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Vascular Prostheses with Covalently Bound Gentamicin and Amikacin Reveal Superior Antibacterial Properties than Silver-impregnated Ones – An *In Vitro* Study

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Abstract *Objective:* This study aims to compare the antibacterial activities of vascular prostheses: silver-impregnated and modified with covalently immobilised antibiotics.
Materials and Methods: Six types of protein-sealed vascular prostheses were modified with amikacin and gentamicin according to the method described in the Polish Patent Office. Their antimicrobial properties were estimated against 14 reference and clinical strains and compared with those of InterGard™ Silver grafts. Cytotoxicity of the tested grafts was estimated against human skin fibroblasts.
Results: Prostheses modified with antibiotics in a stable covalent mode were found to be much more effective against bacterial growth and biofilm formation, as well as in case of methicillin-resistant *Staphylococcus aureus* (MRSA), than InterGard™ Silver. They inhibited the bacterial growth for at least 30 days, without losing higher than 10% of the initial amount of its drug content. They were also good, non-toxic matrices for growth of human skin fibroblasts.
Conclusions: Prostheses modified with covalently immobilised antibiotic according to our technique are much more effective than InterGard™ Silver at protection against bacterial growth. They are also compatible with human skin fibroblasts.

Vascular surgery of the 21st century still faces many problems, including those concerning the postoperative infections of implanted vascular prostheses. Reports present a low incidence of vascular graft infections (0.5–5% of all cases)[1,2]; however, they are associated with 12–27% mortality and 10–15% limb amputation rates when it concerns infrarenal and femoral arteries.[3] Microbial attack is specially dangerous in relation to cell adhesion and biofilm formation on the implant surface. The cells entrapped within the biofilm form complex communities[4] of enhanced

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resistance to antibiotics; they become 10—1000 times less sensitive to antimicrobial agents than do planktonic cells.[5] Conventional management of infected vascular grafts includes total graft excision combined with debridement of adjacent native artery, replacement by a new prosthesis or bypass grafting and aggressive systemic antibiotic administration. Effectiveness of antibiotic therapy, however, depends on location, extent of infection, the type of the infecting microorganism and its sensitivity to the applied antibiotic.[6,7] Early-onset infections are generally caused by *Staphylococcus aureus* (a representative of coagulase-positive staphylococci), while late-onset infections are caused by coagulase-negative biofilm-producing *Staphylococcus epidermidis*, although recently it was observed that mixed infections were more prevalent.[8] In current estimations, *S. aureus*, *S. epidermidis* and *Escherichia coli* are responsible for approximately 75% of wound/graft infections.[9] Moreover, remember that antimicrobial resistance among bacteria is an increasing threat.[10] Among *Staphylococci*, for example, methicillin-resistant *S. aureus* (MRSA) is responsible for over 50% of infections in vascular surgery procedures, open reduction of fracture of long bones and limb amputation cases.[11]

Graft infections may be reduced by using modified vascular prostheses of increased antimicrobial activity. Such grafts are produced by soaking commercially available prostheses into antibiotics solutions (e.g., in case of rifampicin-soaked protein-sealed grafts[12]). However, the drug is quickly eluted from such prostheses when incubated in liquid. Some research groups attempted to increase the antibacterial activity of vascular prostheses by prebonding of polyester grafts previously activated by succinylated gelatin with two antibiotics (rifampin and tobramycin).[13] The effect of antibiotics absorbed into vascular grafts may also be increased by simultaneously applying lipopeptides bearing antimicrobial activity against a large number of Gram-positive cocci.[14] However, despite these promising concepts, there is still a need for producing prostheses which would retain significant antibacterial activity for longer than 1 month, thus preserving them from early as well as late bacterial infections.

An alternative strategy for enhancing antimicrobial effects of vascular grafts is their impregnation by silver salts. Silver ions exert bactericidal activity on DNA, protein and membrane levels through several mechanisms.[15,16] Efficacy of silver-impregnated prostheses in combating vascular implant infections was reported in numerous articles.[17—21] On the other hand, there are also some reports stating that the positive role of silver in preventing infections is rare or controversial.[22—26] Bactericidal properties of silver salts are even increased in the presence of rifampin in comparison with the antibiotic alone.[27] However, the long-term activity of silver-coupled grafts seems to be inefficient, similarly to antibiotic-soaked prostheses that quickly elute the majority of adsorbed drug.

We have already reported the effectiveness of prostheses modified with covalently immobilised aminoglycoside antibiotics,[28,29] which showed a long-lasting antibacterial activity. Some of their advantages have been highlighted: stability during sterilisation and long-term storage, cytocompatibility and others. In some *in vitro* experiments, we have found that these prostheses are much more efficient at protecting themselves and the surrounding medium against bacterial attack than not just non-modified grafts but also commercially available silver-impregnated prostheses used in vascular surgery as one of the most frequently applied modified matrices showing antibacterial properties. This study presents the results of comparison of antibacterial properties between protein-sealed vascular prostheses with covalently immobilised aminoglycoside antibiotics and silver-impregnated prostheses.

Materials and Methods

Media and strains

Bacterial growth inhibition was tested on Müeller—Hinton (M—H) broth, Müeller—Hinton (M—H) agar II medium (both from Oxoid Ltd., England) or liquid Luria—Bertani (LB) broth (Biocorp, Poland).

Bacterial strains were stored in microbanks (PRO-LAB Diagnostics, UK). Reference strains were: *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *S. aureus* ATCC 25923. Clinical strains were isolated in the Clinical Hospitals in Lublin, Poland: *E. coli* from ear, urinary tract and faeces; *E. coli* 262 amikacin-resistant (MIC 500 $\mu g\,ml^{-1}$); *P. aeruginosa* from eyelid; ESBL (extended-spectrum β-lactamase) *P. aeruginosa*; *S. aureus* from throat and faeces; *S. aureus* MR3 (methicillin-resistant); *S. aureus* S1 (ampicillin-resistant); *S. epidermidis* from skin. The choice of the bacterial strains was based on literature data, as the strains are claimed to be responsible for majority of vascular graft infections.[30,31]

The vascular prostheses used in the experiments are listed in Table 1.

Gentamicin sulphate and amikacin sulphate (Biodacyna) for immobilisation (40 $mg\,ml^{-1}$ and 250 $mg\,ml^{-1}$, respectively) were supplied by KRKA (Croatia) and Bioton (Poland), respectively. Standards for gentamicin and amikacin measurements were obtained from Fluka (1 $mg\,ml^{-1}$) and the Institute of Biotechnology and Antibiotics, Warsaw (Poland), respectively.

Reagents for culture tests were supplied by Sigma (USA), unless otherwise stated.

Table 1 Description of prostheses used in experiments.

Prosthesis	Manufacturer	Material
1. Hemashield Gold™	Boston Scientific, USA	Polyester + collagen
2. Wovex™	Bard, Cardial, France	Polyester + collagen
3. Gelsoft™	Vascutek, Scotland	Polyester + gelatin
4. UniGraft® DV straight	Braun Melsungen AG, Germany	Polyester (PET) + gelatin
5. Tricogel®	Tricomed, Poland	Polyester (PET) + gelatin
6. InterGard™ Silver	InterVascular, France	Polyester + collagen/silver

All other reagents (of analytical or HPLC grade) were obtained from Merck (Germany) and Fluka (Switzerland).

Covalent immobilisation of antibiotics on vascular prostheses and drug release

Covalent immobilisation of gentamicin and amikacin on commercial protein-sealed vascular prostheses (Table 1) was performed according to the procedure described in the Polish Patent no. P358934.[32] Briefly, the first step of the reaction included the prosthesis activation with glutaraldehyde; second, antibiotic reaction with activated matrix, followed by reduction of Schiff bases by $NaBH_4$. An antibiotics solution of 2.5 mg ml^{-1} in 12 ml per 1 g of prosthesis was used for immobilisation. The amount of antibiotics both bound to prosthesis and released to phosphate-buffered solution (PBS) in drug release tests was estimated by high performance liquid chromatography (HPLC) or ultraviolet (UV) spectrophotometric assays, as described elsewhere.[28,29]

In vitro drug release test was performed on five pieces (50 mg) of modified prosthesis with 20 ml sterile PBS, pH 7.4, shaken in Erlenmayer flasks at 37 °C for 30 days. The PBS was exchanged every week and assayed for antibiotic content. The test was performed in triplicate.

Inhibition of bacterial growth and biofilm formation

Antibacterial activity of the selected prostheses modified by antibiotics or impregnated with silver (InterGard™ Silver) was estimated by the following methods:

- using spot test, as described elsewhere,[28] with an exception that M–H agar II medium was used.
- by incubation of a 1-cm^2 fragment of modified prosthesis in 20 ml sterile liquid LB broth supplemented with bacterial inoculate to appropriate final CFU (colony forming units) ml^{-1}, for 30 days, under gentle shaking (35 rpm) at 37 °C. The medium was exchanged every 7 days with simultaneous supplementation with appropriate bacterial inoculate. Concentration of bacteria in medium samples was estimated every week using the overall count of bacteria by a serial dilution method. All experiments were performed in triplicate.

Subsequently, all 1-cm^2 fragments of vascular prostheses covalently modified with antibiotics or InterGard™ Silver, incubated with bacteria-containing media for 30 days, were estimated for biofilm presence using 2,3,5-triphenyltetrazolium chloride (TTC). For this purpose, they were extensively washed with sterile distilled water to remove all non-adhered cells and placed in sterile tubes containing 5 ml M–H broth supplemented with 15 μl 1% TTC solution. The tubes were then incubated for 18 h at 37 °C and estimated for appearance of red formazan dye, indicating the reduction of TTC by living bacterial cells.

Stability of antibacterial activity of vascular prostheses

Stability of antibacterial activity was assayed against reference bacterial strains: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923. Liquid LB medium (5 ml) containing 1-cm^2 sterile fragment of each prosthesis was supplemented with 5 μl of 1×10^6 inoculum of each type of bacteria. Media were incubated for 30 days at 37 °C with quotidian exchange of medium and inoculation with a fresh dose of bacteria. OD_{550nm} was measured every week as a function of turbidity caused by bacterial propagation.

Cell culture

Human skin fibroblasts (HSFs) were grown in a culture medium (Dulbecco's minimum essential medium (DMEM) containing 10% foetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin). Pieces of vascular prostheses modified with gentamicin and amikacin were placed in a 24-well microplate (Nunc, Denmark) and incubated in a culture medium at 37 °C, 5% CO_2, overnight. The medium was then removed and replaced by 3×10^4 HSF suspension in the culture medium. Starting at 48 h, the culture medium was changed daily. Cytotoxicity of the tested prostheses was estimated in the culture medium after 24 h of growth, as a function of LDH activity released from the cells in the presence of prostheses, using LDH Cytotoxicity Detection Kit (Roche Diagnostics, Switzerland). Presence of HSF on prostheses (after 120 h of culture) was detected by confocal microscopy, after washing twice in sterile PBS and subsequently staining with 3,3′-dihexyloxacarbocyanine iodide. Specimens were examined using confocal microscope LSM-5 (Zeiss, Germany) at 514 nm.

Results

Covalent immobilisation of antibiotics on vascular prostheses and drug release

The results of amikacin and gentamicin immobilisation to six types of protein-sealed vascular prostheses showed that, among the tested grafts, gelatin-sealed matrices were able to bind more drug than collagen-coated matrices (Table 2). It was also shown that amikacin was bound to matrices in higher amounts than gentamicin, which was demonstrated for each prosthesis (Table 2). UniGraft® was the optimal matrix for binding of both antibiotics (Table 2).

Table 2 Results of gentamicin and amikacin immobilisation to protein-sealed vascular prostheses.

Type of prosthesis	Gentamicin bound to prosthesis (mg/g)	Amikacin bound to prosthesis (mg/g)
1. Hemashield Gold™	1.14 ± 0.7	2.48 ± 0.03
2. Wovex™	2.52 ± 0.9	3.12 ± 0.65
3. Gelsoft™	4.02 ± 0.7	5.12 ± 0.6
4. UniGraft® DV straight	4.02 ± 0.6	6.24 ± 0.7
5. Tricogel®	3.64 ± 0.3	4.64 ± 0.8

As the best results of antibiotics immobilisation were obtained for UniGraft® prosthesis, the results of other tests were demonstrated for these prostheses with covalently immobilised amikacin and gentamicin and compared with those of InterGard™ Silver.

Drug release test revealed that the much of the immobilised antibiotics was stable while only some amount of drug had been released to the surrounding medium during 30 days of the tests (Table 3). Antibiotics immobilised to prostheses, according to the patented method for creation of covalent bonds, remained on the matrix in approximately 90% of the initial amount after 30 days.

Inhibition of bacterial growth and biofilm formation

Inhibition of bacterial growth by prostheses modified with antibiotics and InterGard™ Silver was demonstrated in contact with both solid and liquid media. The prosthesis selected for these tests was UniGraft®, showing the best results of antibiotic binding among all tested biomaterials. Reaction of vascular graft fragments with infected agar medium mimicked the conditions of direct prosthesis—bacteria contact (Fig. 1). It was shown that both gentamicin- and amikacin-modified prostheses revealed the ability to inhibit bacterial growth, as demonstrated by the presence of growth inhibition zones (Fig. 1, A1—3 and B1—3). The fact that these zones are of small dimensions is in agreement with the results of drug release tests, which showed that the majority of antibiotic was bound by strong covalent bonds and only approximately 10% by weak and easily diffusing interactions. On the contrary, silver-impregnated prostheses did not evoke such effect and no bacterial growth inhibition zone was observed around these grafts (Fig. 1 and C1—3). However, when fragments of InterGard™ Silver prostheses were lifted above the infected agar medium, the places free of bacteria appeared on the surface of the medium (Fig. 1, Insert C1′).

A comparative study on antibacterial activity of tested prostheses was also performed in liquid media supplemented with reference bacterial strains or clinical isolates from surgical tracts. The general tendency has shown that covalently modified UniGraft® prostheses, containing either gentamicin or amikacin, were able to inhibit growth of tested bacterial strains, also clinical isolates, with just a few exceptions (Tables 4 and 5). They concerned two reference strains: *S. aureus*, propagating in presence of both prostheses and *P. aeruginosa*, growing in presence of amikacin-modified graft. This effect, however, was observed only when huge bacterial concentration (4×10^8) was applied. Moreover, it should be noted that, despite the bacterial growth, the inhibiting activity of released antibiotic has been observed in the early period of experiment (Table 4). Amikacin-modified prostheses were unable to inhibit bacterial propagation only in case of two *E. coli* clinical isolates, while those containing gentamicin revealed bactericidal activity against all tested clinical strains. By contrast, InterGard™ Silver revealed only scarce antibacterial activity, mainly limited to the first day of experiment; subsequently, all tested bacteria grew abundantly in presence of InterGard™ Silver grafts (Tables 4 and 5).

Biofilm presence on tested prostheses was estimated after extensive washing to remove all non-adsorbed bacterial cells prior to placing in fresh TTC-supplemented medium. Therefore, presence of formazan could have appeared only in case of prostheses colonised by bacterial biofilm. It was found that biofilm was detected on control non-modified UniGraft® and InterGard™ Silver grafts while covalently modified UniGraft prostheses, containing gentamicin and amikacin, remained untouched by biofilm.

Stability of antibacterial activity of vascular prostheses

Stability of antibacterial activity of InterGard™ Silver and antibiotic-modified UniGraft® prostheses showed that, despite quotidian change of medium with simultaneous supplementation with bacterial inoculate gentamicin- and amikacin-modified grafts were able to continuously inhibit

Table 3 Comparison of amounts of antibiotics remaining on covalently modified PET vascular prostheses during elution (30 days, 37 °C, PBS) and rifampicin-adsorbed prostheses cited in literature. 1—2 — UniGraft® prostheses covalently modified according to our method, 3*—5* — rifampicin-adsorbed prostheses (results cited by Lovering & MacGowan[12]).

Days	Amount of antibiotic remaining on prosthesis (mg/g) after elution				
	Own results		(Results cited by Lovering & MacGowan[12])		
	1	2	3*	4*	5*
	Gentamicin; gelatin-sealed UniGraft, Germany	Amikacin; gelatin-sealed UniGraft, Germany	Rifampicin; albumin-sealed, Vasculour, USA	Rifampicin; collagen-sealed Hemashield, USA	Rifampicin; collagen-sealed Cardial, USA
0[AI]	11.01	6.79	10.689	7.174	7.424
1	10.90	6.75	0.0853	0.0022	0.024
7	10.87	6.53	0.0042	0.0008	0.0007
14	10.87	6.53	0	0	0
21	10.85	6.53	0	0	0
30	10.85	6.53	0	0	0

AI amounts observed directly after immobilization.

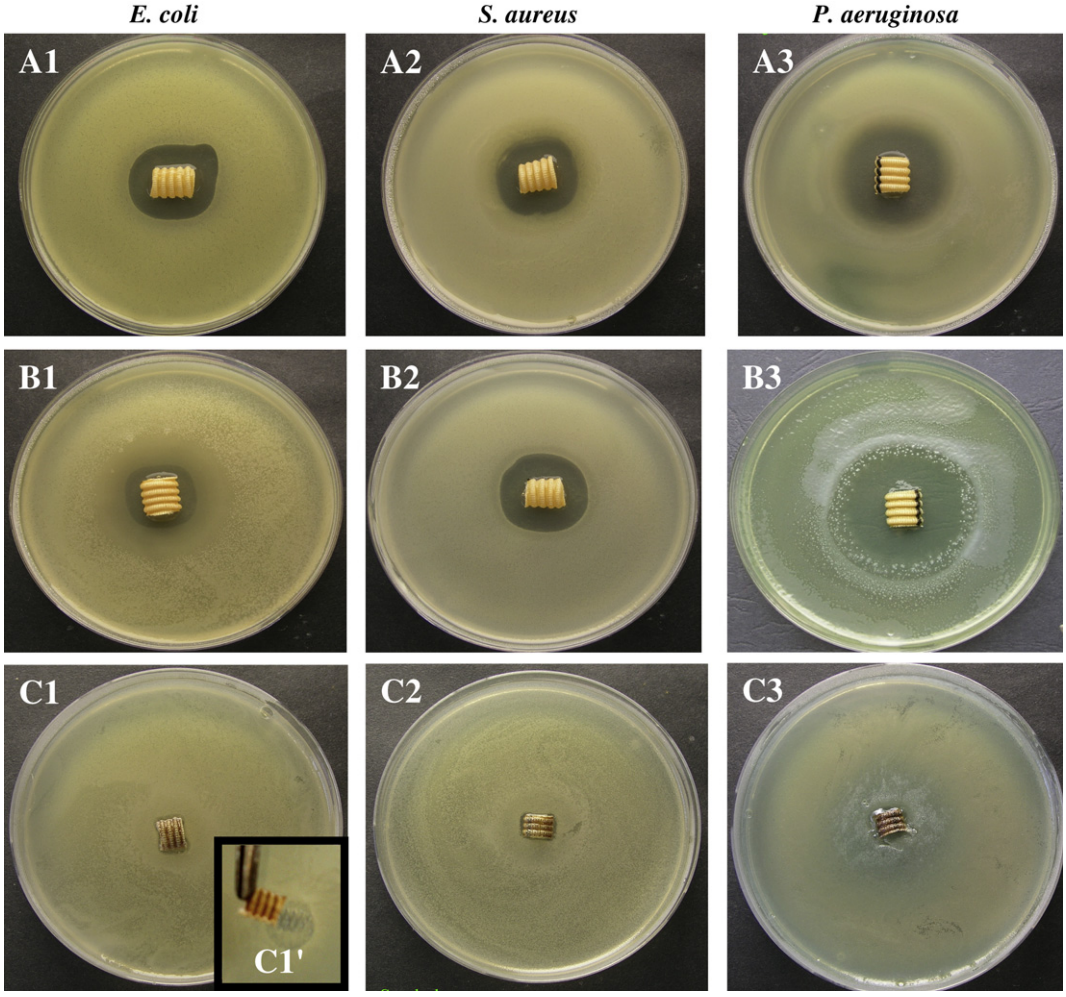

Figure 1 Inhibition of bacterial growth by UniGraft® prostheses covalently modified with gentamicin (A1—A3) and amikacin (B1—B3) versus InterGardTM Silver (C1—C3) grafts. 1 — *E. coli* ATCC 25922; 2 — *S. aureus* ATCC 25923; 3 — *P. aeruginosa* ATCC 27853. Insert C1′ - plate with the fragments of prosthesis lifted up above infected agar medium, showing the places where bacterial growth was inhibited.

the growth of reference strains. Under the same conditions, InterGard™ Silver prostheses revealed only slight antibacterial effects; their bactericidal activity was significantly limited in comparison with covalently modified prostheses (Fig. 3).

Cell culture

The ability of human skin fibroblasts to grow on vascular prostheses was tested in our experiments only for grafts covalently modified with gentamicin and amikacin, as

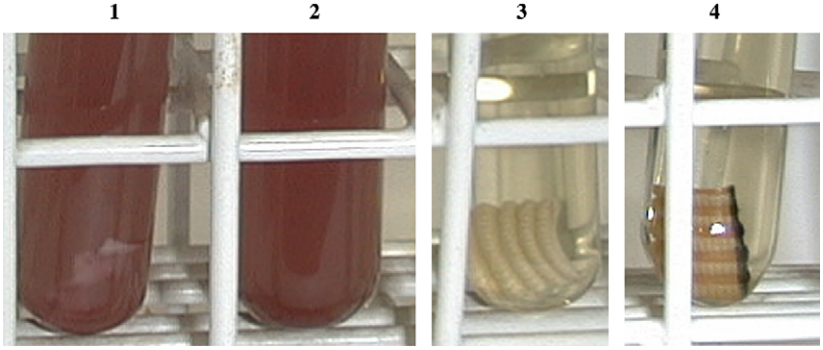

Figure 2 *E. coli* ATCC 25922 biofilm formation on vascular prostheses. (TTC reduction method, 24 h, 37 °C). 1 — UniGraft® no modified control; 2 — InterGardTM Silver; 3 — amikacin-modified UniGraft®; 4 — gentamicin-modified UniGraft®.

Table 4 Inhibition of growth of reference bacterial strains in presence of antibiotic-modified grafts versus silver-impregnated prostheses. Medium inoculated to a final bacteria concentrations as shown in the Table was exchanged every week. Bacteria: *E. coli* ATCC 25922; *P. aeruginosa* ATCC 27853; *S. aureus* ATCC 25923. (MICs in brackets).

Type of prosthesis	Strain (MIC μg/ml)	Initial CFU/ml	Concentration of bacteria (CFU/ml)				
			Day 1	Day 7	Day 14	Day 21	Day 30
UniGraft® modified by gentamicin (9.5 mg/g prosthesis)	*E. coli* (5)	1×10^4	0	0	0	0	0
		4×10^8	0	0	0	0	0
	P. aeruginosa (10)	1×10^4	0	0	0	0	0
		4×10^8	0	0	0	0	0
	S. aureus (10)	1×10^4	0	0	0	0	0
		4×10^8	$1.2 \times 10^7 \pm 0.15$	$1.3 \times 10^8 \pm 0.15$	$1.3 \times 10^8 \pm 0.15$	$1.3 \times 10^8 \pm 0.15$	$1.3 \times 10^8 \pm 0.15$
UniGraft® modified by amikamicin (9.7 mg/g prosthesis)	*E. coli* (5)	1×10^4	0	0	0	0	0
		4×10^8	0	0	0	0	0
	P. aeruginosa (10)	1×10^4	0	0	0	0	0
		4×10^8	0	0	$6.6 \times 10^7 \pm 0.15$	$6.6 \times 10^7 \pm 0.15$	$6.6 \times 10^7 \pm 0.15$
	S. aureus (10)	1×10^4	0	0	0	0	0
		4×10^8	0	0	$7.0 \times 10^7 \pm 0.2$	$7.0 \times 10^7 \pm 0.2$	$7.0 \times 10^7 \pm 0.2$
InterGard™ Silver	*E. coli*	1×10^4	$1.2 \times 10^4 \pm 0.1$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$
		4×10^8	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$
	P. aeruginosa	1×10^4	0	$7.2 \times 10^8 \pm 0.2$	$7.2 \times 10^8 \pm 0.2$	$7.2 \times 10^8 \pm 0.2$	$7.2 \times 10^8 \pm 0.2$
		4×10^8	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$
	S. aureus	1×10^4	$1.2 \times 10^5 \pm 0.1$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$
		4×10^8	$1.2 \times 10^5 \pm 0.1$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$	$1.2 \times 10^9 \pm 0.15$

Table 5 Inhibition of growth of clinical bacterial isolates in presence of antibiotic-modified grafts versus silver-impregnated prostheses. Medium inoculated to a final bacteria concentrations as shown in the Table was exchanged every week. (MICs in brackets). Initial CFU/ml of all bacterial suspensions in Luria–Bertani medium: 1×10^5.

Type of prosthesis	Strain (MIC μg/ml)	Concentration of bacteria (CFU/ml)				
		Day 1	Day 7	Day 14	Day 21	Day 30
UniGraft® modified by gentamicin (9.5 mg/g prosthesis)	*E. coli*, ear (5)	0	0	0	0	0
	E. coli, urinary tract (20)	0	0	0	0	0
	E. coli, faeces (5)	0	0	0	0	0
	P. aeruginosa, eyelid (5)	0	0	0	0	0
	S. aureus, throat (5)	0	0	0	0	0
	S. aureus, faeces (5)	0	0	0	0	0
	S. aureus MR3[a](5)	0	0	0	0	0
	S. epidermidis, skin (2)	0	0	0	0	0
UniGraft® modified by amikamicin (9.7 mg/g prosthesis)	*E. coli*; ear (5)	0	$5.0 \times 10^8 \pm 0.2$	$>10^9$	$>10^9$	$> 10^9$
	E. coli; urinary tract (20)	0	0	0	0	0
	E. coli; faeces (5)	0	0	0	0	0
	E. coli; AR 262[b] (5)	$5.0 \times 10^8 \pm 0.2$	$>10^9$	$>10^9$	$>10^9$	$> 10^9$
	P. aeruginosa; ESBL[c] (10)	0	0	0	0	0
	S. aureus S1[d] (5)	0	0	0	0	0
	S. aureus MR3 (10)	0	0	0	0	0
	S. epidermidis; skin (5)	0	0	0	0	0
InterGard™ Silver	*E. coli*; ear	$1.2 \times 10^5 \pm 0.1$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$
	E. coli; urinary tract	$1.4 \times 10^5 \pm 0.1$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$	$1.2 \times 10^9 \pm 0.2$
	E. coli; faeces	$1.4 \times 10^5 \pm 0.1$	$1.4 \times 10^9 \pm 0.2$	$1.4 \times 10^9 \pm 0.2$	$1.4 \times 10^9 \pm 0.2$	$1.4 \times 10^9 \pm 0.2$
	E. coli; AR 262	$8.4 \times 10^4 \pm 1.0$	$8.4 \times 10^8 \pm 2.0$	$8.4 \times 10^8 \pm 2.0$	$8.4 \times 10^8 \pm 2.0$	$8.4 \times 10^8 \pm 2.0$
	P. aeruginosa; ESBL	$2.4 \times 10^2 \pm 0.5$	$6.8 \times 10^8 \pm 2.0$	$6.8 \times 10^8 \pm 2.0$	$6.8 \times 10^8 \pm 2.0$	$6.8 \times 10^8 \pm 2.0$
	S. aureus; S1	0	$5.6 \times 10^8 \pm 2.0$	$5.6 \times 10^8 \pm 2.0$	$5.6 \times 10^8 \pm 2.0$	$5.6 \times 10^8 \pm 2.0$
	S. aureus; MR3	0	$6.8 \times 10^8 \pm 2.0$	$6.8 \times 10^8 \pm 2.0$	$6.8 \times 10^8 \pm 2.0$	$6.8 \times 10^8 \pm 2.0$
	S. epidermidis; skin	0	$2.2 \times 10^8 \pm 2.0$	$2.2 \times 10^8 \pm 2.0$	$2.2 \times 10^8 \pm 2.0$	$2.2 \times 10^8 \pm 2.0$

[a] *S. aureus* methicillin-resistant.
[b] *E. coli* 262 amikacin-resistant.
[c] *P. aeruginosa* extended-spectrum β-lactamase.
[d] *S. aureus* ampicilin-resistant.

InterGard™ Silver prostheses was carefully examined by manufacturer as a matrix for human cell attachment. The results showed that HSF cells grew easily on both non-modified and antibiotic-modified UniGraft® prostheses (Fig. 4). The cytotoxicity test on osteoblasts cultured in the presence of vascular grafts did not show any statistically significant increased release of LDH to the surrounding medium (103%) in comparison with control containing HSF without prostheses (100%). This indicated the lack of cytotoxicity of the tested antibiotic-modified biomaterials.

Discussion

Vascular aortic infection is one of the most serious complications in postoperative arterial surgery treatment. Short-term infection is easier to fight when antimicrobially modified (with silver or antibiotic) biomaterials are used

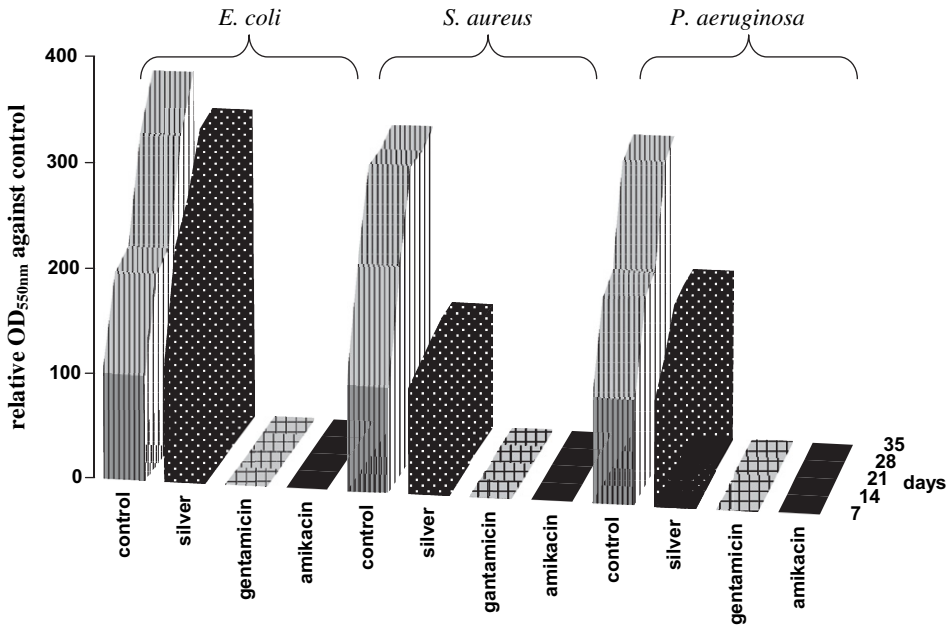

Figure 3 Stability of antibiotic-modified UniGraft® vascular prostheses against InterGardTM Silver grafts. Bacterial strains tested: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853; control — medium infected with bacteria without prostheses. Medium was exchanged daily and inoculated with bacteria to 1 × 102 CFU/ml. SDs were less than 8.9% in all cases.

instead of non-modified ones. The disadvantage of this concept lies in the fact that the currently proposed modified matrices usually undergo quick elution of antibacterial agent from implanted matrix, thus leaving the material unprotected against bacterial attack in the late postoperative period.[12]

Apart from antibiotics, silver is also frequently applied as another bactericidal substance. Silver is one of the microbial agents that are active against virulent bacteria, including MRSA, which are the leading cause of vascular graft infections.[11,33] Silver is also effective against burns, chronic osteomyelitis and various infections[15] and is commercially used for decades for protection of vascular prostheses.

In this study, we compared the antibacterial activity of silver-impregnated vascular prostheses with grafts covalently modified with aminoglycoside antibiotics. The superiority of these prostheses over antibiotic-soaked materials were shown in our research as they are able to retain the majority of bound antibiotic on the prostheses even after 30 days of elution (Table 3). In comparison, rifampicin-soaked prostheses released the whole amount of the attached drug during the first 7 days of release test (Table 3). Similarly, covalently modified vascular grafts were found to be much more effective against many reference and clinical bacteria than commercially available InterGard™ Silver (Tables 4 and 5, Fig. 1). The diversity of clinical isolates tested in our experiments seems to confirm the antibacterial effectiveness of patented biomodification of implantable materials, thus suggesting that they may fight postoperative infections caused by highly aggressive strains muted at surgical tracts in hospitals. The resistance of studied prostheses against MRSA strain is also highly promising. It is worth noting that InterGard™ Silver, tested against MRSA strain in our experiments, revealed only short-lasting antibacterial effect, disappearing just after

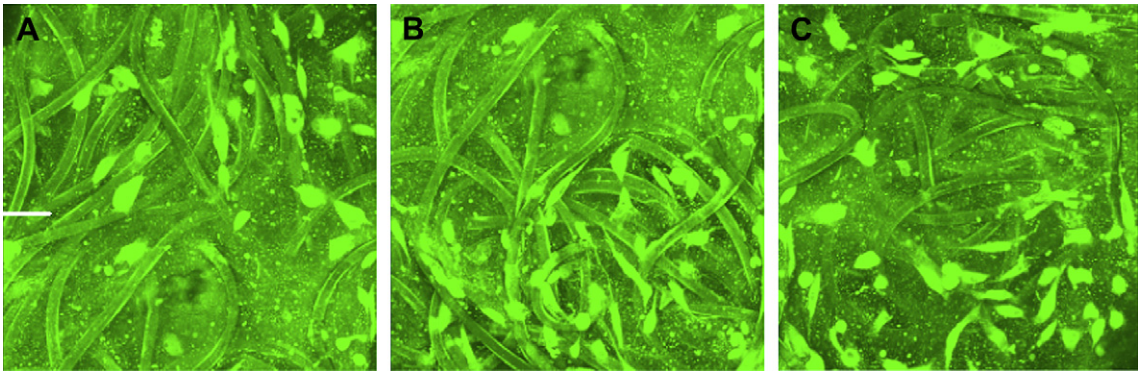

Figure 4 Growth of human skin fibroblasts (HSF) on UniGraft® vascular prostheses non-modified (A) and covalently modified with gentamicin (B) and amikacin (C). Mag. 50×.

7 days of continuous contact with bacterial suspension (Table 4). Covalently (amikacin and gentamicin) modified prostheses also protected the surrounding liquid (represented by the lack of biofilm on their surfaces; Fig. 2) from bacterial attack for at least 1 month, without losing more than 10% of the initially bound drug content in contrast to InterGard™ Silver graft (Tables 4 and 5, Figs. 1—3). These results are in agreement with the other study concerning comparison of rifampin-bonded gelatin-sealed and silver/collagen-impregnated prostheses and their antibacterial activity. The authors found that InterGard™ Silver grafts were less resistant against bacterial colonisation than rifampin-soaked ones, despite the fact that rifampin was only weakly attached to the prostheses.[34] As antibacterial effect of silver ions occurs only when the metal ions diffuse from the matrix, thus penetrating bacterial cell walls and turning DNA into a condensed form and denaturing enzymes by attaching to thiol groups,[15] it clearly appears that majority of silver ions is released from the prostheses within few days.

Some clinical observations confirm that InterGard™ Silver prostheses are ineffective in combating heavy infections. Surgeons suggest that they could be used in primary operations, but vascular prostheses infected after the implantation should be replaced using allogenic implants.[35,36]

Gentamicin and amikacin have been rarely in use in the recent years for their ototoxicity and renal toxicity and also because stronger antibiotics such as rifampicin and vancomycin were commonly used. However, emergence of muted bacterial strains, resistant even to vancomycin, is often a limiting factor in antibacterial prophylaxis. On vascular surgery tracts in the Hospital Henri Mondor, France, the use of rifampin in the procedure room is no longer allowed because of the rise in rifampin-resistant *S. aureus* strains.[27] In a dog model described in their study, rifampin released from prostheses reached a subminimal bactericidal concentration within several days after the implantation, thereby creating an environment for the development of resistance to this antibiotic.[27] Moreover, as vancomycin has recently become the first-line drug for the treatment of MRSA; however, vancomycin-resistant strains of MRSA are now emerging.[37] Therefore, gentamicin and amikacin could be effective in combating the postoperative bacterial infections caused by vancomycin- and rifampin-resistant microorganisms. Stable covalent modification of vascular grafts by amikacin and gentamicin may reopen an old gate to effective eradication of bacteria from graft-surrounding tissues for months, and not just for a few days after the implantation.

One may say that silver-impregnated grafts are thus ineffective in comparison with those by antibiotics and their production and used should be aborted. We would not agree with such a statement. As a compound with a mode of antibacterial action unlike that of antibiotics, it can play a supporting role as a substance co-immobilised with stable-bound antibiotics on the same prosthesis. Recently, a concept of two antimicrobial agents used together on vascular prostheses emerged as effective in antibacterial protection of implantable biomaterials.[13,14] In addition, silver and rifampin adsorbed to vascular prostheses were suggested to demonstrate a synergistic action with efficacy against all Gram-positive and -negative bacteria.[27] The modes of the action of antibacterial agents used in presented experiments seemed to work in union to decrease the risk of infection. It is possible that our future work, concerning the uploading of silver-impregnated prostheses with covalently immobilised aminoglycoside antibiotics, could lead to creation of a more effective tool for prevention of bacterial infections. Moreover, it is clear that further tests concerning *in vivo* behaviour of covalently modified antibiotics-bonded prostheses versus silver-impregnated ones are necessary to fully evaluate their potency at bacterial prevention during surgical operations.

Conflict of Interest

None.

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