Antibacterial Activity of Gentamicin-bonded Gelatin-sealed Polyethylene Terephthalate Vascular Prostheses

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Objectives*. To create an antibiotic-modified vascular prosthesis with a prolonged bactericidal activity, susceptible to endothelialisation.*

Methods*. We used a covalent method of gentamicin sulphate immobilisation to polyethylene terephthalate prosthesis sealed with gelatin. Antibacterial activity was assayed in Luria-Bertani medium against* Staphylococcus aureus, Escherichia coli *and* Pseudomonas aeruginosa *strains. Prosthesis endothelialisation was performed using bovine aorta endothelial cells (BAEC).*

Results*. Gentamicin was bound to vascular prostheses in the amount of 12 g per kg of prosthesis. Ninety-seven percent of antibiotic bound in covalent way and remained on the biomaterial for at least 30 days during shaking in PBS solution. Gentamicin-modified prostheses exerted bactericidal or bacteriostatic effect on growth of clinical and reference bacterial strains, prevented biofilm formation and were highly susceptible to endothelialisation. BAEC viability exceeded 90%, which indicated that gentamicin-vascular prostheses were not toxic for these cells.*

Conclusions*. Covalent gentamicin immobilisation resulted in effective antibacterial protection of vascular prostheses against clinical and reference strains of* S. aureus, E. coli *and* P. aeruginosa *and allowed for a strong adherence of endothelial cells to antibiotic-modified prostheses.*

Keywords: Gelatin-sealed vascular prostheses; Covalent immobilisation; In vitro bacterial adherence; Endothelial cell proliferation.

The reported incidence of vascular graft infections ranges between 0.5 and 5%.[1] They are potentially devastating complications of reconstructive arterial surgery.[2] Vascular prosthetic infections are associated with a high rate of limb loss and mortality (up to 79 and 75% of cases, respectively)[1,3] and may be caused by perioperative contamination, postoperative wound infection or systemic bacteraemia.[4] Reduction of graft infections may be obtained by impregnation of the commercial prostheses with antibiotics (or any other antiseptic chemicals) to prevent the bacterial adherence.[5] This effect was achieved by antibiotics adsorption to prostheses sealed with gelatin, collagen or albumin.[6,7] An example of such procedures was rifampicin ionic bonding to protein-sealed biomaterials; however, it was shown that this antibiotic was quickly eluted to perigraft tissues.[2,7,8]

The antibiotic-prostheses hybrids produced so far lack the appropriate stability because their antibacterial protection is limited to several days, depending on the antibiotic and biomaterial type.[5,9–11] To overcome this problem, our group has been recently, working on covalent gentamicin immobilisation to protein-coated or silanized silica materials. We have shown *in vitro* the stability of covalent bonds between the antibiotic and keratin-coated silica matrices.[12] Strong chemical stability was observed for gentamicin-modified gelatin-sealed polyethylene terephthalate prostheses, with the majority of antibiotic bound covalently.[13] In this paper, we present the long-lasting antibacterial properties of gelatin-sealed polyethylene terephthalate vascular

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prostheses with covalently immobilised gentamicin and their high susceptibility to endothelialisation.

Materials and Methods

Materials

Gentamicin sulphate for immobilisation was obtained from KRKA (Croatia) and used as solution at concentration of 40 mg/ml in 0.1 M phosphate-buffered saline (PBS), as described in The British Pharmacopoeia.[14] Gentamicin solution for standard curve preparation was supplied by Fluka (Switzerland) at concentration of 1 mg/ml. Methanol and 2-propanol (HPLC grade) were obtained form Merck, Germany. All other reagents and solvents for HPLC were of analytical grade (Merck, Germany). Phthaldialdehyde reagent was freshly prepared by dissolving 200 mg of *o*-phthaldialdehyde (OPA, Fluka, Switzerland) in 1.5 ml methanol, adding 15 ml borate buffer (2.5% boric acid adjusted to pH 10.4 by 40% KOH), 0.5 ml mercaptoacetic acid and finally, by adjusting pH to 10.4 with 40% potassium hydroxide. Tricogel® vascular prostheses (Tricomed, Poland) were made of polyethylene terephthalate fibres sealed with pig gelatin.

Gentamicin immobilisation

Covalent immobilisation of gentamicin on 1 cm^2 pieces of Tricogel® prostheses was performed in triplicate according to procedure described in our patent pending No P-358934.[15] Quantitative gentamicin estimation was performed according to the method described in The British Pharmacopoeia[16] using a HPLC procedure (Gilson HPLC system, USA). Chromatographic analysis was performed using Nova-Pak® RP-18 (150×3.9 mm^2; particle size: 5 um) column (Waters, USA). Absorbance measurements were made at wavelength 330 nm.

In vitro drug release

Gentamicin-modified prosthesis samples (5×0.05 g) were incubated in portions (20 ml) of 0.1 M PBS pH 7.4 in Erlenmayer flasks at 37 °C in a water bath shaker for a 30-days period. Gentamicin concentration in removed PBS was estimated every 7 days. The experiment was performed in triplicate.

Assay of antibacterial activity

Antibacterial activity of vascular prostheses modified by covalent gentamicin immobilisation was tested according to two procedures:

by spot test: pieces of prostheses were put on agar Luria-Bertani (LB) (Difco, USA) plates and covered with semisolid (0.7%) LB medium containing bacteria (1×10^6 cfu (colony forming units)/ml). The plates were incubated at 37 °C for 18 h.
by incubation in liquid LB medium with gentle shaking (35 rpm) at 37 °C with bacteria of concentrations indicated in Table 2.

Tests were performed against clinical bacterial isolates from patients of Public Clinical Hospital no 4, Lublin, Poland (*Escherichia coli* from ear and urinal tract, *Staphylococcus aureus* from throat and stools, *Pseudomonas aeruginosa* from eyelids) and reference strains (*E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853). Prostheses with and without gentamicin were sterilised by UV-light prior to experiments (Philips lamp, 30 W/cm^2, 0.5 h, from distance of 15 cm). All experiments were performed in triplicate. The average of three experiments (±SD) is presented in overall count of bacteria by the serial dilution method.

Test of bacterial adhesion to gentamicin-modified vascular prostheses

Bacterial adhesion to the prostheses was investigated using the suspension of bacterial cells in LB broth (1×10^6 cfu/ml). Pieces of gelatin-sealed vascular prosthesis (control) or the gentamicin-modified prosthesis were incubated with bacterial broth using gentle agitation (35 rpm; 37 °C, 48 h). Prosthesis pieces were subsequently taken out from the broth and washed out three times with 50 ml of sterile distilled water to remove all non-adhered bacteria.

Prosthesis samples for scanning electron microscopy were prepared according to method of Mercier *et al.*[17] Prostheses were fixed in 3% (v/v) glutaraldehyde (Sigma, USA) in 0.1 M PBS with 8% sucrose for 2 h at 4 °C and kept overnight in 0.1 M PBS at 4 °C. The prostheses were subsequently washed three times for 10 min in 0.2 M cacodyl buffer (Sigma, USA) pH 7.4. Then the samples were fixed in 1% (w/v) OsO$_4$ for 1 h at 4 °C and dehydrated (each step for 15 min at R/T) in series of alcohol–water mixtures (70, 95, 100% ethanol) and then in series of ethanol–acetone mixtures (30, 50, 70, 100% acetone). Dehydrated samples were dried in

a CO_2 atmosphere in a model CPD 030 critical-point dryer (Bal-Tec, Switzerland), coated with gold–palladium by a high resolution sputtering system Polaron SC 7640 (Polaron, UK) and examined using a scanning electron microscope Tesla BS300 (Tesla, Czech Republic) operated at 15 kV. A Satellite TC computer program (Tescan, UK) was used to perform image analysis.

Bovine aorta endothelial cell culture

Bovine aorta endothelial cell culture (BAEC) was prepared as previously described.[18] Vessels isolated from freshly slaughtered steers and heifers were clumped before dissection from surrounding tissue, excised, rinsed with PBS and filled with 0.25% trypsin solution. After 30 min incubation at 37 °C, the endothelial cell suspension was collected and centrifuged at 250g for 10 min. The cell pellet was resuspended in culture medium and transferred into the 75 ml tissue culture flasks (Nunc, Denmark). Culture medium consisted of Dulbecco's modified Eagle medium (DMEM) (Sigma, USA), 10% fetal bovine serum (FBS) (Life Technologies, Germany), penicillin (100 U/ml) and streptomycin (100 U/ml) (Sigma, USA). The cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air. Endothelial cell morphology was confirmed by typical cobblestone appearance as well as by staining for von Willebrand factor (Dakopatts, Denmark). For experiments, BAEC at passage 5–8 were used.

Cell test design

Pieces of vascular prostheses were placed in a 24-well microplate (Nunc, Denmark) and incubated in culture medium at 37 °C overnight. The prosthesis samples were inoculated with 2.5×10^4 BAEC suspended in a fresh culture medium. Starting at 48 h, culture medium was changed every day. Cell growth was assessed after 48, 72, 96 and 144 h. Cells were detached from prosthesis surface by 0.25% trypsin-EDTA solution (Sigma, USA) and counted in a Thoma hemocytometer. Cell viability was determined by 0.4% Trypan Blue exclusion. For visualisation, prosthesis fragments were washed in PBS, fixed in cold methanol for 5 min and stained with Giemsa for 30 min.

Confocal microscopy

Prosthesis pieces covered with BAEC (obtained in the previous experiment) were washed twice in PBS and subsequently stained with 3,3' dihexyloxacarbocyanine iodide ($DIO_{3(6)}$) (5 uM/ml) for 10 min in the dark. Specimens were examined under the confocal microscope (LSM-5, Pascal, Zeiss, Germany) at 514 nm.

Results

Gentamicin immobilisation

We demonstrated that each of four predominate structural forms (C1, C1a, C2, C2a) of gentamicin bound to gelatin-coated prosthesis in different amounts (Table 1). Using 5.2 mg/ml of gentamicin solution we found the prosthesis to contain 12,620 mg of the antibiotic per kg, with immobilisation yield of 40.15%.

In vitro drug release

About 3% of the gentamicin initially bound to Tricogel® prostheses was released from the biomaterial during the 1st 7 days of shaking in buffered saline (PBS) at 37 °C (which was equal to 380 mg of antibiotic released from 1 kg of prosthesis). Ninety-seven percent of the antibiotic remained on the matrix for at least 30 days (Fig. 1).

Assay of antibacterial activity

Estimation of antibacterial properties of gentamicin-modified prostheses was performed using two procedures. In the spot test, the zones of growth inhibition around these prosthesis samples were measured for reference bacterial strains: 37±1 mm for *E. coli*, 35± 1 mm for *S. aureus* and *P. aeruginosa*. The zones observed for clinical isolates of these bacteria ranged between 8 and 10 mm. In a second procedure, the growth of the bacterial cells in liquid LB medium with the antibiotic-modified prostheses was tested for 28 days. The results (Table 2) show that the

Table 1. Gentamicin immobilisation on gelatine-sealed vascular prostheses

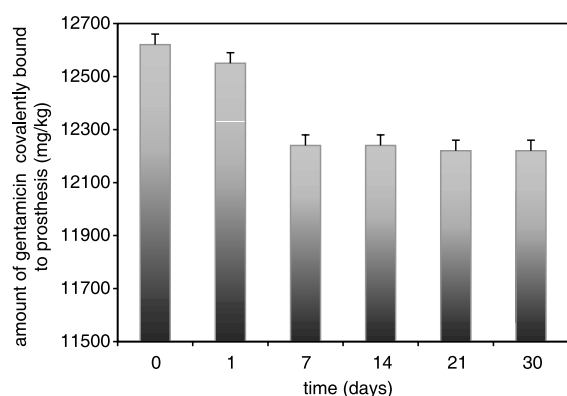| Gentamicin forms	Concentration of gentamicin (mg/ml)	
	Before immobilisation	After immobilisation
C1	2.973	1.811
C1a	1.146	0.666
C2a	0.490	0.300
C2	0.632	0.359
Total amount of gentamicin	5.242	3.138

Fig. 1. Release of gentamicin from vascular prosthesis during 30 days of incubation in PBS with shaking at 37 °C.

immobilised gentamicin exerted strong inhibitory effects on all bacteria used in these experiments. This effect was bactericidal for each tested bacterial strain (complete inhibition of bacterial growth). Only in the case of a very high initial concentration of reference bacterial strain did the effect of immobilised drug become bacteriostatic (Table 2).

Test of bacterial adhesion to gentamicin-modified vascular prostheses

SEM photographs and microbiological tests on agar plates revealed that the surfaces of gentamicin–Tricogel® prostheses after incubation with three bacterial strains were free both of bacterial cells and biofilm, contrary to control prostheses without antibiotic. Fig. 2 presents this effect for *S. aureus*—a representative bacterial species responsible for biofilm formation.[19] During microscopic observations of gentamicin-modified prostheses, only single bacterial cells were found on their surfaces. However, these bacteria were unviable as was confirmed by microbiological tests with these prosthesis samples incubated in liquid

LB medium. Similar results were obtained when gentamicin-modified prostheses were incubated with *E. coli* and *P. aeruginosa* (data not shown).

Prostheses endothelialisation

Growth of BAEC on gentamicin-modified and control (without the antibiotic) biomaterial samples was tested *in vitro*. The results indicated that BAEC attachment and growth on both gentamicin-modified and control prostheses were similar (Fig. 3). The cell numbers increased within culture time for both prostheses (with and without antibiotic) reaching the highest values at 144th h of culture. The experiment was terminated at 144 h as the May–Grünwald–Giemsa of prosthesis samples with and without gentamicin showed confluent monolayer on the prosthetic surfaces (data not shown). During the experiment, the cell viability exceeded 90% (Table 3). The observation using confocal microscope showed typical cobblestone morphology of growing endothelial cells on surfaces of both prostheses (Fig. 3).

Discussion

The problem of vascular grafts infection may cause devastating complications in reconstructive arterial surgery. Therefore, vascular surgeons are interested in new solutions in this field. Covalent immobilization of antibiotics on vascular prostheses seems to be the most effective solution providing stable complexes of durable antibacterial activity. According to Earnshaw,[5] it is not clear whether the antibiotic should be bound tightly to the graft in high concentration or it should be released in controlled way into perigraft tissues. The optimal antibiotic (of a broad or narrow spectrum) and duration of its attachment to the prostheses are not

Table 2. Inhibitory effect of immobilised gentamicin bacterial growth in LB medium with shaking at 37 °C for 28 days

| Strain	MIC of gentamicin (μg/ml)	Initial number of bacteria (cfu/ml)	Number of bacteria (cfu/ml)$\times 10^7 \pm$SD
Escherichia coli			
(a) Isolated from ear	1.0	4.0×10^6	0
(b) Isolated from urinary tract	1.5	4.0×10^6	0
(c) ATCC 25922	4.0	4.0×10^6	0
(d) ATCC 25922	5.0	4.0×10^8	8.0 ± 2.0
Staphylococcus aureus			
(a) Isolated from throat	1.0	1.9×10^7	0
(b) Isolated from stools	1.0	1.9×10^7	0
(c) ATCC 25923	3.0	1.9×10^7	0
(d) ATCC 25923	4.5	1.9×10^9	9.5 ± 1.5
Pseudomonas aeruginosa			
(a) Isolated from eyelids	3.0	5.9×10^6	0
(b) ATCC 27853	0.5	5.9×10^6	0
(c) ATCC 27853	0.8	5.9×10^8	2.9 ± 0.5
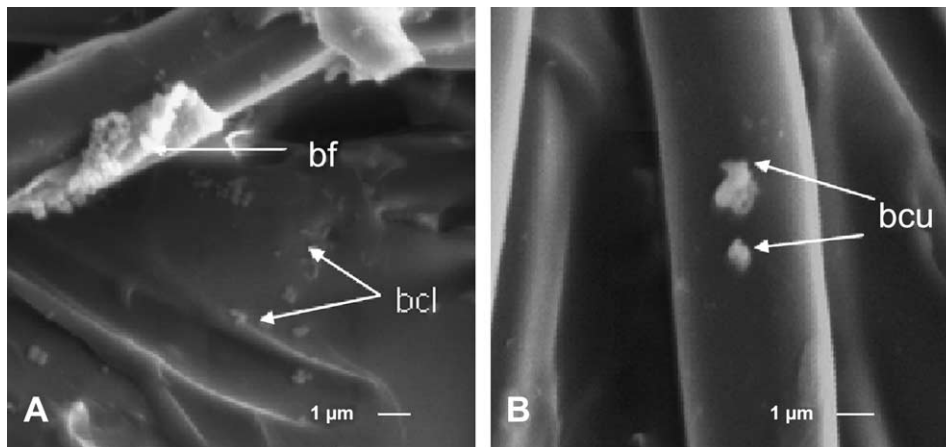
Fig. 2. Test of adhesion of *Staphylococcus aureus* to vascular prostheses modified by gentamicin (A, control prostheses; B, prostheses with antibiotic; bf, biofilm; bcl, living bacterial cells; bcu, unviable bacterial cells).

defined but it is suggested that the antibiotic should be present on the prostheses for at least several days and reveal an activity against the graft infecting pathogens.

Up to now, the majority of research on this topic describes rifampicin bound to gelatin-, collagen- or albumin-sealed vascular prostheses in passive or ionic mode.[1,2,4,7,8,11] However, prostheses soaking in antibiotic solution (passive adsorption) is an ineffective process as the drug is eluted from the grafts within at last 4–7 days after the implantation by circulating blood and other fluids.[20,21] Ionic rifampicin bonding to biomaterial is also ineffective and allows for the antibacterial protection of prostheses only up to 10 days.[20,21] Then, the amount of antibiotic remaining on prosthesis drops below minimal inhibition concentration (MIC).[2,9,11,20,22] Despite this, Strachan and coworkers[9] argued for the use of antibiotic-impregnated grafts for patients of increased infection risk. *In vivo* experimental studies showed that reinfection rates were slightly reduced when the rifampicin-impregnated grafts were used, though MRSA infections caused more serious difficulties.[21,23]

Our results obtained for covalently-modified prostheses show that during the 1st days after immobilization only 3% of antibiotic was released from the biomaterial. This small quantity of drug may allow

protection of the perigraft tissues just after the prosthesis implantation. Simultaneously, the amount of antibiotic remaining on the prosthesis is sufficient to protect the graft for a long time because the gelatin is gradually digested and detached from the graft together with the antibiotic. According to our results, this modification may protect the prosthesis surface against bacterial cell attachment and biofilm formation. It should be stressed that this procedure of gentamicin immobilisation did not influence the properties of the vascular prosthesis and allowed for BAEC growth. These highly promising results require further studies using human endothelial cells and fibroblasts.

It seems that covalent antibiotic-modification of knitted prostheses made of polyethylene terephthalate is of practical significance because it may minimise bacterial growth and adherence to the implantable biomaterial, particularly in cases of high risk patients (with diabetes, distal skin necrosis or previously subjected to surgery.[5]) This is of special importance because infections of these prostheses are more frequent than these of prostheses made of polytetrafluoroethylene (PTFE).[24] These results require confirmation by further *in vivo* experiments concerning clinical research on animals and in case of positive results—also on people. However, we may assume that covalent antibiotic modification of protein-sealed vascular prostheses is a highly promising technique in graft infections prophylaxis.

Table 3. Evaluation of endothelial cells growth on vascular prostheses without and with immobilised gentamicin

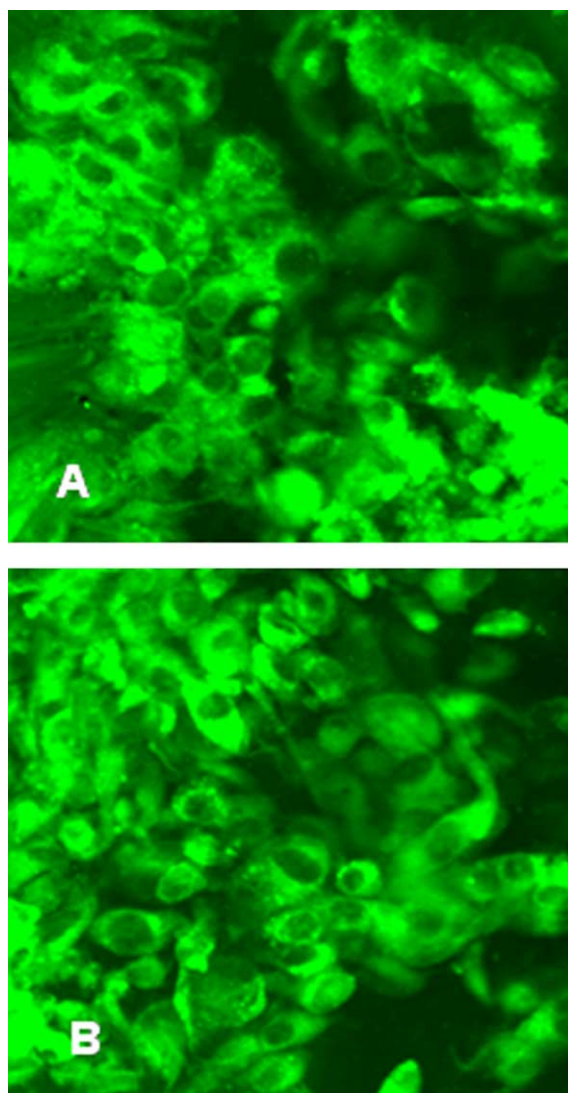| Time (h)	Cell number $\times 10^4 \pm$ SEM	
	Control prostheses	Prostheses with gentamicin
48	5.0±2.0	5.0±2.0
72	13.5±0.5	12.0±7.0
96	26.5±4.5	26.5±2.5
120	32.75±1.75	36.25±2.75
144	59.0±0.0	58.0±0.0

SEM, standard error of mean.

Fig. 3. Graphs of endothelial cells using a confocal microscophy technique (A, control prosthesis+BAEC; B, gentamicin-modified prosthesis+BAEC). Mag. 200×.

endothelial cells photographs by confocal microscopy technique.

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