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Amikacin-loaded vascular prosthesis as an effective drug carrier

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Abstract

Strong covalent immobilization of amikacin on Uni-Graft® DV straight vascular prostheses made of gelatine-sealed poly(ethylene terephthalate) fibres was performed according to procedure described in the Polish Patent No. P-358934. The concentrations of amikacin in sample solutions were estimated either by HPLC or by UV spectroscopy method previously optimized for amikacin measurements. A high correlation was found between these two methods. It was found that the antibiotic was bound in mixed-type way via three types of interactions: strong covalent bonds (dominating amount: 81.84%) and weak interactions: physical adsorption and ionic bonds (18.19%). Even when total amount of physically and ionically attached drug has been released, the remaining covalently bound amount still locally protected the prostheses *in vitro* against bacteria. The release test was conducted in PBS at pH 7.4 at 37 °C and showed that about 15% of total drug amount was eluted from the matrix during the first 7 days of shaking, then no more antibiotic was released. It suggested that about 85% of amikacin attached to prosthesis modified in mixed-type mode was bound via covalent interactions. A bacterial inhibition test on *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 showed inhibition of growth for all strains at low inoculum concentrations up to 30 days as well as high inoculum concentration for *E. coli*. At high concentrations of *S. aureus* and *P. aeruginosa*, the modified prostheses showed slight bacteriostatic effect since 10th day of experiment. Amikacin-modified vascular prostheses might therefore be protected against bacterial infection locally, without long-lasting drug release to human system.

Keywords: Amikacin; Prosthetic biomaterial; Antibacterial capabilities; Drug release

1. Introduction

Infections of vascular prostheses, appearing after the implantation, belong to serious complications in vascular surgery. Cost analysis of treatment during vascular graft infections (Szilagyi et al., 1972) (infections concern the graft, surrounding tissues and vascular anastomosis) suggests the necessity of immediate re-operation of the patient (Nevelesten et al., 1997; Pipinos et al., 2000) because systemic antibiotic therapy and local treatment often result in appearance of sepsis or massive haemorrhage. The risk of graft infections is estimated to be only 1–5% of operation cases, regardless the treatment method and antibiotic prophylaxis (Hernández-Richter et al., 2003). However, the mortality in these cases ranges between 25% and 75% (in USA—10–15%), depending on many factors, while amputations of infected limb is necessary in 15–60% (Moore and Cole, 1991; Kikta et al., 1987; Huh et al., 1998). Therefore, the infected graft is usually surgically replaced by a new one (silver-coated or antibiotic-soaked) or by vascular auto- and allografts in order to lower the re-infection rate (Chiesa et al., 1998; Nevelesten et al., 1995; Novali, 2001). This remains the most accepted practice in cases of aortic graft infections or infected aortic aneurysm. However, the problem of autogeneic and allogeneic material supply and high operation risk led to the common use of prosthetic biomaterials. Therefore, the modification of these biomaterials in order to prevent their infection and/or re-infection may be of primary importance to resolve this problem. The antibiotic-loaded (either adhesively by electrostatic or hydrophobic interactions or ionically) biomaterials were proposed (Belt et al., 2001; Haverich et al., 1992; Gahtan et al., 1995; Vicaretti et al., 1998). According to Frutos et al. (2000), the choice of antibiotic used for chemical modification of biomaterial depends on its germicidal activity, antibacterial spectrum and stability both at human body temperature and during the immobilization procedure. Also antibiotic

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release rate is dependent on type of sealing agent (gelatine, collagen or albumin) and antibiotic used. For example, pefloxacin and glicopeptide antibiotics are usually quickly eluted, with total loss of activity within several minutes and 24 h of incubation, respectively (Galdbart et al., 1996).

In our earlier results, we have shown the possibility of strong chemical immobilization of gentamicin to gelatin-sealed poly(ethylene terephthalate) vascular prosthesis (Ginalska et al., 2005a,b). This affected the antibacterial biomaterial activity allowing for its significantly prolongation in comparison with commonly used antibiotic-soaked grafts (Avramovic and Fletcher, 1991). Therefore we decided to make an attempt to immobilize another drug, of stronger bactericidal activity, on these grafts. Among the aminoglycoside antibiotics, amikacin is one of the most popular in medicine and veterinary, due to its broad spectrum against Gram-positive and Gram-negative bacteria including *Pseudomonas*, *Escherichia coli*, *Proteus*, *Klebsiella*, *Serratia*, *Staphylococcus* and mycobacteria (Gringauz, 1997; Ristuccia and Cunha, 1985; Sánchez-Martinez et al., 2004). Also, it is less susceptible to enzymatic degradation than other aminoglycoside antibiotics.

Our earlier work (Ginalska et al., 2005a) did not concern the precise characterization of created gentamicin–biomaterial interactions nature. This article is an attempt of deeper explanation of this problem and experimental confirmation that drug immobilization is mainly of chemical, covalent nature. We wanted also to estimate the stability of interactions between immobilized antibiotic and prostheses modified according to our method. However, this work concerns immobilization of amikacin—aminoglicoside antibiotic of a newer generation than gentamicin.

2. Materials and methods

2.1. Materials

Standard amikacin sulphate USP 24 was obtained from Institute of Biotechnology and Antibiotics (Warsaw, Poland). Commercial Biodacyna® solution containing 250 mg of amikacin disulphate per 1 mL from Bioton (Warsaw, Poland) were used for immobilization process. *o*-Phthaldialdehyde (OPA) was obtained from Fluka (Switzerland). Methanol and 2-propanol (both of HPLC grade) and all other reagents and solvents of analytical grade were obtained from Merck (Darmstadt, Germany). The water used in the experiments was double distilled. Phosphate-buffered saline (PBS) solution at pH 7.4 prepared according to the British Pharmacopoeia (1999) was used for dissolving and diluting the amikacin.

Derivatizing (phthalaldehyde) reagent was freshly prepared according to the method described elsewhere (Ginalska et al., 2005a) and was stable in the dark at 4 °C, over a period of 12 h.

2.2. Immobilization process

Immobilization of amikacin was performed in triplicate on 1 cm^2 segments of Uni-Graft® DV straight vascular prostheses made of poly(ethylene terephthalate) (PET) fibres and sealed with gelatine (Braun Melsungen AG, Germany). First step of the reaction included the prosthesis activation with glutaraldehyde; the second—amikacin reaction with activated matrix, followed by reduction of Schiff bases by $NaBH_4$. The detailed procedure was described in the Polish Patent No. P-358934 (Ginalska et al., 2004).

For optimization of the immobilization process, amikacin solutions at concentrations of 10.0 mg/mL (S1), 5.0 mg/mL (S2), 2.5 mg/mL (S3), 1.25 mg/mL (S4), 0.625 mg/mL (S5), 0.3125 mg/mL (S6) were prepared by diluting the commercial Biodacyna® preparation.

2.3. HPLC and UV spectrophotometric assays

Chromatographic analysis was carried at λ 329 nm on an Gilson HPLC-DAD system equipped with a Nova-Pak® RP-18 (3.9 mm × 150 mm, 5 μm) column from Waters. A mixture of methanol, water and glacial acetic acid (50:45:5, v/v/v) containing 10 mM/L sodium hexanesulphonate was used as mobile phase at a flow rate of 1.5 mL/min.

Derivatization procedure: a 0.5 mL of 2-propanol and 0.5 mL of phthalaldehyde reagent were added to an aliquot of 1.0 mL of each calibration solution or sample solutions and mixed accurately. The mixtures were heated in a thermostatically controlled water bath at 60 °C for 10 min, cooled in an ice bath and analyzed in duplicate by UV spectroscopy and for the control by HPLC. The concentration of amikacin in sample solutions was calculated from the calibration equation after multiplying by the dilution factor.

UV spectrophotometric analysis was carried out at 335 nm on a Cecil CE 7200 double-beam UV–vis spectrophotometer (Cecil Instruments Limited, England) at the scan speed: 3000 nm/min, bandwidth: 2 nm, path length: 1 nm as the operating conditions.

Calibration amikacin solutions in the concentration range from 0.1 to 0.5 mg/mL and amikacin samples were prepared in the manner described in previous report (Ginalska et al., 2005a).

2.4. Scanning electron microscopy (SEM)

SEM experiments were performed as previously described (Ginalska et al., 2005a).

2.5. In vitro drug release

Percentage of amikacin bound to prostheses by physical adhesion, ionic interaction and covalent bonds was estimated. For this purpose, the amikacin attached by physical adsorption was released from the prostheses during mechanical shaking with 15 mL of the distilled water (five times for 15 min); then the prostheses were shaken five times with 15 mL of NaCl solution for 15 min for ionic interactions removal. The concentration of NaCl used in this experiment was previously optimized in the range between 0.1 and 1 M. The amount of amikacin covalently bond to the Uni-Graft® prosthesis was calculated from the difference between the total amount of amikacin associated with the prosthesis and the sum of the antibiotic released from the immobilized prosthesis during the washing with water and with

solution of sodium chloride. The experiment was performed in triplicate.

Presence of three types of amikacin-prosthesis interactions was confirmed by antibacterial activity test on *E. coli* ATCC 25922. Inocula (1° in McFarland scale) were kept under sterile conditions on Mueller-Hinton Agar II (Lab M^{TM}, IDG plc, UK) (on Petri dishes, 37 °C, 24 h). Prostheses were sterilized by UV-light prior to experiments (Philips lamp, 30 W/cm^2, 0.5 h, from distance of 15 cm). The following Uni-Graft® samples used in the experiments: (1) Uni-Graft® piece coupled with amikacin in mixed-type mode (containing passive, ionic and covalents bonds), (2) Uni-Graft® piece coupled with amikacin bound by ionic and covalent interactions (after eluting with water) and (3) Uni-Graft® piece containing amikacin covalently bound (after eluting with water and afterwards with 1 M NaCl). One-centimetre square pieces of vascular prostheses were directly placed on agar. The plates were incubated for 2 h at room temperature, then for 18 h at 37 °C. Subsequently, the zones of *E. coli* growth inhibition were measured.

Stability of amikacin bonds was estimated in as follows: the prostheses modified with amikacin in mixed-type and control prostheses soaked with 2.5 mg/mL amikacin solution were incubated in 20 mL portions of 0.1 M PBS pH 7.4 at 37 °C for 30 days. On 1st, 3rd, 7th, 14th, 21st and 30th day of shaking, the PBS was removed and replaced by a fresh portion of saline. Amikacin concentrations in removed PBS were estimated as described above. The experiment was performed in triplicate.

2.6. Minimum inhibitory concentrations (MICs)

Determination of MICs was performed in duplicate according to modified method described by Lőwdin et al. (1998). Determination of killed bacteria was performed as follows: samples were taken out, diluted in PBS and spread onto agar plates. The plates were incubated at 37 °C, and the colonies were counted after 24 h. For determinations of CFU, only the plates with 10–500 colonies per plate were taken into account. The measurements were performed in triplicate.

2.7. Assay of antibacterial activity

Assay was performed against reference strains *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853. Before testing, inocula of all bacteria were grown in Luria-Bertani (LB) medium. The inocula, in infecting doses at concentrations indicated in Table 3, were mixed with 20 mL of LB medium containing mixed-type amikacin-modified Uni-Graft® pieces (1 cm^2). During 30-day incubation period (shaking: 35 rpm at 37 °C), the optical density in LB medium was measured (o.d.) at 550 nm. The average values of three experiments (±S.D.) were presented in overall count of bacteria by the serial dilution method. All experiments were performed in triplicate.

3. Results and discussion

The UV spectrophotometric method was developed for quick estimation of results of amikacin immobilization on the surface of vascular prosthesis. Estimation of degree of drug binding to the prosthesis was afterwards confirmed by HPLC method.

HPLC assay selected for present work is described in gentamicin sulphate monograph of British Pharmacopoeia (1999) and it was used in our previous paper for the analysis of gentamicin concentration (Ginalska et al., 2005a). This analytical procedure has been adapted for determination of amikacin after introduction of some modifications. The applied methods were based on the reaction of amikacin with *o*-phthalaldehyde and the mercaptoacetic acid (at pH 10.4) to form a highly fluorescent thioisoindole derivative which is detected at 329 nm. In view of very weak absorption properties of amikacin, derivatization of this aminoglycoside antibiotic was necessary. Optimization of derivatization conditions showed that molar ratio between amikacin (0.5 mg/mL) and *o*-phthalaldehyde significantly influences the derivatization yield and large excess of derivatizing reagent caused the decrease of the response. Temperature in the range 50–70 °C and reaction time in the range 5–30 min had no significant effect on analytical response (Fig. 1). As a result, *o*-phthalaldehyde reagent at concentration of 5 mg/mL, temperature of 60 °C and reaction time of 10 min were selected to be the optimum derivatization conditions for the assay. Because the amikacin–OPA derivative was unstable, heated samples were rapidly cooled and their absorbance was determined over a period of 2 h.

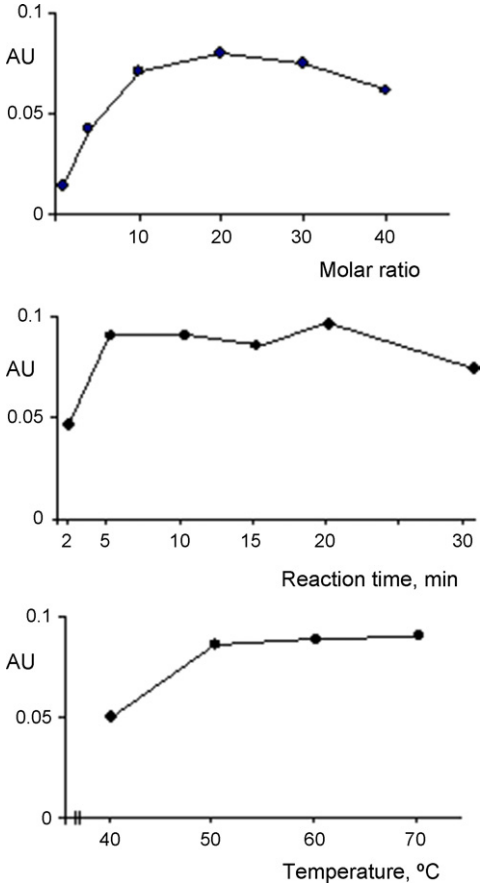

Fig. 1. Effect of the molar ratio (amikacin (0.5 mg/mL): OPA), reaction time and temperature on the amikacin derivatization yield in HPLC method.

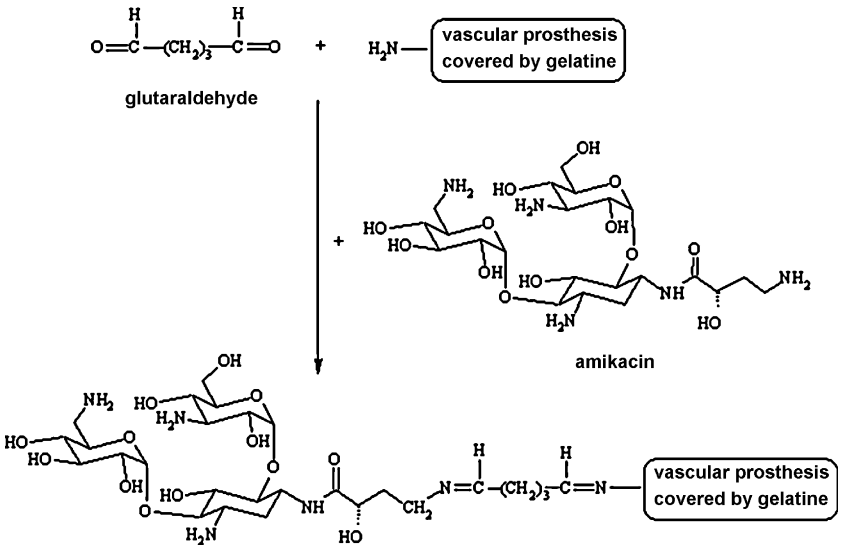

Fig. 2. Hypothetical mechanism of covalent amikacin bonding to vascular prostheses.

For immobilization of antibiotic, surfaces of Uni-Graft® prosthesis were modified by glutaraldehyde in order to obtain stable covalent binding of amikacin. According to mechanism presented in Fig. 2, we assume that bifunctional agent used in experiments – under optimised conditions – allowed for creation of reversible Schiff bases between free primary amino groups of biomaterial and amikacin. Resulting Schiff bases were then reduced with $NaBH_4$ to secondary amines. Such a mechanism is similar to a process of ligands immobilization in preparation of matrices for affinity chromatography (Hermanson et al., 1992).

Scanning electron micrographs of gelatine-sealed vascular prosthesis without or with antibiotic were shown in Fig. 3A and B, respectively. Modification by antibiotic immobilization did not affect the structure of prostheses which was confirmed by presence of unchanged gelatin fibers on graft surfaces.

The estimation of amikacin binding to the Uni-Graft® prosthesis surface was carried out through an indirect method by calculating the difference in amikacin concentrations before and after immobilization. The representative HPLC chromatogram was shown in Fig. 4. The derivatized amikacin was eluted as two major components (mean ± S.D., $n = 10$): at 7.77 min ± 0.70 (symmetrical peak) and 10.06 min ± 1.44 (tailing peak). The other small peaks present in the samples containing amikacin may correspond to impurities related to semisynthetic amikacin (British Pharmacopoeia, 1999) or degradation products resulting from antibiotic heating. To evaluate the amikacin binding results, we took into account the first, well shaped peak because we found better correlation between the peak height (or peak area) response and the calibration concentrations for this peak ($r = 0.998$) in comparison with that for the second peak ($r = 0.96$). No interfering peaks were found in antibiotic-free sample obtained after immobilization (for method selectivity test).

The percentage of antibiotic associated with matrix (immobilization yield) was calculated using formula: $(A - B/A) \times 100$, where A and B represent the initial and final concentrations of amikacin, respectively. The obtained results, summarised in Table 1, demonstrate that amounts of amikacin bound to Uni-Graft® prosthesis increased with the increasing antibiotic concentrations in solutions prepared for immobilization. The amounts of amikacin bound to matrix expressed in mg/g or % versus the drug concentration before immobilization were

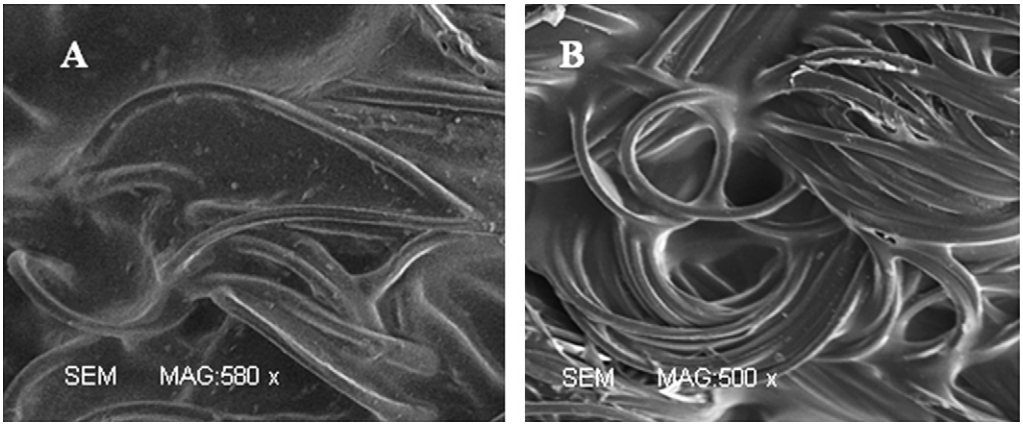

Fig. 3. Scanning electron micrographs (SEMg) of Uni-Graft® without (A) and with (B) immobilized amikacin.

Table 1
Concentration of amikacin before (samples A) and after (samples B) immobilization determined by use of the HPLC method on the basis of regression equation

Experimental series	Samples A (mg/mL)	Samples B (mg/mL)	Amikacin bound to prosthesis from 1 mL of sample		Total amount of amikacin bound to prosthesis (mg/g prosthesis)	Immobilization yield (%)
			mg	R.S.D.%[a] ($n = 5$)		
S1	7.9563	6.0261	1.9302	13.34	15.4416	24.26
S2	4.9068	3.9599	0.9469	14.56	7.5751	19.30
S3	2.7114	1.7038	1.0076	25.71	8.0607	37.16
S4	1.2464	0.7214	0.5250	21.95	4.2003	42.12
S5	0.7537	0.2074	0.5463	22.39	4.3703	72.48
S6	0.3769	0.0408	0.3360	11.54	2.6883	89.17

[a] R.S.D.: relative standard deviation.

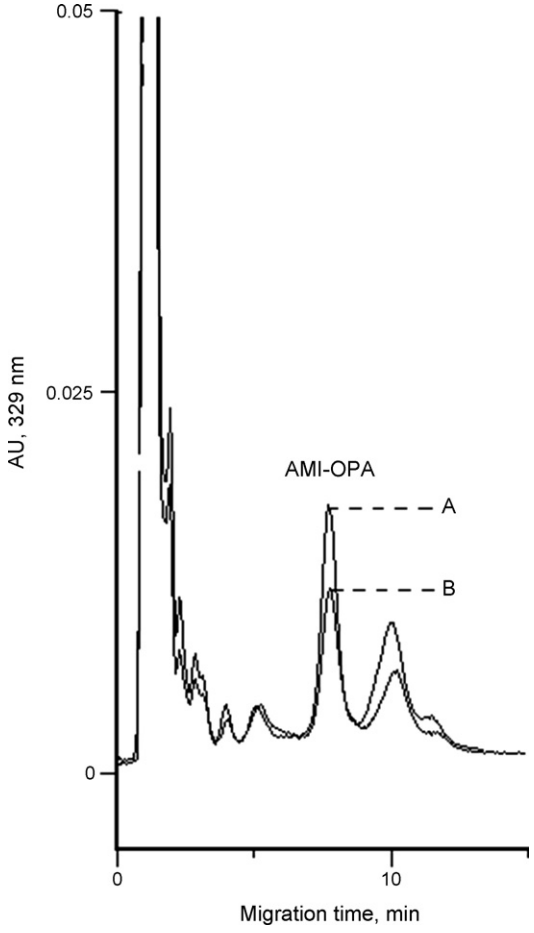

Fig. 4. Chromatogram of the amikacin (AMI)–OPA derivative obtained from analyses of samples before (A) and after (B) immobilization.

graphically shown in Fig. 5. It can be observed (Fig. 5) that significant increase of amikacin immobilization was correlated with an increase of the initial antibiotic concentration within the range 0.31–2.5 mg/mL. The further increase of amikacin concentration up to 10 mg/mL did not significantly influence the increase of the coupled antibiotic amount. Simultaneously, the immobilization yield decreased when the initial amikacin concentration increased (Fig. 5). Therefore, amikacin concentration of 2.5 mg/mL was selected as optimum for drug immobilization to Uni-Graft® prosthesis, taking into account the yield of this process (37.16%), the quantity of antibiotic bound (approximately 8.06 mg/g of prosthesis) and microbiological analysis of such obtained implantable material. The similar results were obtained when the derivatized amikacin samples were determined by UV spectroscopy. The regression analysis (data not shown) allowed for a conclusion that correlation between HPLC and spectrophotometric methods was sufficient (with the correlation coefficient of 0.95), comparing the amount and percentage of immobilized antibiotic. This suggests the feasibility of these procedures for amikacin binding control during production of drug-modified prosthesis; therefore, the majority of amikacin concentration measurements were performed by cheaper spectrophotometric method.

The results of experiments showed that the antibiotic has been attached to Uni-Graft® prostheses via strong covalent bonds but also weak, non-covalent (passive or ionic) interactions. The last were created due to a porous structure of PET biomaterial and interactions between oppositely charged chemical groups of gelatin and amikacin. The percentage of particular amikacin-graft interactions was therefore subjected to estimation. It was found that passively bound antibiotic can be removed by eluting with water and ionically bound—by NaCl solution. Concentration of NaCl was previously optimized (Fig. 6) showing that 0.7–1 M NaCl completely removes ionically bound amikacin from Uni-Graft® prostheses. Therefore, 1 M NaCl was chosen for further experiments. It should be noted that 0.15 M (0.9%) NaCl (physiological salt) elutes approximately 50% of ionically bound drug amount. It is therefore probable that *in vivo* the modified prosthesis may release to blood a significant amount of ionically bound antibiotic.

The experiment showed that dominating amount of antibiotic (81.84% of total amount) was covalently bound to prostheses.

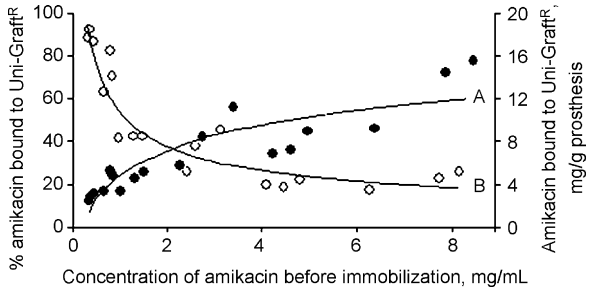

Fig. 5. The amounts of amikacin bound to Uni-Graft® prosthesis (A) vs. the percentage of amikacin bound to Uni-Graft® prosthesis as a function of the initial concentrations of this antibiotic (B).

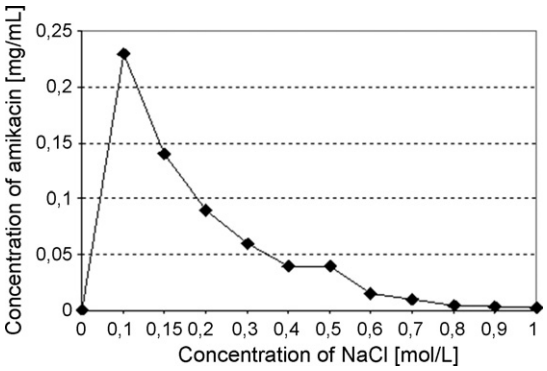

Fig. 6. Optimization of NaCl concentration for a release of ionically bound amikacin.

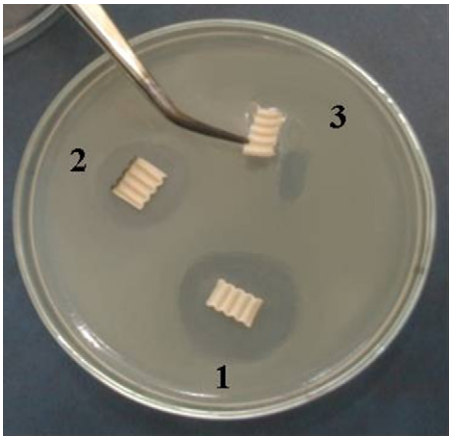

Fig. 7. Inhibition zones of *E. coli* growth (at 37 °C for 18 h) for following samples: (1) Uni-Graft® piece coupled with amikacin in mixed-type mode (containing passive, ionic and covalents bonds), (2) Uni-Graft® piece coupled with amikacin bound by ionic and covalent interactions (after eluting with water) and (3) Uni-Graft® piece containing amikacin covalently bound (after eluting with water and afterwards with 1 M NaCl). The piece 3 was lifted to show the inhibition of bacterial growth under the sample.

Only 18.13% of total amount of attached antibiotic was bound by physical adsorption and ionic interactions (Table 2).

Described model of amikacin–matrix interactions differentiation can be defined as an indirect proof of their existence. However, this hypothesis is based on nature and stability of chemical bonds under different conditions. NaCl solutions of optimized concentrations are commonly known reagents used for removal of ionically bound ligands, specially in ion-exchange chromatography techniques (Millesime et al., 1995).

Microbiological test provided a direct proof of presence of three types of interactions between amikacin and gelatin-sealed vascular prostheses. The idea of this test was based on the method described by Anhalt (1991) and in third edition of European Pharmacopoeia (1997). Presence of *E. coli* growth inhibition zones was observed around the pieces of prosthesis containing three or two types of interactions (Fig. 7) which phenomenon resulted from antibiotic release to salt containing agar broth. In a case of prosthesis containing exclusively strong covalent bonds, the inhibition of *E. coli* growth was found only within the zone of direct contact between bacteria and matrix, which confirm the lack of passively or ionically bound antibiotic.

Prostheses modified with amikacin were subjected to the test of drug–biomatrix bonds stability for 30 days. In case of control (amikacin-soaked) prostheses it was found that ≅80% of total antibiotic amount was released to PBS just during the first day of shaking; after 7 days of experiment the drug was completely eluted from biomatrix. In case of the prosthesis modified with antibiotic in mixed-type mode, about 15% of total drug amount was released from the matrix during the first 7 days of shaking, then the amount of bound antibiotic remained stable without further release to the medium (Fig. 8). It suggested that about 85% of amikacin attached to prosthesis modified in mixed-type mode was bound via covalent interactions, which confirms the results obtained in previous experiment (Table 2).

Stigter et al. (2004) tested the process of several antibiotics release from hydroxyapatite-coated titanium implants, in PBS pH 7.4 at 37 °C. He stated that rate of antibiotics release depended on presence of free functional groups which can create chelates or bonds with the biomaterial. He also found that cefalothin (antibiotics with free carboxyl groups) is released in ≈70% during 16 h, cefamandol and tobramycin – in 80–90% during 8 h and gentamicin – in 100% during 1 h.

Estimation of antibacterial properties of amikacin-modified prostheses was performed against bacterial strains (*E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853) in liquid LB medium with the antibiotic-modified prostheses for 30 days. The results (Table 3) showed that immobilized

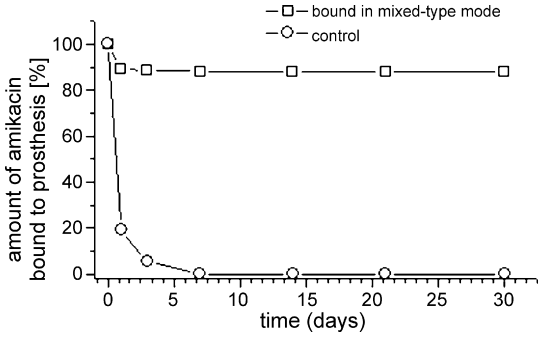

Fig. 8. Release of amikacin from vascular prostheses during 30 days of continuous shaking in PBS at 37 °C. Tested prostheses were: soaked in amikacin solution (control) and modified by amikacin in mixed-type mode.

Table 2
Estimation of the amikacin release from the surface of the drug-modified Uni-Graft® prosthesis by UV spectrophotometric assay

Total amount of amikacin bound to prosthesis (mg/g prosthesis/%)	Amount of amikacin released from prosthesis with water (physical adsorption) (mg/%)	Amount of amikacin released from prosthesis with 1 M NaCl (ionic binding) (mg/%)	Amount of amikacin covalent bound to prosthesis (mg/g prosthesis/%)
9.75 ± 3/100	0.11 ± 0.02/1.13	1.66 ± 0.1/17.0	7.98 ± 0.07/81.8

Table 3
Inhibitory effect of mixed-type immobilized amikacin (6.32 mg/g prostheses) on bacterial growth in LB medium at 37 °C over the course of 30 days

Strain	Initial number of bacteria (CFU/mL)	Number of bacteria (CFU/mL)						
		20 h	Day 4	Day 7	Day 10	Day 14	Day 21	Day 30
Escherichia coli, ATCC 25922, MIC 5 μg/mL	1×10^2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4×10^6	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4×10^8	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pseudomonas aeruginosa, ATCC 27853, MIC 3 μg/mL	1×10^2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1×10^4	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	4×10^7	0.00	0.00	0.00	2.1×10^7	2.1×10^7	2.1×10^7	2.1×10^7
	4×10^9	0.00	0.00	0.00	6.6×10^7	6.6×10^7	6.6×10^7	6.6×10^7
Staphylococcus aureus, ATCC 25923, MIC 5 μg/mL	1×10^2	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	1×10^4	0.00	0.00	0.00	4.4×10^7	4.4×10^7	4.4×10^7	4.4×10^7
	4×10^6	0.00	0.00	0.00	5.9×10^7	5.9×10^7	5.9×10^7	5.9×10^7
	4×10^8	0.00	0.00	0.00	7.0×10^7	7.0×10^7	7.0×10^7	7.0×10^7

amikacin exerted inhibitory effect on all strains used in this experiment. This effect was bactericidal for *E. coli* strain (complete inhibition of bacterial growth). Similar effect was observed in case of low initial concentrations of *P. aeruginosa* (10^2 and 10^4 CFU/mL) and *S. aureus* (10^2 CFU/mL) cells. In case of higher concentrations of their inocula, amikacin-loaded prostheses inhibited the growth of bacterial strains up to 7th day of experiment (Table 3). During next stage of experiment, *P. aeruginosa* and *S. aureus* cells underwent propagation; however, a weak bacteriostatic activity of immobilized antibiotic was still observed.

Controlled drug release provides localized delivery and lowers the risk of systemic side effects. However, according to available literature, present research focus mainly on biomaterials impregnation with antibiotics solutions which results in creation of weak non-covalent interactions between drug and matrix (Vicaretti et al., 1998; Strachan et al., 1991; Lovering and MacGowan, 1996; Galdbart et al., 1996; Earnshaw, 2000). The majority of antibiotic is usually eluted within first days after such prosthesis implantation; therefore, their antibacterial activity is not sufficient enough to protect patients against early bacterial infections. Graft protection via ionic attachment of antibiotic, although more efficient, is not very usual because it requires additional prosthesis modifications. The only example of such modified commercial prosthesis (supplied by Vascutec, Scotland) was succinylated gelatin-sealed biomaterial for coupling with rifampicin (Strachan et al.,1991).

Our results of mixed-type amikacin immobilization on Uni-Graft® vascular prostheses showed that this drug remained on the biomaterial surface at high concentrations for at least 30 days. During this period, the prostheses revealed antibacterial activity against three bacterial strains playing a crucial role in vascular graft infections and released the antibiotic slowly, thus allowing for a longer *in vitro* antibacterial protection than that described by other authors (Haverich et al., 1992; Vicaretti et al., 1998; Strachan et al., 1991; Earnshaw, 2000).

It is worth noting that mixed-type immobilized amikacin may lower the side effects in comparison with free antibiotic systemically delivered to patients organism (Omri and Ravaoarinoro, 1996). Thus, the use of mixed-type amikacin modified prostheses may help to avoid this problem. We suppose that *in vivo*, the physically and ionically bound amikacin will be released from prostheses during first several days after the implantation and the remaining covalently bound antibiotic will locally protect the implant. We also assume that this type of local antibacterial protection will remain until human or bacterial proteolytic enzymes (*e.g.* gelatinases) degrade the gelatin coating of prosthesis. However, *in vivo* research is still required to evaluate these hypothesis.

4. Conclusions

According to obtained results, amikacin may be applied for mixed-type vascular prostheses modification to protect them from Gram-positive and Gram-negative bacterial infections. As the antibiotic is coupled to grafts mainly by covalent bonds, these prostheses may probably be locally protected from bacterial infection and biofilm formation. This is very important in case of knitted PET prostheses, specially susceptible to bacterial which settle between their fibers. We imply that this method of amikacin-vascular prosthesis coupling should be developed for further evaluation in *in vivo* experimental studies. These will help to show the apparent antibacterial activity of chemically modified implants in human body.

Continued work in engineering of these systems offers promise in prevention of clinical vascular graft infections.

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