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ORIGINAL ARTICLE

Impregnation of catheters with anacardic acid from cashew nut shell prevents *Staphylococcus aureus* biofilm development

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anacardic acid, antimicrobial agent, biofilm, catheter, staphylococcus.

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Abstract

Aim: The effect of anacardic acid impregnation on catheter surfaces for the prevention of *Staphylococcus aureus* attachments and biofilm formations were evaluated.

Methods and Results: Silicon catheter tubes were impregnated using different concentrations of anacardic acids (0·002–0·25%). Anacardic acids are antibacterial phenolic lipids from cashew nut (*Anacardium occidentale*) shell oil. Anacardic acid-impregnated silicon catheters revealed no significant haemolytic activity and were cytocompatible against fibroblast cell line (L929). Sustained release of anacardic acids was observed for 4 days. Anacardic acid-impregnated silicon catheters efficiently inhibited *S. aureus* colonization and the biofilm formation on its surface. The *in vivo* antibiofilm activity of anacardic acid-impregnated catheters was tested in an intraperitoneal catheter-associated medaka fish infection model. Significant reduction in *S. aureus* colonization on anacardic acid-impregnated catheter tubes was observed.

Conclusions: Our data suggest that anacardic acid-impregnated silicon catheters may help in preventing catheter-related staphylococcal infections.

Significance and Impact of the Study: This study opens new directions for designing antimicrobial phytochemical-coated surfaces with ideal antibiofilm properties and could be of great interest for biomedical research scientists.

Introduction

Catheter-related biofilm infections are considered to be a significant reason behind morbidity and mortality in patients requiring catheterization (Gahlot *et al.* 2014; Nicolle 2014). *Staphylococcus aureus* is the most common causative agent for the catheter-related biofilm infections (Esposito *et al.* 2013), which are very hard to eradicate using standard antibiotic regimens (Götz 2002; Otto 2008; Bhattacharya *et al.* 2015).

Staphylococcus aureus biofilms are community of sessile bacteria encased in an extracellular matrix called polysaccharide intercellular adhesion and consist of microbial cells, nutrients, polysaccharides, extracellular DNA and proteins (Vu *et al.* 2009; Kiedrowski *et al.* 2011; Arciola *et al.* 2015; Schwartz *et al.* 2016). Biofilm-embedded *S. aureus* are highly resistant to antimicrobial agents. Within the circulatory system, the host proteins such as fibrin, fibronectin, fibrinogen and collagen get deposited on the surface of the inserted catheters which is further enhanced by *S. aureus* biofilm formation (Archer *et al.* 2011; Donlan 2002, 2011).

Thus, to prevent the incidence of catheter infections, it is essential to inhibit the initial *S. aureus* attachment and the subsequent formation of biofilm on the surfaces of catheters (Bhattacharya *et al.* 2015). Several strategies to prevent biofilm formation on the catheters have been developed and clinically evaluated. To reduce the risk of

bacterial attachment and infection on catheter, antiseptics and antibiotics have been applied topically at the insertion site (Miller and O'Grady 2012). Lately, the utilization of antimicrobial flush solutions has been proposed (Raad *et al.* 2007). However, coating of catheters with antimicrobial agents are considered to be more protective against bacterial attachment and infection, particularly if both the internal and external surfaces of the device are coated (Hanna *et al.* 2006; Zhang *et al.* 2013; Singha *et al.* 2017). Currently, several types of antibiotic-coated catheters have been tested in clinical trials: vancomycin-, teicoplanin-, cefazolin-, silver-, chlorhexidine-/silver sulfadiazine- and minocycline-/rifampin-coated catheters (Kamal *et al.* 1991, 1998; Hannan *et al.* 1999; Farr 2001; Donlan 2011). Among these, minocycline-/rifampin- and chlorhexidine-/silver sulfadiazine-covered catheters appeared to reduce the rate of catheter-related infections (Yücel *et al.* 2004).

Recently, phytochemicals are showing substantial interest as leads for tracking new pharmaceutical agents. Among numerous antimicrobial plant compounds, anacardic acids, totarol, anthemic acid, ferulic acids, gallic acid and plumbagin were reported to possess antistaphylococcal activity (Muroi and Kubo 1996; Borges *et al.* 2013; Nair *et al.* 2016). The objective of the present research is to evaluate the potential of anacardic acid (AA; Fig. 1a) as antibiofilm agent on medical catheters. AA is phenolic lipids from cashew nut (*Anacardium occidentale*) shell oil and consists of a mixture of closely related compounds of a salicylic acid substituted with saturated or unsaturated 15–17 carbon alkyl chain. The safety of AA was previously tested in *in vivo* mice model where subacute dose of AA was found to be nontoxic till 300 mg kg^{-1} (Carvalho *et al.* 2011). Antimicrobial activity of AA has been demonstrated against *Propionibacterium acnes*, *Helicobacter pylori*, *Streptococcus mutans* and *S. aureus* (Muroi and Kubo 1993, 1996; Hollands *et al.* 2016). The bactericidal and antimicrobial activities of AA against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) have already been reported. AA has a minimum inhibitory concentration of 6·25 $\mu g\ ml^{-1}$ against *S. aureus* (Muroi and Kubo 1996). Here, we demonstrate that silicon catheter tubes can be easily be impregnated with AAs and the AA-impregnated catheters can be used to decrease the incidence of catheter-associated *S. aureus* biofilm infections.

Materials and methods

Phytochemicals

AA was purified from petroleum ether extract of cashew nut shell (CNSE) as described previously (Omanakuttan *et al.* 2012) and dissolved in dimethyl sulphoxide (DMSO). CNSE contains a mixture of three similar compounds viz. cardol, cardanol and AA. AA was separated from the other constituents by column chromatography (SiO_2) eluting with petroleum ether containing increasing proportions of chloroform. AAs containing fractions were analysed by HPLC (Phenomenex C-18 reverse phase column, Shimadzu LC-20, monitored at 245 nm). The analysis revealed that the AA fraction contains a mixture of monoene (1·3%), diene (18·3%) and triene (56·2%).

Bacterial strains and growth conditions

SA113-expressing (ATCC 35556) (Iordanescu and Surdeanu 1976) and SA113-expressing green fluorescent protein (SApC*tufgfp*) (Biswas *et al.* 2006) was grown in tryptic soy broth (TSB) overnight at 37°C in a shaking incubator at 120 rev min^{-1}. For biofilm assay, SA113 was grown under static condition at 37°C in TSB supplemented with 0·5% glucose (TSBG) for 48 h. All *S. aureus* clinical isolates were obtained from the Department of Microbiology, Amrita Institute of Medical Sciences and Research Center, Kochi, India. For antimicrobial activity assays, tryptic soy agar (TSA) plates were used.

Antimicrobial activity assays

Agar plate disc diffusion assay

Antimicrobial activity of AA was evaluated against SA113 and its clinical isolates. *S. aureus* clinical isolates were obtained from Department of microbiology, Amrita Institute of Medical Sciences, Kochi, India. Overnight bacterial cultures (100 μl) were spread-plated on TSA plates. Filter paper discs (0·5 cm diameter) containing 10 μg of anacardic acid per disc were placed on the agar plate. DMSO (10 μl) containing filter discs was served as negative control. Following which, the plates were incubated overnight at 37°C in an upright position and the zone of inhibition was measured (Nair *et al.* 2016).

Biofilm assay

To test the biofilm dissolution activity of AA, SA113 (pC*tufgfp*) was grown on coverslips. Coverslips were placed in 24-well microtitre plate containing TSB medium with 0·5% glucose (TSBG) and *S. aureus* were allowed to form biofilm for 48 h. Thereafter, wells were washed using phosphate buffer saline (PBS, pH7.2) and fresh TSBG medium was added. AA was added to wells to achieve a final concentrations of 2 (0·002%), 10 (0·01%), 50 (0·05%) and 250 (0·25%) μg/ml, and microtitre plate was incubated further at 37°C for 24 h. The coverslips were gently washed with PBS and photographed using fluorescence microscope (Biswas *et al.* 2006; Nair *et al.* 2016).

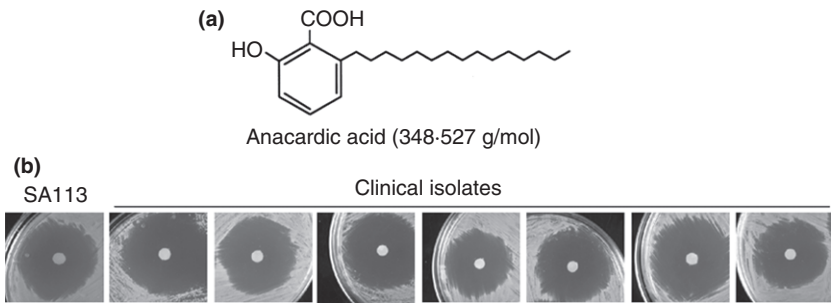

Figure 1 Antimicrobial activity of anacardic acid. (a) Structure of anacardic acid. (b) Agar plate disc (5 mm diameter) diffusion assay demonstrating antibacterial activity of anacardic acid (10 μg per disc) against SA113 and clinical strains.

Impregnation of AA on catheter tubes

The impregnation process was carried out as described previously (Fisher *et al.* 2015; Nair *et al.* 2016). Briefly, AA was diluted in chloroform to give concentrations of 0·002, 0·01, 0·05 and 0·25%. Silicon catheter segments 1 mm × 6 mm were immersed in the AA solutions for 1 h, during which the silicone swelled to approximately twice its volume. The catheter segments were then rinsed in absolute ethanol and then in deionized water. The catheter segments were dried for 24 h at 37°C. During evaporation of chloroform, the catheters returned to their previous dimensions and AA gets impregnated evenly throughout the catheter. The silicon catheter segments were then packaged and sterilized by ethylene oxide sterilization.

In vitro retention of antimicrobial activities of AA-coated catheter tube

100 μl of overnight bacterial cultures was spread-plated on TSA plates. Sterile AA-impregnated catheter tubes were embedded horizontally into the plates. The plates were incubated overnight at 37°C under static condition. Each consecutive day, the zones of inhibitions were measured and the catheter tubes were transferred to freshly inoculated plates. The process was repeated until no zones were observed (Sherertz *et al.* 1993; Bassetti *et al.* 2001). All experiments were carried out in triplicate.

Antibiofilm activity of AA-coated catheters

SA113 was grown in 1 ml TSBG medium overnight. AA-impregnated catheter tubes were added into the SA113 culture, and the tubes were incubated for 48 h at 37°C under static condition. Catheters were then lifted from the tubes with the help of a forceps, washed by dipping in PBS to remove loosely attached bacteria, and imaged using scanning electron microscope (SEM). Alternatively, catheter tubes were placed in 1 ml PBS and vortex for 20 min to detach the catheter tube-attached bacteria. Bacterial suspension was serially diluted and plated in TSA plates. The plates were incubated overnight at 37°C, and bacterial colonies were counted (Nair *et al.* 2016).

Cytocompatibility of AA-coated catheters

Haemolysis assay

5 ml of human blood sample was collected from healthy individuals and mixed with 750 μl of acid citrate dextrose to prevent clotting. This mixture was then diluted with PBS in the ratio of 1 : 10, and 500 μl of diluted blood samples was aliquoted in several 1·5-ml microcentrifuge tubes, and AA-impregnated catheter tubes were added to the centrifuge tubes. These tubes were incubated in 37°C incubator for 24 h. 0·1% (v/v) Triton X-100-treated blood sample was served as positive control. Following incubation, the tubes were centrifuged at 2050 × g for 20 min (MySpin12 Thermo Scientific Centrifuge, USA). Absorbance of supernatants was measured at 540 nm using microtitre plate reader (Synergy H1 Microplate Reader, USA), and the percentage of haemolysis was calculated (Maya *et al.* 2012; Kiruthika *et al.* 2015).

Cell culture

Mouse fibroblast cells (cell line L929) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) foetal bovine serum and 1% (v/v) penicillin–streptomycin at 37°C in 5% CO_2 incubator.

Cell viability

Using Alamar Blue assay, the cytocompatibility of the AA-coated catheter tubes was determined using L929 fibroblast cells (Rampersad 2012; Kiruthika *et al.* 2015; Nimal *et al.* 2016). Briefly, AA-coated catheters were incubated in 1·5-ml microcentrifuge tube containing 200 μl DMEM for 4 days at 37°C for the release of AA into the medium. L929 fibroblast cells were seeded at 8000 cells per well in a 96-well microtitre plate, and the plate was incubated at 37°C for 24 h. 200 μl DMEM containing AA was added to the 96-well plate and incubated

at 37°C for 24 h. L929 cells without any AA treatment were used as a positive control, and 0·1% (v/v) Triton X-100-treated L929 cells were served as negative control. After 24-h incubation, the medium from the wells was removed and wells were washed twice with PBS. 100 μl of 10% (v/v) Alamar Blue solution in DMEM was added to each well and incubated in CO_2 incubator at 37°C for 4 h. Using a micro plate reader (Synergy H1 Microplate Reader, USA), the colour changes in the Alamar Blue solution were quantified by measuring the absorbance at 570 nm and at 600 nm as reference wavelength; the percentage of L929 fibroblast cells' viability was calculated.

In vivo toxicity and efficacy assay in medaka fish

Maintenance

Medaka fish (*Oryzias latipes*) belonging to the QURT strain were obtained from National Bio Resource Project (NBRP), Japan. They were maintained and reared under laboratory conditions at 26–28°C and 60% humidity with 14-h light and 10-h dark cycles. The fish were fed three to four times daily with brine shrimp (Ocean Star International PRO 80, USA). All the experiments were conducted in strict compliance with the Institute Animal Ethics Committee guidelines after obtaining specific approval.

In vivo toxicity study

Four- to five-month-old male medaka (0·2 ± 0·06 g, 2·4 ± 0·15 cm) were used for the experiments. Fishes were anaesthetized in buffered 0·1% tricaine methane sulphonate (MS-222) before implantation (Chatterjee *et al.* 2017). AA-coated catheters were implanted into the intraperitoneal (i.p) cavity and observed for 14 days (six fish per group).

Catheter-associated biofilm assay

Catheter tubes were cut into small pieces (length 0·6 mm; diameter 1·5 mm) and sterilized by dipping into 70% isopropanol. Catheter tubes were washed with sterile deionized water and coated AA by dipping in 0·25% AA solutions, as described above. Fish were anaesthetized in buffered 0·1% MS-222, and abdominal wall of the fish into the intraperitoneal cavity was dissected. AA-coated catheter tube was implanted into the i.p cavity and inoculated with 2 μl of SA113 (six fish per group). Uncoated catheters were used as control. Peritoneal cavity opening was sealed with TruSeal (a skin adhesive for topical wound closure). After 48 h postincubation, the fishes were collected from each treatment group and euthanized by treating with overdose of MS-222. The catheter tubes were taken out, washed with sterile PBS to remove unbound bacteria and immersed into 100 μl of sterile TSB and vortex for 20 min. The bacterial suspensions were serially diluted and plated on TSA plates for selective isolation of SA113 *colonies*. Plates were incubated at 37°C overnight for colony formation (Chatterjee *et al.* 2017).

Statistical analysis

All the experiments were performed in triplicate, and results were expressed as mean ± SD. Statistical significance was calculated with two tailed nonparametric unpaired *t* test and using Graph Pad Prism ver. 5.04 (Graph Pad Software Inc., San Diego, CA). $P < 0{\cdot}05$ (*), $P < 0{\cdot}01$ (**) and $P < 0{\cdot}001$ (***) were considered statistically significant.

Ethical statement

All animal experiments were carried out after obtaining the approval from the Institutional Animal Ethics Committee, Amrita Institute of Medical Sciences, Kochi, India.

Results

AA inhibited the growth of *S. aureus*

Initially, the *in vitro* antimicrobial activity of AA was tested against *S. aureus* strain SA113 and its clinical isolates. Agar plate disc diffusion assay results demonstrated that AA inhibited the growth of SA113 and all clinical isolates (Fig. 1b). Apart from the antimicrobial activity, we have also tested the biofilm dispersion activity of AAs against *S. aureus* (Fig. 2). Preformed biofilms were incubated with increasing concentrations of AAs. Fluorescence microscopy images demonstrated the increased SA113 biofilm dispersion with increasing concentrations of AA (Fig. 2a,b). The biofilm dispersion was 40, 76, 80 and 99·96% when preformed biofilms were incubated with 0·002, 0·01, 0·05 and 0·25% AA solutions (Fig. 2c).

Development of AA-impregnated catheter tubes and its antimicrobial activity

We next attempted to coat catheter tubes with AA to prevent *S. aureus* biofilm development. Silicon catheter tubes were impregnated with AA prior to the biofilm assays (Fig. 3a). The release of AA from the catheter tubes was tested using agar plate disc diffusion assay (Nair *et al.* 2016). Sustained release of AA was observed for 4 days, and it reached to 100% on the fourth day (Fig. 3b). The zone of inhibition was found around the catheter tubes

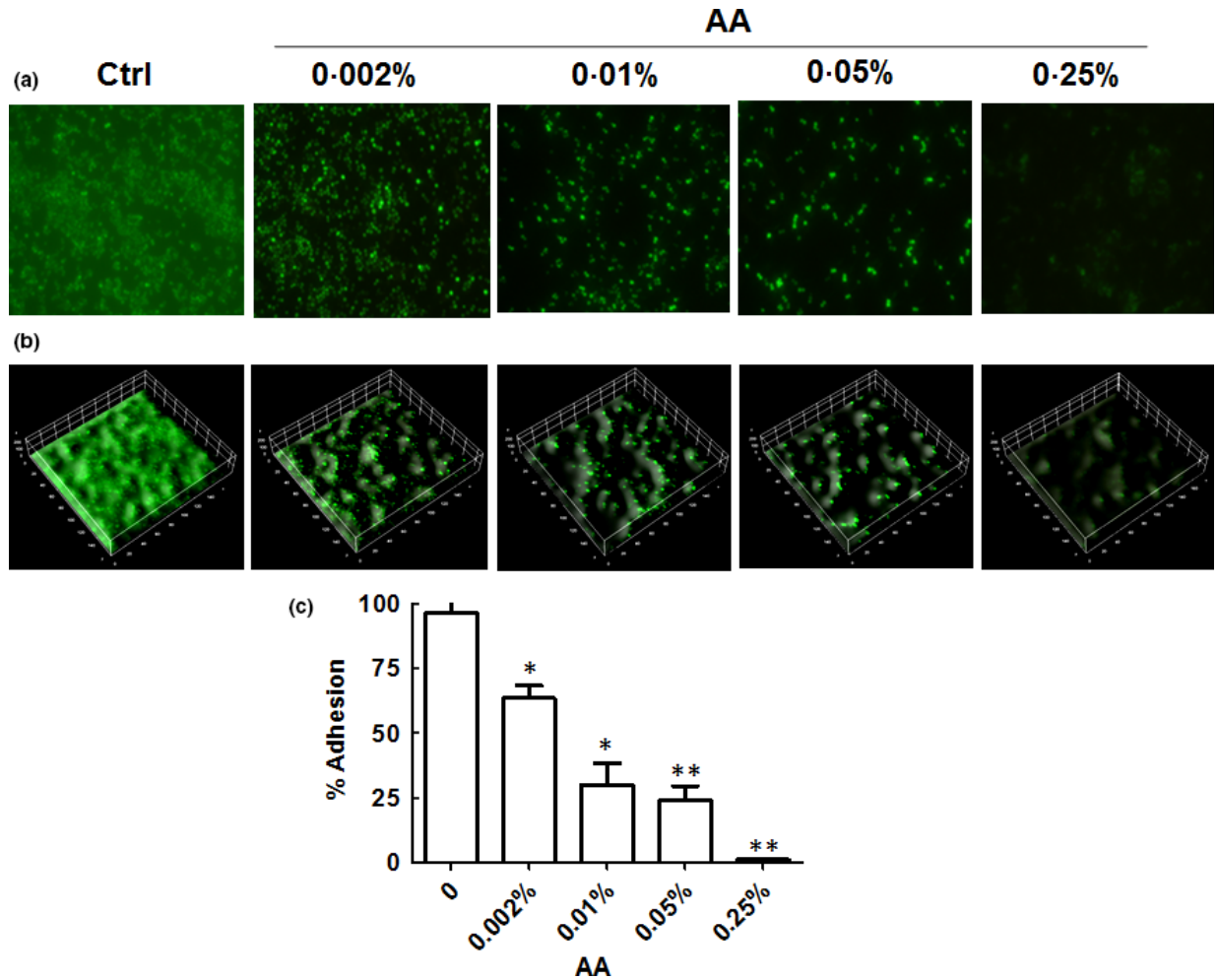

Figure 2 Biofilm dispersion activity of anacardic acid. (a, b) One-dimensional (a) and interactive three-dimensional (b) fluorescence microscopy images demonstrating dispersion of SA113(pC*tufgfp*) biofilms in the presence of different concentrations of anacardic acids. (c) Bar diagram represents adhesion of SA113 with glass surfaces after treatment with anacardic acids. *$P < 0{\cdot}05$, and **$P < 0{\cdot}01$. [Colour figure can be viewed at wileyonlinelibrary.com]

when placed in plates spread with *S. aureus* (Fig. 3c). Each consecutive day, the zones of inhibitions were measured and the catheter tubes were transferred to freshly inoculated plates. Gradual reduction in the zone diameter was observed in every consecutive day till the fourth day and after which no zones of inhibition were observed. Maximum growth inhibition zone was observed in the second day, indicating the highest amount of AA release took place in the second day (Fig. 3c,d). It was expected that gradual release of AA from the catheter surface hinders the biofilm formation of *S. aureus* on catheter surfaces and catheter tube that was coated with increased concentration of AA will cause more inhibition of biofilm development. SEM images confirmed that biofilm development of *S. aureus* on AA-coated catheter tubes was highly impaired (Fig. 4a,b). Microbial attachments were reduced by 20% in 0·002% AA-loaded catheter tubes, 60% in 0·01% AA-loaded catheter tubes and 80% in 0·05% AA-loaded catheter tubes compared to the control. There was no trace of SA113 biofilm on catheter tube that was loaded using a 0·25% AA solution (Fig. 4c). Our results showed that AA could be used for impregnation of catheter tubes to prevent *S. aureus* biofilm development.

In vitro and vivo toxicity assays

Haemolysis and cytotoxicity assays were carried out with different percentage of AA-coated catheter tubes. AA-coated catheters displayed no significant haemolytic (Fig. 5a,b) and cytotoxic activity (Fig. 5c). The erythrocyte lysis was below 5% when treated with AA-coated catheter tubes. The L929 fibroblast cell viabilities were

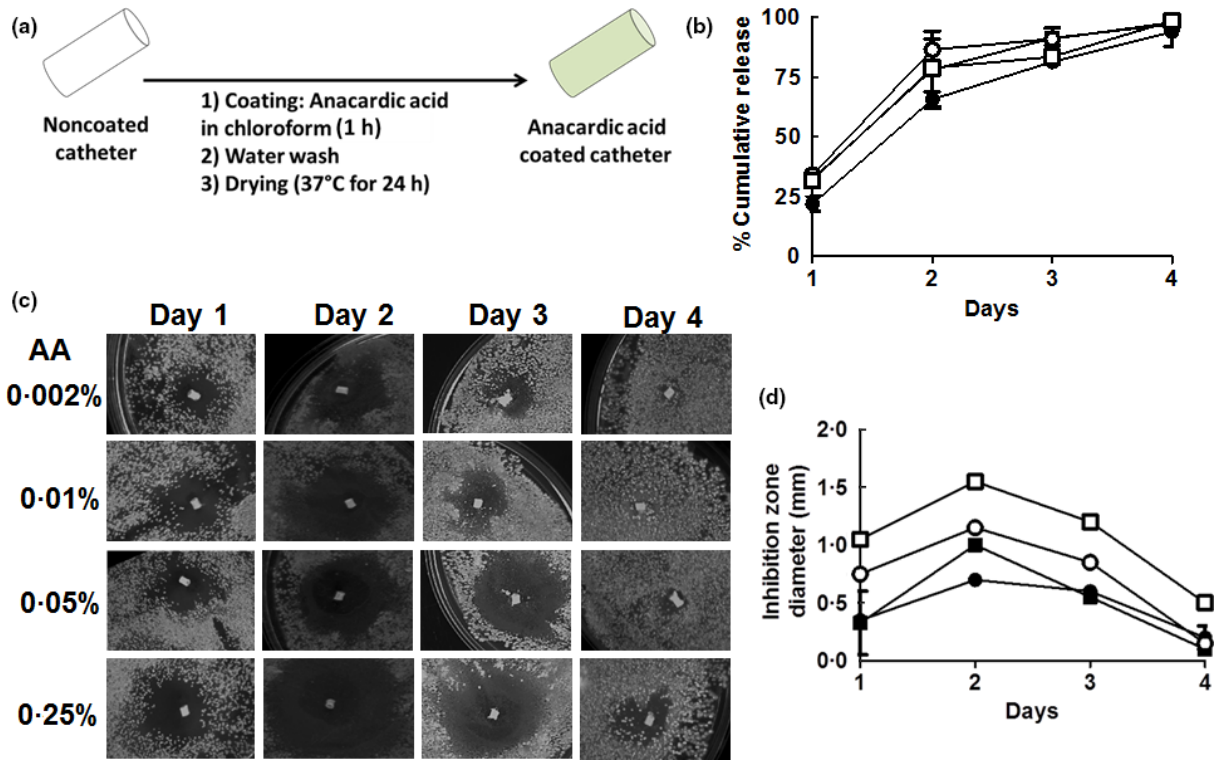

Figure 3 Development of anacardic acid-coated catheter and its antimicrobial activity. (a) Schematic representation of the method used to produce anacardic acid-coated catheters. (b) Cumulative release of anacardic acid from anacardic acid-coated catheter tubes. (c) Agar plate disc diffusion assays using AA-coated catheter segments against *Staphylococcus aureus*. (d) Graphical representation of zone of inhibition produced by AA-coated catheter tubes during agar plate disc diffusion assays. Catheter tubes that were coated using 0·002% (●), 0·01% (■), 0·05% (○) and 0·25% (□) anacardic acid solutions for 1 h were used for the experiments. [Colour figure can be viewed at wileyonlinelibrary.com]

above 75% when treated with releasate from the AA-coated catheters.

To study the toxic effect of AA-coated catheter tube, we used a medaka fish model (n = 6). AA-coated catheters were implanted i.p. in medaka fish and were observed for 14 days. Fish were inspected three times daily. No abnormal behaviour, weight reduction and mortality were observed. Results indicated virtually no toxicity of AA-coated catheters.

Medaka fish intraperitoneal catheter-associated infection model demonstrates reduced initial attachment of *S. aureus* with AA-coated catheter tubes

For *in vivo* biofilm assay, 0·25% AA-coated catheter tubes were implanted into the i.p cavity of medaka fish and infected with SA113 (n = 6 fish per group). The fish were killed 48 h after infection, and the numbers of bacteria associated with the catheters were determined by viable count. The average bacterial counts that were attached with the catheters were reduced by 1·7 $\log_{10}$CFU pr catheter in the presence of AA (Fig. 6). The average *S. aureus* count in uncoated catheter tubes was around 4·5 $\log_{10}$CFU per catheter, whereas the average bacterial count in catheter tubes that were coated using 0·25% AA was around 2·8 $\log_{10}$CFU per catheter.

Discussion

The prevention of *S. aureus* biofilm formation is more desirable than treating biofilm-related infections. Therefore, a number of antibiotics and antiseptics including clindamycin, rifampicin, triclosan, quaternary ammonium species, nitrofurazone, silver sulfadiazine and chlorhexidine were used for coating catheters to prevent *S. aureus* biofilm formation (Danese 2002). However, except for nitrofurazone and silver sulfadiazine, resistance of *S. aureus* against the other antibiotics and antiseptic agents was also reported in the literature (Brenwald and Fraise 2003; Levin *et al.* 2005; Zhou *et al.* 2012; Schlett *et al.* 2014). Thus, there is need for novel antimicrobial agent(s)-impregnated catheter tubes

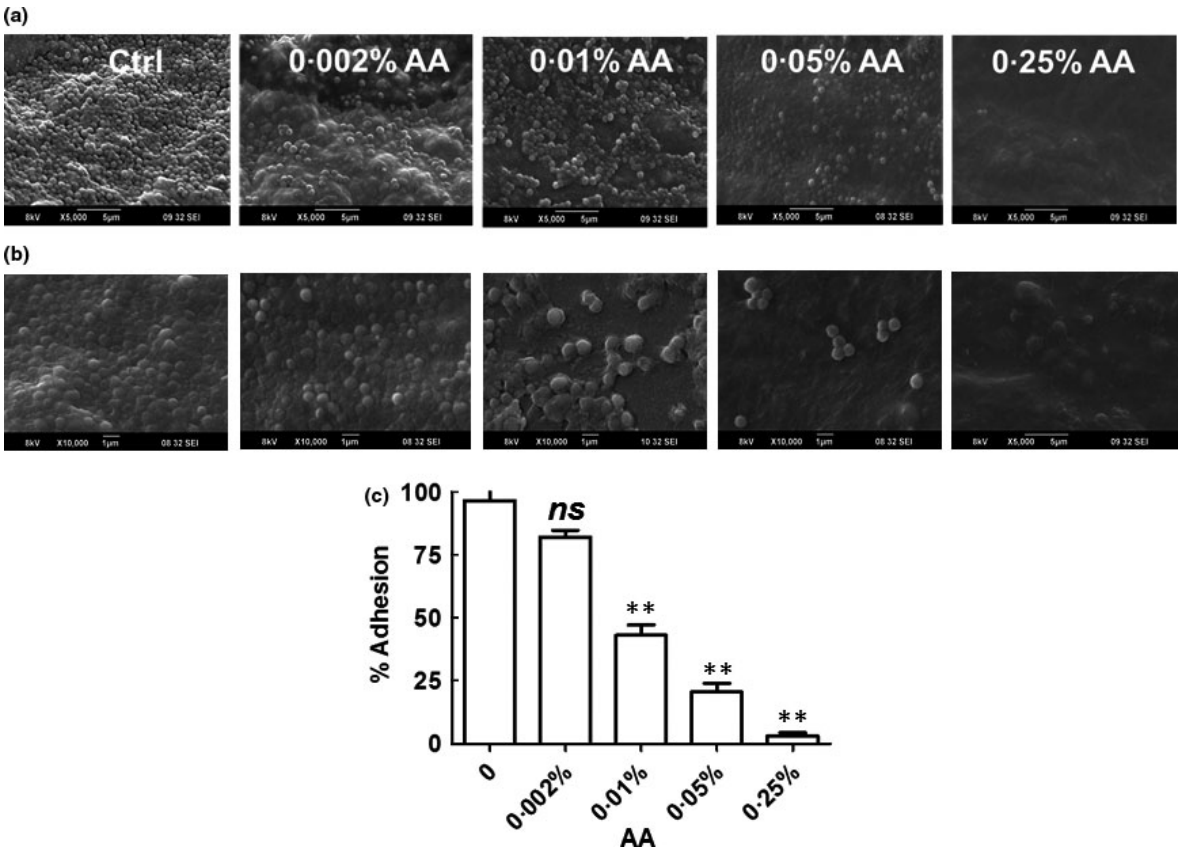

Figure 4 Biofilm inhibitory activity of anacardic acid-coated catheters against *Staphylococcus aureus* after 48 h of incubation. (a, b) SEM images at lower (a) and higher (b) magnifications. (c) Bar diagram demonstrates reduced biofilm development of *S. aureus* on anacardic acid-coated catheters. $^{**}P < 0{\cdot}01$, and *ns* represents data not statistically significant.

to prevent biofilm development of *S. aureus* in a more effective manner.

The antibiofilm activity of AA is due to its unique antimicrobial mechanism of action. Previous studies have demonstrated that AA can enter into the bacterial membrane lipid bilayers where it disrupts the membrane, blocks bacterial NADH oxidase activity and interferes with bacterial electron transport pathway (Kubo *et al.* 2003; Stasiuk and Kozubek 2010). As AA acts on multiple targets, it is very unlikely that *S. aureus* will develop resistance against it. We therefore have selected AA for catheter impregnation.

This study describes the efficacy of AA-loaded catheters in the prevention of *S. aureus* biofilm development. The efficacy of catheter coated with AA can be explained better with following reasons: (1) catheter containing AA produces zone of growth inhibition against SA113 *in vitro,* suggesting significance of antimicrobial activity; (2) sustained release of AA from catheters for 4 days offers protection against *S. aureus* biofilm; (3) AA-impregnated catheters did not display any toxicity which we have validated using *in vitro* haemolysis and cytocompatibility assays and *in vivo* using medaka fish model; and (4) finally, the biofilm inhibitory activity of AA-impregnated catheters was validated using medaka fish i.p implant model.

In this study, we have impregnated AA into the catheter to prevent initial attachment and biofilm formation of *S. aureus* on catheter surfaces. The major drawback of this study is the lower impregnation efficiency of AA into the catheter tubes. Although we have used very high concentration of AA to impregnate catheter tubes, the impregnation efficiency was not sufficient enough to provide the release of AA from these catheter tubes for 4 days. The antibiofilm efficacy of catheters needs to be extended for longer duration. This could be achieved by chemically conjugating AA using a cross-linker to the catheter surfaces, so that the release of AA from the catheter surfaces may not be necessary to achieve its desired antibiofilm activity; at the same time, the growth, attachment and biofilm development of the microbes which come in contact with the catheters could be

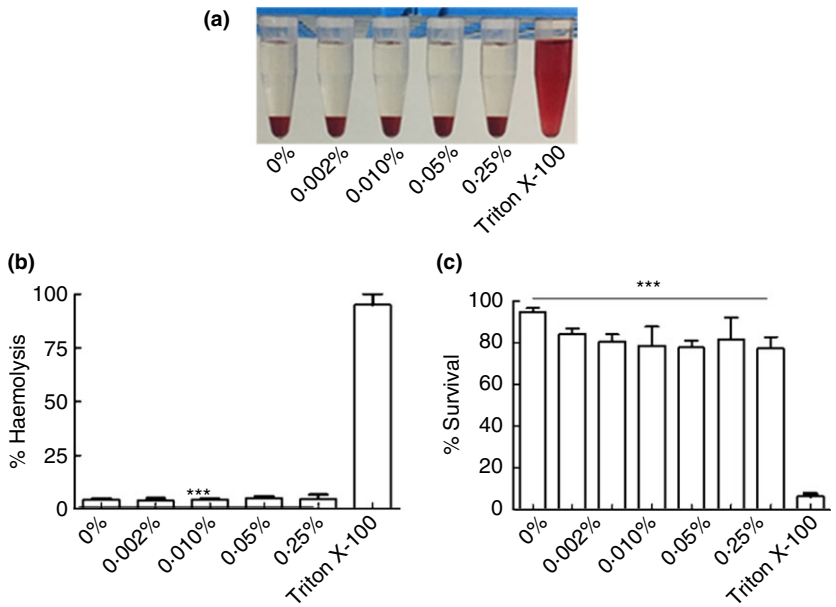

Figure 5 Cytocompatibility of anacardic acid-coated catheters. (a) Test tube image and (b) bar diagram represent percentage haemolytic activity of anacardic acid-coated catheters. (c) Cell viability of L929 fibroblast cell line treated with the releasate of different percentage of anacardic acid-coated catheters. ***$P < 0{\cdot}001$. [Colour figure can be viewed at wileyonlinelibrary.com]

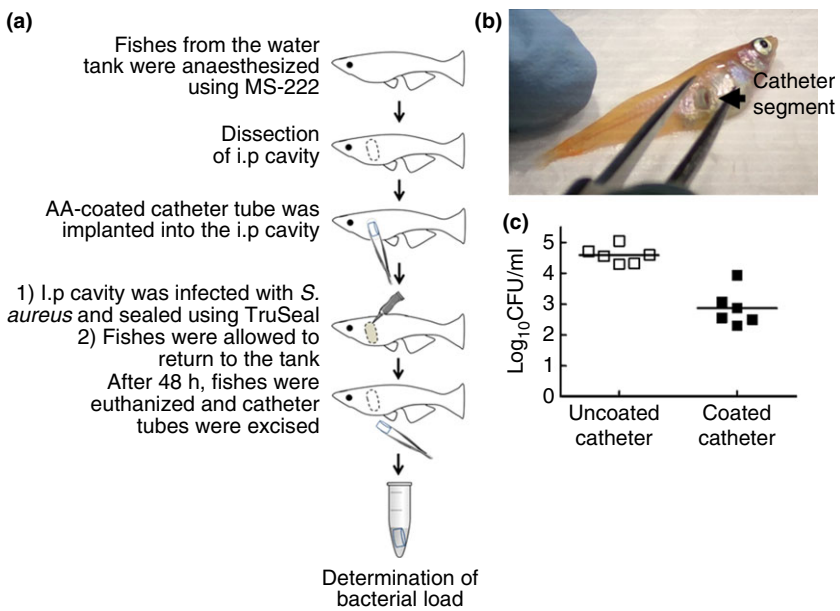

Figure 6 *In vivo* activity of anacardic acid-impregnated catheter in medaka fish intraperitoneal catheter-associated infection model. Anacardic acid was impregnated into the silicon catheter tubes by dipping the catheter segments into 0·25% anacardic acid solution for 1 h. (a) Schematic representation of experimental procedure adopted for the development of medaka fish i.p catheter-associated biofilm infection model. (b) Image of the dissected medaka fish penetonium. Arrow indicates the position of the catheter segment. (c) Reduced *Staphylococcus aureus* attachment on anacardic acid-coated catheters compared to uncoated catheters ($n = 6$). Infected catheters were excised from the peritoneal cavity 48 h after infection, and bacterial load was determined. [Colour figure can be viewed at wileyonlinelibrary.com]

arrested. Nevertheless, here, we have demonstrated AA can be used instead of common antibiotics and antiseptic agents for developing catheters with antibiofilm activities. This work could be of great interest for the medical field, opening new directions for designing phytochemical-coated surfaces.

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Conflict of Interest

The authors declare no conflict of interests.

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