Appl Microbiol Biotechnol
DOI 10.1007/s00253-014-6128-2

Enzymatic synthesis of poly(catechin)-antibiotic conjugates: an antimicrobial approach for indwelling catheters

Idalina Gonçalves · Ana S. Abreu · Teresa Matamá · Artur Ribeiro · Andreia C. Gomes · Carla Silva · Artur Cavaco-Paulo

Received: 6 June 2014 / Revised: 1 September 2014 / Accepted: 4 October 2014

Abstract Biofilm formation in urinary indwelling catheters is one of the most critical issues that patients face. Catheters were coated with poly(catechin)-antibiotic conjugates with enhanced antimicrobial properties. Catechin was conjugated with two antibiotics, namely trimethoprim (TMP) and sulfamethoxazole (SMZ) via activation with *N*,*N*′-disuccinimidyl carbonate (DSC) and subsequent coupling to molecules containing α-amine moieties. Silicone and polyurethane catheters were functionalized in situ through laccase oxidation of catechin-antibiotic conjugates. Four antimicrobial coatings were produced, namely with poly(catechin), poly(catechin)-TMP, poly(catechin)-SMZ and poly(catechin)-TMP-SMZ. The bacterial adhesion reduction was tested on the functionalized devices using gram-negative and gram-positive strains. The most significant reduction in adhesion was observed with poly(catechin)-TMP (gram-negative −85 % and gram-positive −87 %) and with poly(catechin)-TMP-SMZ (gram-negative −85 % and gram-positive −91 %). The cytotoxicity to mammalian cells was tested by indirect contact for 5 days and revealed that all the tested coatings supported more than 90 % of viable cells. A promising approach for the increase of the indwelling catheter lifespan was developed aiming to reduce catheter-associated chronic infections.

Keywords Laccase · Catechin · Trimethoprim · Sulfamethoxazole · Antimicrobial · Catheters

Electronic supplementary material The online version of this article (doi:10.1007/s00253-014-6128-2) contains supplementary material, which is available to authorized users.

I. Gonçalves · A. S. Abreu · T. Matamá · A. Ribeiro · A. C. Gomes · C. Silva (✉) · A. Cavaco-Paulo (✉)
CEB—Centre of Biological Engineering, University of Minho, 4710-057 Braga, Portugal
e-mail: cmpsilva@ceb.uminho.pt
e-mail: artur@deb.uminho.pt

T. Matamá · A. Ribeiro · A. C. Gomes
CBMA (Centre of Molecular and Environmental Biology), Department of Biology, University of Minho, Campus of Gualtar, 4710-057 Braga, Portugal

Present Address:
A. S. Abreu
IPC—Institute of Polymer and Composites/I3N—Institute of Nanostructures, Nanomodelling and Nanofabrication, University of Minho, 4800-058 Braga, Portugal

Present Address:
C. Silva
3B's Research Group, Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, S. Cláudio de Barco, Taipas, 4806-909 Guimarães, Portugal

Introduction

Lengthy hospital stays have been shown to be associated with a higher risk of chronic infection (Eckmann et al. 2013). It is thought that many of these infections result from bacteria growing on the outer and/or inner surface of indwelling devices leading to biofilm formation. The typical strategy to eradicate bacteria is by the use of antibiotics (Hancock 2005). However, this approach has not been efficient enough since bacteria are increasingly adopting resistance mechanisms. Indeed, the initial attachment of planktonic bacteria to a surface may be vulnerable to the antibiotic action, but the additional binding and microcolonization of bacteria that promotes the biofilm formation are associated with high tolerance to them (Høiby et al. 2010; Shafahi and Vafai 2010; Smith 2005). This resistance may occur through antibiotic inactivation by modification of its chemical moiety, the specific modification of the macromolecular target and the prevention of antibiotics from reaching their targets (cell membranes, cell-wall biosynthesis enzymes and substrates, bacterial protein

Published online: 21 October 2014

synthesis and bacterial nucleic acid replication/repair) by reducing uptake or through antibiotic efflux (Alanis 2005; Hancock 2005). In addition, once installed, the biofilms also possess resistance mechanisms such as physical or chemical diffusion barriers to antimicrobial penetration, slow growth of the biofilm due to nutrient limitation and activation of the general stress response and the occurrence of a biofilm-specific phenotype (Mah and O'Toole 2001; Prasanna and Doble 2008). Therefore, the need for a new antimicrobial solution with a distinct action mode from that provided by the existing antibiotics has been the focus of various studies (Cushnie and Lamb 2005; Orhan et al. 2010). Different bactericidal and bacteriostatic approaches have been studied aiming to improve antibiotic therapy including physiochemical modification of the biomaterial surface (Klibanov 2007; Kowalczuk et al. 2010; Tiller et al. 2001), impregnation of antimicrobial agents into medical device polymers (Bayston et al. 2009; Desai et al. 2010; Kohnen et al. 2003) and the use of electric fields (Ahmad et al. 2012; Lee et al. 2005; Lichter and Rubner 2009). Regardless of some clinical success, the effectiveness of these mechanisms is limited and can diverge depending on the bacterial strains. Therefore, the development of antimicrobial alternatives is still an important issue to be addressed by the scientific community (Rodrigues 2011).

Among the natural products, it is possible to find a significant number of antimicrobial agents with a broad spectrum, a low toxicity and acceptable pharmacokinetics which can be useful to functionalize surfaces and subsequently reduce the bacteria growing on invasive devices (Cushnie et al. 2003; Cushnie and Lamb 2005, 2011). Polyphenols, a class of natural compounds with a broad range of pharmacological properties, have been the focus of scientific research aiming to overcome the drawbacks of current drugs and produce more effective products (Cushnie et al. 2003; Ouattara et al. 2011). These highly hydroxylated compounds have well described powerful biological activities and can be classified according to their structure as phenolic acid derivates, flavonoids and tannins (Alberto et al. 2011; Karou et al. 2005). Concerning their antibacterial mechanism of action, it has been reported that polyphenols can interfere with nucleic acid synthesis (topoisomerase inhibition) (Auzanneau et al. 2012; Lambert et al. 2005), cytoplasmic membrane damage (perforation and/or a reduction in membrane fluidity) (Ajuwon et al. 2013), energy metabolism (NADH-cytochrome *c* reductase inhibition) (Moini et al. 1999) and cell wall/membrane synthesis inhibition (Palacios et al. 2014) reducing the bacteria colonization (Cushnie and Lamb 2005, 2011). Moreover, the use of polyphenols in combination with other antimicrobial agents has also been cited as a successful practice to fight the drug resistance problem (Lin et al. 2008; Rizzo et al. 2014). The synergistic effect provided by both may potentiate the antibiotic efficacy at lower concentrations decreasing adverse reactions (Daglia 2012; Jigisha et al. 2012). Oxidized polyphenols also demonstrate their capacity to inhibit bacterial growth since their polymer size can contribute to the microorganism toxification (Karou et al. 2005). Enzyme-induced oxidation and coupling of phenolic functional groups result in polymer surfaces useful for material functionalization (Gonçalves et al. 2013). This process can be supported by the use of laccase (*p*-diphenol:dioxygen oxidoreductase, EC 1.10.3.2), an oxidative enzyme that catalyses the one-electron oxidation of reducing substrates such as phenols and their derivatives or aromatic amines. The ability of laccases to act over a wide range of substrates makes these enzymes extremely useful biocatalysts for distinct biotechnological applications (Mot and Silaghi-Dumitrescu 2012; Sharma et al. 2007).

In a previous study, it was reported that the in situ functionalization of catheters through laccase polymerization of catechin (natural phenol) reduced bacterial adhesion onto the polyurethane and silicone catheter surface (Gonçalves et al. 2013). Herein, the strategy was to in situ enzymatically oxidize catechin-antibiotic conjugates with laccase aiming the bacterial adhesion reduction onto the functionalized catheters. The main advantage of producing conjugates containing antibiotics coupled to poly(catechin) lies on the ability of catechins, and its polymerized derivatives, to act on and disturb the bacterial membranes. Thus, two antibiotics commonly used to treat chronic infections, namely trimethoprim (TMP) and sulfamethoxazole (SMZ), were selected. Both act to inhibit different enzymatic steps of the folic acid pathway, leading to the cessation of thymidine monophosphate bacterial synthesis which is required for the DNA replication (Zander et al. 2010). The bacterial adhesion reduction was studied using gram-positive and gram-negative bacteria, and normal human skin fibroblast cells were selected for the cell viability evaluation of the newly developed antimicrobial systems.

Materials and methods

Materials

Sterile single-lumen polyurethane (PU) and double-lumen silicone catheters (SI) were supplied from Pronefro (Portugal) and Degania Medical (Israel), respectively. Laccase (EC 1.10.3.2) from ascomycete *Myceliophthora thermophila* (17 g protein/L, 560 U/mL at 50 °C), Novozym® 51003, was obtained from Novozymes (Denmark). Mouse fibroblast cells L929 were acquired from ATCC and maintained according to the supplier's recommendations. Mueller-Hinton (MH) broth, Dulbecco's modified Eagle's medium (DMEM), L-glutamine, sodium bicarbonate, sodium pyruvate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulphoxide, catechin hydrate, trimethoprim (TMP), sulfamethoxazole (SMZ), *N,N′*-disuccinimidyl carbonate (DSC), 4-(dimethylamino)-

pyridine (DMAP), methanol, sodium acetate trihydrate, 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Spain). Bradford solution was acquired from Bio-Rad Laboratories GmbH (Germany). Fetal bovine serum (FBS) was obtained from Lonza (Walkersville, Inc., MD, USA). Glacial acetic acid was purchased from Panreac (Spain), and synthetic urine was acquired from Synthetic Urine e.K. (Germany). All of these chemicals were used as supplied without any further purification.

Bacterial strains

Pseudomonas aeruginosa PAO1 was provided by Dr. Pedro Santos from CBMA (University of Minho, Portugal). *Escherichia coli* HB101, *Proteus mirabilis* 933, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 35983 and *Bacillus subtilis* 48886 were obtained from University of Minho (Braga, Portugal).

Methods

Protein concentration and enzymatic activity of laccase

The total protein concentration of laccase was determined following the Bradford's methodology in which BSA was used as standard (Bradford 1976). The enzymatic activity of laccase was evaluated through oxidation of ABTS at 50 °C in 0.1 M sodium acetate buffer with pH 5 (Childs and Bardsley 1975). One unit of laccase (U) was defined as 1 μmol of ABTS oxidized per minute. The protein quantification and enzymatic activity of laccase were measured using a Helios Gamma UV-vis spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Conjugation of catechin with TMP or SMZ

A reaction model for the synthesis of catechin-antibiotic conjugates was developed based on a previous work (Diamanti et al. 2008). Figure 1 shows the chemical structure of all the components involved in the conjugation reaction. The conjugation was initiated by the activation of hydroxyl groups which was performed by mixing solutions of 0.03 M lyophilized catechin with 0.1 M DSC and 0.1 M DMAP in anhydrous 1,4-dioxane. The reaction remained under a nitrogen atmosphere for 24 h. Subsequently, the organic solvent was evaporated at reduced pressure. The white solid obtained was washed with chloroform and filtered in order to remove the DSC and DMAP that did not react. For the catechin-antibiotic conjugation, 1 eq. of TMP or SMZ was added to the activated catechin dissolved in methanol. The process was carried out overnight at room temperature with constant stirring. Finally, the solvent was evaporated at reduced pressure, and the obtained powder was identified by Fourier transform infrared (FTIR) analysis as the catechin-antibiotic conjugate.

Characterization of the catechin-antibiotic conjugates

After the chemical synthesis of catechin-antibiotic conjugates, the reaction products were characterized by FTIR spectroscopy. FTIR measurements were recorded using a PerkinElmer 1600 spectrometer, by averaging 16 individual scans over the range 4,000 cm^{-1} to 450 cm^{-1}. The samples were prepared in potassium bromide (KBr) disks.

Catheter functionalization through in situ polymerization of catechin and catechin-antibiotic conjugates by laccase action

The coating followed an experimental procedure previously optimized for catechin polymerization and attachment onto the PU and SI catheter surface (Gonçalves et al. 2013). Firstly, an alkaline pre-treatment was carried out wherein catheters of 1 cm each were incubated with 2 g/L sodium hydroxide at room temperature for 30 min under orbital agitation. Samples were washed with distilled water. Then, the in situ catheter functionalization was carried out using different concentrations of monomer: (1) 10 mM catechin, (2) 10 mM catechin-TMP, (3) 10 mM catechin-SMZ and (4) 5 mM catechin-TMP plus 5 mM catechin-SMZ. Each compound was previously dissolved in methanol (5 % *v*/*v*) and diluted in 0.1 M sodium acetate buffer (pH 5). The phenolic oxidation proceeded with 2 U/mL laccase at 50 °C under 70 rpm for 2 h under aired conditions. Washing procedures were realized with distilled water in order to remove the non-attached polymer.

Colorimetric measurements of the functionalized catheters

The colour change after catheter functionalization was monitored by colour strength estimation (*k*/*s*) using a Datacolor

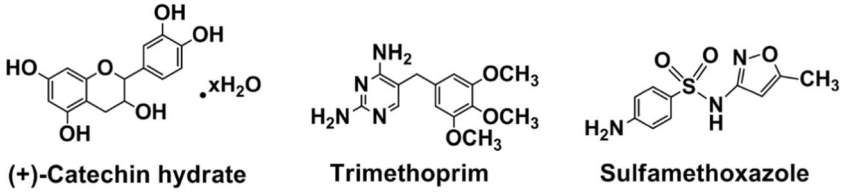

Fig. 1 (+)-Catechin hydrate, trimethoprim (TMP) and sulfamethoxazole (SMZ) structures

apparatus at standard illuminant D65 (LAV/Spec. Excl., d/8, D65/108) with the Kubelka-Munk equation (Eq. 1):

$$\frac{k}{s} = \frac{(1-R)^2}{2R} \quad (1)$$

where k is the absorbance coefficient, s is the scattering coefficient and R is the reflectance ratio. The setting up of a narrow wavelength peak was due to the polymer polydisperse size which contributes differently to the emission spectra. Therefore, the data correspond to the sum of k/s values achieved in the wavelength range of 400–700 nm.

Water uptake measurements

The surface characterization in terms of water uptake of the functionalized catheters was studied by measuring the water contact angle using a Dataphysics instrument (Filderstadt, Germany) and the OCA20 software (Germany). A dosing volume of water droplet was set as 3 μL using a Hamilton 500-μL syringe type. Conditions for measurement were selected as ellipse fitting. The data were determined by averaging values at three different points of each catheter sample.

Leaching profile of the functionalized catheters

The modified catheters were incubated with 0.01 M phosphate-buffered saline (PBS) solutions at pH 7.4 and synthetic urine during 15 days, at 37 °C and 70 rpm of agitation. The leaching profile was studied in terms of colour strength (k/s). Samples were tested in triplicate.

Antimicrobial assays

Determination of MIC

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial agent that inhibited the microorganism growth in a microdilution well. Broth microdilution test (Wiegand et al. 2008) was used to determine the MIC value of each developed conjugate against gram-positive *(S. epidermidis*, *S. aureus* and *B. subtilis*) and gram-negative strains (*E. coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*). Firstly, the inocula were prepared in Mueller-Hinton (MH) broth and grown overnight at 37 °C with orbital agitation. Further, each inoculum was diluted in MH broth until an optical absorbance of 0.05 at 600 nm. An optical absorbance of 0.01 at 600 nm was established only for *Pseudomonas aeruginosa* due to the fast growth of these bacteria. Seven different concentrations of each compound were prepared in MH broth, and 50 μL of each solution was distributed in 96-well plates. Posteriorly, 50 μL of each bacterial isolate was added to the corresponding well, and the plates were incubated at 37 °C for 16 h. Samples were tested in duplicates, and the MIC values were achieved by measuring the optical absorbance at 600 nm in a microplate reader (SpectraMax® Plus384).

Bacteria adhesion assay

The antimicrobial efficiency of the modified catheters was assessed in vitro against the gram-positive and gram-negative strains previously mentioned. The aim was to test the effect of coating on bacterial adhesion without interference of any exterior effect, like proteins from urine. The bacterial attachment onto the catheter surface was determined by an adhesion assay under static conditions (Darouiche et al. 2009; Gonçalves et al. 2013). The inocula were prepared in MH broth and grown overnight at 37 °C with orbital agitation. Further, each inoculum was diluted in MH broth until an optical absorbance of 0.05 at 600 nm. Only for *Pseudomonas aeruginosa* was established an optical absorbance of 0.01 at 600 nm due to the fast growth of these bacteria. Sterilized (ethylene oxide) catheter segments of 1 cm each were individually placed in 2 mL of inoculated MH broth and incubated for 16 h at 37 °C. Afterwards, the samples were washed three times with sterile PBS and transferred to an Eppendorf tube containing 1 mL of PBS. Adherent cells were removed by sonication at 42 kHz, 70-W output for 2 cycles of 30 s, followed by vortexing at 2,500 rpm during 2 min. Bacterial suspensions were serially diluted in PBS and then plated onto MH-agar dishes. Except for *Pseudomonas aeruginosa* and *B. subtilis* which remained at room temperature, all the plates were incubated at 37 °C for 24 h and the colonies were counted. Samples were tested in duplicates, and two independent assays were accomplished for each microorganism. Non-coated catheters were used as control samples for further comparison with all the other coated samples.

Evaluation of the planktonic bacteria growth

The effect of each functionalized catheter in the planktonic bacteria growth was observed. The procedure followed the guidelines mentioned in the bacteria adhesion assay section. After the incubation of modified catheters with each selected bacteria at 37 °C for 16 h, 100 μL of each sample was plated in 96-well plates and the optical absorbance at 600 nm was measured with the microplate reader. Samples were tested in duplicates, and the inoculum without catheter was used as control.

Surface analysis—SEM

The bacteria adhesion onto the catheter surface was confirmed by scanning electron microscopy (SEM). Firstly, the bacteria were fixed at room temperature for 2 h using 2.5 % glutaraldehyde solution prepared in 0.1 M cacodylate buffer (pH 7.2). Then, each sample was washed three times with distilled water and plunged into nitrogen liquid before freeze-drying under vacuum conditions (Baillie and Douglas 1999; Hawser et al. 1998; Steinbrecht and Müller 1987). For SEM analysis, the samples were coated with gold and scanned at different points using an electron microscope model LEICA S360 with a backscattered and secondary electron detector, at ×2,000 and ×10,000 magnification. The catheters non-inoculated with bacteria were also analysed aiming to evidence the polymer attachment onto sample surface.

Cell viability assay—indirect contact

The cytotoxicity evaluation of human cells when exposed to the novel antimicrobial catheters was accomplished in two steps, namely Dulbecco's modified Eagle's medium (DMEM) was pre-conditioned in contact with each sample and the resulting leachables were frozen for posterior application to fibroblast cells. In the first stage, the sterilized (ethylene oxide) catheter pieces (1 cm each) were disposed in 24-well plates and submerged in DMEM containing 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 1 % Pen & Strep and 1 % sodium pyruvate. The incubation was carried out at 37 °C for three different incubation times, namely 1, 3 and 5 days. Subsequently, each inoculated medium was collected into Eppendorf tubes and frozen at −80 °C. Further, the L929 cells were cultured at 37 °C in a humidified atmosphere with 5 % CO_2. The cells were previously seeded in 24-well tissue culture plates at a density of 30,000 cells/well. Just prior to adding the leachables to the cells, they were supplemented with FBS (10 % *v*/*v*) and L-glutamine. Cell viability was evaluated at 37 °C after 24, 48 and 72 h of incubation by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay as a measure of cellular metabolic viability. Cells incubated with dimethyl sulphoxide (DMSO, 15 %) were used as cell death controls, and cells grown with regular culture medium were used as live cell controls. Additionally, non-coated catheters were considered the main control for further comparison with all the other coated samples.

Cell viability—MTT assay

After each incubation, the MTT solution (5 mg/mL, 50 μL) was added to each well, and after 2 h at 37 °C in a humidified 5 % CO_2 atmosphere, the medium was replaced with DMSO/ethanol solution (1:1 *v*/*v*, 500 μL). When the reaction product (MTT formazan) was completely dissolved, 150 μL of each solution was transferred in triplicate to a 96-well plate, and the absorbance was read at 570 and 690 nm using a multiwell plate reader spectrophotometer. The relative cell viability was calculated considering the non-coated catheter incubated for 24 h as reference (100 % viability). Samples were tested in duplicates, and four independent assays were performed for each coating.

Statistical analysis

Data was statistically analysed through one-way ANOVA and two-way ANOVA test, respectively. GraphPad Prism 5.0 for Windows was the software used. Significant differences were identified as $P<0.05$.

Results

Characterization of catechin-antibiotic conjugates

As previously described, the hydroxyl groups present on the natural phenol structure were firstly activated by reacting with DSC and DMAP. Then, each antibiotic was mixed with the modified catechin, and the conjugation was accomplished (Fig. 2). The chemical characterization of each reaction product was carried out by FTIR. Figure 3 shows the FTIR spectra achieved for catechin before and after coupling with the linker (DSC) and with the selected antibiotics, TMP and SMZ. The general spectrum ranging from 4,000 to 500 cm^{-1} corresponding to each compound and an amplification of the most noising part (from 2,000 to 400 cm^{-1}) are presented. By this way, it was possible to verify the spectral differences after each stepping process and comprove the final product achievement.

Catheter functionalization through in situ polymerization of catechin and catechin-antibiotic conjugates by laccase action

The attachment of catechin and catechin-antibiotic conjugates onto the PU and SI catheters was assessed using laccase as biocatalyst. The end products of enzymatic oxidation were detected by UV-visible spectrophotometry. After laccase addition, the intensity of catechin peak at around 270 nm decreased comparatively to the catechin-antibiotic conjugates peak. On the other hand, the poly(catechin) peak at 460 nm was more pronounced than the peak of conjugate (data not shown). Thus, the free form of catechin monomer proved to be more easily oxidized by laccase than when it is conjugated with antibiotics. The in situ functionalization of catheters

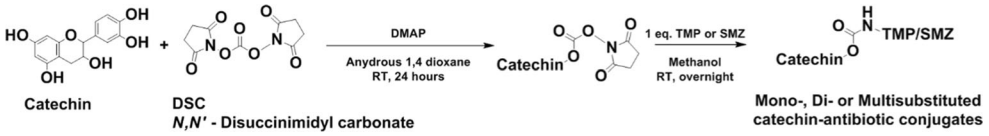

Fig. 2 Reaction scheme of catechin activation followed by its chemical conjugation with trimethoprim (TMP) or sulfamethoxazole (SMZ)

followed the steps represented in Fig. 4. Catechin-antibiotic conjugates were efficiently oxidized by laccase action and attached onto the catheter surface. The colour strength measurements allowed observing the differences between non-functionalized and functionalized samples. Concerning these results, PU coating was more effective than the SI coating since highest *k/s* spectral values were observed (Fig. 5). Besides, it was also noteworthy that poly(catechin) coating retains highest colour strength intensity than the poly(catechin)-antibiotics, confirming the previous mentioned UV-visible results.

Water uptake of functionalized catheters

The PU and SI catheter hydrophobicity decreased after each functionalization process as it was confirmed by the water contact angle reduction relatively to the non-coated catheter. The starting water contact angle of 114.55° and 134.35° for PU and SI catheters, respectively, was reduced in 25° and 2° after the poly(catechin) attachment onto the catheter surface. On the other hand, the highest hydrophobicity decreasing was attained with poly(catechin)-antibiotic conjugate coating yielding less 45° and 8° of water contact angle than the non-

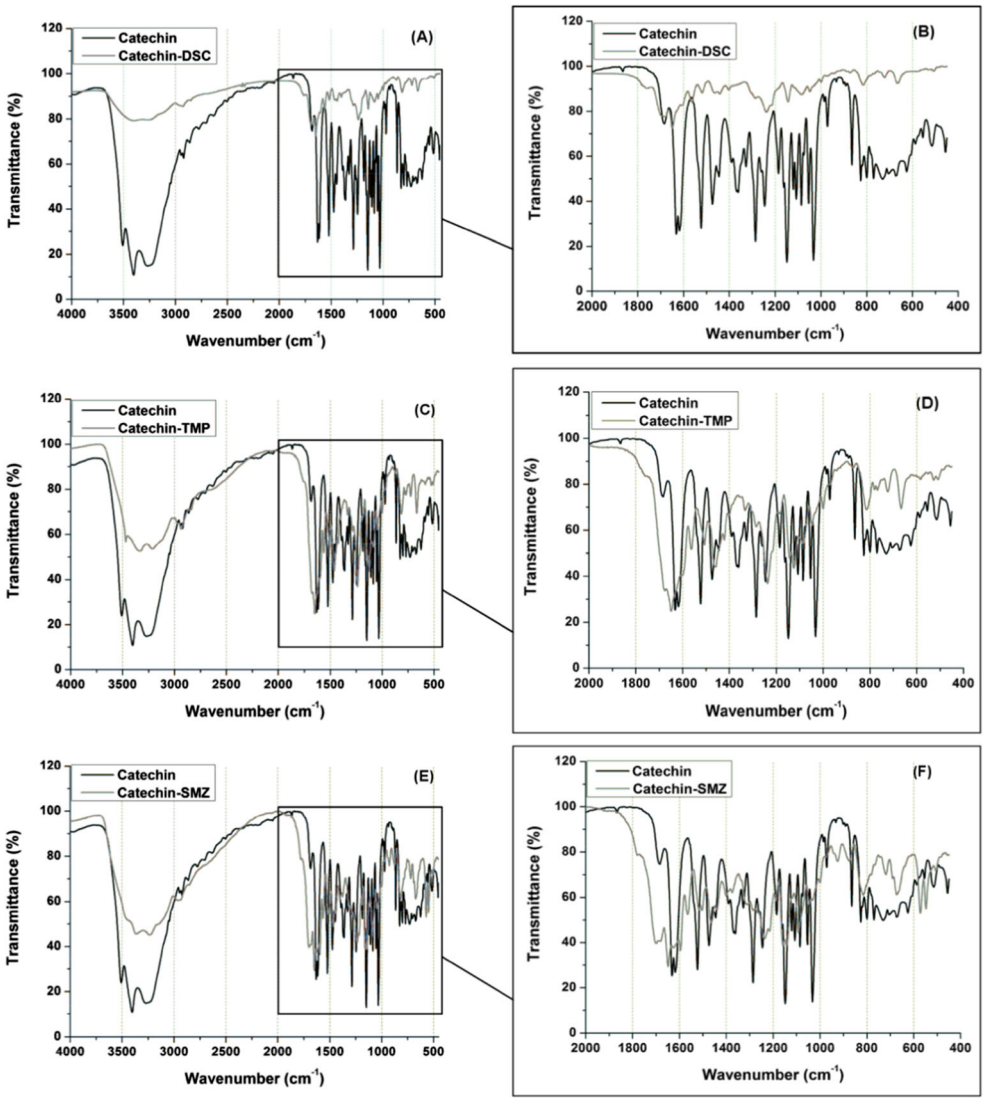

Fig. 3 FTIR spectra obtained for catechin versus catechin-DSC (**a**), catechin versus catechin-TMP (**c**, **d**) and catechin versus catechin-SMZ (**e**) using scales from 4,000 to 500 cm^{-1}. **b**, **d**, **f** are expansions of each spectrum from 2,000 to 400 cm^{-1}

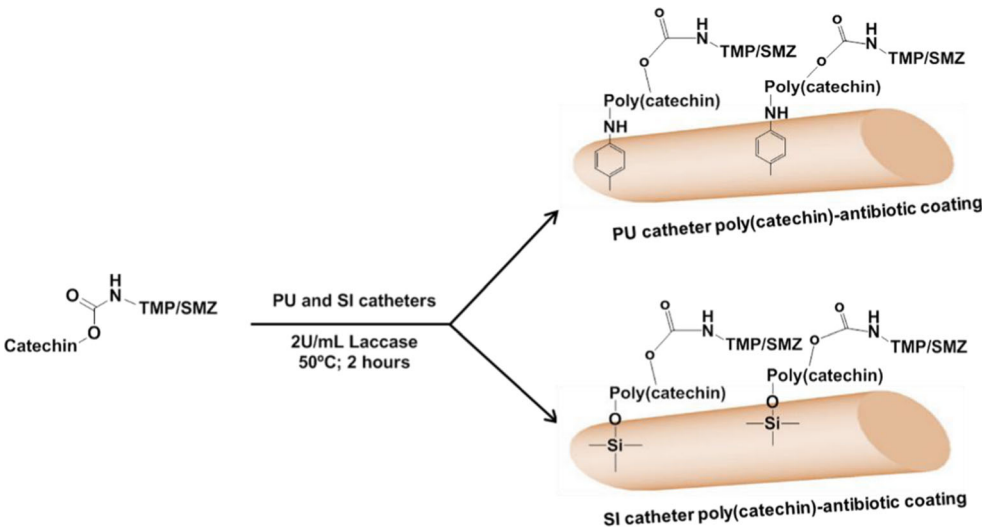

Fig. 4 In situ laccase functionalization of polyurethane (*PU*) and silicone (*SI*) catheters using catechin-TMP and/or catechin-SMZ conjugates (10 mM substrate; 2 U/mL laccase; 0.1 M sodium acetate buffer (pH 5) at 50 °C for 2 h)

coated samples for PU and SI catheters, respectively (data not shown). Therefore, the hydrophobicity reduction was more pronounced on PU catheters than on SI devices. Comparing both poly(catechin)- and poly(catechin)-antibiotic-coated samples, a highest hydrophobicity decrease was observed on the later ones.

Leaching profile of the functionalized catheters

In order to simulate the in vivo conditions for intravenous and urinary applications, all the modified catheters were incubated in PBS and in synthetic urine solutions at 37 °C for 15 days. Figure 6 presents the colour strength measurements for each coated sample before (0 days) and after 15 days of incubation. The loss of polymer attached onto the PU and SI catheter surface was evident for both PBS and synthetic urine solutions. However, the colour strength decreasing was less pronounced on PU devices. Nevertheless, more than a half of the coloration remained in each coated catheter surface, revealing that a high content of antimicrobial polyphenol remains on the medical devices.

Antimicrobial activity of phenolic-antibiotic conjugates

Minimum inhibitory concentrations (MICs)

The development of novel catechin-antibiotic conjugates as well as their enzymatic polymerization leads to structural conformation changes of both TMP and SMZ. Consequently, the antimicrobial profile of free antibiotics was also modified, and for this reason, the MIC values of each synthetized compound were determined (Table 1). In general, the chemical catechin-antibiotic conjugation and the enzymatic oxidation of each conjugate maintained or increased the minimum concentration required to promote bacterial growth inhibition. Only when the SMZ was present in the poly(catechin) conjugate structure, the MIC value for *E. coli* and *Proteus mirabilis* was reduced. Unfortunately, it was not possible to achieve any MIC values of antibiotic conjugates for *Pseudomonas aeruginosa* neither of SMZ conjugates for *S. aureus*.

Bacterial adhesion onto the functionalized catheter surface

As previously mentioned, the strong adhesion on the outer and/or inner surface of indwelling devices is the first stage of the mechanism of biofilm formation (Prasanna and Doble 2008). For this reason, the bacterial adhesion test was performed on each developed coating. Figure 7 shows the effect of poly(catechin) and poly(catechin)-antibiotic conjugates as antimicrobial coating of PU and SI catheters. Overall, the antimicrobial efficiency was more evident for PU devices. Both gram-positive and gram-negative bacteria adhesion to

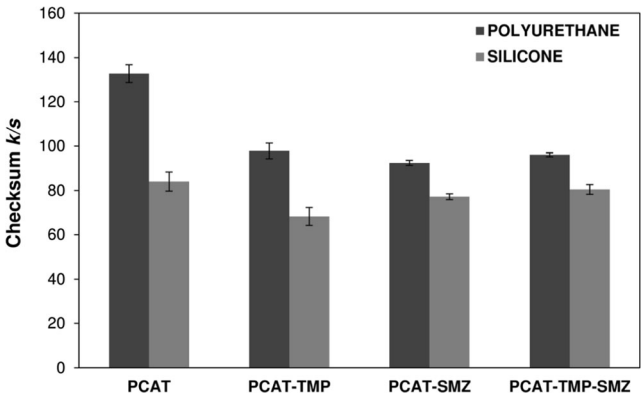

Fig. 5 Colour strength (checksum *k/s*) of each modified polyurethane and silicone catheters with poly(catechin) (*PCAT*), poly(catechin)-trimethoprim (*PCAT-TMP*), poly(catechin)-sulfamethoxazole (*PCAT-SMZ*) or both poly(catechin)-antibiotic conjugates (*PCAT-TMP-SMZ*). Enzymatic functionalization of catheters was carried out using 2 U/mL laccase in 0.1 M methanol/sodium acetate buffer (pH 5) at 50 °C under 70 rpm for 2 h

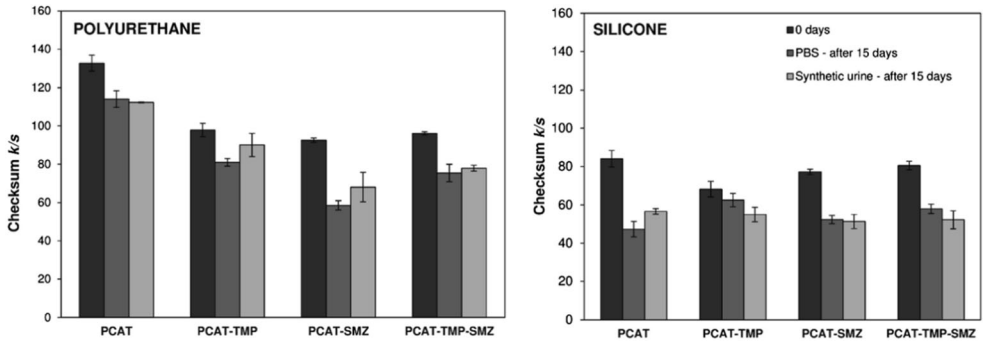

Fig. 6 Colour strength (checksum *k/s*) of each modified polyurethane and silicone catheters before (0 days) and after incubation with PBS and synthetic urine at 37 °C for 15 days with slow agitation. Poly(catechin) (*PCAT*), poly(catechin)-trimethoprim (*PCAT-TMP*), poly(catechin)-sulfamethoxazole (*PCAT-SMZ*) or both poly(catechin)-antibiotic conjugates (*PCAT-TMP-SMZ*) were the developed coating. The functionalization process proceeded using 2 U/mL laccase in 0.1 M methanol/sodium acetate buffer (pH 5) at 50 °C under 70 rpm for 2 h

the catheter surface were affected by poly(catechin) or poly(catechin)-antibiotic conjugate action. The analysis of the four coatings developed revealed that the presence of poly(catechin)-TMP-SMZ promoted the highest reduction of bacterial adhesion. From all the microorganisms tested, *Pseudomonas aeruginosa* was less affected by the catheter surface modification, with only one logarithmic unit of adhesion reduction observed in all the conditions tested.

Effect of the coated catheters in the planktonic culture growth

This study was carried out by incubation of PU-coated catheters with the selected bacterial strains. PU material was chosen for these tests, since it revealed the best coating levels with lowest leaching properties. The inoculum of each coated catheter with each bacterial strain studied revealed that, except for *Pseudomonas aeruginosa*, all the novel antimicrobial catheters inhibited the planktonic culture growth. The poly(catechin) coating inhibited around 21–43 and 13–38 % of planktonic bacteria growth for gram-negative and gram-positive strains, respectively. On the other hand, the most pronounced inhibition was verified with the poly(catechin)-antibiotic conjugate coating. The planktonic culture growth was reduced between 28–55 and 19–80 % for gram-negative and gram-positive strains, respectively (Fig. S1).

Surface analysis—SEM

The bacteria adhesion reduction on functionalized catheter surface was performed by SEM analysis after inoculation of samples with a gram-negative (*E. coli)* and a gram-positive (*S. epidermidis*) strains. Once again, the PU material was selected for these tests, since it revealed the best coating levels, lower leaching and the lowest number of bacteria colony-forming units on the catheter surface. Figure 8 shows the SEM images attained for each inoculated sample as well as evidences of the polymer attachment onto the catheter surface (non-inoculated samples). When the non-treated PU catheters were exposed for 16 h to bacterial suspension, an extremely higher colonization than the functionalized samples was evident for both bacteria. On the devices coated with poly(catechin), a significant decrease of adherent cells was

Table 1 Minimum inhibitory concentrations (mg/L) for each developed conjugate, namely catechin-trimethoprim (PCAT-TMP), poly(catechin)-trimethoprim (PCAT-TMP), poly(catechin)-sulfamethoxazole (CAT-SMZ) and poly(catechin)trimethoprim-sulfamethoxazole (PCAT-TMP-SMZ)

	E. coli	*P.mirabilis*	*P.aeruginosa*	*B. subtilis*	*S.aureus*	*S.epidermidis*
TMP	0.1–0.2	2–4	–	0.25–0.5	1.5–3	0.5–1
CAT-TMP	0.2–0.4	2–4	–	0.5–1	3–6	0.5–1
PCAT-TMP	0.4–0.6	2–4	–	0.25–0.5	6–12	2–4
PCAT-TMP-SMZ[a]	0.4–0.6	4–8	–	0.25–0.5	6–12	2–4
SMZ	4–8	4–8	–	0.06–0.12	–	1–2
CAT-SMZ	2–4	4–8	–	0.12–0.24	–	2–4
PCAT-SMZ	32–64	2–4	–	0.06–0.012	–	2–4
PCAT-TMP-SMZ[b]	2–4	2–4	–	0.06–0.12	–	2 - 4

"–" non-effective inhibitory response

[a] Taking into account the MIC reference value of TMP

[b] Taking into account the MIC reference value of SMZ

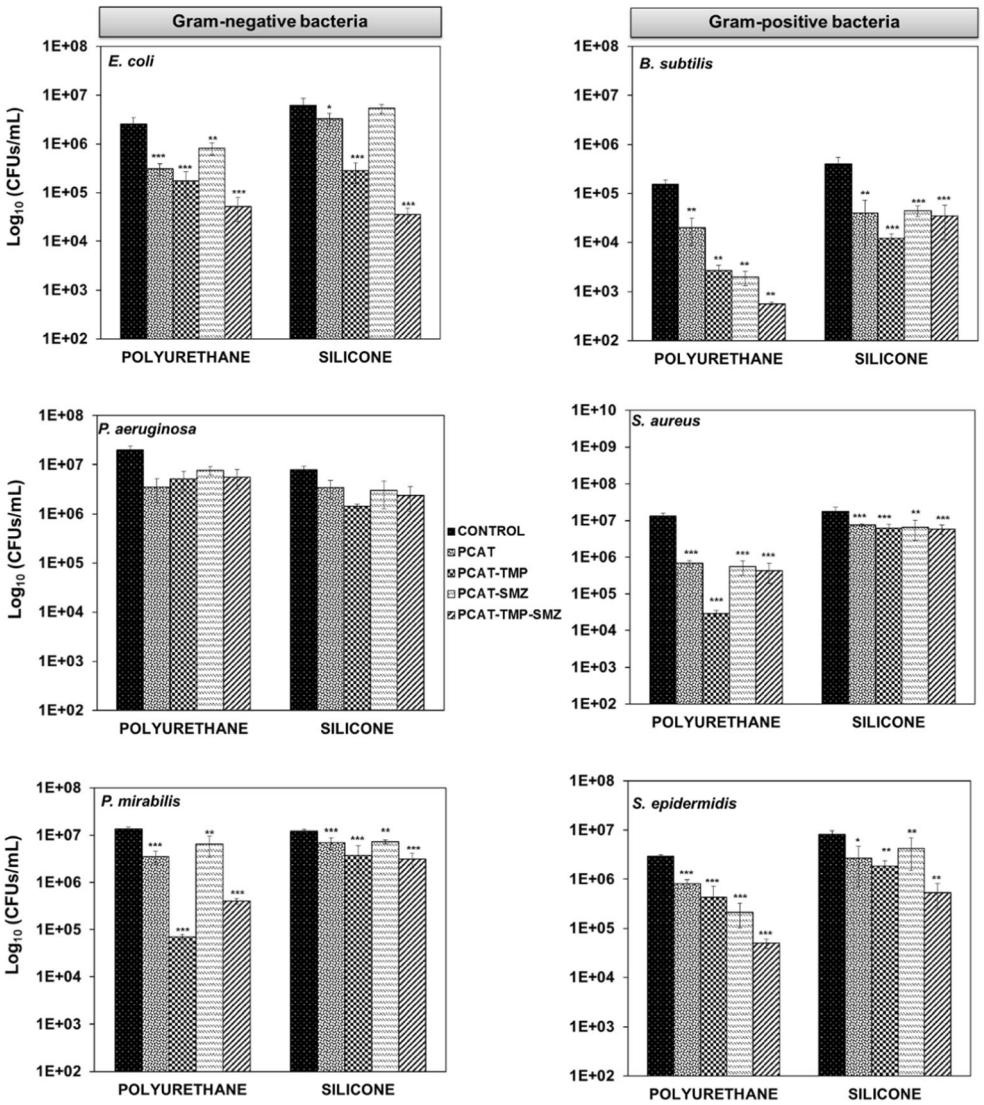

Fig. 7 Bacteria colony-forming units (*CFUs*) adhered on functionalized catheter surface for gram-negative (*E. coli*, *P. aeruginosa* and *P. mirabilis*) and gram-positive strains (*B. subtilis*, *S. aureus* and *S. epidermidis*). Four coatings were analysed: poly(catechin) (*PCAT*), poly(catechin)-trimethoprim (*PCAT-TMP*), poly(catechin)-sulfamethoxazole (*PCAT-SMZ*) and poly(catechin)-trimethoprim-sulfamethoxazole (*PCAT-TMP-SMZ*). Statistically significant differences were achieved by comparing of each coating with non-coated samples (*CONTROL*). ***Amount of adhered cells on coated catheter is significantly different than non-coated sample ($P<0.001$); **amount of adhered cells on coated catheter is significantly different than non-coated sample ($P<0.01$); *amount of adhered cells on coated catheter is significantly different than non-coated sample ($P<0.05$)

notorious. When polycatechin-antibiotic conjugates were applied, the bacteria attachment reduced significantly as well as the number of bacterial colonies.

Coated catheter cytotoxicity towards cultured mammalian cells

The final application for the coated PU and SI catheters, as indwelling devices, demands strict control of their toxicity to cells. Thus, it was important to assess cell viability of mammalian cell cultures exposed to the leachables. For this, biomaterial reference cell line L929 (mouse fibroblasts) was cultured and exposed to solutions, in which the modified catheters were previously conditioned. Non-coated catheters were used as control samples. The effect of all the coating process without phenolic compounds was also verified. Figure 9 demonstrates the relative cell viability profile achieved. Comparing both control and coated devices, it was observed that significant differences on relative cell viability were only observed after 5 days of incubation in DMEM and 48 h in contact with L929 cells. Nevertheless, viability values around 90 % were maintained with all the antimicrobial coatings developed. For both materials studied, the cellular morphology was not compromised (data not shown).

Discussion

The synthesis of polymer-antibiotic conjugates for therapeutic applications has been the target of various research studies (Kopeček 2013; Pasut and Veronese 2007). In this study, a strategy based on the enzymatic polymerization of monomer-antibiotic conjugates was developed. The monomer selected was catechin, a natural flavonoid which previously proved to be easily polymerized by laccase oxidation showing

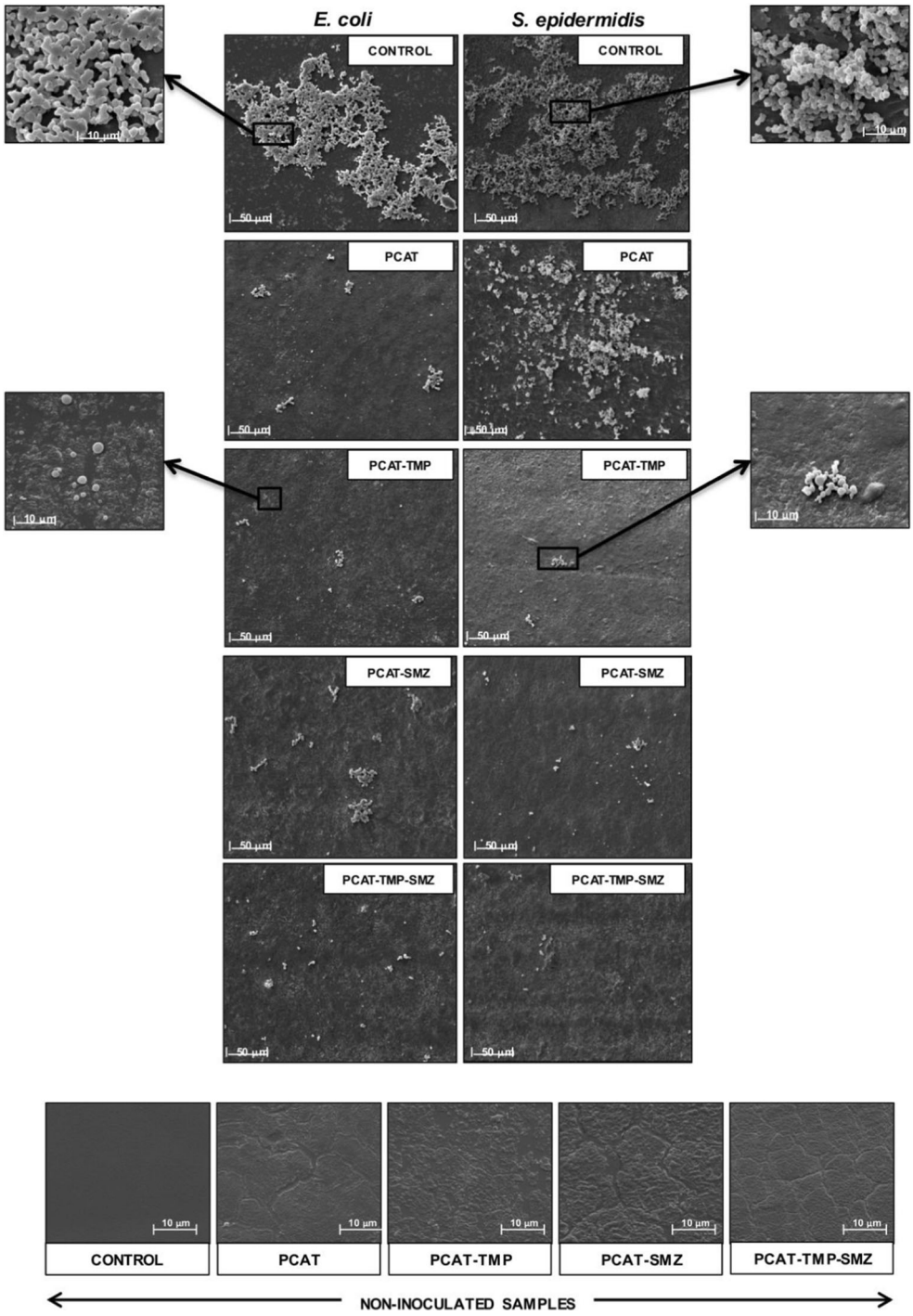

Fig. 8 Scanning electron microscopy (SEM) analysis of each modified polyurethane catheter after inoculum with *E. coli* and *S. epidermidis* at 37 °C for 16 h. Poly(catechin) (*PCAT*), poly(catechin)-trimethoprim (*PCAT-TMP*), poly(catechin)-ulfamethoxazole (*PCAT-SMZ*), poly(catechin)-trimethoprim-sulfamethoxazole (*PCAT-TMP-SMZ*) and the non-coated sample (*CONTROL*) were observed. The images were recorded with a magnitude of ×2,000 and ×10,000

antimicrobial efficiency when used as catheter coating (Gonçalves et al. 2013). The antibiotics chosen were TMP and SMZ usually recommended by clinical guidelines for empirical treatment of chronic infections and used as commercial antibiotic in the market named as Bactrim® (Jancel and Dudas 2002; Miller and Tang 2004). In catechin-antibiotic conjugation, the phenolic compound affinity to the antibiotics was prompted through hydroxyl group activation (Aldwin et al. 1990). The reaction yield was improved by using DMAP which deprotonated the hydroxyl group making it more reactive towards DSC. Then, the activated hydroxyl group reacted with the electrophilic carbonyl of DSC leading to the activated succinimidyl group. Posteriorly, the activated catechin was coupled with amino groups present on the TMP and SMZ structure immersing the modified monomer into antibiotic solution (Diamanti et al. 2008). The final products, namely catechin-DSC, catechin-TMP and catechin-SMZ, were characterized by FTIR analysis. On catechin spectrum,

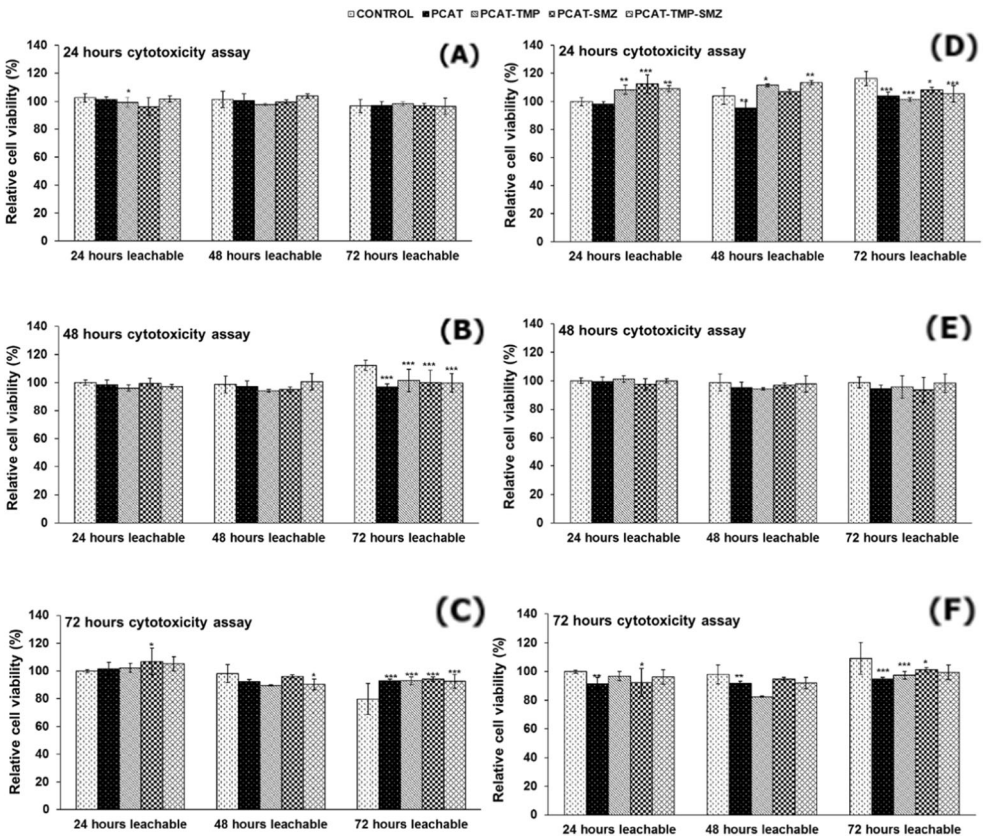

Fig. 9 Relative L929 cell viability after 24, 48 and 72 h of culture with DMEM solutions pre-inoculated with functionalized polyurethane (**a**–**c**) and silicone (**d**–**f**) catheters for 1, 3 and 5 days. Non-coated sample was used as CONTROL. Poly(catechin) (*PCAT*), poly(catechin)-trimethoprim (*PCAT-TMP*), poly(catechin)-sulfamethoxazole (*PCAT-SMZ*) and poly(catechin)-trimethoprim-sulfamethoxazole (*PCAT-TMP-SMZ*) were the coated samples. Data were achieved in relation to the control for one incubation day and show the mean values of three replicates from four experiments carried out independently. Statistical significant differences are indicated. ***Significantly different from the control ($P<0.001$); **significantly different from the control ($P<0.01$); *significantly different from control ($P<0.05$)

the characteristic absorption regions of hydroxyl group O-H (3,508–3,270 cm^{-1}), aromatic rings by C=C group (1,634 cm^{-1}) and also C-O group around 1,149–1,033 cm^{-1} are evidenced (Maoela et al. 2009). When the hydroxyl groups of catechin were activated by reacting with DSC, the intensity of hydroxyl group O-H band decreased, and a prominent triple peak appeared around 1,570 cm^{-1} proving the formation of a succinimidyl ester. Concomitantly, a stretching vibration of aromatic nitro N-O groups occurred at 1,290 cm^{-1}. Also a new peak came up around 1,770 cm^{-1} corresponding to the carbonyl C=O groups evident on DSC structure as well as on the ester linkage (Baca et al. 2009; Jo et al. 2000; Sam et al. 2011). Afterwards, the coupling reaction of activated catechin with antibiotics was assessed, and consequently, some new peaks were observed such as the N-H bending vibration of primary amines around 1,650 cm^{-1} and the C-N stretching vibration of aliphatic amines in the region 1,140–1,080 cm^{-1}, both characteristic of the TMP structure. Moreover, the presence of methoxy (O-CH_3) group was identified by a small stretch peak at 2,940 cm^{-1} as well as by the methyl (C-H) vibrations at 1,460 cm^{-1} (Coates 2000; Stuart 2004; Zade et al. 2011). Around 1,560–1,510 cm^{-1}, it was possible to observe the non-symmetrical substitutional aromatic ring present on TMP with N as a substitutional atom (Franklin et al. 2009; Ungurean et al. 2013). As a result of catechin-SMZ coupling, the presence of imino C=N groups which exhibit absorption at 1,595 cm^{-1}, the stretching in the sulphate S=O bond around 1,260–1,160 cm^{-1} characteristic from the SMZ compound, and the equivalent to thiol C-S and C-S-H stretching vibrations between 720 and 550 cm^{-1} was confirmed. In addition, the amines N-H bending around 1,650 cm^{-1} and the C-N stretching vibration at 1,150–1,090 cm^{-1} was also evident. The nitro N-O stretching bands occurred around 1,565–1,465 cm^{-1} as well as around 1,320–1,240 cm^{-1} accordingly to the corresponding symmetry (Coates 2000; Silva et al. 2009).

Once the successful development of catechin-antibiotic conjugates was confirmed, the in situ laccase oxidation of the phenolic monomer part was assessed for PU and SI catheter functionalization. As it was reported (Gonçalves et al. 2013; Guimarães et al. 2011; Jeon et al. 2012; Silva et al. 2011), the phenols can be directly oxidized by laccase yielding the corresponding phenoxy free radicals that become quinone intermediates. These compounds can react among each other through either coupling-based polymerization or radical rearrangement giving the dead-end products. On this work, the polymerization yield was monitored by UV-visible spectrophotometry wherein the band intensity of each compound is directly proportional to concentration values. Both unmodified and conjugated catechins were efficiently oxidized, however, in a different polymerization degree. The non-modified monomer was more easily converted into the

corresponding polymer than the conjugate compounds (data not shown). This behaviour may be due to the modified catechin possessing less hydroxyl groups available for the oxidative coupling reactions (Guimarães et al. 2011; Silva et al. 2011). The poly(catechin) or poly(catechin)-antibiotic conjugate attachment onto the catheter surface can be mostly attributed to the reaction of the intermediate quinones with highly reactive hydroxyl −OH and amine −NH groups resulting from the alkaline pre-treatment of SI and PU catheters, respectively (Chattopadhyay and Raju 2007; Gonçalves et al. 2013; Gopalakrishnana and Sujathaa 2011). However, the catheter coating by means of adsorption mechanisms must also be considered. The coloration observed after coating results from the nature of the phenol that changed from colourless (SI) or white (PU) to dark yellow when oxidized. The colour strength measurements performed allowed the effectiveness comparison of each coating since this parameter is directly related with the amount of polymer covering the catheter surface. PU devices were more efficiently coated with poly(catechin) and poly(catechin)-antibiotic conjugates than the SI catheters. Indeed, the previous alkaline hydrolysis of PU catheters yielded hydrophilic carboxylic −COOH and amine −NH groups available for reaction with quinone. On the other hand, only one type of hydrophilic group (−OH) was attained and available after SI sample hydrolysis. This would decrease the attachment yield of polymerized phenolic compounds onto the catheter surface. Comparing both, polycatechin and poly(catechin)-antibiotic conjugate, it is clear that the higher coverage of the catheter surface was achieved with poly(catechin). The lower number of hydroxyl groups available on the conjugates for enzymatic polymerization and the subsequent attachment could reduce the final amount of polymer obtained onto the catheter surface.

The coatings developed onto the catheter devices promoted chemical and physical modifications at the surface level which are essential to study. The water uptake is one of the key parameters that could influence the bacterial adherence and its proliferation onto the catheter surface. It is known that hydrophobic organisms have preference for hydrophobic surfaces. When specific functional groups are applied as a coating, the hydrophobicity and surface charge can be modified reducing the microorganism attachment, generating a more biocompatible material (Prasanna and Doble 2008). Herein, the poly(catechin) and poly(catechin)-antibiotic conjugates coating reduced the hydrophobic character of both PU and SI catheters. The highest hydrophobicity reduction was attained on PU catheters coated with poly(catechin)-antibiotic conjugates. This can be explained by the highest amount of poly(phenol) attached onto the PU catheter surface as previously mentioned. Poly(catechin) possesses hydroxyl groups on its structure conferring hydrophilic character to the material surface. The presence of amine groups on both antibiotic structures also contributes to decrease the hydrophobic nature of catheters justifying the results obtained for poly(catechin)-antibiotic conjugates.

The highest coating life-time was observed on the PU catheters. The high amount of groups (carboxylic −COOH and amine −NH) at their surface, available to react with the poly(phenols), increases coating strength and thus contributes to the life-time of the antimicrobial device. Nevertheless, more than half of poly(catechin) or poly(catechin)-antibiotic conjugates amount remained on both PU and SI catheter surface. Therefore, the functionalized catheters address to the need of invasive catheters for longer periods.

The synthesis and characterization of the developed coatings were followed by the conjugation process effect in the MIC values of free antibiotics. As it was expected, once combined to other molecules of those antibiotics, MIC values changed. Indeed, the coupling reaction between catechin and TMP or SMZ occurred through the nitrogen of the amine groups reducing the tertiary amine moiety. Therefore, the antimicrobial activity of the compounds was directly compromised by the conjugation processes (Radjenović et al. 2009). Although the produced catechin and poly(catechin)-antibiotic conjugates still reduced bacterial growth, two antimicrobial agents were successfully combined resulting in a novel model to prevent bacterial infections. Moreover, both poly(catechin) and poly(catechin)-antibiotic conjugate coatings gave a reduction in the bacterial adhesion as well as in the planktonic culture growth for each microorganism studied. It is known that flavonoids do not kill bacterial cells but merely induce the formation of bacterial aggregates and thereby reduce the colony number in viable counts (bacteriostatic activity) (Cushnie and Lamb 2005). Catechins are a group of flavonoids that have been demonstrating activity against gram-positive and gram-negative bacteria damaging them by two possible mechanisms: firstly, catechins may perturb the lipid bilayers by directly penetrating the membrane and disrupting the barrier function; alternatively, these phenolic compounds may cause membrane fusion which consists in a process that results in intramembranous material leakage and consequent aggregation (Cushnie and Lamb 2005; Ikigai et al. 1993). Herein, the reduction of bacterial adhesion was more pronounced using poly(catechin)-antibiotic conjugates. Although lower coating levels were achieved with conjugates, the amount attached onto the catheter surface was sufficient to reduce the bacteria adhesion. Comparing with non-treated catheters, SEM analysis shows the bacteria colony reduction for each developed coating. It is our assumption that, depending on the strength of antibiotic-polymer interaction, the release of TMP or SMZ could arise promoting its diffusion into the culture medium. In addition, enzymes secreted by bacteria could also support the drugs release (Kostakioti et al. 2005; Lee and Schneewind 2001; Wandersman 1992). The combination of two antibiotics

in the same conjugate revealed to improve the antibacterial efficacy of the coating preventing the microbial resistance appearance (Monzón et al. 2001; Worthington and Melander 2013). A synergistic effect can be reached when the action mechanisms of each compound, bactericidal or bacteriostatic, are well defined (Hancock 2005). TMP and SMZ are bacteriostatic antibiotics which inhibit enzymatic steps on the bacterial folate biosynthesis and together allow a broad bacterial spectrum of action. Indeed, SMZ inhibits dihydropteroate synthetase (DHPS) responsible for the production of dihydrofolate from *p*-aminobenzoic acid. Subsequently, TMP inhibits dihydrofolate reductase (DHFR) which catalyses the formation of tetrahydrofolate from dihydrofolate (Eliopoulos and Huovinen 2001; Fresta et al. 1996; Vickers et al. 2009). The main advantage to produce conjugates containing antibiotic coupled to poly(catechin) lies on the ability of catechins and its polymerized derivatives, to act on and disturb the bacterial membranes. This property would potentiate the antibiotic diffusion into cell interior causing a strong bacterial reduction on the catheter surface (Fresta et al. 1996). Aiming future medical applications, the viability of mammalian cells exposed to leachables of the developed antimicrobial coatings was evaluated. Even with some punctual interference on cellular metabolism observed, the rule was that L929 cells maintained more than 90 % viability even in longer incubations which is a very good indicator of the cytocompatibility of the devices. This behaviour was similar for both PU and SI catheters.

From this work resulted four novel antimicrobial coatings for medical devices. Taking the advantage of poly(catechin) and antibiotics properties, antimicrobial conjugates were designed, and the obtained synergism allowed the inhibition of planktonic bacteria growth and the bacterial adhesion reduction onto the coated surfaces. This synergism was more evident on poly(catechin)-TMP and poly(catechin)-TMP-SMZ where a higher bacteria reduction was observed. Moreover, the cellular metabolism assays demonstrate that the antimicrobial coatings developed are not harmful for the cellular metabolism, maintaining more than 90 % of cell viability after the highest incubation time tested. A promising approach for extending the life-span of indwelling catheters was developed. Concomitantly, the catheter-associated infections could be reduced and even improving the life quality of catheterized patients.

Acknowledgments The authors would like to acknowledge Pronefro (Portugal) and Degania (Israel) for supplying polyurethane and silicone catheters, respectively. The author Idalina Gonçalves would like to acknowledge the NOVO project (FP7-HEALTH-2011.2.3.1-5) for funding. This work was partly supported by FEDER through POFC–COMPETE and by Portuguese funds from Fundação para a Ciência e a Tecnologia (FCT) through the project PEst-OE/BIA/UI4050/2014. The authors Carla Silva and Teresa Matamá would like to acknowledge FCT for their scholarships SFRH/BPD/46515/2008 and SFRH/BPD/47555/2008.

References

Ahmad Z, Vargas-Reus MA, Bakhshi R, Ryan F, Ren GG, Oktar F, Allaker RP (2012) Antimicrobial properties of electrically formed elastomeric polyurethane copper oxide nanocomposites for medical and dental applications. In: Methods in Enzymology. vol 509. American Print, San Diego, pp 87–99

Ajuwon OR, Katengua-Thamahane E, Van Rooyen J, Oguntibeju OO, Marnewick JL (2013) Protective effects of rooibos (*Aspalathus linearis*) and/or red palm oil (*Elaeis guineensis*) supplementation on tert-butyl hydroperoxide-induced oxidative hepatotoxicity in Wistar rats. Evid Based Complement Alternat Med 2013:19. doi:10.1155/2013/984273

Alanis AJ (2005) Resistance to antibiotics: are we in the post-antibiotic era? Arch Med Res 36(6):697–705. doi:10.1016/j.arcmed.2005.06.009

Alberto MR, Canavosio MAR, Nadra MCMd (2011) Antimicrobial effect of polyphenols from apple skins on human bacterial pathogens. Electron J Biotechn 9(3):205–209. doi:10.2225/vol9-issue3-fulltext-1

Aldwin L, Gauney S, Groves ES, Nitecki DE, Rudolph AA, Winkelhake JL (1990) Polymer/antibiotic conjugate. US Patent 1990003252, California, US

Auzanneau C, Montaudon D, Jacquet R, Puyo S, Pouységu L, Deffieux D, Elkaoukabi-Chaibi A, De Giorgi F, Ichas F, Quideau S, Pourquier P (2012) The polyphenolic ellagitannin vescalagin acts as a preferential catalytic inhibitor of the α isoform of human DNA topoisomerase II. Mol Pharmacol 82(1):134–141. doi:10.1124/mol.111.077537

Baca AG, Brown J, Buckley DN, Nam P, O'Dwyer C, Etcheberry A (2009) State-of-the-art program on compound semiconductors 50 (SOTAPOCS 50) -and- processes at the semiconductor solution interface 3, vol 19. The Electrochemcial Society, New Jersey

Baillie GS, Douglas LJ (1999) *Candida* biofilms and their susceptibility to antifungal agents. In: Ron JD (ed) Methods in Enzymology. vol 310. Academic Press, pp 644–656

Bayston R, Fisher LE, Weber K (2009) An antimicrobial modified silicone peritoneal catheter with activity against both Gram positive and Gram negative bacteria. Biomaterials 30(18):3167–3173. doi:10.1016/j.biomaterials.2009.02.028

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1–2):248–254. doi:10.1016/0003-2697(76)90527-3

Chattopadhyay DK, Raju KVSN (2007) Structural engineering of polyurethane coatings for high performance applications. Prog Polym Sci 32(3):352–418. doi:10.1016/j.progpolymsci.2006.05.003

Childs RE, Bardsley WG (1975) The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochem J 145(1):93–103

Coates J (2000) Interpretation of infrared spectra, a practical approach. In: Meyers RA (ed) Encyclopedia of analytical chemistry. Wiley, Chichester, pp 10815–10837

Cushnie TPT, Lamb AJ (2005) Antimicrobial activity of flavonoids. Int J Antimicrob Ag 26(5):343–356. doi:10.1016/j.ijantimicag.2005.09.002

Cushnie TPT, Lamb AJ (2011) Recent advances in understanding the antibacterial properties of flavonoids. Int J Antimicrob Ag 38(2):99–107. doi:10.1016/j.ijantimicag.2011.02.014

Cushnie TPT, Hamilton VES, Lamb AJ (2003) Assessment of the antibacterial activity of selected flavonoids and consideration of discrepancies between previous reports. Microbiol Res 158(4):281–289

Daglia M (2012) Polyphenols as antimicrobial agents. Curr Opin Biotech 23(2):174–181. doi:10.1016/j.copbio.2011.08.007

Darouiche RO, Mansouri MD, Gawande PV, Madhyastha S (2009) Antimicrobial and antibiofilm efficacy of triclosan and

DispersinB® combination. J Antimicrob Chemoth 64(1):88–93. doi:10.1093/jac/dkp158

Desai DG, Liao KS, Cevallos ME, Trautner BW (2010) Silver or nitrofurazone impregnation of urinary catheters has a minimal effect on uropathogen adherence. J Urol 184(6):2565–2571. doi:10.1016/j.juro.2010.07.036

Diamanti S, Arifuzzaman S, Elsen A, Genzer J, Vaia RA (2008) Reactive patterning via post-functionalization of polymer brushes utilizing disuccinimidyl carbonate activation to couple primary amines. Polymer 49(17):3770–3779. doi:10.1016/j.polymer.2008.06.020

Eckmann C, Wasserman M, Latif F, Roberts G, Beriot-Mathiot A (2013) Increased hospital length of stay attributable to *Clostridium difficile* infection in patients with four co-morbidities: an analysis of hospital episode statistics in four European countries. Eur J Health Econ 14(5):835–846. doi:10.1007/s10198-013-0498-8

Eliopoulos GM, Huovinen P (2001) Resistance to trimethoprim-sulfamethoxazole. Clin Infect Dis 32(11):1608–1614. doi:10.1086/320532

Franklin S, Bhuvana KP, Balasubramanian T (2009) Vibrational, optical and microhardness studies of trimethoprim DL-malate. Cryst Res Technol 44(12):1329–1332. doi:10.1002/crat.200900056

Fresta M, Furneri PM, Mezzasalma E, Nicolosi VM, Puglisi G (1996) Correlation of trimethoprim and brodimoprim physicochemical and lipid membrane interaction properties with their accumulation in human neutrophils. Antimicrob Agents Ch 40(12):2865–2873

Gonçalves I, Matamá T, Cavaco-Paulo A, Silva C (2013) Laccase coating of catheters with poly(catechin) for biofilm reduction. Biocatal Biotransfor Early online:1–11. doi:10.3109/10242422.2013.828711

Gopalakrishnana S, Sujathaa R (2011) Ageing studies of cardanol based polyurethanes. Res J Pharm Biol Chem Sci 2(3):1069–1080

Guimarães C, Kim S, Silva C, Cavaco-Paulo A (2011) In situ laccase-assisted overdyeing of denim using flavonoids. Biotechnol J 6(10):1272–1279. doi:10.1002/biot.201100201

Hancock REW (2005) Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infect Dis 5(4):209–218. doi:10.1016/S1473-3099(05)70051-7

Hawser SP, Baillie GS, Douglas LJ (1998) Production of extracellular matrix by *Candida albicans* biofilms. J Med Microbiol 47(3):253–256. doi:10.1099/00222615-47-3-253

Høiby N, Bjarnsholt T, Givskov M, Molin S, Ciofu O (2010) Antibiotic resistance of bacterial biofilms. Int J Antimicrob Ag 35(4):322–332. doi:10.1016/j.ijantimicag.2009.12.011

Ikigai H, Nakae T, Hara Y, Shimamura T (1993) Bactericidal catechins damage the lipid bilayer. BBA-Biomembranes 1147(1):132–136. doi:10.1016/0005-2736(93)90323-R

Jancel T, Dudas V (2002) Management of uncomplicated urinary tract infections. Western J Med 176:51–55

Jeon J-R, Baldrian P, Murugesan K, Chang Y-S (2012) Laccase-catalysed oxidations of naturally occurring phenols: from in vivo biosynthetic pathways to green synthetic applications. Microbiol Biotechnol 5(3):318–332. doi:10.1111/j.1751-7915.2011.00273.x

Jigisha A, Nishant R, Navin K, Pankaj G (2012) Green tea—a magical herb with miraculous outcomes. Int Res J Pharm 3(5):139–148

Jo S, Engel PS, Mikos AG (2000) Synthesis of poly(ethylene glycol)-tethered poly(propylene fumarate) and its modification with GRGD peptide. Polymer 41(21):7595–7604. doi:10.1016/S0032-3861(00)00117-8

Karou D, Dicko MH, Simpore J, Traore AS (2005) Antioxidant and antibacterial activities of polyphenols from ethnomedicinal plants of Burkina Faso. Afr J Biotechnol 4(8):823–828

Klibanov AM (2007) Permanently microbicidal materials coatings. J Mater Chem 17(24):2479–2482. doi:10.1039/b702079a

Kohnen W, Kolbenschlag C, Teske-Keiser S, Jansen B (2003) Development of a long-lasting ventricular catheter impregnated with a combination of antibiotics. Biomaterials 24(26):4865–4869. doi:10.1016/S0142-9612(03)00379-X

Kopeček J (2013) Polymer-drug conjugates: origins, progress to date and future directions. Adv Drug Deliver Rev 65(1):49–59. doi:10.1016/j.addr.2012.10.014

Kostakioti M, Newman CL, Thanassi DG, Stathopoulos C (2005) Mechanisms of protein export across the bacterial outer membrane. J Bacteriol 187(13):4306–4314. doi:10.1128/jb.187.13.4306-4314.2005

Kowalczuk D, Ginalska G, Golus J (2010) Characterization of the developed antimicrobial urological catheters. Int J Pharm 402(1–2):175–183. doi:10.1016/j.ijpharm.2010.10.014

Lambert JD, Hong J, G-y Y, Liao J, Yang CS (2005) Inhibition of carcinogenesis by polyphenols: evidence from laboratory investigations. Am J Clin Nutr 81(1):284S–291S

Lee VT, Schneewind O (2001) Protein secretion and the pathogenesis of bacterial infections. Genes Dev 15(14):1725–1752. doi:10.1101/gad.896801

Lee D, Cohen RE, Rubner MF (2005) Antibacterial properties of Ag nanoparticle loaded multilayers and formation of magnetically directed antibacterial microparticles. Langmuir 21(21):9651–9659. doi:10.1021/la0513306

Lichter JA, Rubner MF (2009) Polyelectrolyte multilayers with intrinsic antimicrobial functionality: the importance of mobile polycations. Langmuir 25(13):7686–7694. doi:10.1021/la900349c

Lin R-D, Chin Y-P, Hou W-C, Lee M-H (2008) The effects of antibiotics combined with natural polyphenols against clinical methicillin-resistant *Staphylococcus aureus* (MRSA). Planta Med 74(08):840–846. doi:10.1055/s-2008-1074559

Mah T-FC, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9(1):34–39. doi:10.1016/S0966-842X(00)01913-2

Maoela MS, Arotiba OA, Baker PGL, Mabusela WT, Jahed N, Songa EA, Iwuoha EI (2009) Electroanalytical determination of catechin flavonoid in ethyl acetate extracts of medicinal plants. Int J Electrochem Sci 4:1497–1510

Miller LG, Tang AW (2004) Treatment of uncomplicated urinary tract infections in an era of increasing antimicrobial resistance. Mayo Clin Proc 79(8):1048–1054. doi:10.1016/S0025-6196(11)62580-9

Moini H, Arroyo A, Vaya J, Packer L (1999) Bioflavonoid effects on the mitochondrial respiratory electron transport chain and cytochrome *c* redox state. Redox Rep 4(1–2):35–41. doi:10.1179/135100099101534729

Monzón M, Oteiza C, Leiva J, Amorena B (2001) Synergy of different antibiotic combinations in biofilms of *Staphylococcus epidermidis*. J Antimicrob Chemother 48(6):793–801. doi:10.1093/jac/48.6.793

Mot AC, Silaghi-Dumitrescu R (2012) Laccases: complex architectures for one-electron oxidations. Biochem Mosc 77(12):1395–1407. doi:10.1134/S0006297912120085

Orhan DD, Özçelik B, Özgen S, Ergun F (2010) Antibacterial, antifungal, and antiviral activities of some flavonoids. Microbiol Res 165(6):496–504. doi:10.1016/j.micres.2009.09.002

Ouattara MB, Konaté K, Kiendrébéogo M, Ouattara N, Compaore M, Meda R, Millogo-Rasolodimby J, Nacoulma OG (2011) Antibacterial potential and antioxidant activity of polyphenols of *Sesbania grandiflora*. Cur Res J Biol Sci 3(4):351–356

Palacios L, Rosado H, Micol V, Rosato AE, Bernal P, Arroyo R, Grounds H, Anderson JC, Stabler RA, Taylor PW (2014) Staphylococcal phenotypes induced by naturally occurring and synthetic membrane-interactive polyphenolic β-lactam resistance modifiers. PLoS One 9(4):e93830. doi:10.1371/journal.pone.0093830

Pasut G, Veronese FM (2007) Polymer–drug conjugation, recent achievements and general strategies. Prog Polym Sci 32(8–9):933–961. doi:10.1016/j.progpolymsci.2007.05.008

Prasanna SS, Doble M (2008) Medical biofilms—its formation and prevention using organic molecules. J Indian Inst Sci 88(1):27–35

Radjenović J, Godehardt M, Petrović M, Hein A, Farré M, Jekel M, Barceló D (2009) Evidencing generation of persistent ozonation

products of antibiotics roxithromycin and trimethoprim. Environm Sci Technol 43(17):6808–6815. doi:10.1021/es900965a

Rizzo A, Carratelli CR, Losacco A, Iovene MR (2014) Antimicrobial effect of natural polyphenols with or without antibiotics on *Chlamydia pneumoniae* infection *in vitro*. Microb Drug Resist 20(1):1–10. doi:10.1089/mdr.2013.0024

Rodrigues LR (2011) Inhibition of bacterial adhesion on medical devices. In: Linke D, Goldman A (eds) Bacterial adhesion. Advances in experimental medicine and biology, vol 715. Springer, Netherlands, pp 351–367

Sam SS, Chazalviel J-NJ, Gouget-Laemmel ACA, Ozanam FF, Etcheberry AA, Gabouze N-EN (2011) Peptide immobilisation on porous silicon surface for metal ions detection. Nanoscale Res Lett 6(1):412. doi:10.1186/1556-276X-6-412

Shafahi M, Vafai K (2010) Synthesis of biofilm resistance characteristics against antibiotics. Int J Heat Mass Tran 53(15–16):2943–2950. doi:10.1016/j.ijheatmasstransfer.2010.04.004

Sharma P, Goel R, Capalash N (2007) Bacterial laccases. World J Microbiol Biotechnol 23(6):823–832. doi:10.1007/s11274-006-9305-3

Silva FEB, Ferrão MF, Parisotto G, Müller EI, Flores EMM (2009) Simultaneous determination of sulphamethoxazole and trimethoprim in powder mixtures by attenuated total reflection-Fourier transform infrared and multivariate calibration. J Pharmaceut Biomed 49(3):800–805. doi:10.1016/j.jpba.2008.12.011

Silva C, Matamá T, Kim S, Padrão J, Nugroho Prasetyo E, Kudanga T, Nyanhongo GS, Guebitz GM, Casal M, Cavaco-Paulo A (2011) Antimicrobial and antioxidant linen via laccase-assisted grafting. React Funct Polym 71(7):713–720. doi:10.1016/j.reactfunctpolym.2011.03.011

Smith AW (2005) Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems? Adv Drug Deliv Rev 57(10):1539–1550. doi:10.1016/j.addr.2005.04.007

Steinbrecht R, Müller M (1987) Freeze-substitution and freeze-drying. In: Steinbrecht RA, Zierold K (eds) Cryotechniques in biological electron microscopy. Springer, Berlin, pp 149–172

Stuart B (2004) Infrared spectroscopy: fundamentals and applications. Wiley, New York

Tiller JC, Liao C-J, Lewis K, Klibanov AM (2001) Designing surfaces that kill bacteria on contact. P Natl Acad Sci 98(11):5981–5985. doi:10.1073/pnas.111143098

Ungurean A, Leopold N, David L, Chiş V (2013) Vibrational spectroscopic and DFT study of trimethoprim. Spectrochim Acta A 102:52–58. doi:10.1016/j.saa.2012.10.026

Vickers AA, Potter NJ, Fishwick CWG, Chopra I, O'Neill AJ (2009) Analysis of mutational resistance to trimethoprim in *Staphylococcus aureus* by genetic and structural modelling techniques. J Antimicrob Chemoth 63(6):1112–1117. doi:10.1093/jac/dkp090

Wandersman C (1992) Secretion across the bacterial outer membrane. Trends Genet 8(9):317–322. doi:10.1016/0168-9525(92)90264-5

Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3(2):163–175

Worthington RJ, Melander C (2013) Combination approaches to combat multidrug-resistant bacteria. Trends Biol 31(3):177–184. doi:10.1016/j.tibtech.2012.12.006

Zade GD, Dhoble SJ, Raut SB, Pode RB (2011) Synthesis and characterization of chlorine-methoxy-diphenylquinoline (Cl-MO-DPQ) and chlorine-methyl-diphenylquinoline (Cl-M-DPQ) blue emitting organic phosphors. J Mod Phys 2:1523–1529

Zander J, Besier S, Faetke S, Saum SH, Müller V, Wichelhaus TA (2010) Antimicrobial activities of trimethoprim/sulfamethoxazole, 5-iodo-2′-deoxyuridine and rifampicin against *Staphylococcus aureus*. Int J Antimicrob Ag 36(6):562–565. doi:10.1016/j.ijantimicag.2010.08.007