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Characterization of the developed antimicrobial urological catheters

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ABSTRACT

Antimicrobial urological catheters were developed by the mixed, covalent and non-covalent binding of sparfloxacin (SPA) to heparin (HP) film which was first deposited on the latex surface of biomaterial. The SPA-HP modified surface was characterized by SEM analysis and ATR-Fourier transform infrared spectroscopy. For the antimicrobial prevention, SPA as an antibiotic with a broad antimicrobial spectrum was chosen. Antimicrobial activity of antibiotic-modified catheter against *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Escherichia coli* strains was assessed using various procedures. On the basis of the inhibition zone and diffusion assays the efficacy around the modified catheters was demonstrated. The test samples clearly showed an antibacterial activity against all tested bacterial stains for a least one month. Inhibition of the bacterial colonization on the modified catheter surface was proved by the biofilm test. Antimicrobial activity of SPA-treated catheter surface was also quantitatively evaluated according to standard method of ISO based on JIS. The R-values were found to be higher than 3.8. The performed research indicated that the immobilization of SPA on the catheter surface by means of the mixed-type bonds resulted in stable antibacterial protection of the urological catheters for a long time.

1. Introduction

Four most common causes of hospital acquired infections are urinary tract infection, surgical site infection, respiratory tract infection and bloodstream infection. Urinary tract infection is the most frequently reported nosocomial infection, accounting for up to 40% of infections. A significant percentage of these infections are associated with urinary catheters. Indwelling urinary catheters, which are used in 15–25% of short-term care patients during their hospitalization, confer a predisposition to bacteriuria. 1–5% of catheter-associated bacteriuria episodes produce secondary bloodstream infection, a serious risk of life-threatening complication (Chenoweth and Saint, 2005; Johnson et al., 2006; Nicolle, 2005; Saint et al., 2002).

The use of urinary catheters can interfere with the normal defences of urinary tract, allowing bacterial colonization which lead to the biofilm formation on their inner and outer surfaces and infections, which are difficult to eradicate by antibiotics (MIC of antimicrobial agents, necessary to kill microorganisms, is significantly higher for biofilm-entrapped bacteria than for planctonic ones). *Staphylococcus aureus* and *Staphylococcus epidermidis* are the microorganisms most frequently involved in catheter-related infections (Chenoweth and Saint, 2005; Choong et al., 2001; Pascual, 2002; Tenke et al., 2006; Warren, 2001). Urinary catheter biofilms are unique because some organisms in the biofilm, such as *Proteus* spp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Providencia* spp. synthesize urease able to hydrolyze urea in the urine to free ammonia. In the alkaline environment, crystals of struvite and hydroxyapatite (the ionic components in the urine) precipitate and then deposit into a bacterial biofilm layer. Progression of the encrustation can completely block the catheter flow (Chenoweth and Saint, 2005; Choong et al., 2001; Tenke et al., 2006).

The presence of urinary catheter biofilm has important implications for prevention and treatment of urinary tract infection in the catheterized patient. The catheter colonized by bacteria frequently must be replaced resulting in increased morbidity for the patient and increased cost to the healthcare system. From a prevention standpoint, the need to develop catheters with materials that prevent microorganism attachment and biofilm formation is paramount.

The available literature has exhibited that various modifications with hydrophilic outer layers, antimicrobial coated surfaces, low surface energy materials, highly biocompatible substances, biodegradable materials, and cell or protein grafted surfaces showed good capability against microbial colonization of biomaterials. Numerous reports demonstrated that coating of biomaterials surface with heparin (Aksoy et al., 2008) chitosan (Shu-Hua Yang et al., 2007), a combination of heparin and chitosan (Fu et al., 2005), cationic charge (Rose et al., 2005), mucin (Shi et al., 2000) or cranberry-derived proanthocyanidins (Eydelnant and Tufenkji, 2008) substantially reduced the adhesion and colonization of bac-

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teria. Due to antithrombogenic properties of heparin, it was also widely used for the surface modification of biomaterials through either chemical binding or physical absorption (Murugesan et al., 2008).

Many attempts have been made to fight catheter-related infections mainly by impregnation with antimicrobials. The papers report that antimicrobial agents such as furanones (Baveja et al., 2004), silver oxide (Johnson et al., 1990), antibiotics (ciprofloxacin, ofloxacin, norfloxacin) (DiTizio et al., 1998; Reid et al., 1994), a combination of antibiotics (minocycline with rifampin) (Raad et al., 1996), titanium dioxide (Sekiguchi et al., 2007) or a combination of silver oxide with titanium dioxide (Yao et al., 2008) exhibited promising results with respect to reducing or inhibiting of bacterial colonization of urinary catheters or potential urological biomaterials.

The aim of the present study was to characterize the developed antimicrobial urinary and prostatic catheters and evaluate the ability of these anti-infective latex biomaterial (heparin and sparfloxacin-containing) to prevent bacterial colonization and biofilm formation, and thereby to resolve the existing important clinical problem of catheter-associated urinary tract infections.

To obtain the antimicrobial urological catheter, sparfloxacin was used. This quinolone antibiotic was selected because of its physico-chemical and antimicrobial properties. Sparfloxacin is a broad-spectrum antimicrobial agent, very effective against a number of gram-positive (staphylococci, streptococci, enterococci) and gram-negative microorganisms (*Enterobacteriaceae*, *Pseudomonas* spp., *Haemophilus influenzae*) and also various species of mycoplasmas, legionellas, chlamydiae, and mycobacteria. The bactericidal action of sparfloxacin results from inhibition of bacterial topoisomerase II (DNA gyrase) and topoisomerase IV, which control DNA topology and assist in DNA replication, transcription, repair and recombination. Besides, sparfloxacin is characterized by a relatively stabile chemical structure (important property during an immobilization process).

2. Materials and methods

2.1. Materials

Pure sparfloxacin (SPA) was obtained from Rhône-Poulenc Rorer (Paris, France). Heparin sodium salt (HP) and all other reagents used for immobilization process were obtained from Sigma–Aldrich (Deisenhofen, Germany). Two-way Foley urinary catheters (Tyco Healthcare/Kendall, UK) or (Unomedical Sdn. Bhd., Malaysia) and three-way Foley prostatic catheter (Coloplast, Denmark) were obtained from a local medical supplier (Skamex, Poland). Acetonitrile of analytical grade were achieved from POCh (Gliwice, Poland). The water used in the experiments was double distilled. Phosphate buffer solution was prepared by dissolving 34 g of potassium dihydrogen phosphate in 500 ml water, adjusting the pH to 7.0 with 1 mol/l sodium hydroxide and making up to 1000 ml with water. Physiological saline was prepared by dissolving 8.5 g of sodium chloride in 1000 ml of water. Phosphate-buffered physiological saline was prepared by diluting phosphate buffer solution with the physiological saline to an 800-fold volume. Washing solution was physiological saline.

2.2. Methods

2.2.1. Preparation of SPA-treated catheters with the mixed binding of SPA

The commercially available urinary or prostatic catheters made of a natural latex, coated with silicone fluid (latex siliconized) or with silicone elastomer (chemically bonded to the inner and outer surface of the latex) were subjected to modification process according to procedure described in the Polish Patent Pending P.389488 (Ginalska and Kowalczuk, 2010).

In the first step, small specimens of 1.0 cm in length of catheter were coated with sulfatized mucopolysaccharide–heparin (HP test samples) and then modified by use of two immobilization methods. *In the method 1*, heparin-coated surfaces were directly oxidized with sodium periodate and the activated matrices were coupled with sparfloxacin in organic medium to form a Schiff base (SPA-HP test samples). *In the method 2*, heparin-coated surfaces were activated with glycidol to introduce the diol groups, then oxidized with sodium periodate and linked with sparfloxacin in organic medium to form a Schiff base (SPA-HP test samples). In both methods, the immobilization of SPA on the heparinized and then oxidized surface was performed in the SPA solutions prepared in acetonitrile at the high concentration of 1.0 mg/ml (SPA-H solution) and at the low concentration of 0.1 mg/ml (SPA-L solution). The resulting catheter specimens were rinsed with water, dried at 50 °C and subjected to ATR-FTIR analysis, to diffusion assay and zone of inhibition assay.

2.2.2. Washing of SPA-treated catheters to eliminate non-covalent attachment of SPA

A series of the catheter specimens treated with SPA of about 2.0 cm in length were prepared as described above (Section 2.2.1) and were washing with a continuously renewing the washing solution for 2 days. The test specimens were immersed in 300 ml of the washing solution (volume sufficient to cover the surfaces) and were stirring on magnetic stirrer with a changing the washing solution every 30 min, then were kept overnight in the water bath, with stirring. Every time, at renewing the washing solution, one test specimen was withdrawn from the bath and introduced into tube containing 5 ml of Mueller–Hinton broth medium (Oxoid, United Kingdom). Next, the medium was inoculated with 16 μl of 0.5°McFarland of *Escherichia coli* ATCC 25992 strain suspension. After a 18 h incubation time at 37 °C, the growth of bacteria in medium was checked. The washing was continued until the detection of bacterial growth. The catheter specimens washed and dried at 50 °C were subjected to ATR-FTIR analysis and to inhibition zone assay.

2.3. Surface characterization

Chemical structure of the surface was examined with attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) using a Thermo Scientific Nicolet 8700A spectrometer equipped with a single-bounce beam path and a diamond crystal at 45° angle of reflection. The samples were analyzed over the region from 500 to 4000 cm^{-1} with the spectral resolution of 4 cm^{-1}. All spectra were averaged over 32 scans and an advanced ATR correction was applied. ATR spectra were recorded on the inside and outside of the modified samples.

The surface topography of the modified and unmodified samples was investigated by scanning electron microscopy (SEM) using scanning electron microscope BS-301 (Tesla, Czech Republic) at 15 kV, after coating with gold/palladium by a high-resolution SEM sputter coater SC 7640 (Quorum Technologies Ltd, UK).

2.4. Antimicrobial evaluation

The antibacterial activity of the SPA-treated surface was assessed *in vitro* against *Staphylococcus aureus* ATCC 25923 strain, *Staphylococcus epidermidis* ATCC 12228 strain and *Escherichia coli* ATCC 25992 strain. Prior to microbiological testes, the test samples were sterilized with ethylene oxide.

2.4.1. Evaluation of antibacterial activity around the SPA-treated catheters using diffusion and inhibition zone assays

For diffusion assay, four catheter specimens of about 1.0 cm in length were used: SPA-treated specimen obtained after immobilization in SPA-H solution, SPA-treated specimen obtained after immobilization in SPA-L solution (both prepared according to procedure described in Section 2.2.1), HP-treated specimen (only with HP) and the untreated specimen as a control. All test specimens were introduced into tubes containing 5 ml of Mueller–Hinton broth medium (Oxoid, United Kingdom) and inoculated with 16 μl of 0.5°McFarland of the bacterial test strain suspensions. The medium was replaced every other day by a fresh portion of the broth and reinoculated. The experiment lasted until the detection of microorganisms growth. The concentration of the viable bacteria in the medium were estimated and expressed as number of colony forming unit (CFU) per milliliter.

For inhibition zone assay, four catheter specimens of about 1.0 cm in length were used: two SPA-treated specimens obtained after immobilization in SPA-H solution (both prepared according to procedure described in Section 2.2.1), the first one after 5 times washing, the second one completely washed, and HP-treated specimen (only with HP), and the untreated specimen as a control. All test specimens were placed on Petri plates with Mueller–Hinton agar medium (Oxoid, United Kingdom), previously inoculated with 1.5×10^8 CFU/ml (0.5°McFarland) of the bacterial test strains). After incubation at 37 °C for 18 h, inhibition zones of microbial growth surround the tested biomaterials were measured.

2.4.2. Evaluation of biofilm formation on the surface of SPA-treated catheters

SPA-treated specimens obtained after immobilization in SPA-H solution and in SPA-L solution (both prepared according to procedure described in Section 2.2.1), HP-treated and the untreated specimens as controls were subjected to the diffusion assay described above. When the bacterial growth was detected in the broth medium, these pieces were subsequent tested for the presence of viable bacteria (biofilm) on their surfaces. All specimens were incubated with 0.001% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, USA) in Mueller–Hinton broth medium at 37 °C for 72 h. In the presence of viable cells, the tetrazolium salt was converted to its colored TTC-formazan product that was monitored visually. The appearance of a slightly red, red or dark red color on the surface of catheter samples proved the presence of bacterial biofilm.

2.4.3. Measurement of antibacterial activity on the surface of SPA-treated catheters

2.4.3.1. Preparation of the test specimens. Nine catheter specimens treated with SPA measuring 35 mm × 30 mm were obtained after immobilization in SPA-H solution according to procedure described in Section 2.2.1. Each of 9 pieces were separately washed by stirring on magnetic stirrer with 15 quantities, each of 200 ml, of physiological saline (with a changing the washing solution every 30 min) and then kept overnight in the water bath for 15 h, with stirring. Prior to test, 18 specimens from the untreated catheter measuring 35 mm × 30 mm were washed 3 times with water and kept overnight in the water bath.

2.4.3.2. Procedure. The experiments were carried out according to two version of procedure.

The first version: The antimicrobial activity was determined accurate according to the standard method ISO 22196:2007 based on JIS Z 2801:2000. In brief, a cell suspension of *E. coli*, *S. aureus* or *S. epidermidis* (each 6×10^5 cells/ml^{-1}) was prepared in the diluted 1/500 nutrient broth (Tryptone Soya Broth, Oxoid, United Kingdom). The test specimens (3 specimens treated with SPA and 6 untreated specimens) were placed in Petri dishes and inoculated with 0.1 ml of a bacterial culture containing 6×10^4 CFU/ml. The inoculum was covered with a sterile polyethylene film (20 mm × 20 mm on a test piece), and the Petri dishes with 3 specimens treated with SPA and with 3 of the 6 specimens of the untreated catheter were incubated at 35 °C for 24 h under humid conditions to prevent desiccation. After incubation, cover film and the cell suspension remaining on the surface were completely rinsed with 10 ml of sterile SCDLP broth (prepared by adding 1.0 g of lecithin from Fluka and 7.0 g of polysorbate from Fluka per liter of Tryptone Soya Agar from Oxoid, UK) using a pipette. 3 specimens from the untreated catheter were also processed in this manner prior to incubation to provide base-line data. Subsequently, serial dilutions of SCDLP broth recovered from test specimens in phosphate-buffered physiological saline were spread onto separate agar plates (using the spread plate method) in duplicate and incubated at 35 °C for 40 h. After incubation, colonies in these plates were counted visually, and the numbers of colony forming units were recorded.

The second version: The antimicrobial activity was determined according to the standard method ISO 22196:2007 based on JIS Z 2801:2000 as described in the first version but without dilution of the nutrient broth (Tryptone Soya Broth) in order to create conditions to the growth of bacteria.

3. Results and discussion

Antimicrobial urological catheter based on the latex matrix (known to have cytotoxic properties) was obtained by coating of the catheter surface with heparin (an anticoagulant and biocompatible agent, known also to have anti-inflammatory and antimicrobial properties) to introduce the hydroxyl groups and subsequently coupling with sparfloxacin in organic medium to form a relatively stable Schiff base, using two immobilization methods. The used methods are well-known and often applied techniques allowing to create covalent bonds, but the application these methods for the obtainment of the antimicrobial urological latex catheter with sparfloxacin is a new conception being aimed at preventing bacterial colonization, biofilm formation and thus inhibiting of catheter-associated urinary tract infections. As a result, as the ATR-FTIR and microbiological tests shown, we achieved the antimicrobial urological catheter with sparfloxacin which was linked to the matrix surface by means of the mixed-type bonds, covalent and non-covalent (adsorption) bonds.

3.1. Surface characterization

SEM images of the test samples subjected to modification process are shown in Figs. 1–3. The noticeable differences in the surface topography of the tested samples can be observed in Figs. 1 and 2. The SEM image of latex film (Fig. 1A) shows relatively small, conspicuous, irregular shaped particles. After coating with HP layer (Fig. 1B) and especially with SPA layer (Fig. 1C), the particles have became badly noticeable. The HP and SPA coatings strengthened spatial irregularities and roughness (Fig. 2; the same test samples, but shown in three-dimensional format). The SEM image in Fig. 3 shows the cross-section of the modified catheter. From this image, it can be concluded that thickness of the modified heparin layer is approximately 100 μm.

The changes in chemical structure on the surface of the modified catheter samples were investigated by attenuated total reflection-Fourier transform infrared spectroscopy. The ATR-FTIR spectra of the test samples after each surface modification step (A, B, C) and the ATR-FTIR spectra of pure substances of HP and SPA are shown in Fig. 4.

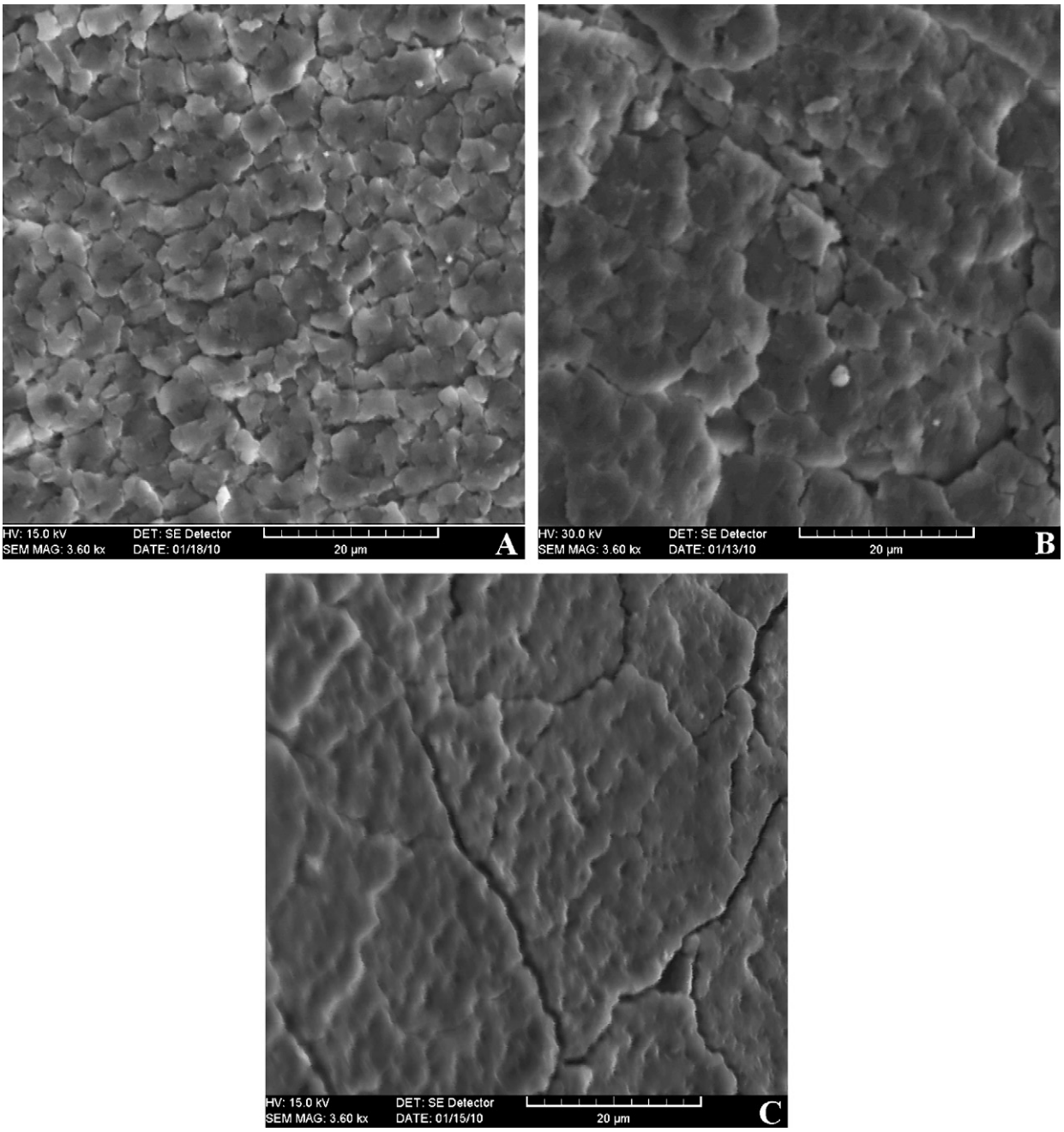

Fig. 1. SEM images of the untreated catheter (A), the catheter with HP (B), and catheter with HP and SPA (C) at magnification 3.6k×.

Heparin is a highly acidic mucopolysaccharide consists of a variably sulfated (N-sulfated and O-sulfated) repeating disaccharide units that include the residues of D-glucosamine and D-glucuronic- or L-iduronic-acids. The ATR-FTIR spectrum of pure heparin (HP in Fig. 4) used for the surface modification of the urological catheters shows the broad, intensive absorption band at 3441 cm^{-1} related to stretching vibrations of hydroxyl groups and the peaks at about 1609 cm^{-1} and 1418 cm^{-1} assigned to asymmetric and symmetric deformations of carboxylate anions, respectively. Comparison obtained peaks and literature data (Sushko et al., 1994) permits to make an assumption that the peaks in the 1300–1100 cm^{-1} region appeared due to S=O asymmetric stretching vibrations of O-sulfate and N-sulfate groups. The absorption bands in the 1100–900 cm^{-1} region can be assigned to S=O symmetric stretching vibrations of O-sulfate and N-sulfate groups. The very characteristic band of heparin occurring at about 890 cm^{-1} due to C–O–C stretching vibrations of sugar ring. The absorbance appearing at about 800 cm^{-1} can be attributed to sulphate half-esters.

Sparfloxacin is synthetic chemical compound derived from fluoroquinolone (5-amino-1-cyclopropyl-7-[-3,5-dimethylpiperazin-1-yl]-6,8-difluoro-4-oxoquinoline-3-carboxylic acid). The ATR-FTIR spectrum of pure sparfloxacin (SPA in Fig. 4) shows numerous absorption bands. On the basis of literature data (Dorofeev, 2004), the most characteristic and predominant of them can be assigned to N–H asymmetric and symmetric stretching vibrations of primary amine at 3460/3340 cm^{-1}, stretching vibrations of carboxy carbonyl ($\nu_{C=O}$ in COOH) at 1714 cm^{-1}, stretching vibrations of carbonyl ($\nu_{C=O}$) in position 4 of quinoline ring at 1639 cm^{-1}, and aromatic C=C stretching vibrations ($\nu_{C=C}$) at 1585/1558 cm^{-1}, 1531/1493 cm^{-1}, 1436 cm^{-1} (with N–H deformations of $-NH_2$ group). The strong absorption band observed at 1436 cm^{-1} can also correspond to the C–H deformations of $-CH_2-$ groups and

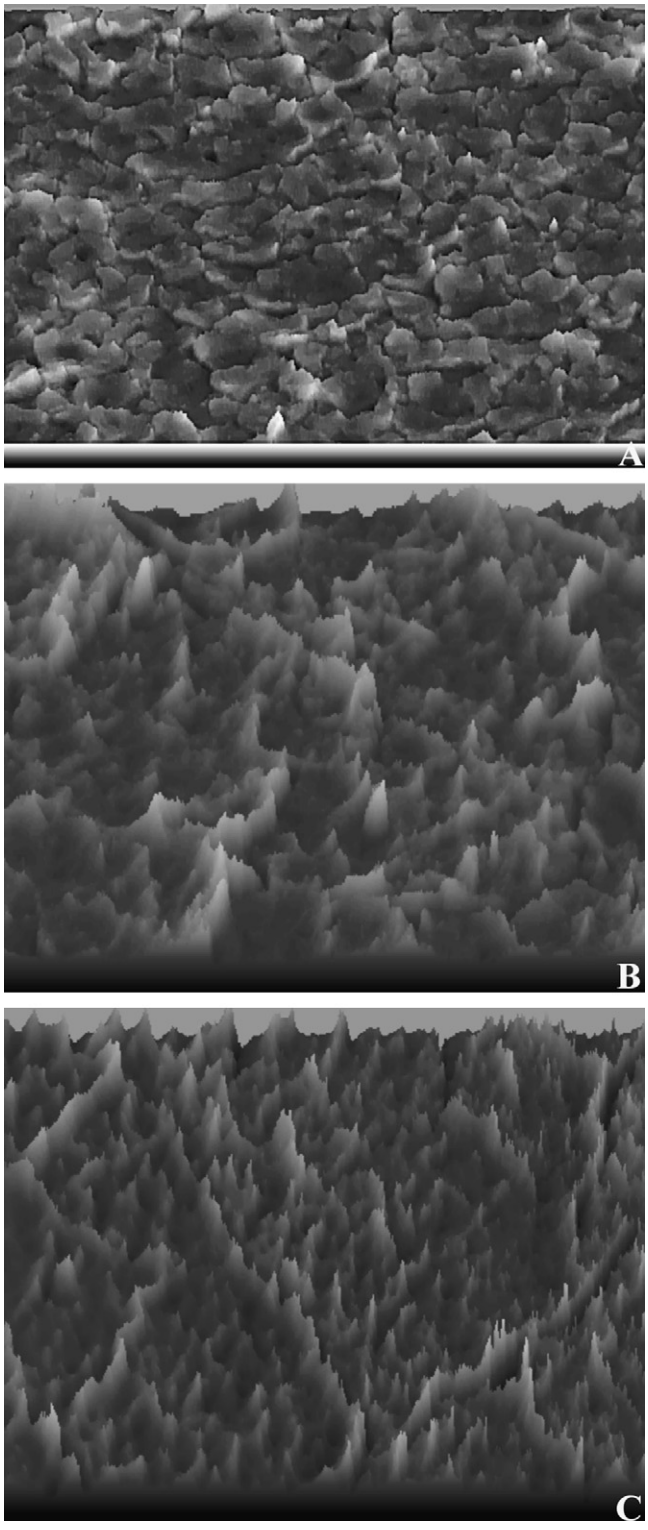

Fig. 2. SEM images of the same catheter samples as in Fig. 1 shown in three-dimensional format.

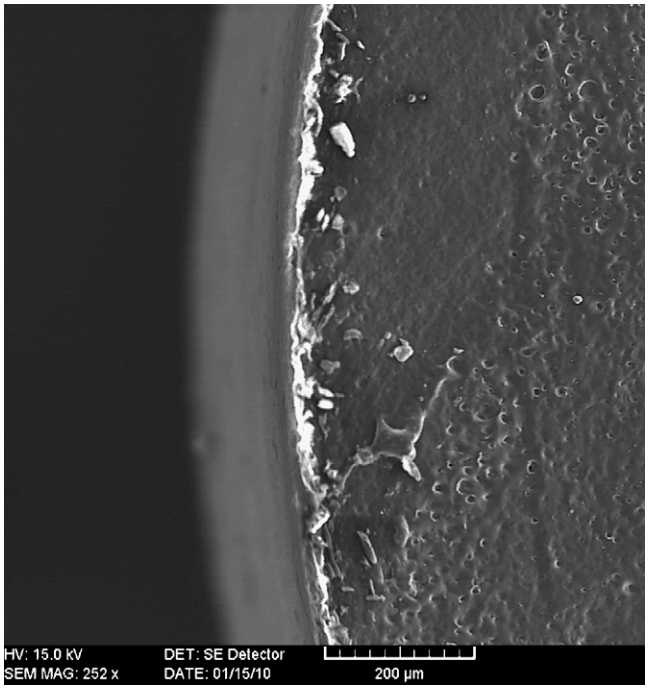

Fig. 3. SEM images of the cross-section of catheter sample coated with HP and modified with SPA at magnification 252×.

$-CH_3$ groups. The further peaks in SPA spectrum can be assigned to C–N (in ring and primary amine) and C–O (in COOH) stretching vibrations in the 1350–1260 cm^{-1} region, C–F stretching vibrations in the 1250–1100 cm^{-1} region, and N–H wagging vibrations at 909 cm^{-1}.

In the spectrum of the untreated latex film (A in Fig. 4), the major absorbance peaks in regions of 3000–2850 cm^{-1}, 1680–1600 cm^{-1}, 1450–1375 cm^{-1} and at 839 cm^{-1} can be attributed to C–H stretching vibrations from CH_3 and CH groups, C=C stretching vibrations, C–H deformations and C–H out of plane deformations, respectively.

The spectra of HP-coated latex film (B in Fig. 4) and of the untreated latex film (A in Fig. 4) are similar. However, in the case of the composite spectrum B, some structurally important features of heparin can be observed: the significant increased intensity of the broad peak at 3440 cm^{-1} corresponding to hydroxyl groups and the additional absorption band at 1552 cm^{-1} with the change of the spectral band in the 1650–1580 cm^{-1} region related to carboxylate anion stretching vibrations. Moreover, the change of the spectral band in the 1300–1000 cm^{-1} region can be caused by the presence of sulphate groups.

The ATR-FTIR spectrum of HP-coated SPA-attached latex film (C in Fig. 4) is similar to the spectrum of HP-coated latex film. However, the binding of sparfloxacin to the oxidized heparin resulted in a decrease of the absorption of heparin hydroxyl bands at 3440 cm^{-1} and in an increase of the spectral intensity at 1725 cm^{-1}, 1634 cm^{-1}, 1442 cm^{-1} and in the 1258–1033 cm^{-1} region. Such absorptions are characteristic for carbonyl, aromatic C=C and C–F stretching vibrations of sparfloxacin. A relatively strong increase of the spectral band in the 1680–1620 cm^{-1} region can result from the formation of the CH=N group (Schiff base) between NH_2 group of SPA and aldehyde group of the oxidized heparin (covalent binding of SPA) as well as from the presence of non-covalent attached SPA. In order to show the formation of covalent binding of SPA, the SPA-treated catheters were subjected to washing with a continuously renewing the washing solution to eliminate non-covalent binding of SPA. The exemplary ATR-FTIR spectra obtained for SPA-treated, five times washing catheter (A) and for SPA-treated, completely washed catheter (B) are shown in Fig. 5. The intensity and form of the spectral band at 1634 cm^{-1}, in the initial spectrum of the tested catheter, were changing after repeated washings. As a result, new peak appeared at 1649.6 cm^{-1}, overlapping with the peak of carbonyl group at 1635.6 cm^{-1}, which can be assigned to the C=N vibrations characteristic of imines.

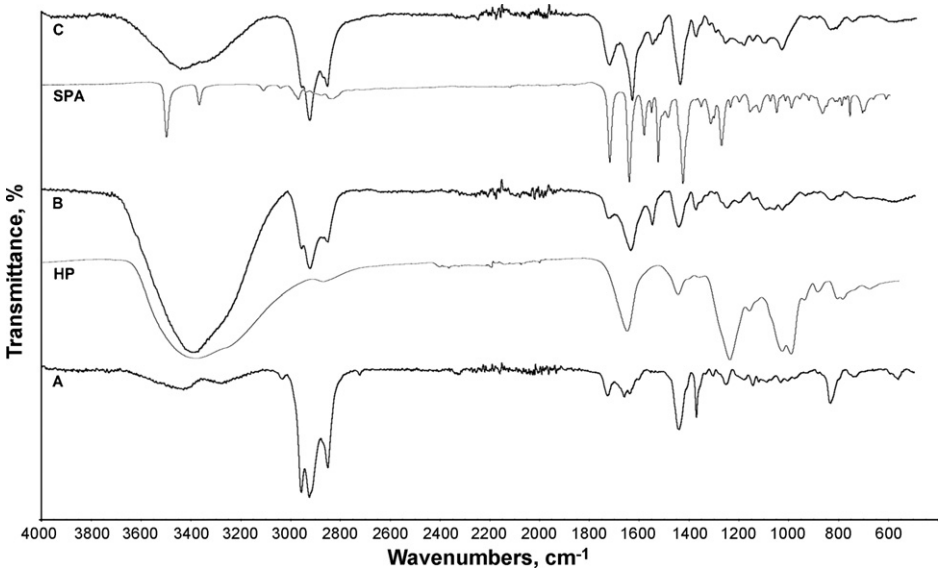

Fig. 4. Overlaid FTIR spectra of the untreated latex film (A), HP-coated latex film (B) and HP-coated SPA-attached latex film (C) both obtained according to the patented method as well as pure substances of sparfloxacin (SPA) and heparin (HP).

3.2. Evaluation of antibacterial activity around the SPA-treated catheters (diffusion and inhibition zone assays)

Antimicrobial activity of SPA-treated catheters was examined by use of qualitative antibacterial test based on diffusion assay to determine the ability of the treated catheter to prevent microbial growth. The modified catheters treated with SPA at different concentrations (0.1 mg/ml and 1.0 mg/ml) were introduced into tubes containing Mueller–Hinton broth medium and examined for their effect against *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228 and *E. coli* ATCC 25992 strains. The tested microorganisms are susceptible to sparfloxacin, MIC: 0.12 μg/ml for *S. aureus*, 0.25 μg/ml for *S. epidermidis*, ≤0.06 μg/ml for *E. coli* (Visser et al., 1991). The SPA-catheter system obtained after immobilization in a solution of 0.1 mg SPA/ml (low concentration of antibiotic) was protected from microbial colonization for at least 7 days (*E. coli*: 10 days, *S. aureus*: 10 days, *S. epidermidis*: 7 days), whereas the SPA-catheter system obtained after immobilization in a solution of 1.0 mg SPA/ml (high concentration of antibiotic) was protected from microbial colonization for at least 27 days (*E. coli*: 34 days, *S. aureus*: 30 days, *S. epidermidis*: 27 days). In the case of control catheters, the bacterial growth was observed just after first day of assay. The lack of bacterial growth for about 30 days can suggest that a part of the antibiotic immobilized via non-covalent bonds was able to diffuse to the surrounding culture medium and that antibiotic was active.

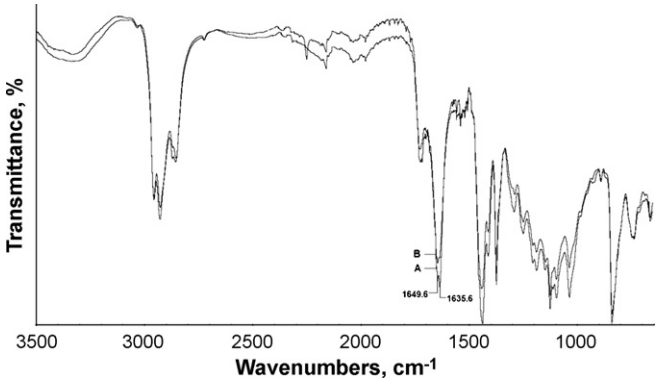

Fig. 5. The typical FTIR spectra obtained for SPA-treated catheter after five times washing (A) and for completely washed SPA-treated catheter (B).

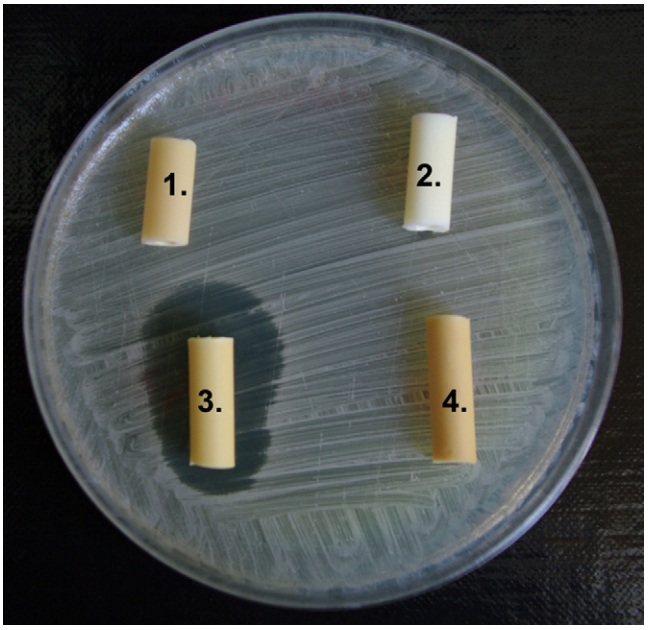

Fig. 6. Inhibition zones around the test samples as their effect against *E. coli*; SPA-treated catheter sample, completely washed (4), SPA-treated catheter sample obtained after 5 times washing (3), HP-coated latex sample (2), the untreated sample (1).

The inhibition zone assay was used for testing leachable SPA from SPA-treated catheters. A zone of inhibition formed around the tested specimens and the diameter of inhibition zone was a measure of film efficacy against *S. aureus*, *S. epidermidis* and *E. coli* strains. The zones of inhibition around the SPA-treated catheter specimen, measuring at beginning of assay about 40 mm, were gradually decreased when this specimen were subjected to subsequent washing. No inhibition zone was observed around the completely washed specimen (these effects can be observed in Fig. 6). However, when this specimen was lifted above, the place free of bacteria appeared on the agar surface. This effect can suggest that a part of the antibiotic immobilized via covalent bonds remained on the surface and that antibiotic was active. Catheters without antibiotic (as control) showed no inhibition zone

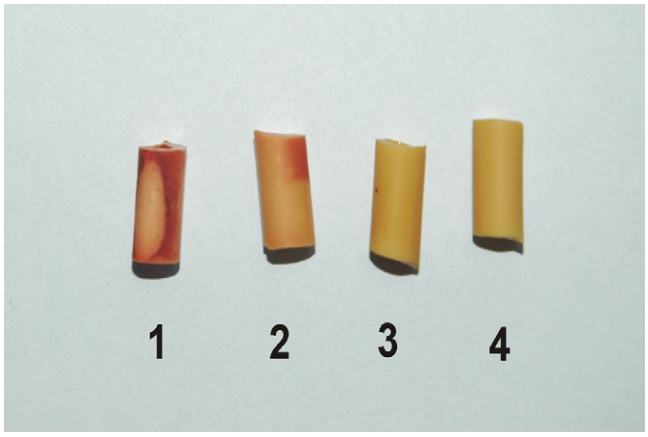

Fig. 7. Bacterial biofilm formation on the test catheter surfaces: samples with SPA immobilized in solutions of 1 mg/ml (4) and 0.1 mg/ml (3), sample with HP (2), the untreated sample (1).

against all tested strains, similarly as catheters coated with heparin.

3.3. Evaluation of biofilm formation

The continuation of the previous diffusion assay was the evaluation of biofilm formation (the presence of viable bacteria) on the surfaces of the tested catheters when the bacterial growth was detected in the broth medium, around these catheters, in the absence of SPA diffusion (for instance in the case of *E. coli* broth: after 34 days for SPA-HP sample coated with SPA in SPA-H solution, after 10 days for SPA-HP sample coated with SPA in SPA-L solution, after 1 day for HP sample and control sample). Bacterial biofilm presence on the surface of SPA-treated and the untreated catheters was estimated after extensive washing to remove all non-absorbed bacterial cells prior to placing in fresh TTC-supplemented medium by use of TTC – reduction test (Bhupathiraju et al., 1999). The exemplary effect of TTC – reduction test obtained for test samples incubated in *E. coli* medium is shown in Fig. 7.

The presence of the colored formazan could have appeared only in case of catheters colonized by bacterial biofilm. It was found (Table 1) that biofilm was detected on the untreated catheters while covalently modified catheters remained untouched by biofilm. The surfaces containing SPA, in contrast to the surfaces without SPA, were free from microbial flora. The bigger amount of antibiotic immobilized on catheter surface protected the tested biomaterial from bacterial colonization more affectively (a lack of TTC-formazan) than the smaller amount of antibiotic (single, viable bacteria cells were presence on the surface; slightly red color of TTC-formazan). Through a lack of antibiotic on catheters (control sample and sample with heparin), bacteria adhered to catheter surfaces and created the biofilm (dark red or red color of TTC-formazan).

Table 1
Bacterial biofilm formation on the surface of catheters with SPA immobilized in solutions at different concentrations (0.1 mg/ml and 1.0 mg/ml), the catheter with HP and the untreated catheter as a control, detected by use of TTC – reduction test.

Catheter test sample	*S. aureus* adhesion	*S. epidermidis* adhesion	*E. coli* adhesion
Control sample	++++	++++	++++
HP-sample	++++	++++	++
HP-SPA sample (0.1 mg/ml)	+	+	–
HP-SPA sample (1.0 mg/ml)	–	–	–

(+) slightly red, (++) red, (++++) dark red color.

From microbiological tests, it can be deduced that the antimicrobial activity of SPA modified catheters was due to the diffusion of SPA into medium (non-covalent attached SPA) and to the presence of SPA on the surface (covalent attached SPA).

3.4. Measurement of antibacterial activity on catheter surface according to ISO and JIS

For the quantitative evaluation of antimicrobial activity of SPA-treated catheters was adopted standard methods described by the International Organization for Standardization (ISO 22196:2007) and based on Japanese Industrial Standard (JIS Z 2801:2000). The SPA-treated catheters, completely washed to remove non-covalent bonds of SPA, was inoculated with bacterial cell suspension of *E. coli*, *S. aureus* and *S. epidermidis*, and held in intimate contact for 24 h at 35 °C with the surface that contained SPA. The antibacterial effect was measured by comparing the survival of bacteria on the treated catheters with that achieved on the untreated catheters and expressed as average Log reduction (*R*-value) and the percent reduction (% reduction) of bacteria from treated versus untreated samples. The *R*-value was calculated by the difference in the logarithmic value of viable cell counts between antimicrobial catheters and untreated catheters.

In the first version of experiment, bacterial cell suspensions of *E. coli*, *S. aureus* and *S. epidermidis* were prepared in the nutrient broth diluted 1/500 with water, accurate according to recommendation of standard methods. In this conditions, bacteria did not survive on the SPA-treated catheters, but also they did not survive on the untreated latex catheters, after 24 h incubation time. In the absence of the control samples calculation of *R*-values was impossible.

A lack of bacterial growth on the untreated latex catheters was probably due to a toxic effect produced by substances eluted into the medium from latex material. The observed toxic effect of latex agrees with the published data (López-López et al., 1991). But, anti-adhesive and toxic properties of latex catheters is not completely sufficient to protect against biofilm formation and against catheter-associated urinary tract infections. Such protection gives SPA-treated catheters, which antimicrobial activity was clearly demonstrated in the second version of experiment using the modified standard methods.

In the second version of experiment, the test inoculum was prepared in undiluted nutrient broth in order to create the favorable conditions to growth of bacteria on the latex untreated samples. Besides, creating the conducive conditions to bacterial growth, taken into account fact that catheters will be used in vivo in similar conditions (in contact with urine). In the undiluted nutrient broth, bacteria did not survive on the SPA-treated catheters. The population of bacteria on the untreated latex catheters after 24 h incubation time was large (about 2.00E+07), whereas it should not be greater than 2 orders of magnitude lower than population recovered prior to incubation. In this connection, *R*-values were calculated using the population of bacteria recovered from the untreated material prior to incubation (JIS Z 2801:2000 V1.0).

A quantitative assessment of antimicrobial activity of the tested samples, the *R*-values and % reduction are shown in Table 2. Fig. 8 presents graphical comparison of counts of viable cell found on antimicrobial and control catheters. On the basis of the antimicrobial activity values which were found to be higher than 3.8, SPA-containing catheters can be categorized as effective antimicrobial surfaces (according to JIS Z2801:2000, the bacterial Log reduction value should not be less than 2.0). Besides, bacterial cell suspensions recovered from SPA-treated catheters and remained overnight did not show any increase (all bacterial cells were killed by SPA bound to the catheter surface), whereas bacterial cell suspensions recovered from the untreated catheters and remained

Table 2
Antimicrobial activity of the catheters with covalent binding of SPA after washings (the treated specimens) and of latex catheter as a control sample (the untreated specimens), tested by using standard methods according to International Organization for Standardization ISO 22196:2007E.

Bacterial strain	Mean of the number of viable bacteria (CFU/cm^2)[a]			Antimicrobial activity[b] (Log CFU reduction)[c]	% reduction[d]
	The untreated specimens after inoculation	The untreated specimens after 24 h	The treated specimens after 24 h		
Escherichia coli	1.80E+04	2.50E+07	<1	3.86	>99.99
Staphylococcus aureus	1.75E+04	2.00E+07	<1	3.85	>99.99
Staphylococcus epidermidis	1.65E+04	1.80E+07	<1	3.82	>99.99

[a] The values for the untreated specimens after 24 h and the treated specimens after 24 h were significantly different according to the Students' t test ($P < 0.05$; $n = 3$).
[b] The values calculated with reference to population of bacteria recovered from the untreated specimens after inoculation (prior to incubation).
[c] The log 10.
[d] % difference in the populations prior to and after incubation.

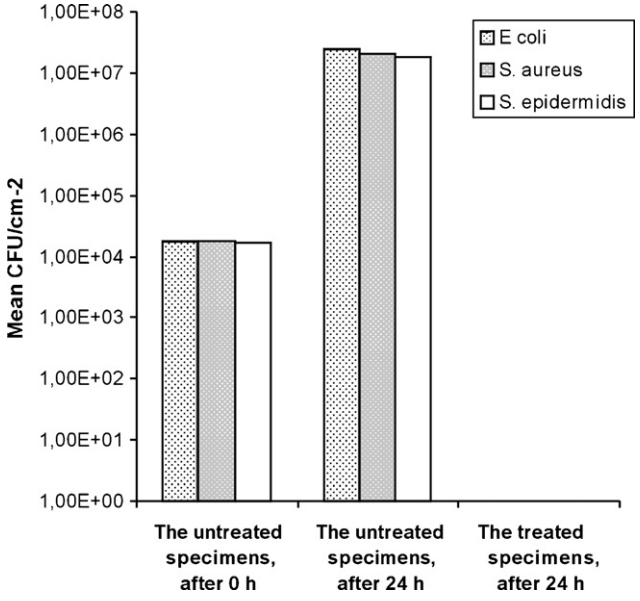

Fig. 8. A quantitative assessment of antimicrobial activity of the tested samples measured according to ISO and JIS methods. Comparison of counts of viable cell found on SPA-treated and control catheters.

overnight showed an intensive increase. The results demonstrate that the latex catheters containing covalent immobilized SPA are characterized by the significant antibacterial activity against *E. coli*, *S. aureus* and *S. epidermidis*.

4. Conclusion

The SPA-HP film was prepared by covalent and non-covalent binding of sparfloxacin to heparin film which was first deposited on the latex surface of catheter and then oxidized with sodium periodate. The presence of the modified heparin layer was confirmed by the observation of surface topography using scanning electron microscopy. The presence of sparfloxacin on the catheter surface and its mixed, non-covalent and covalent (via CH=N group) binding, was concluded from ATR-FTIR analysis. The antibacterial activity of the modified catheters containing SPA against gram-positive and gram-negative microorganisms was confirmed by the diffusion assay, by quantitative estimation of the ability to reduce the viable bacterial cells according to procedures ISO and JIS, and by inhibition of biofilm formation. After over month period of the antibacterial efficiency against bacterial strains in the broth medium, which was due to the diffusion of SPA into this medium, the modified catheters showed still the protection from microbial colonization and biofilm formation.

It seems that the duration of the indicated antibacterial protection is sufficient for potential clinical applications of such modified catheters.

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References

Aksoy, A.E., Hasirci, V., Hasirci, N., 2008. Surface modification of polyurethanes with covalent immobilization of heparin. Macromol. Symp. 269, 145–153.
Baveja, J.K., Willcox, M.D., Hume, E.B., Kumar, N., Odell, R., Poole-Warren, L.A., 2004. Furanones as potential anti-bacterial coatings on biomaterials. Biomaterials 25, 5003–5012.
Bhupathiraju, V.K., Hernandez, M., Landfear, D., Alvarez-Cohen, L., 1999. Application of tetrazolium dye as an indicator of viability in anaerobic bacteria. J. Microbiol. Methods 37, 231–243.
Chenoweth, C.E., Saint, S., 2005. Infections associated with urinary catheters. In: Seifert, H., Jansen, B., Farr, B. (Eds.), Catheter-related Infections. Marcel Dekker, New York, pp. 551–585.
Choong, S., Wood, S., Whitfield, H., 2001. Catheter-associated urinary tract infection and encrustation. Int. Antimicrob. Agents 17, 305–310.
DiTizio, V., Ferguson, G.W., Mittelman, M.W., Khoury, A.E., Bruce, A.W., 1998. A liposomal hydrogel for the prevention of bacterial adhesion to catheters. Biomaterials 19, 1877–1884.
Dorofeev, V.L., 2004. Infrared spectra and the structure of drugs of the fluoroquinolone group. Pharm. Chem. J. 38, 693–697.
Eydelnant, I.A., Tufenkji, N., 2008. Cranberry derived proanthocyanidins reduce bacterial adhesion to selected biomaterials. Langmuir 24, 10273–10281.
Fu, J., Ji, J., Yuan, W., Shen, J., 2005. Construction of anti-adhesive and antibacterial multilayer films via layer-by-layer assembly of heparin and chitosan. Biomaterials 26, 6684–6692.
Ginalska, G., Kowalczuk, D., 2010. A method of antibacterial matrix obtainment via chemotherapeutic agent immobilization onto bioactive-surface. Biuletyn Urzędu Patentowego 9, 5.
ISO 22196:2007 (E), 2007. Plastics – measurement of antimicrobial activity on plastics surfaces, International standard, International Organization for Standardization.
JIS Z 2801:2000 (E), 2001. Antimicrobial products – test for antimicrobial activity and efficacy, Industrial standard, Japanese Standard Association.
JIS Z 2801:2000 V1.0, 2005. Technical White Paper, Industrial Microbiological Services LTD.
Johnson, J.R., Kuskowski, M.A., Wilt, T.J., 2006. Systematic review: antimicrobial urinary catheters to prevent catheter-associated urinary tract infection in hospitalized patients. Ann. Intern. Med. 144, 116–126.
Johnson, J.R., Roberts, P.L., Olsen, K.A., Moyer, K.A., Stamm, W.E., 1990. Prevention of catheter associated urinary tract infection with a silver oxide coated urinary catheter: clinical and microbiologic correlates. J. Infect. Dis. 162, 1145–1150.
López-López, G., Pascual, A., Martínez-Martínez, L., Perea, E.J., 1991. Effect of a siliconized latex urinary catheter on bacterial adherence and human neutrophil activity. Diagn. Microbiol. Infect. Dis. 14, 1–6.
Murugesan, S., Xie, J., Linhardt, R.J., 2008. Immobilization of heparin: approaches and applications. Curr. Top Med. Chem. 8, 80–100.
Nicolle, L.E., 2005. Catheter-related urinary tract infection. Drugs Aging 22, 627–639.
Pascual, A., 2002. Pathogenesis of catheter-related infections: lessons for new designs. Clin. Microbiol. Infect. 8, 256–264.
Raad, I., Darouiche, R., Hachem, R., Mansouri, M., Bodey, G.P., 1996. The broad-spectrum activity and efficacy of catheters coated with minocycline and rifampin. J. Infect. Dis. 173, 418–424.
Reid, G., Sharma, S., Advikolanu, K., Tieszer, C., Martin, R.A., Bruce, A.W., 1994. Effects of ciprofloxacin, norfloxacin, and ofloxacin on in vitro adhesion and survival of *Pseudomonas aeruginosa* AK1 on urinary catheters. Antimicrob. Agents Chemother. 38, 1490–1495.
Rose, S.F., Okere, S., Hanlon, G.W., Lloyd, A.W., Lewis, A.L., 2005. Bacterial adhesion to phosphorylcholine-based polymers with varying cationic charge and the effect of heparin pre-adsorption. J. Mater. Sci. Mater. Med. 16, 1003–1015.

Saint, S., Lipsky, B.A., Goold, S.D., 2002. Indwelling urinary catheters: a one-point restraint? Ann. Intern. Med. 137, 125–127.

Sekiguchi, Y., Yao, Y., Ohko, Y., Tanaka, K., Ishido, T., Fujishima, A., Kubota, Y., 2007. Self-sterilizing catheters with titanium dioxide photocatalyst thin films for clean intermittent catheterization: basis and study of clinical use. Int. J. Urol. 14, 426–430.

Shi, L., Ardehali, R., Caldwell, K.D., Valint, P., 2000. Mucin coating on polymeric material surfaces to suppress bacterial adhesion. Colloids Surf. B Biointerfaces 17, 229–239.

Yang, S.-H., Yu-Sheng, Lee, J., Lin, F.-H., Yang, J.-M., Chen, K.-S., 2007. Chitosan/poly(vinyl alcohol) blending hydrogel coating improves the surface characteristics of segmented polyurethane urethral catheters. J. Biomed. Mater. Res. Part B Appl. Biomater. 83, 304–313.

Sushko, N.I., Firsov, S.P., Zhbankov, R.G., Tsarenkov, V.M., Marchewka, M., Ratajczak, C., 1994. Vibrational spectra of heparins. J. Appl. Spectrosc. 61, 704–707.

Tenke, P., Kovacs, B., Jäckel, M., Nagy, E., 2006. The role of biofilm infection in urology. World J. Urol. 24, 13–20.

Visser, M., Rozenberg-Arska, M., Beumer, H., Hoepelman, I.M., Verhoef, J., 1991. Comparative in vitro antibacterial activity of sparfloxacin (AT-4140; RP 64206), a new quinolone. Antimicrob. Agents Chemother. 35, 858–868.

Warren, J.W., 2001. Catheter-associated urinary tract infections. Int. J. Antimicrob. Agents 17, 299–303.

Yao, Y., Ohko, Y., Sekiguchi, Y., Fujishima, A., Kubota, Y., 2008. Self-sterilization using silicone catheters coated with Ag and TiO_2 nanocomposite thin film. J. Biomed. Mater. Res. Part B Appl. Biomater. 85, 453–460.