with low vacuum at 10 keV with spot size 4.5 µm, pressure 0.6 torr and count rate 5000–6000 cps and dead time 20 %. The counts collected by the detector in the EDS spectra were compared as weight percentages of the total count of all elements using a standard method by the NSS X-ray microanalysis software (Thermo Scientific, UK). Biofilm samples are imperfect for EDS analysis because the samples are not flat, polished and homogenous; therefore the results are semi-quantitative, and the normalized elemental weight percentage was used. Experiments were performed with triplicate biofilm and control samples for each stain.

Differences between the amounts of the various metals (as percentages) in the biofilm were statistically evaluated by comparing means using a two-sample, two-tailed t-test. Differences were considered significant for *P*<0.05.

**Biofilm growth in central venous catheter sections.** To demonstrate the efficacy of the primary candidate contrast agent from initial studies, assays growing static biofilms were performed to demonstrate the potential for this optimized µCT protocol to detect and quantify biofilms associated with clinical specimens. *S. epidermidis* ATCC 35984 biofilms were grown in 1 cm sections of a representative CVC (5 French Lifecath PICC). A sterile scalpel was used to cut the CVC into 1 cm sections, which were placed in individual wells in a 24-well plate (Fisher Scientific, UK) and inoculated via syringe with 1.5 ml of *S. epidermidis*. The inoculated sections were immersed in the remainder of the inoculum and incubated at 37 °C, in 5 % CO\(_2\) for periods of either 2, 12, 72 or 120 h (5 days) with media replacement every 12 h. Two triplicate sets of
Biofilms were grown at each time point. One set was designated for c.f.u. determination and the duplicate set was designated for biofilm detection by µCT. For c.f.u. analysis, at each time point, the CVC sections were removed from the growth medium and gently rinsed in PBS three times by pipette aspiration. Rinsed samples were further sectioned using a sterile scalpel into 1–2 mm transverse pieces to facilitate recovery of the bacteria from the surface. The sectioned pieces were then sonicated and c.f.u. counts performed as previously described with the data expressed as c.f.u. cm\(^{-1}\) length and cm\(^{2}\) of CVC.

**Optical profilometry of central venous catheters.** Optical profilometry, a non-contact interferometric-based method for characterizing surface topography, was used to establish the CVC material roughness to ensure that µCT processing excluded any CVC material surface roughness features from the biofilm. Optical profilometry (Alicona Infinite Focus, USA) was performed on the inner lumen surfaces from five sterile CVCs (5 French Lifecath PICC) using Nano gold and silver nitrate stains were pre-performed during the scans. Reconstructions were undertaken using a filtered-back projection algorithm implemented within XMReconstructor software package (Xradia, USA).

To segment biofilm from µCT scans, a series of image-processing steps were undertaken. First, global thresholding of µCT data was undertaken to generate a mask containing both biofilm and catheter information. Due to the poor contrast between biofilm and catheter, a novel segmentation tool written in MATLAB was used to separate the two constituents from the mask. The MATLAB algorithm (see supplemental materials) took individual 2D slices of masked data to identify the centre of the catheter and subtracted the wall thickness of the catheter at each slice location surrounding the central point; this left behind the biofilm information.

**Statistics.** One-way ANOVA at 95% confidence interval (CI) was used (Minitab 16, USA) to compare differences between the material surface roughness of the five CVCs measured by profilometry. The log-transformed data were normally distributed, as tested by the Ryan–Joiner test for normality (P<0.05).

To test the difference between the biofilm volume and percentage volume occlusion detected by X-ray µCT at the different in vitro biofilm growth periods, a two-sample two-tailed t-test at 95% CI was used.

The sensitivity of X-ray µCT to detect biofilm biomass and the correlation between the biofilm volume detected by µCT with c.f.u. was estimated by performing the Pearson correlation coefficient (www.socscistatistics.com/tests/pearson) between geometric (log\(_{10}\) transformed) c.f.u. and µCT in vitro biofilm volume data (from

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**Fig. 1.** X-ray µCT tomographs (a, d, f) and corresponding SEM micrographs (b, e, g) of *S. epidermidis* ATCC 35984 central venous catheter-(CVC) flow biofilm model samples, demonstrating the ability of this technique to differentiate the degree of biofilm CVC colonization. Inset (c) is a representative high magnification SEM of the biofilm within a CVC flow model showing the associated cocci of *S. epidermidis*. Scale bars, 1 mm, except inset (c) which is 5 µm.
samples stained by dual staining with nano gold and silver nitrate) at the various growth periods, thus covering the complete range of biofilm development stages from attachment and growth, to full maturation.

RESULTS

In vitro central venous catheter-flow biofilm model

Initial assays to observe the ability of μCT to identifying biofilms in CVCs were conducted on an in vitro flow model using osmium tetroxide and uranyl acetate to stain the biofilm. The CVC-flow biofilm model accumulated heterogeneous amounts of biofilm throughout the catheter (Fig. 1). The distal tip of the CVC had the most biofilm accumulation compared with the middle and transcutaneous/inlet sections. The mean volume of individual biofilm aggregates in the tip were significantly greater than those in the inlet section (Mann–Whitney test, CI at 95 %, P=0.0001) but there was no difference between the tip and middle sections (P=0.742, Fig. 2).

Coverslip and central venous catheter biofilm growth curves

The glass coverslip biofilms grown for 5 days had $8.8 \times 10^{7} \pm 1.7 \times 10^{7}$ c.f.u. cm$^{-2}$ (mean and 1 SD, n=3), whereas there were $1 \times 10^{6} \pm 2.4 \times 10^{6}$ c.f.u. cm$^{-2}$ (approximately $2.6 \times 10^{6} \pm 4.9 \times 10^{6}$ c.f.u. cm$^{-2}$) in the CVC biofilm after 5 days of growth. Biofilm growth within the CVC increased from 2 to 72 h, after which growth reduced at 5 days (120 h, Fig. S4). The maximum c.f.u. count was $3.3 \times 10^{7}$ c.f.u. cm$^{-2}$ after 72 h, which would equate to approximately $8.3 \times 10^{7} \pm 1.38 \times 10^{7}$ c.f.u. cm$^{-2}$ (mean and 1 SD, n=3) of inner surface.

Biofilm staining screen – high-vacuum SEM/EDS

The SEM micrographs of the coverslip biofilms used in the single-stain assay demonstrated significant biofilm growth (Fig. 3). All of the replicate biofilms were similar in extent and morphology, and none of the contrast agents used for staining caused any noticeable changes to the overall biofilm structure, as compared with the unstained control (Fig. 3a, b).

Osmium tetroxide, followed by phosphotungstic acid and silver nitrate (measured by the elemental composition of Os, W and Ag, respectively), provided the greatest metal uptake by the coverslip biofilm with signals of 23, 20 and 13 %, respectively (Fig. 3). The elemental proportion of osmium from the osmium tetroxide was significantly greater than metal uptake from all of the other stains apart from tungsten from phosphotungstic acid (P<0.05).

Biofilm staining screen – low-vacuum SEM/EDS

The scanning electron micrographs of the dual-stained biofilms were similar to those from the first run and consisted of clusters of cocci, interspersed by a monolayer of single cells (Fig. 4b). Again, after staining, the biofilms were similar in extent and morphology to the unstained control, regardless of the type of contrast stain (Fig. 4c–j). Importantly, EDS of the clean coverslip controls with no biofilm showed that the metals in single- and dual-staining preparations did not bind to the glass (Fig. 4a) and the...
the combination of silver nitrate and 10 nm gold gave the highest combined (Au + Ag) elemental weight percentage of 38.9±1.67 %, which was significantly greater than the other dual stains, which ranged between 26.3 and 27.0 % (P<0.05).

### Optical profilometry to measure central venous catheter roughness for automated segmentation

Optical profilometry revealed that the polyurethane Lifecath CVCs had an average roughness of 206±29.07 nm (n=15). There was no statistically significant difference between the roughness of any of the CVC sections (P=0.211). From the roughness measurement of the Lifecath PICC, we assumed that by applying a 2-pixel median (equivalent to a roughness element of 4 µm) filter during μCT image analysis, we would remove any CVC material, as surface asperities from the MATLAB-segmented biofilm.

### X-ray μCT of in vitro central venous catheter during biofilm development

Using an optimized staining protocol, utilizing 10 nm gold and silver nitrate, μCT with comparative SEM was used to assess biofilm formation in CVC in a static growth model. The time series of the progression of biofilm accumulation during biofilm development was quantified in terms of biofilm architecture is shown in Fig. S5 via a reconstructed tomographic image.

Although the sum volumes of biofilm were similar at any one time point (ranging from 1.87×10⁶ to 3×10⁷ µm³ after 2 h of growth, 3.45×10⁶ to 2.04×10⁷ µm³ after 12 h, 7.12×10⁶ to 3.52×10⁷ µm³ at 72 h, and finally, from 4.36×10⁷ to 4.71×10⁸ µm³ after 120 h of growth) for the negative control (clean catheter), there was almost no signal, suggesting minimal debris or background staining of the catheter wall (Fig. 5a, b). After 2 h, there were sparse single cocci and a small number of cell clusters of cocci observed in the catheter lumen (Fig. 5c, d). By 12 h, the monolayer had become denser, and the clusters were larger (between 10 and 20 µm) and more numerous (Fig. 5e, f). By 72 h, the biofilms were similar to those seen on the coverslips after the same growth period, and consisted of larger cell clusters over 100 µm in diameter, separated by interstitial channels in which there was a dense monolayer of cells (Fig. 5g, h). Finally, after 120 h, thick biofilm clusters of up to approximately 400 µm in length were seen protruding from the thinner base layer (Fig. 5i, j), with some bare patches, indicating biofilm sloughing. The level of detail afforded by X-ray μCT in terms of biofilm architecture is shown in Fig. S5 via a reconstructed tomographic image.

Fig. 3. Representative scanning electron micrographs of biofilms grown on glass slides, demonstrating that staining with different metals did not affect biofilm structure, in this example (a) osmium tetroxide and (b) phosphomolybdic acid. Scale bars, 10 µm. Graph shows the percentage of metal constituent of each stain (silver nitrate, Ag; osmium tetroxide, Os; uranyl acetate, U; iodine, I; phosphotungstic acid, Wo; and phosphomolybdic acid, Mo) retained by the biofilm measured by energy dispersive X-ray spectroscopy (mean and 1 SD, n=3). The samples stained with osmium tetroxide had the highest percentage of metal uptake, followed by phosphotungstic acid and silver nitrate.

spectra only gave peaks associated with the glass elements, silica, silicon, oxygen, sodium and aluminium (data not shown).

Of the low-vacuum single-stained biofilm samples, PTA measured as tungsten had the greatest metal uptake (or best X-ray signal) elemental weight percentage (25.70 ±0.83 %, n=3) compared with 60 nm gold particles and silver nitrate (measured as Ag) (6.94±1.09 % and 3.26 ±0.07 %, respectively, Fig. 4). When the dual stains were tested, 10 nm gold and silver nitrate provided the greatest metal uptake by the biofilm of all the single and dual stains by between 144 and 151 %. For the single stains, tungsten in PTA had an elemental weight percentage of 25.7±1.4 %, which was significantly higher than Ag (3.3 ±0.12 %) from silver nitrate or Au (6.94±1.89 %) as 60 nm particles (P<0.01).

There was no additive effect on metal proportion from any of the dual staining with PTA (P>0.05); however,
sections, the biofilm was composed of a small number of large aggregates, while others were composed of many smaller aggregates (Fig. 6a). Interestingly, over time, there was generally a decrease in the distribution of smaller clusters, suggesting the formation of a more confluent biofilm. The overall biofilm volume increased over time, as expected.

At each consecutive time point (apart from 12 and 72 h), there was a statistically significant difference ($P<0.05$) suggesting that µCT was able to detect the differences between the biofilm volumes at each time point. Between 12 and 72 h, the difference in sum biofilm volume was not statistically significant ($P=0.636$). When looking at this alongside the culture data, it could suggest that the biofilm cells were increasing, but that the total volume remained constant, whereas after 72 h, the cell numbers decreased, but the total volume increased.

The sum volume measured by µCT was higher at each point compared with the viable c.f.u. measured by enhanced culture through sonication and solid-agar plating. For culture, the maximum number of viable cells was seen at 72 h of growth, with a reduction thereafter.
(as discussed before). In contrast, the sum volume of the biofilm measured by µCT was \(5.3 \times 10^8 \pm 1.3 \times 10^7\) c.f.u. cm\(^{-2}\) after 72 h (approximately \(1.3 \times 10^8 \pm 3.24 \times 10^7\) c.f.u. cm\(^{-2}\) of inner surface) and \(2.1 \times 10^8 \pm 1.4 \times 10^7\) c.f.u. cm\(^{-2}\) (approximately \(5.3 \times 10^8 \pm 3.64 \times 10^7\) c.f.u. cm\(^{-2}\)) after 120 h of growth. Therefore, the total biofilm volume continued increasing, whereas the viable c.f.u. reached its maximum after 72 h. There was a strong positive correlation between biofilm biomass measured as log c.f.u. and the log of the sum volume of deposit from µCT (Pearson correlation coefficient \(R=0.9828\), \(R^2=0.9659\) and \(P=0.003\), Fig. 6b).

Fig. 5. Representative scanning electron micrographs (a, c, e, g, i) and corresponding X-ray µCT images (b, d, f, h) of catheter biofilms at various time points of 0 h (a and b), 2 h (c and d), 12 h (e and f), 72 h (g and h) and 120 h (i and j). (a and b) No bacterial attachment was observed at 0 h. (c and d) After 2 h, there were sparse single cocci and infrequent small clusters of cocci on the surface of the catheters. (e and f) By 12 h, the monolayer had become denser and the clusters were larger (between 10 and 20 µm) and more numerous. (g and h) After 72 h, the biofilms were similar to those seen on the glass coverslips after the same growth period and consisted of larger cell clusters separated by interstitial channels within which was a dense monolayer of cells. (i and j) At 120 h, the biofilm was denser in parts, but some bare patches were now evident, suggesting biofilm sloughing. Scale bars of all images, 1 mm, except inset images, where scale bars are 10 µm.

Fig. 6. The total volume of biofilm and the volume distribution of individual aggregates (a) and correlation between the biofilm c.f.u. as determined by culture (b) and in 2 mm sections of catheters from triplicate (A, B and C), independent experiments by X-ray µCT. (a) Although the sum volumes of biofilm were similar at any one time point for the replicate samples, there were sometimes notable differences in the size distribution of biofilm clusters inside the catheters. In some cases, the biofilm was composed of a small number of large clusters (i.e. 120 h A) while others were composed of many smaller clusters (i.e. 2 h C). (b) There was a good log–log correlation between biofilm c.f.u. and the volume of biofilm detected by µCT, as shown by linear regression of the log–log data (inset).
DISCUSSION

Preliminary assays using X-ray μCT were highly effective at detecting catheter-associated S. epidermidis biofilms in a CVC-flow model using osmium tetroxide and uranyl acetate as contrast agents. This method also demonstrated sufficient sensitivity to be able to detect differences in biofilm distribution throughout a CVC. Whilst the combination of osmium tetroxide and uranyl acetate was sufficient for biofilm detection providing acceptable contrast, due to toxicity issues, other stains were investigated as safer and more practical alternatives. For this work, EDS was used to semi-quantitatively measure the relative abundance of stain uptake into the biofilm as a measure of staining efficacy. All the samples containing biofilm showed significantly larger carbon peaks and smaller silica peaks. As a data example, the sample stained with 10 nm nano gold and silver nitrate showed a gold peak at 2.12 keV and a silver peak at 3.35 keV, showing that the stains were retained within the biofilm. The higher count rate seen in the control sample was because EDS has greater efficacy on smooth, polished samples; thus the X-rays received from ‘non-perfect’ biofilm samples do not give such high signals. For this reason the count rate could not be used and the data were analysed by element weight percentage. EDS determined that 10 nm gold and silver nitrate was the best suited of the stains tested as the contrast agent for detecting biofilms within CVCs.

Initially, single-metal contrast stains were tested and PTA outperformed nano gold and silver nitrate. However, because it is common practice to use more than one stain for enhancement in microscopy (Lackie, 1996; Lackie et al., 1985; Scopsi et al., 1986), we decided to apply two stains. Applying two stains resulted in higher signals, with 10 nm gold and silver nitrate producing the highest X-ray signal. 10 nm gold as a primary stain gave the same signal compared with 60 nm gold, and yet 10 nm gold resulted in 1.73 times as much silver as the samples with 60 nm gold as a primary stain. The difference may have been because more 10 nm gold particles could be deposited, creating a bigger surface area for silver deposition.

Other stains were considered for analysis but excluded for practical reasons. Barium sulphate was previously used by Davit et al. (2011), but due to the fact that samples were stacked on top of each other and scanned in duplicate for about an hour each, movement of barium sulphate was a high probability. Movement during scanning does not allow precise calculation of the centre of rotation, therefore inhibiting the 3D reconstruction of data. In addition, as EDS required ethanol dehydration and critical-point drying, a barium sulphate suspension would not have been possible. Potassium iodide was used in the infancy of testing X-ray μCT for biofilm detection within CVCs but did not provide sufficient contrast (data not shown). Iodine in ethanol was also tested using EDS, but again, did not result in a high count rate. Previous studies have also used silver-coated microspheres to provide contrast. The microspheres had an average diameter of 10 μm, which was considered too large and therefore was not considered for this study (Iltis et al., 2011). EDS analysis in this study used smaller 10 nm particles which, in combination with silver nitrate, were demonstrated to provide the greatest metal uptake by the biofilm and therefore were determined to be the most effective of the stains evaluated. Of potential benefit for future in vivo studies, silver nitrate is an inorganic chemical with antiseptic activity, which may potentially be of benefit if used for staining in a CVC lock (Peng et al., 2012).

While a limitation of the current study is the focus on a single bacterial species, albeit one of significant clinical importance in CRI management in S. epidermidis, X-ray μCT represents a non-specific method of bacterial identification which is not subject to species identification bias. Importantly, studies have demonstrated X-ray μCT identification of Gram-negative bacteria using silver microspheres as a contrast agent (Iltis et al., 2011), and multispecies water biofilms using barium sulfate and propidium iodide (Davit et al., 2011). Other studies have also used gold and silver nanoparticles to identify yeast species by surface-enhanced Raman scattering (Fakhrrullin et al., 2009), highlighting that these contrast stains, combined with X-ray μCT, are applicable across a wide range of potential pathogens. Importantly, also for clinical application, silver and gold nanoparticles also lend themselves to conjugation with oligonucleotides, which may allow bacterial-specific labeling capable of resolving bacteria from thrombus and other occlusions, which future studies into clinical application will need to address (Tauran et al., 2013). However, additionally, further studies are also required to investigate in vivo toxicity. While significantly less toxic than traditional contrast agents, such as osmium tetroxide, gold nanoparticle studies have raised concerns about biocompatibility and cytotoxicity, dependent on particle size, shape, charge and the cell type in question, although studies are contradictory, as with silver nanoparticles (Alkilany & Murphy, 2010; Tauran et al., 2013). Consequently, there is scope for future studies to further optimize this staining protocol to reduce the risk of adverse toxic effects for potential in vivo use.

Using this optimized staining method, we were able to detect catheter-associated biofilms after as little as 2 h post-inoculation, as corroborated by SEM, demonstrating a higher degree of sensitivity in the methodology in its capacity to detect early bacterial cell colonization of a substratum. At each time point we would expect X-ray μCT detection of total CVC contamination to be greater than the same assessment by c.f.u. culture, as the stain is not bacterial specific. Consequently, X-ray μCT would detect not only viable bacteria, as identified by culture, but non-viable bacteria as well as the biofilm matrix. However, there was no difference in the sum volume, as detected by μCT and c.f.u. at all time points, with the exception of 120 h. This suggests that total biovolume during biofilm development in the CVC at 2 h, 12 h and 72 h could be attributed to bacterial cell proliferation, whereas beyond this, at 120 h, matrix production predominated in the absence of bacterial cell proliferation as...
the CVC system reached carrying capacity (Lorenz & Wackernagel, 1994).

Similarly to Ilitis et al. (2011), analysis on the data was performed using Avizo Fire. In contrast, Davit et al. (2011) did not analyse their data and instead used the CT scans for qualitative purposes. We were able to capture data in our study at a higher resolution than both of these previous studies. The pixel resolution of the data presented was between 2.1 and 2.6 µm, whereas Ilitis et al. (2011) had pixel resolutions of 4.5 and 11.8 µm, despite the use of synchrotron sources. Davit et al. (2011) also used a benchtop CT system similar to the equipment used in this study, although they had a much lower pixel resolution of 12 µm. Often, however, the resolution is limited by the equipment and sample size, which also has implications for scan time, a limitation to this technique that future advances in technology may overcome.

This technology has been demonstrated as capable of imaging and quantifying biofilms in CVCs and other radio-opaque, clinically relevant surfaces (e.g. stents). It demonstrates high sensitivity, with the ability to identify the location and extent of infecting biofilm with increased sensitivity over existing culture methods, which are often associated with false-negative results due to difficulties culturing biofilm infections. X-ray µCT can be used qualitatively and quantitatively, and avoids user bias, which can often be attributed to 'line-of-sight' techniques such as confocal laser scanning microscopy and SEM. Additionally, this study also demonstrates that novel, less-toxic contrast stains are equally sensitive as the more conventionally used stains with higher cytotoxicity. Consequently, this work significantly advances the existing body of literature by illustrating the potential of harnessing X-ray µCT techniques to study bacterial biofilms via direct, non-invasive, non-destructive technology on medical devices, where biofilms are often difficult to study in situ. As such, X-ray µCT has future potential in the study of biofilms on medical devices and for clinical application for the diagnosis and assessment of biofilm formation.

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