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The Release Kinetics, Antimicrobial Activity and Cytocompatibility of Differently Prepared Collagen/Hydroxyapatite/Vancomycin Layers: Microstructure vs. Nanostructure

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Abstract

The aim of this study was to develop an osteo-inductive resorbable layer allowing the controlled elution of antibiotics to be used as a bone/implant bioactive interface particularly in the case of prosthetic joint infections, or as a preventative procedure with respect to primary joint replacement at a potentially infected site. An evaluation was performed of the vancomycin release kinetics, antimicrobial efficiency and cytocompatibility of collagen/hydroxyapatite layers containing vancomycin prepared employing different hydroxyapatite concentrations. Collagen layers with various levels of porosity and structure were prepared using three different methods: by means of the lyophilisation and electrospinning of dispersions with 0, 5 and 15wt% of hydroxyapatite and 10wt% of vancomycin, and by means of the electrospinning of dispersions with 0, 5 and 15wt% of hydroxyapatite followed by impregnation with 10wt% of vancomycin.

The maximum concentration of the released active form of vancomycin characterized by means of HPLC was achieved via the vancomycin impregnation of the electrospun layers, whereas the lowest concentration was determined for those layers electrospun directly from a collagen solution containing vancomycin. Agar diffusion testing revealed that the electrospun impregnated layers exhibited the highest level of activity. It was determined that modification using hydroxyapatite exerts no strong effect on vancomycin evolution. All the tested samples exhibited sufficient cytocompatibility with no indication of cytotoxic effects using human osteoblastic cells in direct contact with the layers or in 24-hour infusions thereof. The results herein suggest that nano- structured collagen-hydroxyapatite layers impregnated with vancomycin following cross-linking provide suitable candidates for use as local drug delivery carriers.

Keywords

Coating; Controlled release; Drug delivery system; Nanoparticle; Pharmacokinetics; Polymeric drug delivery system

1. Introduction

The infection of implanted endoprostheses represents a serious problem [1, 2] with respect to orthopaedic and trauma surgery and one of the ways in which to increase the efficacy of therapy consists of the application of a local antibiotic delivery system [3, 4, 5]. The local delivery of antibiotics maximises target tissue concentration while minimising the risk of systemic toxicity.

Glycopeptide [6] vancomycin is one of the most commonly used local delivery antibiotic vehicles [7]. Since the 1950s vancomycin has been used to treat severe infections caused by gram-positive bacteria. Vancomycin-resistant *Staphylococcus aureus* (VRSA) [8, 9] is most common in elderly patients, especially those patients with a history of vancomycin-resistant enterococci. One possible explanation as to why infections caused by VRSA are becoming more commonly reported is that vancomycin breaks down over time to form crystalline degradation products (CDP-1s) [10, 11] which are antimicrobially ineffective. During *in vitro* exposure at a temperature of 20–25°C, up to 50% of vancomycin is converted to CDP-1s within 16 hours and 90% of vancomycin is converted to CDP-1s within 40 hours. In addition, an acidic pH of 4.1 to 4.2 contributes towards the formation of the two conformational isomers of CDP-1s: CDP-1M (major) and CDP-1m (minor).

The most extensively studied and earliest commercially available device designed for the controlled release of antibiotics was developed in the 1970s according to Buchholz and Engelbrecht's [12] innovative idea of releasing antibiotics from polymethylmethacrylate (PMMA) bone cement. This device is still widely used, however, it enables only a small fraction of the loaded drug to diffuse through the polymer pores and provides an initial burst release of antibiotics with the larger part of the loaded antibiotic remaining within the cement. Since PMMA is not biodegradable, secondary surgery is subsequently necessary to remove the PMMA before new bone can regenerate. Thus, with a view to overcoming these disadvantages, various biodegradable devices made from both natural and synthetic polymers have been produced by means of various processes in recent years [13]. Biodegradable polymers can be modified by means of calcium phosphate nanoparticles and tailored for a specific application [14-16] and local antibiotic delivery systems can be prepared in a number of final forms. Over the past decade, researchers have developed biodegradable polymeric scaffolds [17-19], degradation beads [20], sheets [21] and membranes [22] for use in the treatment of bone infections. A further alternative method with respect to the treatment of osteomyelitis involves the use of a hydrogel structure that is easily administered (injectable) [23-25] and, moreover, is not particularly invasive. Antibiotic-loaded implant coatings provide a straightforward approach to the prevention of implant-associated infections [26-30]. This “soft matter on hard matter” method provides an immediate response to the threat of implant contamination and, moreover, does not require the use of any other carrier than the orthopaedic implant itself.

The loading of drugs can be conducted via the use of a number of different techniques, the most simple of which consists of the straightforward mixing of the polymer and antibiotics in the form of a dry powder [20] or solution [19,21,23,31,32]. Although all the approaches employed are generally successful in terms of providing for the long-lasting release of therapeutic antibiotic concentrations, drug loading is often conducted by means of the mixing of the drug with polymers sometimes dissolved in harsh solvents. A further method involves the soaking of the antibiotics by means of immersion in a drug-containing solution [25,33-39]. Electrospinning, a promising processing technique that utilises electrical forces to produce ultrafine polymeric fibres from polymer solutions is seen as having great potential in terms of the development of nano-structured biomedical materials. Nanofibers are particularly efficient drug delivery agents due to their high surface-area-to-

volume ratios, high porosities and 3D open porous structures [40]. The resulting electrospun fibres have been successfully investigated with respect to their use as matrices containing antibiotics [41], sandwich structures for the repair of infected wounds [22,42,43] and the electrospun vancomycin-loaded coating for titanium implants [44,45]. Ying-Chao Chou et al. [46] have used electrospinning for the preparation of an artificial periosteum that incorporates biodegradable drug-embedded nanofibers so as to provide an adequate level of drug release capacity as well as biodegradable stents for the mimicking of the mechanical properties of the periosteum in connection with the management of open fractures. A further promising technique concerning the bioactive modification of the surface of titanium implants consists of electromechanically-assisted deposition by means of which collagenous or chitosan interfaces can be created [26-29,47].

The technology and conditions concerning composite preparation are of particular importance since they are able to significantly affect the final microstructure of the composite and, consequently, the vancomycin release profile. The fact that vancomycin can be washed out during the rinsing and cross-linking processes is often not taken into consideration [31]. In addition, the fact that the temperature applied during the preparation of the composite [20] may lead to the transformation of vancomycin into its microbiologically inactive products is very often neglected; indeed, the monitoring of the antibiotically inactive forms of vancomycin is a very important issue which is, unfortunately, often ignored [22,45]. The analysis of vancomycin concentrations continues to be performed by means of UV/VIS assay [26,43,47]; however, this method is incapable of determining the inactive degradation products of vancomycin. Only the high performance liquid chromatography (HPLC) method is able to provide an effective tool for the quantitative and qualitative analysis of such products [11,48].

The aim of this study is to compare biodegradable composite layers prepared using three different techniques, the main requirement of which is that they are capable of releasing the active form of vancomycin with an initial burst release which eliminates the development of a biofilm and, for at least 3 weeks at concentrations exceeding the minimum inhibitory concentration for VRSA, ensure that such vancomycin release rates are not toxic for the participating cells. Nano- and micro- structured layers based on collagen (type I, isolated from calf skin) and 0, 5 and 15wt% of hydroxyapatite nanoparticles were prepared employing either the lyophilisation or electrospinning of the dispersions with or without the presence of 10wt% vancomycin hydrochloride and were subsequently cross-linked with N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) [49], a commonly used cross-linking agent. The pure cross-linked collagen/hydroxyapatite electrospun mats obtained were subsequently impregnated with 10wt% vancomycin. The *in vitro* release rates of the vancomycin and its inactive degradation products were characterised by means of the HPLC method. The antimicrobial effects of the layers were determined using the agar diffusion testing technique against four different clinical isolates. The *in vitro* biological evaluation was conducted using SAOS-2 cells in direct contact with the layers or their 24 h infusions. The multidisciplinary results of the comparison of the three different techniques concerning the preparation of a collagen/hydroxyapatite/antibiotic drug delivery system suggest that the electrospinning of collagen/hydroxyapatite in combination with antibiotic impregnation provides a promising method with respect to creating a drug delivery system for use in orthopaedic surgery which exhibits a higher level of effectiveness than does the direct electrospinning of antibiotic solutions.

2. Materials and Methods

2.1 Materials, Preparation and Characterisation

Nano- and micro-structured layers were prepared based on collagen (type I, VUP Medical, Czech Republic), 0wt%, 5wt% or 15wt% of hydroxyapatite nanoparticles (avg. 150nm, Sigma Aldrich, Germany) and vancomycin (VANCO, vancomycin hydrochloride, Mylan S.A.S, France) in the amount of 10wt% of the total weight of collagen (COL) with hydroxyapatite (HA). The micro-structured layers (L0, L5 and L15) were prepared by means of the lyophilisation of a COL/HA/VANCO dispersion in a phosphate buffer (PBS)/ethanol solution (the preparation process has been described elsewhere [11]). The nano-structured layers were prepared via the electrospinning of an 8wt% collagen PBS/ethanol solution modified by 8wt% (to COL) polyethylene oxide (PEO; Mr 900,000, Sigma Aldrich, Germany) with dispersed HA particles. The vancomycin was applied using two different procedures, i.e. direct introduction to the COL solution prior to electrospinning (E0, E5 and E15) or the subsequent impregnation of the electrospun COL/HA cross-linked layers (EI0, EI5 and EI15). Both types of electrospun mats were prepared using a high voltage level of 45kV and the feeding rate was set at 130 □l.min-1, the temperature at 24°C and the relative humidity at 20-25% (4SPIN, Contipro, Czech Republic). The production rate of the nanofibrous collagen mats was increased via the application of electroblowing [50]; the flow rate of the preheated air (25°C) was set at 30l.min-1. All the electrospun mats were collected on a static continual collector (22 x 29cm) and then cut to the appropriate sizes using a scalpel following cross-linking. The stability of all the collagen layers was enhanced by means of cross-linking with a 95% ethanol solution containing EDC and NHS at a weight ratio of 4:1; the EDC and NHS (Sigma Aldrich, Germany) were used as received. Following a reaction period of 24 hours at 37°C, all the layers were washed in 0.1M Na2HPO4 (2 x 45 min), followed by rinsing using deionised water (30 min). They were then frozen at -15°C for 5 h and lyophilised at - 105°C at a pressure level of approximately 1.3Pa (BenchTop 4KZL, VirTis, Czech Republic). Following cross-linking, the PEO and NaCl were fully leached out. The electrospun COL/HA cross-linked layers were impregnated with a vancomycin ethanol solution and dried at room temperature in a laminar box until a constant weight was achieved (up to 2 hours).

The structure of the prepared samples was evaluated by means of attenuated total reflection infrared spectrometry (FTIR) using a Protégé 460 E.S.P. infrared spectrometer (Thermo Nicolet Instruments, USA) equipped with an ATR device (GladiATR, PIKE Technologies, USA) with a diamond crystal. All the spectra were recorded in absorption mode at a resolution of 4 cm−1 and 128 scans. The areas of the bands (integral absorbencies) were determined using OMNIC 7 software. The samples were also characterised using scanning electron microscopy (SEM) (QUANTA 450, FEI, USA).

2.2 Preliminary Evaluation of Antimicrobial Susceptibility and Vancomycin Release

An investigation was conducted of the in vitro release of vancomycin from COL/HA/VANCO layers prepared by means of three different methods. Six samples of each type of layer were placed on a sterile gauze pad and firmly caulked prior to being transferred to separate test tubes containing a weight/volume ratio of 200mg/20ml of PBS (pH 7.4) which were placed in an incubator at a temperature of 37°C. The solid phase extraction method and HPLC analysis (HPLC on an Agilent 1200 series system equipped with a DAD diode array detector - Agilent Technologies) were then employed in order to characterise the in vitro release rates of the vancomycin and its crystalline degradation antibiotically inactive products over a 21-day period. Details of the HPLC analysis are described in an article written by Melicherčík et al. [48].

A methiciline-resistant Staphylococcus aureus (MRSA) isolate was used for the preliminary selection of the optimal COL/HA/VANCO layer preparation method. Model isolates were retrieved by means of the examination of hospital (General University Hospital, Prague, Czech Republic) patient specimens. The disc diffusion test was performed as described below.

2.3 Comprehensive Evaluation of Antimicrobial Susceptibility

Following the selection of a suitable method for the preparation of the COL/HA/VANCO layers, the evaluation of the antimicrobial susceptibility of the EI0, EI5 and EI15 samples was conducted by means of the use of four types of isolate retrieved from the hospital patient specimens mentioned above. The group consisted of one MRSA isolate, one Staphylococcus epidermidis isolate (gentamicin- resistant) and two Enterococcus faecalis isolates, one of which consisted of an E. faecalis gentamicin- resistant isolate and the other of E. faecalis acquired as a result of the analysis of an infected joint replacement. Each inoculum was produced from an 18-24 h pure culture of the test isolates via a Mueller-Hinton agar medium. Suspensions were prepared from 1 - 4 colonies of the test isolates (only well-isolated, morphologically similar colonies grown on a non-selective medium) in 2ml of sterile saline solution and the turbidity was adjusted (using a Densi 2, Erba Lachema densitometer, Brno, Czech Republic) to 0.5 of the McFarland turbidity standard (approximately 1-2x108 CFU/ml). Disc diffusion was performed using a Mueller-Hinton agar medium (Oxoid Ltd., Hampshire, UK; batch LOT1381672) with the test samples. Sterile cotton swabs were used to spread the inoculum evenly over the agar plates in three directions. Discs with a diameter of 6mm (n=7) were firmly applied to the dried surface of the inoculum agar plates using a sterile needle within a 15-minute time period and further incubated at 37°C for 24 hours. It was expected that the careful application of the inoculum and the streaking of the plates would result in even growth without the occurrence of separate colonies. The inhibition zones were read off using a ruler while holding each agar plate approximately 30cm from the eye. Standard 6mm antibiotic discs were used for positive control (PC) purposes, i.e. vancomycin 30□g for S. aureus and S. epidermis, and vancomycin 5□g for both Enterococci, and discs made up of electrospun mats without vancomycin impregnation served for negative control purposes (analogically with 0, 5 and 15wt% HA).

2.4 Evaluation of Vancomycin Release in Blood Plasma

In addition, samples prepared by means of electrospinning followed by impregnation with vancomycin (EI0, EI5, and EI15) were subjected to an investigation of the in vitro release of vancomycin in human blood plasma. The samples (n=6) were placed in separate test tubes with a weight/volume ratio of 200mg/20 ml of human blood plasma (16 donors of different blood group, sex and age) and incubated at 37°C in a 5% CO2 atmosphere (DH CO2 incubator, Thermo Scientific) with antibiotics (penicillin and streptomycin) for 6 h and for 1, 3, 10, 15 and 30 days. Solid phase extraction and HPLC analysis were conducted as described above.

2.5 Evaluation of Structural and Mechanical Stability

Structural and mechanical stability were further analysed with respect to the electrospun impregnated layers (EI0, EI5 and EI15) by means of the testing of degradation in blood plasma under the conditions described above (at 37°C, 5% CO2 atmosphere, penicillin/streptomycin, for 6 hours and for 1, 3, 10, 15 and 30 days). The volume of the medium was maintained at a weight/volume ratio of 30mg/15ml. The extent of in vitro degradation was calculated according to the following equation: $D = (W_0-W_t)/W_0*100\%$, where D is the mass loss, W_0 is the initial dried weight of the sample and W_t is the dried weight of the sample after degradation (n=6). The swelling ratio (Esw) was calculated using the following equation: $Esw = (W_{sw}-W_0)/W_0*100\%$, where W_0 is the initial dried weight of the sample and W_{sw} is the weight of the swollen sample (n=6). The weight of the swollen samples was measured

following the removal of each sample from the medium and after a 1-minute delay and the removal of any excessive medium surrounding the sample; the dried weight of the samples was measured following lyophilisation.

The mechanical properties prior to and following immersion in blood plasma were evaluated by means of the uniaxial tensile testing of rectangular strips of the layers (the average width and length of the samples was approximately 10mm and 40mm respectively). During the test procedure the value of strain at failure (the maximum strain sustained by the material before breaking, where strain is defined as the ratio of the elongation of the sample to reference length), the ultimate tensile strength (the maximum nominal stress sustained by the material; nominal stress is defined as the ratio of applied force to the reference cross-section of a sample) and the modulus of elasticity (the slope of the tangent made to a stress-strain relationship on the initial linear part) were determined. Tensile tests were conducted using a Zwick/Roell multipurpose testing machine equipped with a built-in video extensometer. By using contrasting marks on the surface of samples, the video extensometer automatically determined the reference length and elongation of the samples. Tensile experiments were conducted at a constant clamp velocity of 0.1mm/s. The loading force was measured by a U9B (±250N, HBM, Germany) force transducer.

Degradation tests under physiological conditions were used as an alternative way in which to assess the stability of the collagenous layers, and ultraviolet-visible spectrophotometry was employed for the quantification of the free amino groups released during the degradation of the samples of 50mg (n=3) immersed in the PBS (37°C, pH 7.4). PBS was collected after 2, 6, 96, 240, 360, 528, 720, 1536 and 2400 hours. The PBS collected (2.5ml) was mixed with 2ml of 0.1M $NaHCO_3$ and 1ml of 0.01% aqueous solution of 2,4,6-trinitrobenzenesulphonic acid (TNBS, Sigma-Aldrich). The mixture was incubated at 40°C for 2 hours. Subsequently, 1ml of a 10% solution of sodium dodecyl sulphate (Sigma-Aldrich) and 0.5ml of 1M HCl were added. Absorbance at 340nm was measured using a Unicam UV 500 spectrophotometer and correlated to the concentration of free amino groups using a calibration curve obtained with L-lysine (Sigma-Aldrich) and L- -amino-n-butyric acid (Lachema, Czech Republic).

2.6 Biological Evaluation of Cytotoxicity and Cytocompatibility

The biological evaluation of the electrospun impregnated layers (EI0, EI5 and EI15) was conducted under in vitro conditions. The aim of the in vitro tests was to verify whether eluted doses of vancomycin exert a negative effect on bone-like cell behaviour. In addition to the effect of antibiotics on bone cells, the influence of the composition of the COL/HA/VANCO layers (EI0, EI5, EI15) and layers containing no vancomycin (N0, N5, N15) on cell behaviour was investigated.

2.6.1 Cells Culture Conditions

SAOS-2 cells (a human osteoblast-like cell line derived from osteosarcoma, obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (GmbH), Germany) were cultured at 37°C in a 5% CO_2 atmosphere and in McCoy's 5A medium without phenol red (PromoCell, Germany) supplemented with 15% heat-inactivated FBS (PAA, Austria), penicillin (20U/ml, Sigma-Aldrich, USA) and streptomycin (20µg/ml Sigma-Aldrich, USA).

2.6.2 Layer Infusions

The nano-structured layers (EI0, EI5 and EI15) on a 48-well plate (Thermo Scientific, USA) were fixed in wells using CellCrown™ inserts (Sigma-Aldrich, USA) following which fully supplemented McCoy's 5A medium (800ul) was added to each layer type and incubated at 37°C and in a 5% CO_2 atmosphere for 24 h in order to obtain layer infusions. The resulting infusions were transferred (400 l) to pre- seeded

cells on a 48-well plate; the cells had been seeded 20 h prior to the addition of the infusions at a concentration of 20 000 cells/cm2. Cell metabolic activity (described below) was measured following 24 h of cell cultivation with the layer infusions.

2.6.3 Cell Cultivation on Layers

The nano-structured layers (EI0, EI5, EI15 and N0, N5, N15) used for cell cultivation purposes were fixed in the wells of a 48-well plate (Thermo Scientific, USA) by means of CellCrown™ inserts (Sigma-Aldrich, USA). The cells were seeded onto structured layers at a concentration of 15 000 cells/cm2 for 2-day and 8-day (after 4 days, fresh medium was added to the cells) cultivation periods, following which both cell metabolic activity and the number of cells on the structured layers were determined.

2.6.4 Determination of Cell Metabolic Activity

The cell metabolic activity test (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, MTS, Promega, USA) was performed according to the standard protocol: the absorbance (490nm and 655nm as reference values) of soluble formazan obtained by means of metabolically active cellular dehydrogenases was determined after 24 h (layer infusions) and 2 and 8 days (directly on the layers) of cell cultivation with respect to both the layer infusions and directly on the layers. Absorbance was determined using a multi-detection micro-plate reader (SynergyTM 2, BioTek, USA). The results were normalised (in percentage) with respect to the control cells with no layer infusions and cells cultivated on control tissue culture polystyrene.

2.6.5 Fluorescence Staining of the Cells

Those cells incubated for 2 and 8 days on structured layers were fixed in 4% paraformaldehyde in PBS at room temperature (RT) for 15 minutes following the measurement of cell metabolic activity. The cells were permeabilised using 0.1% Triton X-100 in PBS (Sigma-Aldrich, USA) at RT for 20 minutes and stained using fluorescence dyes: the cell nuclei were stained with DAPI at RT for 45 minutes (1:1000; Sigma-Aldrich, USA) and the actin filaments with Phalloidin-Alexa Fluor 488 also at RT for 45 minutes (1:500; Life Technologies, USA).

2.6.6 Imaging of Fluorescently Stained Cells

Wide-field images of the cells on the structured layers were obtained using an Eclipse Ti-S microscope and a DS-U2 Digital Camera (Nikon, Japan). The images were acquired using 10x and 40x lenses and adjusted by means of ImageJ software (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2015) and Cell Profiler (Broad Institute, USA) software.

2.7 Statistical Evaluation

The subsequent statistical analysis was performed using statistical software (STATGRAPHICS Centurion XV, StatPoint, USA). The normality of the data was verified primarily by means of the Shapiro-Wilk's and Chi-Squared tests; outliers were identified via either the Grubbs' or Dixon's tests. The mean values and variability of normally distributed numerical data were expressed as the arithmetical mean and the standard deviation (SD) while non-normally distributed numerical data was expressed as the median and interquartile range (IQR). Homoscedasticity was verified by means of the Levene's and Bartlett's tests. Non-parametric analysis was employed since either the assumption of normality or homoscedasticity were violated and, consequently, the Kruskal-Wallis test for multiple comparison or

the Mann-Whitney W test (as a post hoc test) were performed. Statistical significance was accepted at $p \leq 0.05$.

3. Results and Discussion

3.1 Characterisation of the COL/HA/VANCO Layers

Representative SEM images of micro and nanostructured COL/HA/VANCO samples prepared using different techniques are shown in the Supplement (Supplement_1). FTIR spectroscopy was employed in order to characterise the composition of the samples and so as to verify the theoretical presence of HA and vancomycin following the application of the three different preparation procedures. The electrospun impregnated layers clearly exhibited vancomycin peaks in the collagen spectra (Fig. 1 A, B), which remained visible even in the presence of HA (data not shown). Vancomycin peaks following impregnation were easily identifiable with respect to all the electrospun impregnated samples (EI0, EI5, EI15) principally in the 1585, 1420 to 1425, 1227 and 1060 cm-1 positions. The IR spectra of the electrospun (E0) samples illustrate the effect of the cross-linking procedure on the presence of vancomycin (Fig. 1 A). From a comparison of the spectra of pure collagen and the E0 samples prior to and following cross-linking (data not shown) it follows that during the 24-hour cross-linking procedure in an ethanol solution, a considerable amount of vancomycin is probably simply washed out - principally in the case of the electrospun samples. Further, the advantageous high surface-area- to-volume ratios of the nanostructured samples exerts a negative role at this stage of preparation. It can be concluded, therefore, that the addition of vancomycin in the final stage of layer preparation presents an effective way in which to deposit antibiotics in detectable amounts. The FTIR spectra in Figure 1 B illustrate the steps involved in the preparation of the electrospun impregnated layers, from pure collagen lyophilisate through electrospinning and cross-linking to the final stage, i.e. vancomycin impregnation. The PEO is fully leached out following the cross-linking procedure. The FTIR spectra further illustrate the presence of vancomycin following impregnation.

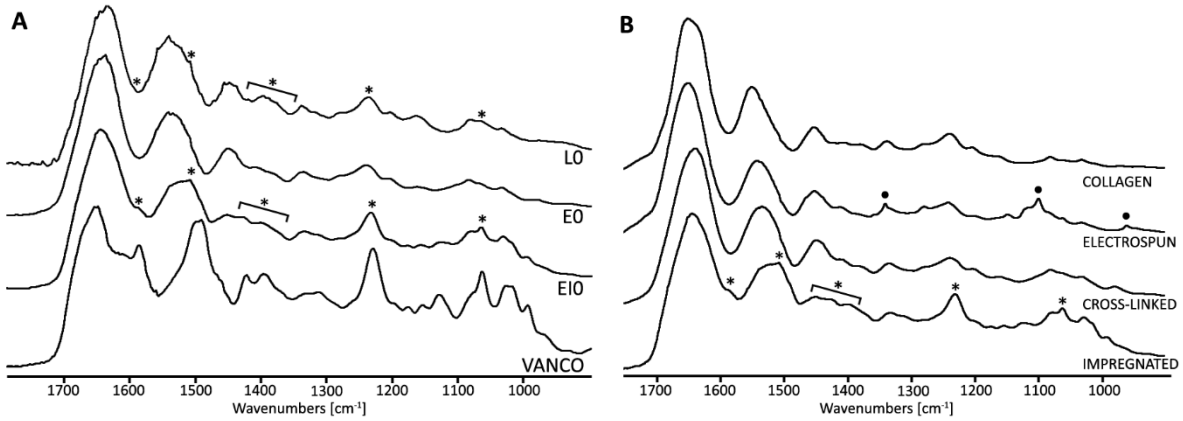
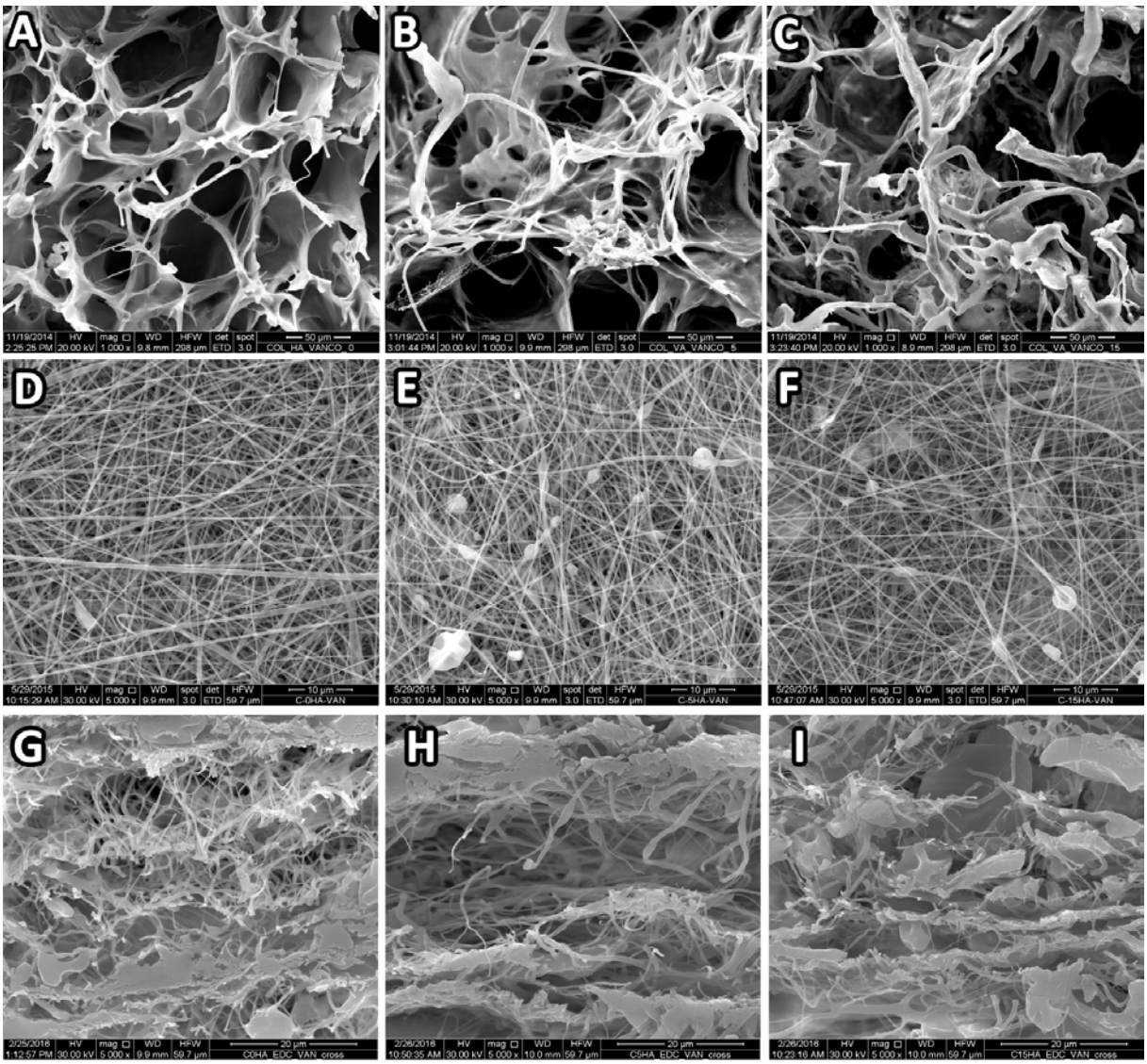


Fig. 1. Illustration of the presence of vancomycin in layers prepared in different ways. (**A**): The FTIR spectra of pure vancomycin (VANCO) lyophilised (L0), electrospun (E0) and electrospun impregnated (EI0) layers without HA. (**B**): The FTIR spectra of E0 samples in each of the preparation stages from pure collagen, following electrospinning, following cross-linking and following impregnation. * denotes typical vancomycin bands, ● denotes PEO bands.



SUPPLEMENT_1. Representative SEM images of micro structured COL/HA/VANCO samples (mag.1000x) with 0wt% (A), 5wt% (B) and 15wt% (C) of hydroxyapatite, nano structured COL/HA/VANCO electrospun layers (mag.5000x) with 0wt% (D), 5wt% (E) and 15wt% (F) of hydroxyapatite, and nano structured COL/HA/VANCO electrospun impregnated layers (mag.5000x) with 0wt% (G), 5wt% (H) and 15wt% (I) of hydroxyapatite.

3.2 Preliminary Evaluation of Antimicrobial Susceptibility and Vancomycin Release

The effect of the preparation and structural properties of COL/HA/VANCO on the kinetics of vancomycin release was further analysed by means of the HPLC method (Fig. 2). Initial measurements after 3 hours revealed average concentrations of up to 18±1mg/l (mean±SD) for the lyophilised (L0) samples [11], up to 48±13mg/l for the electrospun samples (E15) and up to 696±208mg/l for the electrospun impregnated (EI0) samples (Fig. 2). The highest average concentration of vancomycin was

achieved with respect to the lyophilised samples after 8 days (approximately 250mg/l), the electrospun samples after 24 hours (approximately 60mg/l) and the electrospun impregnated samples after just 3 hours (approximately 700mg/l). The average concentration of CDP-1M (a major degradation product) was found to be similar to that of the active form of vancomycin with respect to the lyophilised samples after 14 days (~180mg/l), the electrospun samples after 10 days (~30mg/l) and the electrospun impregnated samples after 12 days (~310mg/l) of incubation, data not shown. In all cases, the vancomycin was converted into its degradation products (CD-1M, CD-1m) at a much slower rate than that reported by Melicherčík et al. (6 days) [48]. Despite the considerable tendency of vancomycin degradation towards crystalline thermal degradation products, levels of the released active form of vancomycin remained above the MIC for VRSA (16mg/l) for more than 3 weeks, with the exception of the E15 samples (15.85mg/l, 21st day). Wachol-Drewek [51] determined that vancomycin release from collagen was characterised by a rapid bolus release; at least 90% of the antibiotic was released within the first day with complete elution occurring within 4 days. Similar results were obtained by Tu et al. [47] who studied vancomycin release from mineralised collagen coatings (dense and porous). They found that 80% of the vancomycin was released in the first 10 hours and the remaining 20% over a period of up to 4 days. A burst release was evident with concern to both the dense and porous collagen coatings involving the release of more than 85% of the antibiotic over 16 hours. The maximum concentration of the released active form of vancomycin exceeded the MIC by up to 17 times (lyophilised samples), 4 times (electrospun samples) and up to 44 times (electrospun impregnated samples). By the end of the experiment, the MIC had been exceeded by up to 6 times (lyophilised), up to 12 times (electrospun impregnated) and was approximately equal in the case of the electrospun samples. The addition of hydroxyapatite exerted only a minor effect on vancomycin release.

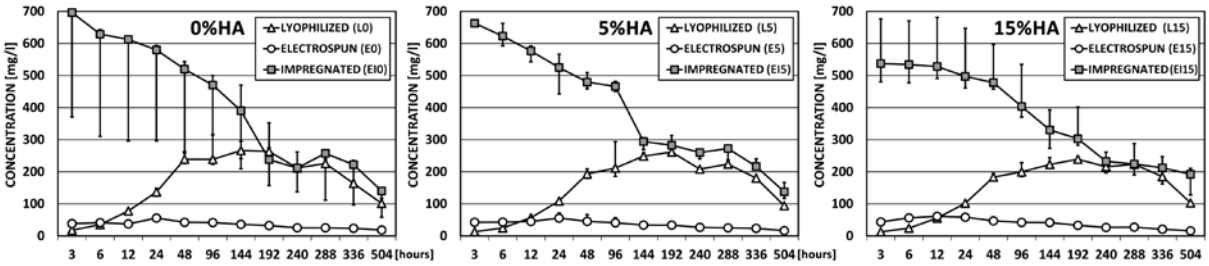
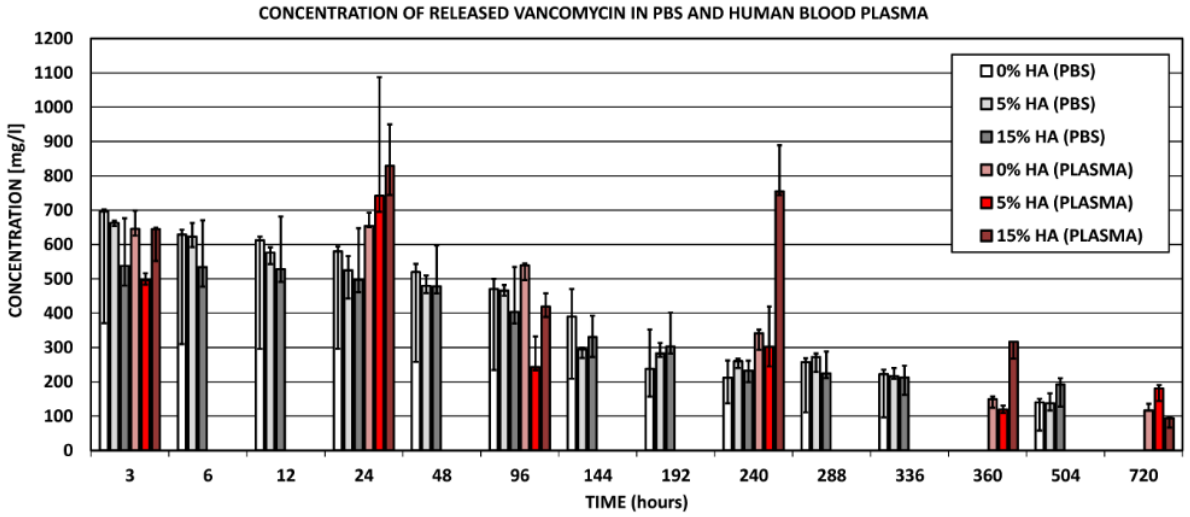


Fig. 2. The concentration of the released active form of vancomycin (median, IQR) with respect to samples with 0, 5 and 15wt% of HA prepared using different methods [2].

The results of the preliminary evaluation of antimicrobial susceptibility are in agreement with the results of vancomycin release chromatography analysis (Fig. 3). The highest rates of antimicrobial activity were determined with respect to the electrospun impregnated samples (EI0, EI5, and EI15). Similar to previous results, no statistically significant differences were detected between the electrospun impregnated samples containing different amounts of hydroxyapatite. The electrospun impregnated COL/HA/VANCO layers were subsequently selected for the comprehensive evaluation of antimicrobial susceptibility and vancomycin release as well as for the evaluation of structural and mechanical stability.



SUPPLEMENT_2. The concentration of vancomycin released from COL/HA/VANCO electrospun impregnated in PBS and human blood plasma.

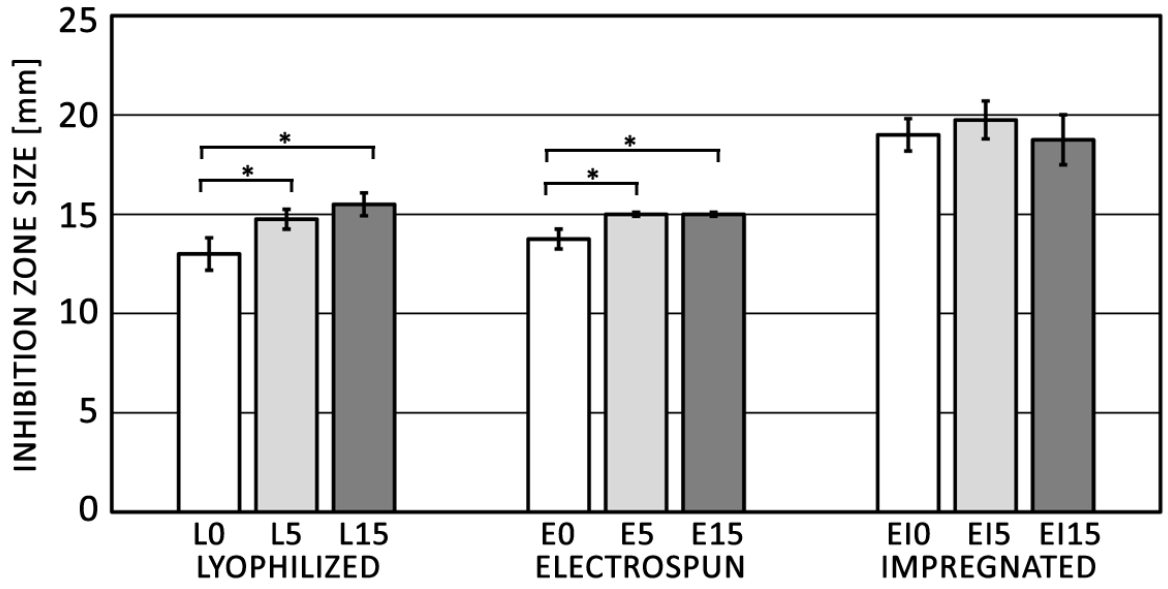


Fig. 3. Inhibition zone sizes of vancomycin loaded lyophilised, electrospun and electrospun impregnated samples with different amounts of hydroxyapatite in contact with MRSA isolates. * denotes statistically significant differences (mean, SD, Mann-Whitney, 0.05).

3.3 Evaluation of Vancomycin Release in Human Blood Plasma

PBS contains only inorganic ions and solely the hydrolytic degradation of the material can be expected compared to blood plasma, with respect to which other types of degradation are enabled, e.g. enzymatic. A comparison of vancomycin release and its degradation in such differing environments consisting of a complex of inorganic and organic components might be beneficial. Therefore, an investigation was conducted of the in vitro release of vancomycin from EI0, EI5 and EI15 layers immersed in human blood plasma for up to 30 days (Fig. 4). Initial measurements after 6 hours revealed average vancomycin concentrations (mean±SD) of up to 655±46mg/l (EI0), 497±22mg/l (EI5) and

609±62mg/l (EI15). The average concentration of vancomycin peaked after 24 hours in the case of all the samples, i.e. 703±108mg/l (EI0), 872±225mg/l (EI5) and 863±146mg/l (EI15). The average concentration of CDP-1M was found to be higher than that of the active form of vancomycin after 30 days (EI0: 280±119mg/l, EI5: 360±223mg/l, EI15: 260±173mg/l). The vancomycin concentrations in blood plasma exhibits higher variances than concentrations in PBS (comparison of release kinetics in PBS and blood plasma is illustrated in Supplement_2). This can be caused by the potential interaction of vancomycin with various blood plasma components. Generally, the vancomycin release profiles of both plasma and PBS are similar and in both cases the vancomycin released remained above MIC for a period of at least three weeks. With respect to immersion in human blood plasma, vancomycin was converted into its degradation products at a relatively slow rate: levels of the released active form of vancomycin remained above the MIC for VRSA for as long as 4 weeks. The maximum concentration of the released active form of vancomycin exceeded the MIC by up to 60 (EI0), 46 (EI5) and 75 (EI15) times.

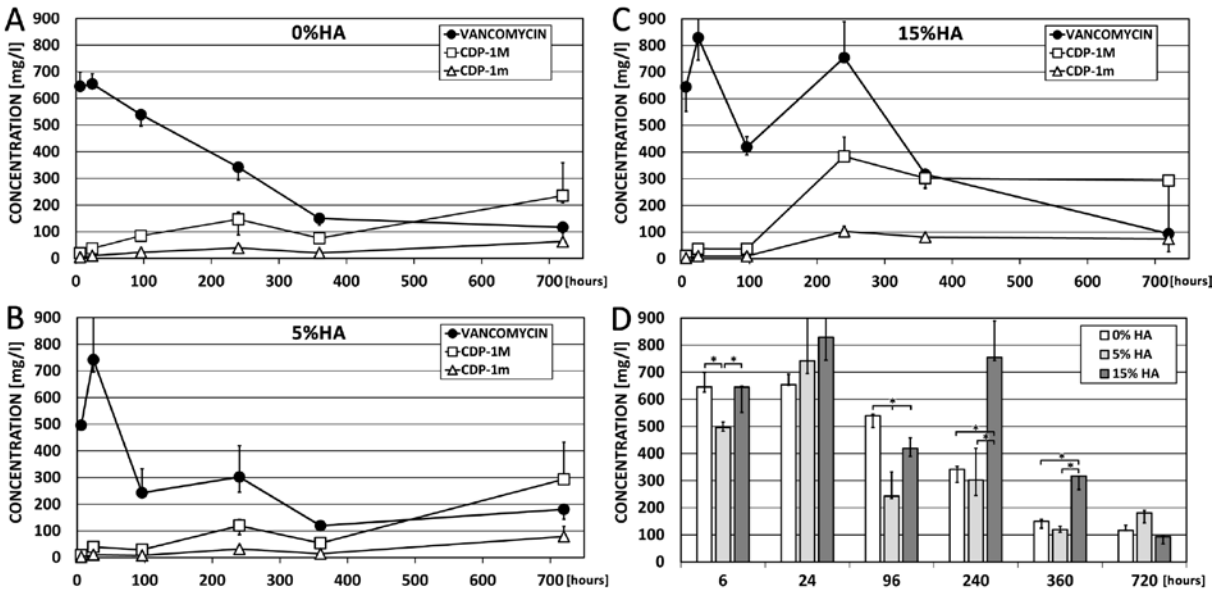


Fig. 4. Evaluation of vancomycin release from electrospun impregnated samples immersed in human blood plasma. **(A, B, C):** The concentration of the released active form of vancomycin and its degradation products CDP-1M and CDP-1m (median, IQR). **(D):** The concentration of the released active form of vancomycin (median, IQR). * denotes statistically significant differences (Mann-Whitney, 0.05).

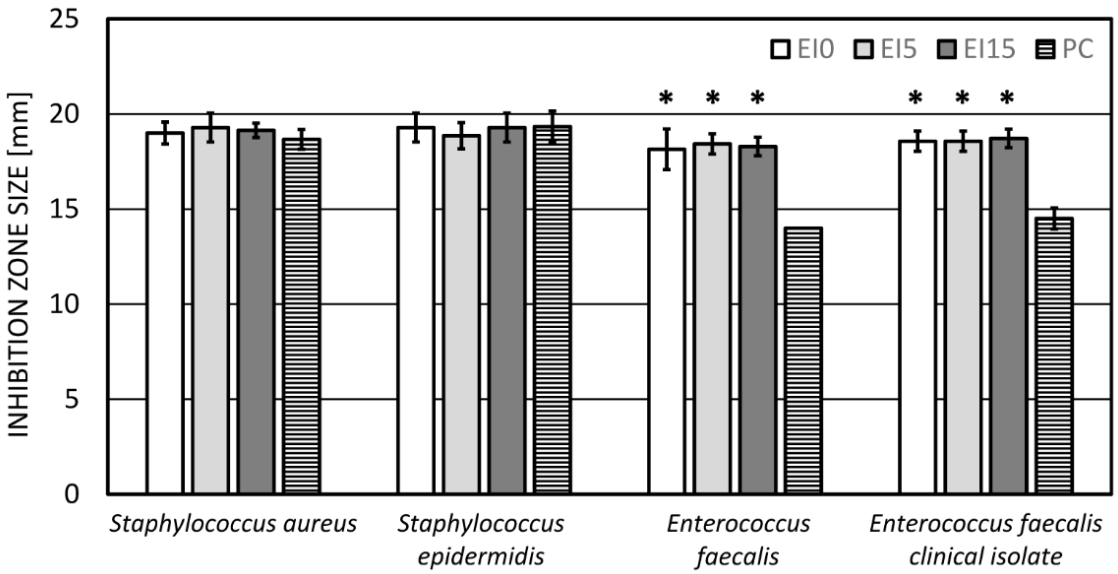


Fig. 5. Inhibition zone sizes of the electrospun impregnated samples with different amounts of hydroxyapatite, and the positive controls. The vancomycin-free negative controls evinced zero inhibition zones (data not shown). * denotes statistically significant differences (mean, SD, Mann-Whitney, 0.05) in comparison with the PC for each of the examined isolate species.

3.4 Comprehensive Evaluation of Antimicrobial Susceptibility

The antimicrobial activity of the EI0, EI5 and EI15 samples against the four bacteria strains were assessed by means of the presence or absence of inhibition zones (disk diffusion test, Fig. 5). All the materials examined exhibited potential antibacterial activity with respect to both Staphylococci isolates and gentamicin-resistant isolates, while the vancomycin-free samples (negative control) exhibited no activity at all with respect to any of the bacteria species tested. The sizes of the inhibition zones surrounding the samples investigated were comparable to those of standard antibiotic discs (PC), i.e. no statistically significant differences (p=0.05) in the case of S. aureus and S. epidermis, and statistically significantly higher in the case of both of the Enterococci species. The size of the inhibition zones around the discs containing different amounts of hydroxyapatite, as a possible vancomycin binder, appeared to have no antibacterial influence.

3.5 Evaluation of Structural Stability

The degradation rates expressed as mass loss and the swelling ratios of the cross-linked collagen samples with various HA concentrations are depicted in Fig. 6. Negative values of degradation indicate a weight increase which can be explained by the adsorption of various components (proteins, saccharides and vitamins) of the blood plasma immediately following exposure (within 6 hours). Conversely, the microstructured lyophilised samples with the same composition studied in [20] exhibited weight losses. The difference in behaviour between the nanofibrous layers and the lyophilised samples can be explained by the high specific surface area of the nanofibers. This phenomenon is also apparent in the swelling behaviour (Fig. 6 B). No obvious dependence was detected of increment size on the type of sample or time period (M-W 0.05). It can be concluded from the results of studies on behaviour in physiological environments and from a comparison with samples studied by Suchý et al. [11] that all the electrospun impregnated layers (EI0, EI5, and EI15) achieved the appropriate degree of cross-linking so as to create stable layers. The application of FTIR

spectroscopy (Fig. 6 C) proved the occurrence of only minor local changes within the samples during leaching in plasma. HA content was detected in both the EI5 and EI15 layers after 30 days; however, the vancomycin had been completely released from all the samples. In addition, the collagen as such was found to remain stable within the electrospun impregnated samples during the whole immersion period (up to 100 days) as documented by the very low (approximately 0.014mM) free amino acid concentration at the end of the experiment (Fig. 7).

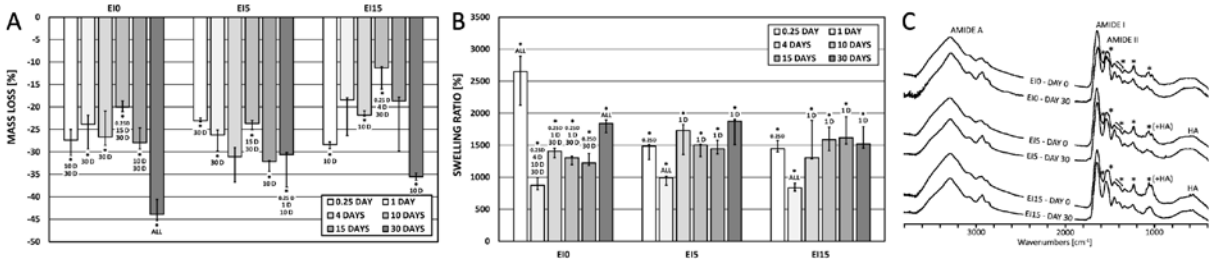


Fig. 6. Evaluation of the structural stability of the electrospun impregnated samples in human blood plasma. **(A):** Degradation rates are expressed as mass loss (median, IQR), **(B):** swelling ratios (median, IQR), **(C):** FTIR spectra before and after exposure to human blood plasma. * denotes statistically significant differences (median, IQR, Mann-Whitney, 0.05).

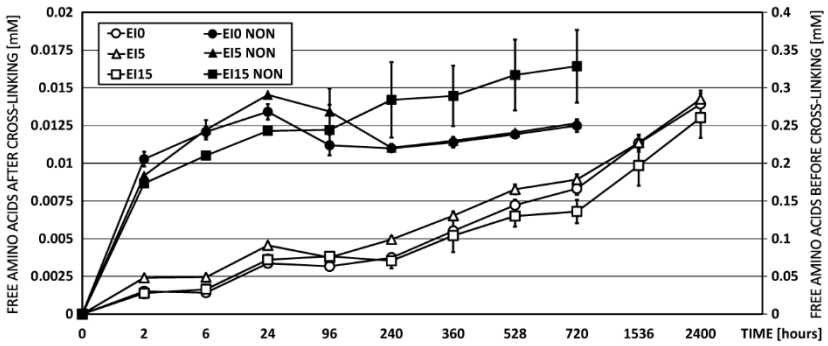


Fig. 7. The concentration of amino acids released from the electrospun impregnated samples (EI0, EI5, EI15) and identical samples prior to cross-linking (NON); note that the opposite axis has a different scale.

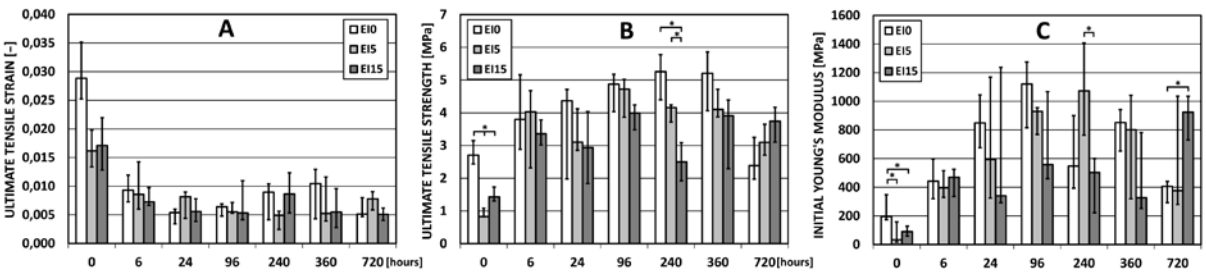
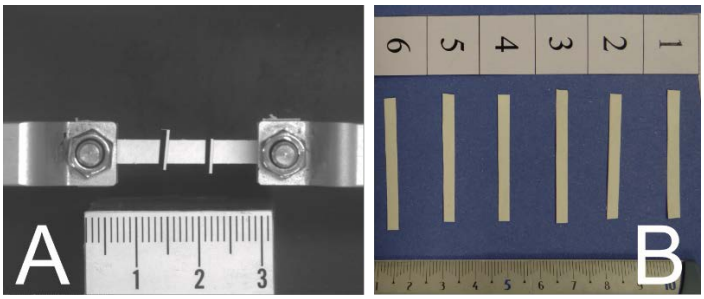
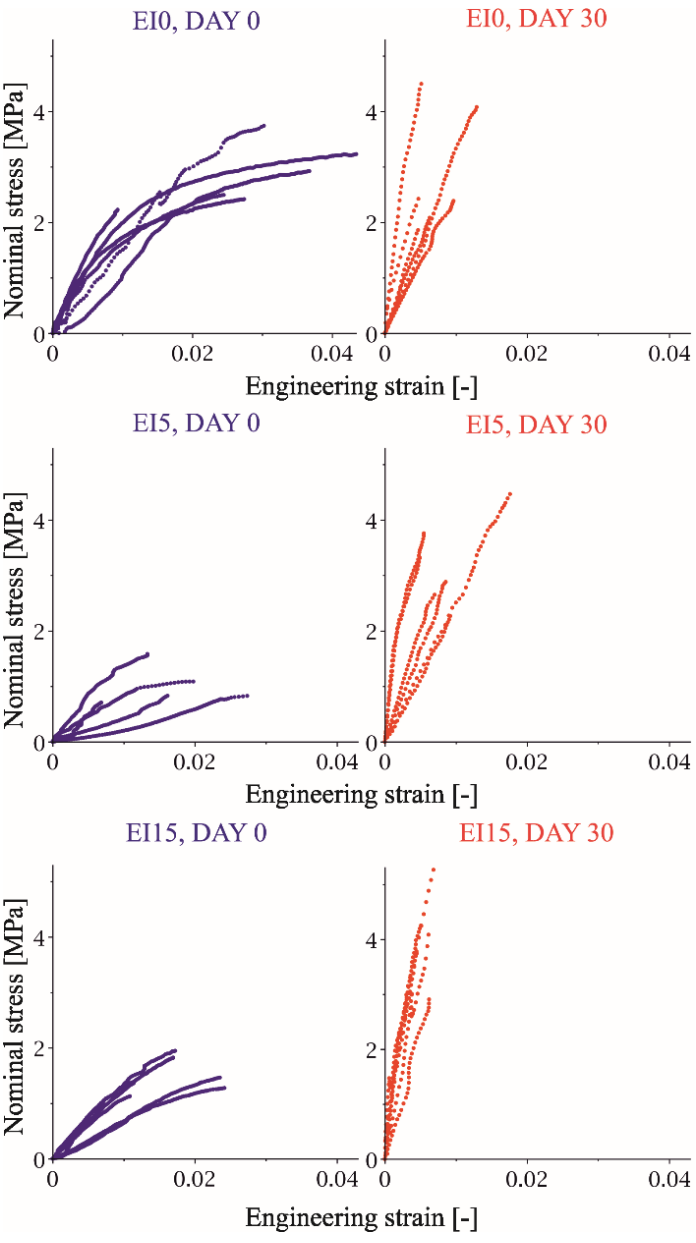


Fig. 8. Mechanical stability of the electrospun impregnated layers. **(A):** ultimate tensile strain, **(B):** ultimate tensile strength, **(C):** initial Young's modulus during 720 hours of exposure in human blood plasma. * denotes statistically significant differences (median, IQR, Mann-Whitney, 0.05).



SUPPLEMENT_3. A sample mounted in the grips of the testing machine (panel A). A group of samples prior to testing (panel B).



SUPPLEMENT_4. Comparison of the uniaxial tensile responses of the nano structured COL/HA/VANCO impregnated electrospun layers before and after 30 days of plasma incubation. The recorded curves document that stress at failure increased and strain at failure slightly decreased during incubation.

3.6 Evaluation of Mechanical Stability

The results obtained from the mechanical tests are shown in Figure 8. All the HA concentrations exhibit an increase in tensile strength after 6 hours of exposure to human blood plasma. From 6 to 360 hours of exposure, the ultimate tensile stress stagnates and after 360 hours a slightly decreasing trend in 0 and 5% of HA can be observed, whereas the tensile strength of the 15% HA samples slightly increases. After 720 hours, the ultimate tensile strength did not decrease to values less than the initial value in either case. In contrast to tensile stress, strain at failure was seen to decrease immediately (6 hours) following plasma exposure (Fig. 8 A). During the whole experiment (24 – 720 hours), ultimate tensile strain practically stagnated. The elastic modulus may, albeit roughly, be considered to be a ratio of tensile stress to strain as confirmed by Figure 8C. The elastic modulus of all the samples increased during 720 hours of blood plasma exposure analogically compared to samples in the dry state (0 hours). The results of the mechanical tests, in accordance with those provided by other stability investigation methods, revealed that the EI0, EI5, and EI15 layers remained stable following leaching in plasma. Comparison of the uniaxial tensile responses of the layers before and after 30 days of plasma

incubation as well as representative images of tested samples can be found in Supplement (Supplement_3, Supplement_4).

3.7. In vitro Evaluation of Cytotoxicity

3.7.1. Evaluation of the Effect of Layer Infusions on Human Cells

The vancomycin impregnated electrospun layers (EI0, EI5 and EI15) and their respective controls without the presence of vancomycin (N0, N5 and N15) were soaked in a cultivation medium for 24 hours. The infusions thus obtained were then transferred to pre-cultivated cells on tissue-culture polystyrene (PS) and incubated together. After 24 hours, the cell metabolic activity was determined (Fig. 9A). The resulting data shows a significant decrease in cell metabolic activity in those cells incubated in infusions obtained from the COL/HA layers with 0wt% and 5wt% of hydroxyapatite (N0 and N5) and from COL/HA/VANCO with 0wt% of hydroxyapatite (EI0) compared to the control infusion from PS. That said, the observed effect was weak (approximately a 10% decrease) and thus cannot be stated to be cytotoxic [52]. Moreover, the effect was no stronger with respect to the vancomycin sample infusions, which suggests an effect of a different character. The metabolic activity of the other infusions was comparable to the control sample, thus it can be concluded that the compounds released from the electrospun layers (active vancomycin, the degradation forms thereof and substances issuing from the COL/HA layer) do not have a cytotoxic effect on the human osteoblastic cell line. Interestingly, those samples with no HA presence were found to be “the most” toxic, independent of the presence of vancomycin; therefore, it might be suggested that certain other compounds are released from this type of layer which negatively influence cell metabolic activity. On the other hand, infusions containing samples with the highest HA concentration (15wt%) were seen to have a significantly more positive effect on cell metabolic activity (again irrespective of the presence of vancomycin). According to the results presented in Fig. 2, the amount of vancomycin released after 24 hours was in the range of 500-600mg/l; thus, it can be concluded that this concentration has no negative effect on osteoblast growth, which is in agreement with data concerning the local administration of vancomycin to osteoblastic cells (MG-63) with respect to which concentrations of 1g/l and less had no negative effect on these cells [53]. However, the concentrations employed in the study described herein led to the creation of the largest inhibition zones with respect to the bacterial strains subjected to testing (as is apparent in Fig. 5).

3.7.2. Evaluation of the Effect of the Cultivation of Osteoblasts on Layers

Osteoblasts were seeded on the nanostructured vancomycin impregnated electrospun layers (EI0, EI5 and EI15) as well as on their respective controls without the presence of vancomycin (N0, N5 and N15) for 2 and 8 days following which their metabolic activity was determined. Fig. 9 (B, C) shows that after 2 days of incubation, the cells on those layers containing 0 and 5wt% of HA (EI0 and EI5) were strongly inhibited by the presence of vancomycin; however, those cells on the layer containing 15wt% of HA (EI15) behaved in a comparable way to the control without the presence of vancomycin. Moreover, most of the cells inhibited by vancomycin were found on layers with 0wt% of hydroxyapatite and the degree of inhibition was seen to decrease with increased amounts of HA in the sample. Following further incubation (8 days), the tendency towards the inhibitory effect of vancomycin on cells deposited on layers with different amounts of HA persisted; nevertheless, the inhibitory effect of vancomycin was not so dramatic. Moreover, the cells cultivated on the COL/HA/VANCO layer with the highest content of hydroxyapatite (15wt%) manifested significantly increased metabolic activity compared to those cells in the control without the presence of the antibiotic.

The data presented in Fig. 9 (B, C) is supported by the fluorescence images of those cells (the staining of the actin cytoskeleton – green and nuclei – blue) cultivated on nanostructured vancomycin impregnated electrospun layers for 2 and 8 days (Fig. 10). After 2 days, the osteoblasts on all the layers tested were seen to be rather round and poorly dispersed: however, they adhered to the layers in reasonable amounts. Fewer cells were apparent with respect to those samples with vancomycin thus confirming the inhibitory effect of vancomycin (Fig. 10). After 8 days, the osteoblasts were mostly well dispersed and exhibited a polygonal-like morphology, thus once more confirming the inhibitory effect of vancomycin on cells cultivated on layers with 0wt% and 5wt% of hydroxyapatite despite the greater number of cells found on these samples. The osteoblasts cultivated on a vancomycin impregnated layer with 15wt% of hydroxyapatite were present in a higher amount thus supporting the results concerning the increased metabolic activity of cells on this sample. The inhibitory effect of vancomycin on cell growth was apparent with respect to all the samples tested; moreover, the effect was more pronounced following a short period of incubation (2 days) and declined (EI0 and EI5) or completely disappeared (EI15) following a longer incubation period (8 days). Importantly, the cells were seen to react not only to vancomycin but also to the properties of the scaffold (differing amount of HA).

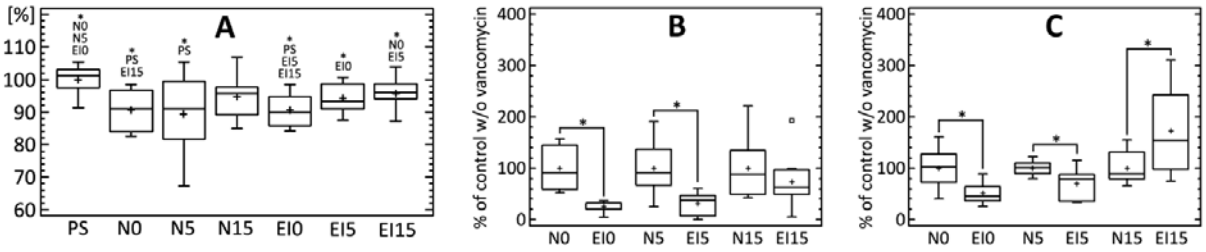


Fig. 9. Metabolic activity of SAOS cells incubated in 24 h infusions expressed as a percentage of the positive control (PS) (A). Metabolic activity of osteoblasts incubated on scaffolds for 2 (B) and 8 (C) days. * denotes statistically significant differences (Mann-Whitney, 0.05).

With respect to the 2-day period, a small number of cells was also visible on the sample without the presence of HA (N0) (Fig. 10); thus, the scaffold itself and the presence of vancomycin were jointly responsible for the lowest cell count provided by the EI0 sample. It might be speculated that cells of an osteoblastic nature which are known to prefer hard substrates for their growth [54] were initially inhibited in terms of growth on the scaffold, which had no content of HA. Moreover, according to the data obtained, this sample exhibited the greatest degree of swelling (Fig. 6 B) and the 24 h-infusion created from this layer also exerted a slight but negative effect on the cells (Fig. 9 A). Thus, the various properties of the EI0 layer may have had a cumulative negative effect on cell behaviour. The observed inhibitory effect of vancomycin in the layers is dependent on and may be modulated by the respective amounts of HA. HA makes up the fundamental component of the bone matrix, thus the observed phenomenon could be the result of the character of the adhesion surface (e.g. HA) and the potential for adhesion [55]. Furthermore, it is important to bear in mind that cell adhesion is mediated by FBS proteins and the interaction of these proteins with the tested layers [56]. The potential for improved adhesion means that the cells are in better condition, which results in increased cell resistance to stress conditions. In addition, the inhibitory effect might be caused by the direct interaction of vancomycin with HA particles in the layer – antibiotic conformation as well as the general level of activity may be altered and HA behaviour (in particular its release) in the layer during incubation may also be critical.

As the results herein indicate, the highest level of vancomycin release was observed with respect to EI15 when compared to the rest of the samples with a maximum incubation period of 24 hours (Fig. 4). At the same time, this layer appears to exert the lowest inhibitory influence on cell metabolic activity after 2 days (as well as 8 days) (Fig. 9 B, C).

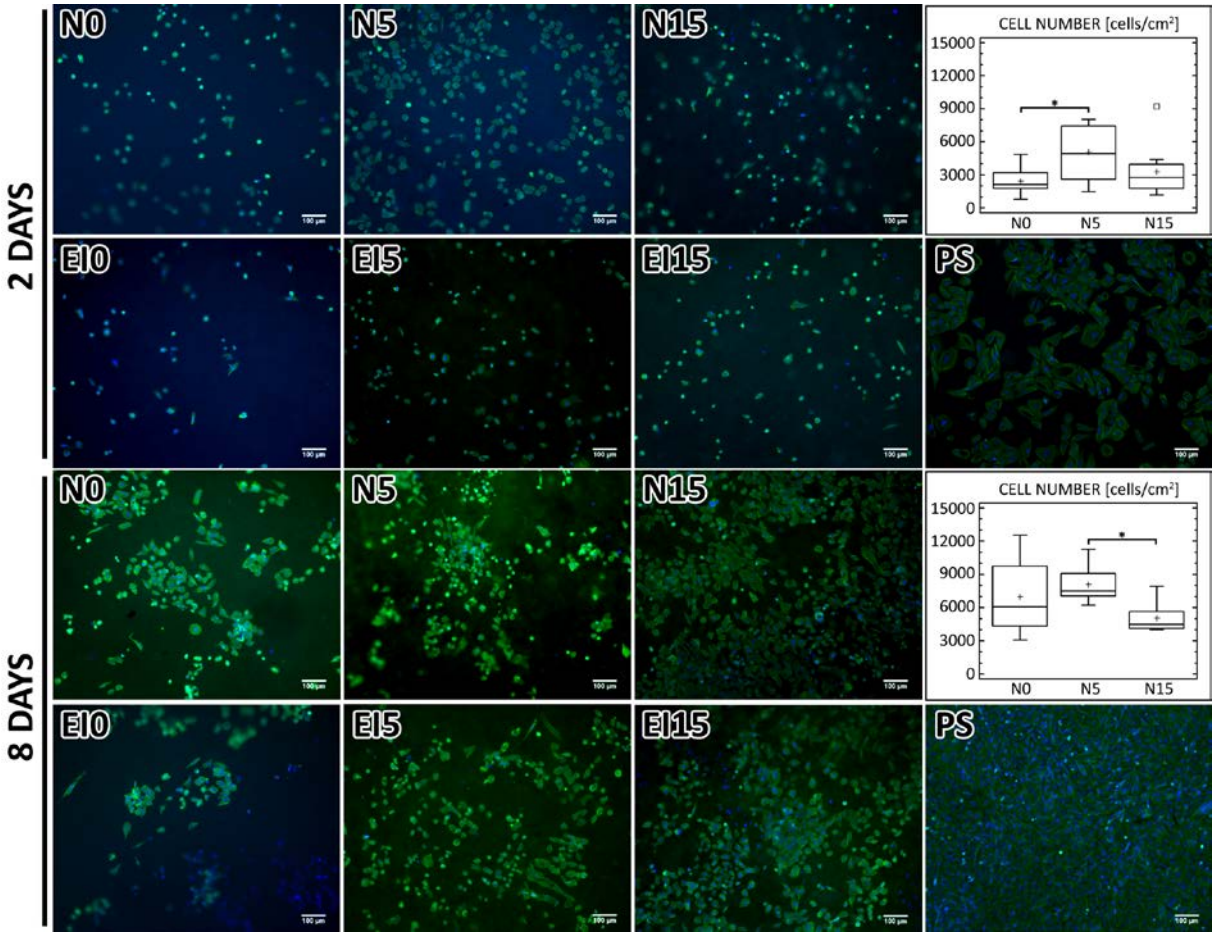


Figure 10. Fluorescence images of osteoblasts cultivated (for 2 and 8 days) on electrospun impregnated vancomycin-free layers (N0, N5, N15) and identical samples impregnated with vancomycin (EI0, EI5, EI15), with the corresponding cell numbers of the vancomycin-free samples. * denotes statistically significant differences (Mann-Whitney, 0.05).

Despite the initial strongly negative effect of the properties of the layers and the presence of vancomycin on the cells, once the osteoblasts had overcome these issues, growth proceeded well. It seems that COL/HA layers become stabilised over time and vancomycin is released at similar levels from all the samples tested; hence, it appears that the cells are very well capable of overcoming stress conditions. Moreover, those cells cultivated on the EI15 layer were seen to grow better than on the same layer without the presence of vancomycin.

4. Conclusion

The most critical element of contemporary implant technology and design consists not of the bulk material from which an implant is produced, but rather its surface, which is required to provide an interface which is suitable for the purposes of post-implantation integration between the bone and the replacement. Inflammatory reactions to both local and systemic infection represent the greatest threat to successful osteo-integration and may lead to the formation of a biofilm which creates a barrier to the binding of the implant. This process may, in turn, result in the loosening and thus suboptimal functioning of the implant and even an indication to re-implantation.

The main goal of the research described herein was to develop a new structured layer based on a collagen-hydroxyapatite composite which can be used (1) as a pro-osteo-integration interface and which, simultaneously, will serve (2) as a local drug delivery system. Three preparation methods were investigated, two of which involved the direct addition of vancomycin to a dispersion from which (A) micro-structured COL/HA layers were obtained by means of lyophilisation and (B) nanostructured layers were obtained via electrospinning. The electrospinning method, which provides a high surface-to-volume ratio to the internal structure of the resulting material, was also utilised for the production of layers without the presence of vancomycin in the source dispersion, but with the addition of the antibiotic to the electrospun material via impregnation. This paper demonstrated that the third method provides layers with the highest concentrations of released antibiotics over the longest time period. The highest average concentration of vancomycin in PBS was achieved with respect to these electrospun impregnated samples after just 3 hours (approximately 700mg/l). The minimum inhibitory concentration was exceeded up to 44 times and the vancomycin released remained above MIC for a period of at least three weeks compared to an initial burst release with complete elution occurring within 4 days as described by previous studies. The average concentration of CDP-1M (a major degradation product) was found to be similar to that of the active form of vancomycin with respect to the electrospun impregnated samples after 12 days (~310mg/l) of incubation. The addition of hydroxyapatite exerted only a minor effect on vancomycin release. The results of the preliminary evaluation of antimicrobial susceptibility was in agreement with the results of vancomycin release chromatography analysis. The highest rates of antimicrobial activity were determined with respect to the electrospun impregnated samples. The in vitro release of vancomycin from the electrospun impregnated COL/HA/VANCO layers immersed in human blood plasma for up to 30 days revealed that levels of the released active form of vancomycin remained above the MIC for VRSA for as long as 4 weeks. In vitro testing against populations of S. aureus, S. epidermis, and E. faecalis confirmed the inhibitory effect of vancomycin released from COL/HA/VANCO layers (EI0, EI5, and EI15). On the other hand, cytocompatibility testing, which employed osteoblasts cells, revealed that these bone-producing cells survive on the layers and that their metabolic activity increases as the degree of mineralisation of the layer increases. This suggests that COL/HA/VANCO layers prepared by means of electrospinning with subsequent impregnation with vancomycin cannot be considered to be cytotoxic for newly-formed bone. Finally, the evaluations of structural and mechanical stability conducted in blood plasma for up to 30 days and in a simulated body environment for up to 100 days revealed that our material retains structural consistency, as proved by the very low free amino acid concentration at the end of the experiment (approximately 0.014mM). Structural stability makes up a necessary precondition for providing a solid scaffold for cell migration and, hence, for the formation of new bone.

We are able to conclude, therefore, that nanostructured layers prepared by means of electrospinning from a COL/HA dispersion, subsequently cross-linked with EDC/NHS and finally impregnated with

vancomycin provide eminently suitable candidates for the preparation of bioactive and pro-osteo-integrating bone-implant interfaces and that they are capable of providing for local drug delivery. It is intended that the next phase of development will be based on the experimental implantation of EI0-15 covered implants in rodents.

Acknowledgements

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