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

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Abstract

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 optimises X-ray micro computed tomography (X-ray µCT) for the quantification and determination of distribution and heterogeneity of biofilms in an *in vitro* central venous catheter (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 optimise 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 negative contrast stains identified 10 nm gold and silver nitrate as the optimum contrast agent for X-ray µCT. This optimised method was then demonstrated to be capable of quantifying biofilms in an *in vitro* static biofilm formation model, with a strong correlation between biofilm detection via SEM and culture.

X-ray µCT has good potential as a direct, non-invasive, non-destructive technology to image biofilms in CVCs, as well as other *in vitro* medical components in which biofilms accumulate in concealed areas.

**Introduction**

The majority of hospital acquired bloodstream infections are attributed to intravenous catheters ([Blot *et al.*, 2005](#_ENREF_2)). Infection of indwelling central intravenous catheters (CVCs) occurs in 5.3 per 1000 catheter days with the mortality rate reaching as high as 35 % ([Kuminsky, 2007](#_ENREF_19); [McGee & Gould, 2003](#_ENREF_25); [Mermel *et al.*, 2001](#_ENREF_26)). Biofilms have been shown to form within 24 hours after CVC insertion and therefore play a major role in catheter related infection (CRI) pathogenesis ([Donlan, 2001](#_ENREF_7); [Fux *et al.*, 2003](#_ENREF_12); [Gotz, 2002](#_ENREF_13); [Raad, 1998](#_ENREF_33)). 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](#_ENREF_10)). 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](#_ENREF_10)).

Biofilms are notoriously difficult to treat due to antibiotic tolerance ([Donlan & Costerton, 2002](#_ENREF_8); [Hall-Stoodley *et al.*, 2004](#_ENREF_15); [Mah & O'Toole, 2001](#_ENREF_24)). 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](#_ENREF_4); [Kristinsson *et al.*, 1989](#_ENREF_18); [Mermel *et al.*, 2009](#_ENREF_27)). However, this method often elicits false negatives, due to the inability of this method 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](#_ENREF_32); [Sherertz *et al.*, 1997](#_ENREF_35); [Zandri *et al.*, 2012](#_ENREF_38)). Frequently bacterial biofilms are identifiable in the absence culture positive results ([Dobbins *et al.*, 1999](#_ENREF_6); [Franson *et al.*, 1984](#_ENREF_11); [Hachem *et al.*, 2009](#_ENREF_14); [Raad *et al.*, 1993](#_ENREF_32)).

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 (CSLM) techniques are limited by the necessity of catheter removal from the patient for accurate diagnosis and are limited 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 100 µm. Optical coherence tomography (OCT) provides quick larger scale 3D imaging and can penetrate further than confocal (1 - 2 mm) but also generally requires an optically clear material ([Li *et al.*, 2016](#_ENREF_22)). In this study we explore the possibility of using X-ray micro-focus computed tomography (µCT) for biofilm imaging at voxel resolutions of 2-4µm 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](#_ENREF_28)). This method has also been used to detect biofilms on model porous media in previous studies ([Davit *et al.*, 2011](#_ENREF_5); [Iltis *et al.*, 2011](#_ENREF_17)). Biofilms were successfully imaged in both studies when X-ray µCT was combined with negative contrast agents. Davit et al. (2011) used a lab-based µCT scanner (Skyscan1174) with a pixel resolution of 9 μm and a medical suspension of barium sulphate whereas Iltis et al. (2011) performed experiments at a dedicated synchrotron beamline (8.3.2 beamline, Advanced Light Source) at a pixel resolution of 4.5 μm using 10 µm hollow silver-coated microspheres as contrast agent. 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](#_ENREF_37)).

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 an 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 enable biofilm detection within CVCs using X-ray µCT scanning was to identify a suitable contrast agent. Energy dispersive X-ray spectroscopy (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 finalised, 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 & in vitro CVC-flow biofilm model*

Cultures of *S. epidermidis* ATCC 35984 (maintained in 1 % peptone and 10 % glycerol in phosphate buffered saline 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, 5 % CO2.

In order to establish the efficacy of µCT as a valid method for identifying biofilm 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 5 French PICC (60 cm of Lifecath polyurethane) was attached to a peristaltic pump using a 25 cm length of silicone tubing (size 13, Masterflex tubing, Fisher Scientific, UK. Half-strength TSB was pumped through the catheter at a flow rate of 1.13 ml/min in a recirculating mode with a reservoir of 100 ml. The growth medium was inoculated with 5 x 105 CFUs per ml of *S. epidermidis.* The complete system was placed in a 37oC 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 the spent media assessed at 24, 48, 72, 90 and 120 h via colony forming unit (CFU) 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 data sets 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 S3.).

*In vitro* biofilm formation

While preliminary results demonstrated the ability of µCT to identify biofilms in CVC, in-human use safety considerations with regards to the stains utilised and their lack of in vivo applicability led investigations to be carried out into alternative negative 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 inoculated with 1 ml of *S. epidermidis* ATCC 35984 at a concentration of 4.8 × 105 colony forming units per ml (CFU/ml) in TSB and incubated at 37 °C, 5 % CO2 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 utilised an environmental SEM (ESEM) with 5 days of biofilm growth. The increase in growth period was 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](#_ENREF_30)). The remaining biofilm coated coverslips were designated for culture to enumerate biofilm (CFUs and assess 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 CFUs

Coverslips were immersed in phosphate buffered saline (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](#_ENREF_16)) onto trypic soya agar (TSA) plates and incubated at 37 oC, 5 % CO2 for 24 h.

Contrast Agent Evaluation with EDS

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 ionisation energies of each element. This technique measures the number and the energy of X-rays produced by an element after high-speed electron bombardment. Consequently, EDS would indirectly compare and give an estimation of which of the contrast stains would more 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 which were to be stained with aqueous 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 hydroquinone reducing 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](#_ENREF_3); [Pacioni *et al.*, 2015](#_ENREF_29)). 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 with 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 SEM 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 to not 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, US) at high vacuum, 10 mm working distance, accelerating voltage 20 keV, spot size of 4.5, pressure of 10−5 Torr at ×1000 magnification with an approximate 1900 counts per second (cps) and dead time of around 20 %. The cps for each metal was normalised against the total counts of all elements.

For the analysis of dual stains (Fig.2.), a more optimised system utilising an Environmental Scanning Electron Microscope (Phillips FEI XL30 ESEM) equipped with an NSS X-ray Microanalysis EDS (Noran, ThermoScientific, 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 of 4.5 µm, pressure of 0.6 Torr and count rate of 5000 to 6000 cps and dead time of 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 normalised 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 (CVC) sections

To demonstrate the efficacy of the primary candidate negative contrast agent from initial studies, assays growing static biofilms were performed to demonstrate the potential for this optimised 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 central venous catheter (5 French Lifecath PICC, Vygon, Swindon UK). The Lifecath PICC is a radiopaque, polyurethane central venous catheter 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. 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, 5 % CO2 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 CFU determination and the duplicate set was designated for biofilm detection by µCT. For CFU 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 to 2 mm transverse pieces to facilitate recovery of the bacteria from the surface. The sectioned pieces were then sonicated and CFUs performed as previously described with the data expressed as CFU per cm length and cm2 of CVC.

Optical profilometry of CVC catheters

Optical profilometry, a non-contact interferometric based method for characterising 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, US) was performed on the inner lumen surfaces from 5 sterile CVCs (5 French Lifecath PICCs) using × 100 magnification. Three profiles were taken from each of the 5 catheters.

X-ray µCT for biofilm detection in CVCs

Based on the results from the EDS screen and safety handling considerations

dual staining was used in order to produce the best contrast of the biofilm for µCT scans. Nano gold and silver nitrate stains were prepared as described previously. The samples were then stained, washed and put through an ethanol dehydration series identical to the EDS glass slide preparation. The stained and ethanol washed *S. epidermidis* CVC samples were placed in carbon fibre reinforced polymer (CFRP) tubes (2 mm inner diameter, 4 mm outer diameter) to support multiple CVC sections vertically during imaging (Fig S1)*.* Up to 25 CVC sections were mounted one on top of the other within a single CFRP tube – this enabled the scans to be set up as an automated batch, with 2 individual specimens fitting within the field of view and scanned simultaneously (Fig S2).Carbon fibre was chosen due to the material stiffness and a relatively low X-ray absorption rendering them transparent with respect to the stained samples. The CFRP tubes were filled with absolute ethanol to prevent sample dehydration, avoid bubble formation and to allow better contrast than would be achieved with water ([Metscher, 2009](#_ENREF_28)). The CFRP tube was sealed and positioned in the scanner. High resolution scans for the CVC-flow biofilm were acquired using a custom-built Nikon Metrology HMX CT. All other scans were acquired with the faster Zeiss Xradia 510 Versa micro-focus CT system. The configuration used in this system comprised of a 160kVp source (Tungsten transmission foil target) together with scintillator and light microscope detector optics – effective pixel dimensions down to 70 nm are possible with true spatial resolution of 700 nm. The settings used in these scans were: 70 kVp, 86 µA, 4x objective, 1.5s exposure, 1601 projections, 1 frame per projection, source to object distance was 21 mm, source to detector distance 54 mm resulting in a voxel resolution of 2.6 µm. Pre-filtration with a 0.15 mm silicon dioxide filter was performed during the scans. 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. Reconstructions were undertaken using a filtered back projection algorithm implemented within XMReconstructor software package (Xradia, USA).

Statistics

One-Way ANOVA at 95 % confidence interval was used (Minitab 16, USA) to compare differences between the roughnesses of the 5 CVCs measured by profilometry. The log transformed data was 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 % confidence intervals was used.

The sensitivity of X-ray µCT to detect biofilm biomass and the correlation between the biofilm volume detected by µCT with CFU was estimated by performing the Pearson Correlation Coefficient (www.socscistatistics.com/tests/pearson) between geometric (log10 transformed) CFU and µCT *in vitro* biofilm volume data (from 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 fully mature.

**RESULTS**

*In vitro CVC-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 to the middle and transcutaneous/inlet sections. The mean volume of individual biofilm aggregates in the tip 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 CVC biofilm growth curves

The glass coverslip biofilms grown for 5 days had 8.8 x 10⁷ ± 1.7 x 10⁷ CFUs/cm2 (mean and 1SD, n = 3), whereas there were 1 x 10⁷ ± 2.4 x 106 CFUs/cm(approximately 2.6 x 107 ± 4.9 x 106 CFUs/cm2) 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 CFU count was 3.3 x 10⁷ CFUs/cm after 72 h, which would equate to approximately 8.3 x 107 ± 1.38 x 107 CFU/cm2 (mean and 1SD, n = 3) of inner surface.

Biofilm Staining Screen - High vacuum SEM / EDS

The SEM micrographs of the coverslip biofilms used in the single stain assays 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 to the unstained control [Fig. 3(a) & (b)].

Osmium tetroxide, followed by phosphotungstic acid and silver nitrate (measured by the elemental composition of Os, W and Ag respectively) provided the most 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.4(b)]. Again, after staining the biofilms were similar in extent and morphology to the unstained control, regardless of the type of contrast stain [Fig.4(c-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.4(a)] and the 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) element weight percentage (25.70 ± 0.83 %, n=3) compared to 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 the combination of silver nitrate and 10 nm gold gave the highest combined (Au + Ag) elemental weight percent of 38.9 ± 1.67 % which was significantly greater that the other dual stains which ranged between 26.3 and 27.0 % (P < 0.05).

Optical profilometry to measure CVC 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 statistical 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 CVC during biofilm development

Using an optimised staining protocol utilising 10nm 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 by SEM over the 120 h growth period is shown in Fig.5. In general the geometric characteristics of the biofilms in regards to size, distribution and location concentration corresponded well between the SEM micrographs and segmented μCT. This offers confidence that the μCT automated segmentation of the biofilm captured these details correctly. For the negative control (clean catheter) there was almost no signal suggesting minimal debris or background staining of the catheter wall [Fig. 5.(a)&(b)]. After 2 h there were sparse single cocci and a small number of cell clusters of cocci observed in the catheter lumen [Fig. 5.(c) & (d)]. By 12 h the monolayer had become denser and the clusters were larger (between 10 and 20 µm) and were more numerous [Fig. 5.(e) & (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. 5 (g) & (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. 5(i) and (j)], with some bare patches were observed 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.

Although the sum volumes of biofilm were similar at any one time point (ranging between 1.87 ×105 µm3 to 3 ×105 µm3 after 2 h of growth, 3.45 ×106 µm3 to 2.04 ×107 µm3 after 12 h, 7.12 ×106 µm3 to 3.52 ×107µm3 at 72 h and finally between 4.36 ×107 µm3 to 4.71 ×105 µm3 after 120 h of growth) for the triplicate repeats [Fig.6(a)], there were notable differences in the distribution of the sizes of biofilm clusters. In some sections the biofilm was composed of a small number of large aggregates while in others were composed of many smaller aggregates [Fig.6(a)]. 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 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 to the viable CFUs measured by enhanced culture by 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 x 10⁷ ± 1.3 x 107 CFUs/cm after 72 h (approximately 1.3 x 108 ± 3.24 x 107 CFUs/cm2 of inner surface) and 2.1 x 108 ± 1.4 x 107 CFUs/cm (approximately 5.3 x 108 ± 3.64 x 107 CFUs/cm2 ) after 120 h of growth. Therefore the total biofilm volume continued increasing whereas the viable CFU’s reached maximum after 72 h. There was a strong positive correlation between biofilm biomass measured as log CFU and the log of the sum volume of deposit from μCT [Pearson Correlation Coefficient R = 0.9828, R2 = 0.9659 and P = 0.003, Fig.6.(b)]

**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 negative contrast agents. This method also demonstrated sufficient sensitivity to be able to detect difference 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 which contained 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 seen 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 was analysed by element weight percentage. EDS determined that 10 nm gold and silver nitrate was 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 of common practice to use more than one stain for enhancement in microscopy ([Lackie *et al.*, 1985](#_ENREF_20); [Lackie, 1996](#_ENREF_21); [Scopsi *et al.*, 1986](#_ENREF_34)), 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 to 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 ([Davit *et al.*, 2011](#_ENREF_5)). 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](#_ENREF_17)). 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 stain of those 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](#_ENREF_31)).

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 as a technique is likely not to be dependent on the species. Importantly, other studies have demonstrated X-ray µCT identification of gram negative ibacteria using silver microspheres as a contrast agent ([Iltis *et al.*, 2011](#_ENREF_17)) and multispecies water biofilms using barium sulfate and propidium iodide ([Davit *et al.*, 2011](#_ENREF_5)). Other studies have also used gold and silver nanoparticles to identify yeast species by surface-enhanced Raman scattering ([Fakhrullin *et al.*, 2009](#_ENREF_9)) highlighting that these negative 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 labelling capable of resolving bacteria from thrombus and other occlusions which future studies into clinical application will need to address ([Tauran *et al.*, 2013](#_ENREF_36)). However, further studies are also required to investigated *in vivo* toxicity. While significantly less toxic than traditional negative contrast agents such as osmium tetroxide, gold nanoparticle studies have raised concerns about biocomptability 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](#_ENREF_1); [Tauran *et al.*, 2013](#_ENREF_36)). Consequently, there is scope in future studies to further optimise this staining protocol to reduce the risk of adverse toxic effects for potential *in vivo* use.

Using this optimised 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 colonisation 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 CFU 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 and bacteria as well as the biofilm matrix. However, there was no difference in the sum volume as detected by µCT and CFUs 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](#_ENREF_23)).

Similar to Iltis 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. The data reported in this study was able to be captured 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 Iltis et al. (2011) had pixel resolution 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 also been demonstrated to be capable of imaging and quantifying biofilms in CVCs and other opaque, clinically relevant surface (eg. stents). This technique demonstrates high sensitivity and the ability to distinguish between the degree of biofilm formation with increased sensitivity over existing culture methods which are often associated with false negative results due to. 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 scanning laser microscopy and SEM. Additionally, this study also demonstrates novel, less-toxic negative contrast stains are equally sensitive as the more conventionally used stains with higher cytotoxicity. Consequently, this work significantly advances the existing body of literature in terms of the possibility of harnessing X-ray µCT techniques to study bacterial biofilms via direct, non-invasive, non-destructive technology on medical devices where biofilm 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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Figure Legends

**Fig.1.** X-ray μCT tomographs (a,d,f) and corresponding SEM micrographs (b, e, g) of *S. epidermidis* ATCC 35984 CVC flow biofilm model samples demonstrating the ability of this technique to differentiate the degree of biofilm CVC colonisation. 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.

**Fig.2.** (a)Plot comparing the biofilm volume distribution of theX-ray μCT scanning CVC flow model samples. As demonstrated by the images in Fig.1., the tip section demonstrated the most biofilm colonisation compared with the middle and transcutaneous sections. (b) Bar chart showing the percentage occlusion of the CVC flow model biofilms scanned with X-ray μCT. The tip section of the CVC was more occluded at 11.6 % compared with the middle (0.016 %) and transcutaneous (0.004 %) sections.

**Fig.3.** Representative scanning electron micrographs of biofilms grown on glass slide 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.

**Fig.4.** Representative scanning electron micrographs of the glass slide biofilms (a-j) and relative proportion of metal uptake of gold and silver from single (white bars) and dual staining (grey bars) (k). (a) A representative micrograph of a clean slide stained (in this case stained with silver nitrate) showing no staining. (b) The unstained control showed that the biofilm was composed of cell clusters interspersed by a monolayer of single cells. Staining with (c) 60 nm gold (Au), (d) silver nitrate (Ag), (e) phosphotungstic acid (W), (f) 10 nm gold (Au) and silver nitrate (Au + Ag), (g) 60 nm gold and silver nitrate (Au + Ag), (h) 10 nm gold and phosphotungstic acid (Au + W), (i) 60 nm gold and phosphotungstic acid (Au + W) and (j) silver nitrate and phosphotungstic acid (Ag + W) did not affect the biofilm structure. Scale bars = 20 μm. k) Percentage of the metal constituent of each stain retained by the biofilm measured by energy dispersive X-ray spectroscopy (mean and 1 SD, n=3). The 10 nm gold and silver nitrate had a significantly greater metal uptake by weight percentage than the other stains (\* indicates P < 0.05).

**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 & b), 2 h (c & d), 12 h (e & f), 72 h (g & h) and 120 h (I and j). (a & 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 in which there 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 CFUs 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 sometime 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 hrs A) while in others were composed of many smaller clusters (i.e. 2 hrs C). (b) There was a good log-log correlation between biofilm CFUs and the volume of biofilm detected by μCT as shown by linear regression of the log-log data (inset).

**Table I.** Various contrast agents screened with EDS and the accompanying materials, concentration and procedures used.