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A chemical method of gentamicin bonding to gelatine-sealed prosthetic vascular grafts

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Abstract

Our aim was to develop a new method of chemical binding of gentamicin to vascular prostheses made of poly(ethylene terephthalate) (PET) fibres and covered with pig gelatine. We estimated (with the HPLC method) the immobilization yield, which equalled 76 or 8% depending on the concentration of the antibiotic used and the amount of gentamicin bound to the prosthesis (1.08–20.6 mg/g of prosthesis). The antibiotic was coupled in two modes: stable covalent binding or weak adhesion. The results confirmed that only a small quantity of the antibiotic (1.03–3.09%) was bound by adsorption. The modification of the prosthesis surface with immobilized gentamicin was visualized with a scanning microscope (SEM). Bacteriostatic properties of bound gentamicin were verified against different concentrations (cfu) of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* strains. We have found lack of growth of these pathogen strains in Luria–Bertani (LB) medium containing pieces of gentamicin-coupled prosthesis during at least 28 days of the experiment. Contrary to that, a control medium containing pieces of prosthesis only soaked with gentamicin allowed a constant growth of bacteria.

Keywords: Biomaterial; Poly(ethylene terephthalate); Covalent immobilization; Antibacterial capabilities; SEM

1. Introduction

Infections of vascular grafts occur despite full sterility during surgical procedures and regardless of perioperative parenteral antibiotic prophylaxis. Such infections may cause a significant danger by increasing the risk of a patient's death. The most common reason for infections is the presence of such bacterial strains as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus* sp. (Huebner and Golabmann, 1999; Lyczak et al., 2000; Puzova et al., 1994). To prevent the colonization of biomaterial after implantation, graft surfaces

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are chemically modified with proteins, silver or antibiotics (Kinney et al., 1991; Gahtan et al., 1995; Phaneuf et al., 2001; Virto et al., 2003; Neut et al., 2003). Prosthetic vascular grafts are prepared with the use of either an active or a passive process of antibiotic loading (Gelabert and Colburn, 1992; Avramovic and Fletcher, 1991; Haverich et al., 1998; Vicaretti et al., 1998). These methods are based on electrostatic or hydrophobic interactions between oppositely charged functional groups of biomaterials and antibiotics, which allow the creation of weak bonds (Belt et al., 2001).

Retention of antimicrobial activity is problematic, and so, it reduces the effectiveness of antibiotic-bound grafts. Appropriate modifications of graft surfaces may result in prolonged (up to 10 days) antibiotic retention (Gahtan et al., 1995; Vicaretti et al., 1998).

In our research, we attempted to resolve the problem of implant infections by covalent binding of gentamicin to gelatine-sealed vascular grafts made of poly(ethylene terephthalate) (PET).

Gentamicin is a mixture of sulphates of structurally-related antimicrobial substances produced by *Micromonospora* species—*purpurea* and *echinospora*—and belongs to amino glycoside family of antibiotics. The mechanism of action of this drug relies on the inhibition of protein biosynthesis by binding to the 30S ribosomal subunit as well as on the resulting blockade of translocation on bacterial ribosomes. At higher concentrations, this aminoglycoside damages also bacterial cell membranes which causes the efflux of some ions and low-molecular mass compounds from cytosol to the outside of cells.

Gentamicin is active against a broad spectrum of bacteria including Gram-positive and Gram-negative bacteria; its high activity against *P. aeruginosa* and *Enterobacteriaceae* (*E. coli*, *Enterobacter*, *Klebsiella*, *Salmonella*, *Shigella*, *Serratia*, *Proteus*) is of particular interest. Gentamicin acts also synergistically with penicillin against *Streptococcus* species (Bowman and Rand, 1980; Gringauz, 1997).

2. Materials and methods

2.1. Materials

Standard solution of gentamicin sulphate at a concentration of 1.0 mg/ml, and *o*-phthaldialdehyde (OPA) were obtained from Fluka Chemie (Switzerland).

A pharmaceutical preparation of gentamicin, at a concentration of 40.0 mg/ml, from KRKA (Croatia) was used for the immobilization process. Phosphate-buffered saline (PBS) solution at pH 7.4 (prepared according to the British Pharmacopoeia, 1998) was used for dissolving and diluting gentamicin. Methanol and 2-propanol (HPLC grade) were obtained from Merck (Germany). All other reagents and solvents used for HPLC analysis were prepared from analytical grade substances obtained from Merck. Phthalaldehyde reagent was freshly prepared by dissolving 200 mg of OPA in 1.5 ml of methanol, adding 15 ml of borate buffer and 0.5 ml of mercaptoacetic acid, and adjusting the produced solution to pH 10.4 with 40% potassium hydroxide solution. Borate buffer was obtained by adjusting the pH of 25% boric acid solution to 10.4 with 40% potassium hydroxide.

2.2. Vascular prostheses

Vascular prostheses—Tricogel® were produced by Tricomed (Poland). They were made of poly(ethylene terephthalate) fibres and covered with pig gelatine.

2.3. Gentamicin immobilization

In order to optimise the process of immobilization, gentamicin solutions at concentrations of 0.25 mg/ml (P1), 0.5 mg/ml (P2), 1.25 mg/ml (P3), 2.5 mg/ml (P4), 5.0 mg/ml (P5), 10.0 mg/ml (P6), 20.0 mg/ml (P7), 30.0 mg/ml (P8) and 40.0 mg/ml (P9) mg/ml were prepared by diluting the commercial preparation of gentamicin (40 mg/ml).

Gentamicin binding to vascular prostheses was performed with the covalent method presented in the Polish Patent no. PL358934 (Ginalska et al., 2003). Vascular PET prostheses were activated with glutaraldehyde according to Lappi et al. (1976).

2.4. Quantification of gentamicin by HPLC

2.4.1. Apparatus

The Gilson (France) HPLC system comprised a 170 Diode Array Detector, Model 306 pumps, a Rheodyne valve with a 20-μl loop, a Model 811C dynamic mixer, a Model 805 manometric module, and a Model 864 degasser. Uni Point™ Software v. 2.1 was used for collecting data. Chromatographic analysis was performed

on a Nova-Pak® RP-18 (150 mm × 3.9 mm, 5 μm particle size) column from Waters. A 5 g solution of sodium hexanesulphonate in a mixture of 650 ml of methanol, 300 ml of water and 50 ml of glacial acetic acid was applied as the mobile phase at a flow rate of 1.0 ml/min at 2500 psi. Measurements were made at a wavelength of 330 nm.

2.4.2. Calibration

Calibration solutions containing increasing concentrations of gentamicin from 0.025 to 1.0 mg/ml were prepared by respective dilution of the standard gentamicin solution (1.0 mg/ml) with water. A 0.6 ml of 2-propanol and 0.4 ml of phthalaldehyde reagent were added to an aliquot of 1.0 ml of each calibration solution and mixed accurately. The mixtures were heated in a thermostatically-controlled water bath at 60 °C for 15 min and cooled (using iced water) prior to injection onto the HPLC column.

2.4.3. Determination of gentamicin in solutions before and after immobilization

One millilitre of each gentamicin solution prepared for immobilization (P1–P9) and obtained after immobilization was analyzed according to the procedure described earlier.

Prior to the determination, the above-mentioned solutions were diluted 1:10 (v/v) (P3–P5) or 1:40 (v/v) (P6–P9) in order to obtain concentrations in the range between 0.25 and 1.0 mg/ml. Solutions P1 and P2 were analyzed directly without diluting. The gentamicin solutions after immobilization were prepared analogously.

2.5. Scanning electron microscopy (SEM)

Samples were coated with gold–palladium by a high-resolution sputtering system Polaron SC 7640 (UK) and examined with a scanning electron microscope (Tesla BS 300, Czech Republic) at 15 kV. A Satellite TC software (Tescan) was used to perform an image analysis.

2.6. The antibacterial test of the Tricogel®

The antibacterial effect of gentamicin was tested using the following bacterial strains: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27833, and *S. aureus* ATCC 25923. Before testing, inocula of each bacterium were grown in Luria–Bertani (LB) medium. The inocula were used in infecting doses as follows:

E. coli—1×10^2, 1×10^4, 4×10^6 and 4×10^8 cfu/ml (colony forming units/ml).
P. aeruginosa—1×10^2, 1×10^4, 1.9×10^7 and 1.9×10^9 cfu/ml.
S. aureus—1×10^2, 1×10^4, 5.9×10^6 and 5.9×10^8 cfu/ml.

The inocula of each bacterial strain were mixed with 5 ml of LB medium containing Tricogel® pieces (1 cm^2). A control contained pieces of Tricogel® soaked in gentamicin solution (4.7 mg/ml). During a 28-day incubation period (shaking: 35 rpm at 37 °C), LB medium samples from each experiment were tested for bacterial growth by measuring the optical density (OD) at 550 nm.

3. Results and discussion

Gentamicin components C1, C1a, C2, C2a were determined by using an analytical procedure (with some modifications introduced) according to the British Pharmacopoeia (1999). The applied procedure was based on the RP-HPLC method with pre-column derivatization with OPA and UV detection at 330 nm. After separation by HPLC, the antibiotic was shown to comprise four peaks corresponding to gentamicin sulphate–OPA derivatives: C1 at $t_R = 2.30 \pm 1.74$ min (R.S.D.%, $n = 10$), C1a at $t_R = 8.78 \pm 5.12$ min, C2a at $t_R = 12.09 \pm 5.45$ min and C2 at $t_R = 13.84 \pm 5.56$ min.

The calibration lines plotted for all gentamicin components at 0.5–0.0125 mg/ml were expressed by the following regression equations [$y = a(\pm\text{S.D.})x + b(\pm\text{S.D.})$]:

$$Y_{C1} = 0.8036(\pm 0.0119)x + 0.0053(\pm 0.0028)$$

$$Y_{C1a} = 0.4069(\pm 0.0053)x + 0.0015(\pm 0.0013)$$

$$Y_{C2a} = 0.1440(\pm 0.0018)x + 0.0006(\pm 0.0004)$$

$$Y_{C2} = 0.2464(\pm 0.0031)x + 0.0010(\pm 0.0007)$$

$$Y_{C1+C1a+C2a+C2} = 1.6009(\pm 0.0219)x + 0.0085(\pm 0.0051)$$

Correlation coefficients were almost identical and all greater than 0.9996. The drug composition expressed as percent of each gentamicin derivative calculated from regression equations (the slope of each line as the percentage of the sum of the slope lines) was found to be C1 = 50.20%, C1a = 25.42%, C2a = 9.00%, C2 = 15.39%.

Surfaces of Tricogel® vascular grafts were modified by glutaraldehyde in order to obtain stable covalent binding of antibiotics. Such an activation resulted in obtaining specific Schiff bases coupling gentamicin. The mechanism of this coupling process is presented in Fig. 1. It is worth noting that all free amino groups of gentamicin may participate (play a role) in covalent binding of this antibiotic to activated grafts.

Scanning electron micrographs of gelatine-sealed vascular grafts without or with the antibiotic are shown in Fig. 2A and B, respectively. Differences in the form of the surfaces of both graft types (with or without gentamicin) may suggest that protein-coated graft surfaces were modified with the antibiotic (Fig. 2B).

The estimation of gentamicin binding to the Tricogel® prosthesis was carried out through an indirect method by finding the difference in gentamicin concentration before and after immobilization. The representative chromatogram is shown in Fig. 3. The percentage of drug associated with the matrix immobilization yield was calculated using the formula: $[(A - B)/A] \times 100$, where A and B represent the initial and final concentrations of gentamicin components established using the corresponding regression equation. The results, summarised in Table 1, demonstrate that the covalent binding of the examined antibiotic to the Tricogel® prosthesis is proportional to the increase of drug concentration during immobilization. Similar conclusions may be drawn on the basis of an analysis of plots exhibited in Fig. 4. Figs. 5 and 6 present the amounts of gentamicin bound to the matrix expressed in mg or %, respectively, as a function of the drug concentration before immobilization. It can be observed (Fig. 5) that the significant increase of gentamicin binding is correlated with an increase of the initial concentration of this drug (range: 0.25–10 mg/ml). Simultaneously, the percentage of binding decreased in relation to initial drug concentrations (Fig. 6). The further increase of gentamicin concentration up to 40 mg/ml did not significantly influence the increase of the antibiotic amount coupled to the prosthesis. Therefore, a gentam-

Table 1
Concentration of gentamicin components before (*A*) and after (*B*) immobilization determined by use of the HPLC method, column *C* presents the amount of the antibiotic bound to the Tricogel® prosthesis

Experimental series	Gentamicin C1			Gentamicin C1a			Gentamicin C2a			Gentamicin C2			Sum of gentamicin compounds			Immobilization yield (%)	Gentamicin bound to prosthesis (mg/g)[a]
	A (mg/ml)	*B* (mg/ml)	*C* (mg/ml)[a]	*A* (mg/ml)	*B* (mg/ml)	*C* (mg/ml)[a]	*A* (mg/ml)	*B* (mg/ml)	*C* (mg/ml)[a]	*A* (mg/ml)	*B* (mg/ml)	*C* (mg/ml)[a]	*A* (mg/ml)	*B* (mg/ml)	*C* (mg/ml)[a]		
P1	0.131	0.035	0.576	0.053	0.012	0.246	0.022	0.004	0.108	0.031	0.005	0.156	0.237	0.056	1.086	76.37	1.08
P2	0.268	0.113	0.930	0.108	0.013	0.570	0.043	0.014	0.174	0.061	0.020	0.246	0.480	0.161	1.914	66.46	1.91
P3	0.589	0.270	1.914	0.238	0.085	0.918	0.100	0.049	0.306	0.145	0.060	0.510	1.072	0.464	3.648	56.72	3.64
P4	1.444	0.720	4.344	0.569	0.258	1.866	0.246	0.122	0.744	0.317	0.159	0.948	2.577	1.260	7.902	51.11	7.90
P5	2.659	1.757	5.412	1.044	0.650	2.364	0.435	0.285	0.900	0.592	0.380	1.272	4.729	3.071	9.948	35.06	9.94
P6	5.393	4.169	7.344	2.158	1.558	3.600	0.899	0.662	1.422	1.222	0.891	1.986	9.673	7.281	14.352	24.73	14.35
P7	10.670	8.921	10.494	3.841	3.391	2.700	1.564	1.402	0.972	2.134	1.996	0.828	18.209	15.710	14.994	13.72	14.99
P8	17.724	15.725	11.994	6.421	5.921	3.000	2.719	2.419	1.800	3.844	3.444	2.400	30.708	27.510	19.188	10.41	19.18
P9	22.222	20.223	11.994	8.420	7.420	6.000	3.468	3.268	1.200	4.844	4.594	1.500	38.953	35.505	20.688	8.85	20.68

[a] Quantity of gentamicin bound to Tricogel® prosthesis (mg/g prosthesis).

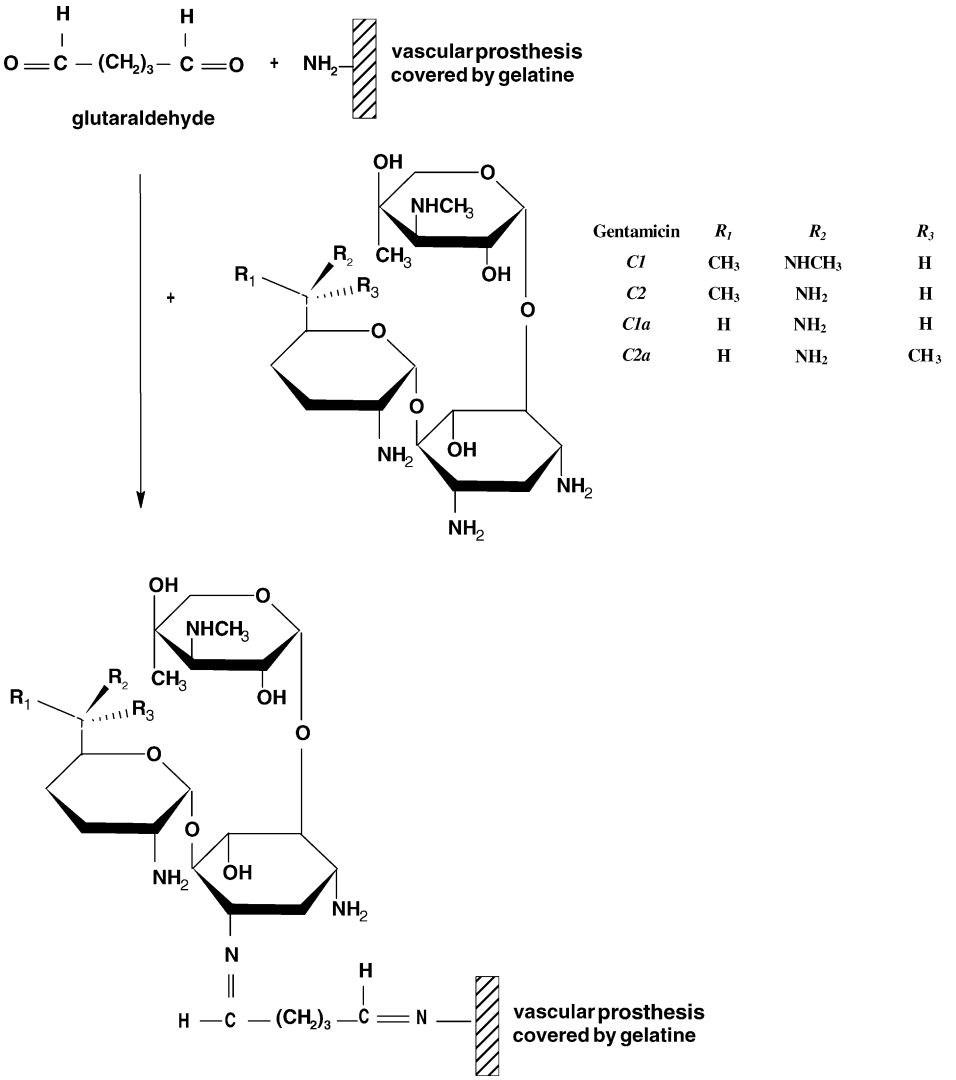

Fig. 1. A hypothetical mechanism of covalent gentamicin bonding to vascular prostheses.

icin concentration of 4.7 mg/ml was selected as optimal for drug immobilization to prosthesis, taking into account the yield of this process (35.09%), the quantity of antibiotic bound to 1.0 g of prosthesis (approximately 9.94 mg) and microbiological analysis of such obtained implantable material.

Moreover, the release of gentamicin from the matrix was studied in order to evaluate the percentage of physical adsorption of this compound. The amount of the drug released from prosthesis during shaking with PBS solution (three times for 30 min) varied from 1.03 to 3.19%. Similarly, in our earlier results concerning the stability of bonds created between gentamicin and gel-PET prostheses (Ginalska et al., 2004a, 2004b, 2004c), we have found that about 3% of immobilized antibiotic was being released from the drug-modified prosthesis during 30 days of continuous shaking in PBS buffer. The results, therefore, show that gelatinised vascular grafts generally bind gentamicin in a chemical mode. Small amounts of this drug eluted from graft surfaces suggest that only a low percentage of the antibiotic was bound to the biomaterial in a non-permanent, passive

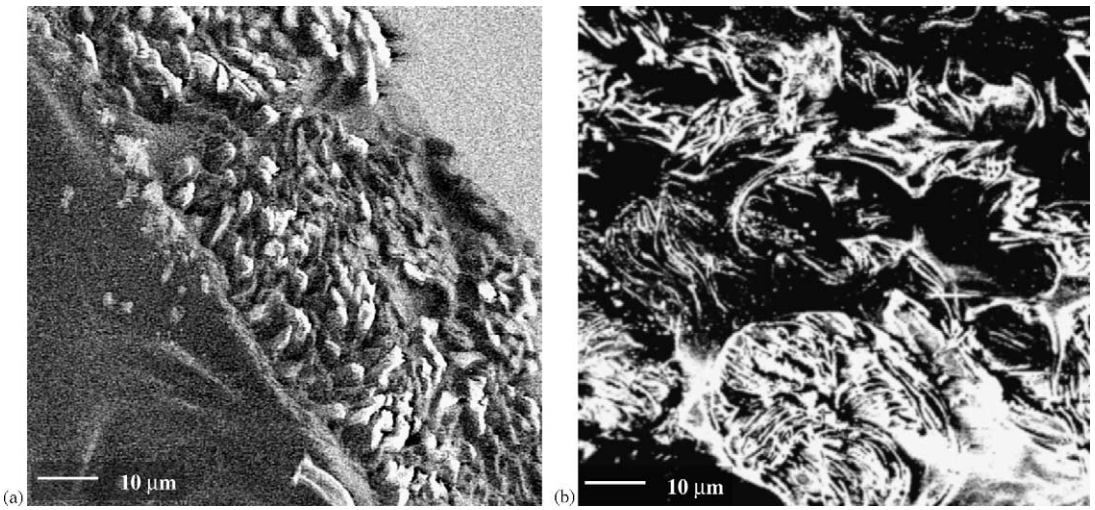

Fig. 2. Scanning electron micrographs of Tricogel® without (A) and with (B) immobilized gentamicin.

or ionic way. However, the covalent binding is the dominant mode of gentamicin attachment to the prostheses.

There is some information in the available literature on coupling of antibiotics (rifampicin, ciprofloxacin, vancomycin, gentamicin) with matrices by ionic interactions (Kinney et al., 1991; Haverich et al., 1998; Ashton et al., 1990). Generally, the passive binding between antibiotics and biomaterials is a predominant means of immobilization (Avramovic and Fletcher, 1991; Goëau-Brissonnière et al., 1999). The meth-

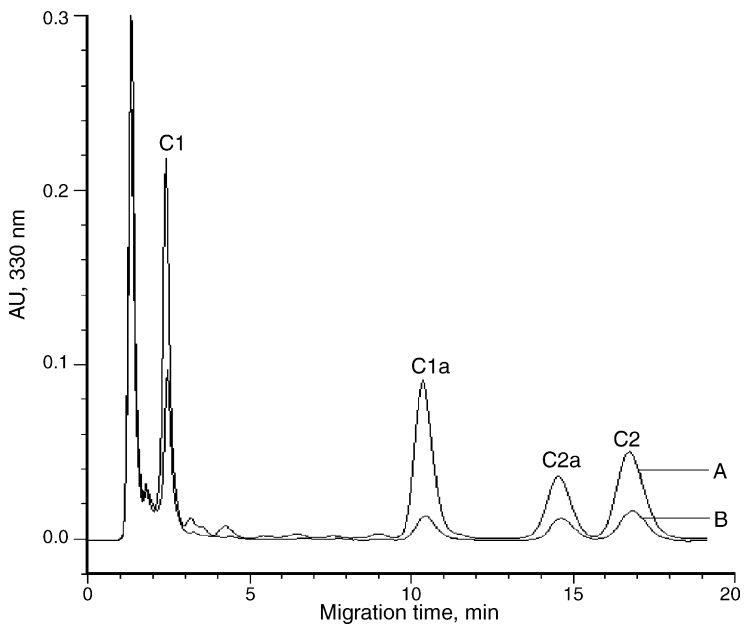

Fig. 3. Chromatogram of the gentamicin sulphate–OPA derivatives obtained from analyses of samples before (A) and after (B) immobilization.

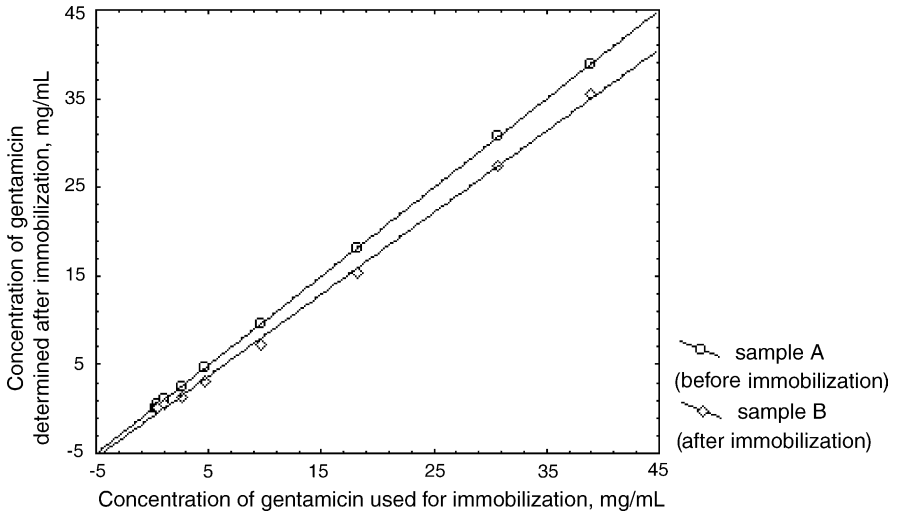

Fig. 4. The determined concentration of the total amount of gentamicin before (sample A) and after (sample B) immobilization in relation to the prepared concentrations of this antibiotic.

ods mentioned above are ineffective because antibacterial protection of such biomaterials is short-lived. The process of total elution of antibiotic from graft surfaces lasts 5–8 or 7–14 days in case of physical adsorption or ionic binding, respectively (Avramovic and Fletcher, 1991). The grafts preserved in this way are fully protected against bacterial infections for up to 10 days; afterwards, the amount of the antibiotic left on these grafts drops below MIC—minimal concentration inhibiting pathogens' growth (Gahtan et al., 1995; Avramovic and Fletcher, 1991; Vicaretti et al., 1998; Belt et al., 2001; Strachan et al., 1991).

During the next stage of the experiments, the bacteriostatic activity of immobilized gentamicin was tested in the presence of *E. coli*, *P. aeruginosa*, and *S. aureus* strains. These strains are responsible for infections during post-operational treatment in hospitals (Huebner and Golabmann, 1999; Lyczak et al., 2000; Puzova et

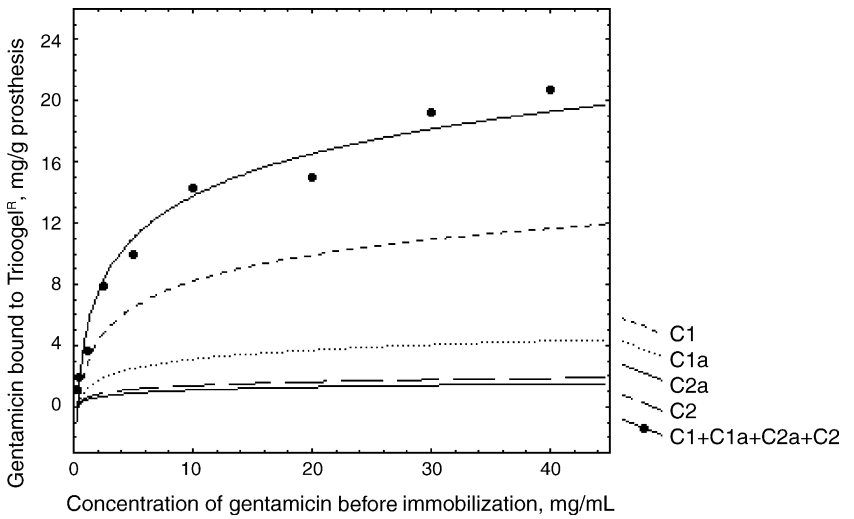

Fig. 5. The amount of gentamicin components bound to Tricogel® prosthesis as a function of the initial concentrations of this antibiotic.

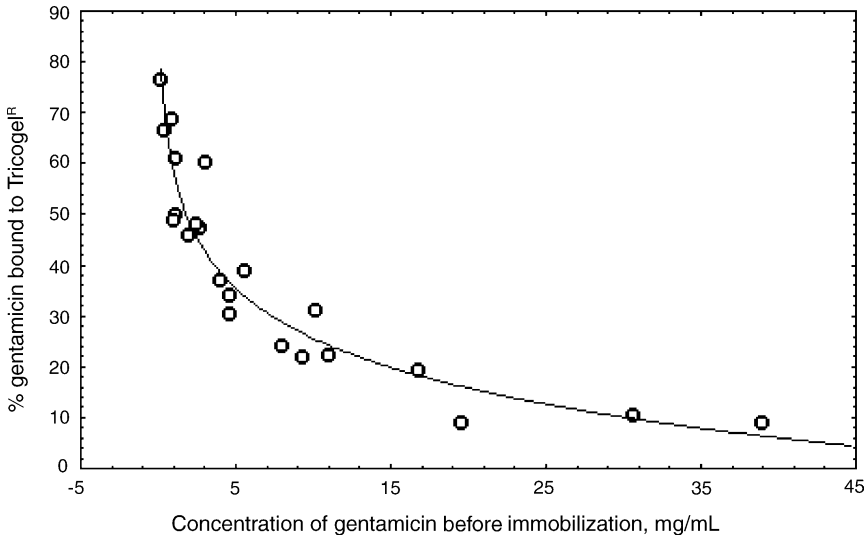

Fig. 6. The percentage of the sum of gentamicin forms bound to Tricogel® prosthesis as a function of the initial concentrations of this antibiotic.

al., 1994). The obtained results (Table 2) suggest that the tested bacteria (in each infecting dose) did not grow (during 21 days) in LB medium while shaking with pieces of gentamicin-bound vascular grafts. Pathogen growth was observed only when bacterial concentration was increased to 10^8–10^9 cfu/ml and only after 28 days of the experiment. In control samples (LB medium shaken with pieces of gentamicin-soaked vascular grafts), a constant growth of bacteria was observed.

Table 2
Optical density measurement of *E. coli*, *P. aeruginosa*, and *S. aureus* incubated in the presence of vascular prostheses containing immobilized gentamicin (9.94 mg/g prostheses) over the course of 28 days in LB medium at 37 °C

Strain	Infecting dose (cfu)	OD at 550 nm measured at		
		Day 7	Day 21	Day 28
E. coli	1×10^2	0	0	0
	1×10^4	0	0	0
	4×10^6	0	0	0
	4×10^8	0	0	0.07 ± 0.05
P. aeruginosa	1×10^2	0	0	0
	1×10^4	0	0	0
	4×10^7	0	0	0
	4×10^9	0	0	0.08 ± 0.06
S. aureus	1×10^2	0	0	0
	1×10^4	0	0	0
	4×10^6	0	0	0
	4×10^8	0	0	0.05 ± 0.01

Bactericidal or bacteriostatic activity of gentamicin-modified vascular prostheses against tested bacterial strains was observed in our present and earlier experiments (Ginalska et al., 2004a, 2004c). This effect is difficult to explain in a view of commonly known mechanism of bactericidal effect of this drug, relying on active NH_2 group's participation. Most probably, only some of gentamicin amino groups are engaged in creation of covalent bonds with gelatine-sealed vascular prosthesis (mostly for steric reasons). Therefore, all remaining NH_2 groups of the drug may potentially participate in bacterial growth inhibition. Moreover, we assume that antibacterial activity of such modified biomaterial was possible due to mixed (covalent and passive) mode of gentamicin immobilization. The antibiotic bound via weak non-covalent interactions was gradually released from prostheses. This protected the biomaterial (as well as surrounding liquids) from bacterial infections and prevented biofilm creation on prosthesis surface (Ginalska et al., 2004c). Simultaneously, the gelatine covering prostheses was being gradually hydrolysed by bacterial proteases; therefore, the gelatine-attached antibiotic was released to the liquid medium. On basis of these observations we assume that in in vivo experiments that effect will be enhanced by activity of patients' own proteases.

4. Conclusions

To decrease the amount of vascular grafts infections, it is essential to eradicate the pathogens from the prosthesis site, both immediately and several days after the implantation. Results of presented experiments seem to promise a great progress in research concerning the prostheses of prolonged bactericidal effect. This is very essential because vascular prosthesis implantation causes violent inflammation, resulting in fibroblasts migration towards the graft and synthesis of connective tissue elements between its fibres. This reduces the risk of infection; however, if bacteria reach the graft before the fibroblasts, they synthesize their protective glycoprotein layer called biofilm which allows for development of infection. Chemical immobilization of gentamicin on gelatine-sealed vascular prostheses enabled the antibiotic to remain on matrix for at least 30 days. This period seems to be sufficient to avoid the biofilm creation and thus reduce the frequency of infections after vascular graft implantation.

For application, the antibiotic-modified biomaterial requires further experimental and clinical investigations.

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