THE INTERRELATIONSHIP BETWEEN THE DRUG EMBEDDED TIZR SURFACE PROPERTIES AND ANTIBACTERIAL EFFECT

MANUELA ELENA VOICU 1, DOINA DRĂGĂNESCU 2*, VALENTINA ANUŢA 3, ANDREI BOGDAN STOIAN 1, DANIELA IONIŢĂ 1, IOANA DEMETRESCU 1,4

1Department of General Chemistry, Faculty of Chemical Engineering and Biotechnologies, University Politehnica of Bucharest, 313 Splaiul Independenţei, 060042, Bucharest, Romania
2Department of Pharmaceutical Physics and Informatics, “Carol Davila” University of Medicine and Pharmacy, 020956, Bucharest, Romania
3Department of Physical and Colloidal Chemistry, “Carol Davila” University of Medicine and Pharmacy, 020956, Bucharest, Romania
4Academy of Romanian Scientists, 3 Ilfov Street, 050044, Bucharest, Romania

*corresponding author: doina.draganescu@umfcd.ro

Manuscript received: September 2022

Abstract

This paper is a report about the elaboration of surfaces coated with PLA nanofibers embedded with gentamicin and its release. The manuscript investigated surface analysis, drug loading and the kinetics of the release. The research design includes FT-IR structural identification of embedded gentamicin, Scanning Electronic Microscopy (SEM) with energy dispersive X-ray spectroscopy (EDX) for morphology and composition and Atomic Force Microscopy (AFM) to measure adhesion force. The antibacterial inhibition index was evaluated against a positive and a negative Gram bacteria such as Staphylococcus aureus and Escherichia coli, respectively. As a conclusion of above determinations positive effect of drug was evidenced and discussed.

Keywords: TiZr, surface properties, embedded drug, antibacterial effect

Introduction

In the last decade investigations on binary titanium alloys especially on the alloys with zirconium were in a continuous increase, leading to more knowledge about their use as alternative for antibacterial commercially pure Ti implant [2, 25, 30]. Especially for the dental applications, TiZr was found nowadays a good choice, having the mechanical and electrochemical stability properties in the case of a Zr proportion in the range of 30 - 50 mol% with competitive merits over pure Ti [16, 18, 33]. An important problem related to biological response of TiZr alloys with both aspects such as biocompatibility and antibacterial effect was the subject of numerous papers [5, 14, 40] presenting coated and uncoated TiZr at micro and nanolevel. Nowadays the bacteria are more and more aggressive [21, 36] due to the antibiotics use which has led to the selection of more resistant bacteria such as MRSA [6, 37, 38]. The implant...
and PLA as polymeric support due to their non-toxic effects, and lower bacterial adhesion is a good selection in all forms [12]. It is used in solid dispersion, nanofibers or hydrogels. With polymers there can be employed various antibiotic types such as beta lactams, fluoroquinolones, aminoglycosides, sulphonamides etc. [28]. Gentamicin sulphate (GS) is one of the most used antibiotics with an effect on the both gram-positive and gram-negative microorganisms [12, 29]. The synergic aspects of combination of a surface treated at nanolevel and a drug have been studied and discussed on Ti [4, 15], but on TiZr which only recently started to be recognized as Ti alternative in implantology the literature in this field is scarce [3]. An enhancement of antibacterial effect on TiZr surface modified at nanolevel with a polymer such as PLA and having a drug incorporated was not investigated until now and this is the novel character of this manuscript. The aim of this paper was GS encapsulation into PLA nanofibers deposited on TiZr samples to evaluate antibiotics properties against *Staphylococcus aureus* and *Escherichia coli*. To the best of our knowledge, the complex inter-relationship between the embedded drug and the PLA nanowires coatings on TiZr surface properties, including adhesion and antibacterial effect is a novelty, worthy of investigation.

**Materials and Methods**

**Sample preparation**

Ti50%Zr alloy samples (20 × 20 × 2 mm) were polished using increased grit SiC paper with a Buehler Beta equipment (Lake Bluff, IL, USA), and cleaned in an ultrasound bath with distilled water (10 min), ethanol (10 min), acetone (10 min) and dried. The samples were dipped in an acid mixture (3HNO₃: 1HF:2H₂O v/v/v) for 10 seconds, followed by washing with distilled water and dried.

**Coating preparation**

The coating was obtained by dissolving PLA (granules size 3 mm, Sigma-Aldrich, St. Louis, MO, USA) in a mixture of chloroform (CHCl₃ 99% - Carl Roth, Germany) and N-Dimethylformamide (DMF 99.9% HPLC grade - AlfaAesar, Haverhill, MA, USA) under magnetic stirring and alternating with ultrasound bath until obtaining a homogeneous solution. The solution was transferred to a 1 mL plastic syringe with a needle used to deposit nanofibers by electrospinning (pump - Legato 180, KD Scientific, Holliston, MA, USA, and voltage power source PS/EJ30P20, Glassman High Voltage, Inc., High Bridge, NJ, USA) with following parameters: voltage 20 kV, flow rate 0.5 mL/h, distance between the collector plate and the needle of the syringe was 10 cm, and deposition time was 30 min. Characterization of the samples was described before in literature in a recent paper which shows surface treatments for suitable coatings [35].

**Drug loading on TiZr-PLA nanofibers**

Gentamicin sulphate (GS) was encapsulated on the PLA nanofibers by immersion of the samples in 10 mL containing 2 g/L GS solution. The GS solution was prepared in a phosphate buffered saline (PBS, pH 5.8) with the composition shown in Table I. All chemicals had analytical grade and were purchased from Sigma-Aldrich. GS absorption was investigated using a spectrophotometer UV-VIS-NIR (model V670, Jasco, Germany), and a 1 cm quartz cuvette. Absorption spectra were recorded hourly for 24 hours.

**Table I**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
</tr>
<tr>
<td>NaHPO₄</td>
<td>1.42</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.24</td>
</tr>
</tbody>
</table>

**Drug release**

Study of GS release behaviour was performed in triplicate by introducing the GS-loaded samples into 10 mL PBS at pH 5.8, and at fixed intervals there were extracted 0.5 mL of receiving medium and the same volume of phosphate buffer saline was added.

**Structural analysis with FT-IR**

PLA nanofibers and GS encapsulated PLA were characterized by Fourier transform infrared spectroscopy (FT-IR) using a Perkin-Elmer Spectrum 100 (Perkin-Elmer, Shelton, USA), in transmittance mode, in the range 450 - 4000 cm⁻¹.

**Contact Angle Measurements**

The measurements were performed as described in a previous work [30] with a contact angle meter CAM 100 equipment.

**Scanning Electron Microscopy (SEM)**

Scaffolds were imaged using a Hitachi TM4000plus tabletop scanning electron microscope (SEM) system (Hitachi High-Tech Corp., Tokyo, Japan). The samples were placed on a conductive graphite sticker and directly analysed. No sputter coating was required. A mixed observation mode (backscattered electron image and secondary electron image) the sample was observed in a low-vacuum mode, using an accelerating voltage of 5 kV.

The evaluation of adhesion forces was performed using an A.P.E. Research A100-SGS atomic force microscope (AFM) and repeating 5 measurements for each sample.

**Evaluation of antibacterial effect**

The antibacterial activity evaluation was performed on sterile TiZr samples uncoated, coated with PLA nanofibers and coated with GS embedded PLA. As bacteria for tests, *Escherichia coli* (K 12-MG1655) and *Staphylococcus aureus* were selected as negative and positive bacteria, respectively. Bacteria were cultured in a tube containing Luria Bertani medium at 37°C (Luria Bertani medium composition: peptone,
10 g/L; yeast extract 5 g/L, NaCl 5 g/L) [19]. According to the known protocol, sterile samples were incubated 24 h of test tubes containing 0.5 mL culture for each bacterium. The optical density was determined after 24 h of incubation in the incubator Laboshake Gerhardt. The optical densities for all samples and control (bacteria culture without sample) were determined by UV-VIS spectrophotometer model V670, Jasco, Germany.

Results and Discussion

Drug loading and release study
The optimization of the drug encapsulation was achieved by immersing the samples in a GS solution of 2 g/L, and was monitored spectrally at λ = 194 nm, using a calibration curve in the concentration range 25 - 300 mg/L, having a coefficient correlation of R² = 0.9999. After 8 h, an encapsulation efficiency of 56.14% is obtained (Figure 1).

The monitorization of the amount of GS released was performed spectrally, using the calibration curve at λ = 194 nm. There is a slow release of GS in the receptor medium, in the first hour 5.60% is released, and after 48 h the release rate is 40.33% (Figure 2).

![Figure 1. Encapsulation efficiency of GS from PLA in PBS pH 5.8](image)

![Figure 2. Release of GS from PLA in PBS pH 5.8](image)

The data obtained from the in vitro release of GS were fitted using five kinetic models: order model 0, order model 1, Higuchi, Peppas-Korsmeyer, and Hixson Crowell [34] The obtained kinetic parameters (velocity constant k, correlation coefficient R², and the release coefficient n) are shown in Table II. Analysing the kinetic parameters for each mathematical model, it is observed that the best correlation coefficient R² was obtained for the Peppas-Korsmeyer model. Thus, the release coefficient n is between 0.45 < n < 0.89, indicating the existence of a non-Fickian diffusion mechanism for nanofibers-encapsulated GS (Table III) [23].

<table>
<thead>
<tr>
<th>Compound</th>
<th>Order model 0</th>
<th>Order model 1</th>
<th>Higuchi</th>
<th>Hixson-Crowell</th>
<th>Peppas-Korsmeyer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%R = k₀⋅t</td>
<td>%R = k₂⋅t</td>
<td>%R = k₃⋅t</td>
<td>%R = k₄⋅√t</td>
<td>%R = k₃⋅√t</td>
</tr>
<tr>
<td>R²</td>
<td>k₀</td>
<td>R²</td>
<td>k₁</td>
<td>R²</td>
<td>k₂</td>
</tr>
<tr>
<td>GS</td>
<td>0.986</td>
<td>3.3609</td>
<td>0.98</td>
<td>0.0413</td>
<td>0.9687</td>
</tr>
</tbody>
</table>

Table II

Kinetic parameters of the five models [34]

Table III

Exponent n values depending on the geometry of the encapsulation system [24]

<table>
<thead>
<tr>
<th>Drug release mechanism</th>
<th>Release speed as a function of time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fickian diffusion</td>
<td>t⁻⁰.⁵</td>
</tr>
<tr>
<td>Non-Fickian diffusion</td>
<td>t⁻¹</td>
</tr>
<tr>
<td>Case II transport</td>
<td>t⁻¹</td>
</tr>
<tr>
<td>Super-II case transport</td>
<td>t⁻¹</td>
</tr>
</tbody>
</table>

The obtained release coefficient (0.64) shows that the release of the drug depends simultaneously on several processes of the erosion of the nanofiber polymer film and the diffusion phenomena.

Scanning Electron Microscopy (SEM)
The SEM images showed homogenous fibres with diameter ranging from 900 - 1200 nm, with no visible cracks or degradation (Figure 3). Gentamicin was grafted on the surface of the fibre, forming a pearl-like structure.

n/Geometry

<table>
<thead>
<tr>
<th>Thin film</th>
<th>Cylinder</th>
<th>Sphere</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td>05 &lt; n &lt; 1.0</td>
<td>0.45 &lt; n &lt; 0.89</td>
<td>0.43 &lt; n &lt; 0.85</td>
</tr>
<tr>
<td>1</td>
<td>0.89</td>
<td>0.85</td>
</tr>
<tr>
<td>n &gt; 1</td>
<td>n &gt; 0.89</td>
<td>n &gt; 0.85</td>
</tr>
</tbody>
</table>

Scanning Electron Microscopy (SEM)
Figure 3.
SEM micrographs of the gentamicin loaded PLA nanofibers, using the mixed (BSE and SE observation mode), at different magnifications: (a) 400x, (b) 600x, (c) 1200x, (d) 1800x

Figure 4.
FT-IR analysis of PLA, GS and encapsulated GS into PLA nanofibers
Fourier transform infrared spectroscopy (FT-IR)
The presence of gentamicin sulphate in PLA nanofibers was evidenced after 24 hours of encapsulated. GS spectrum shows compound specific peaks at wavelengths 1619, 1525 and 1286 cm\(^{-1}\), that are assigned to the N-H primary aromatic amide group, and peaks at 876 and 972 cm\(^{-1}\) are attributed to the C-O-C group, peak at 1037 cm\(^{-1}\) is assigned to the sulphur content as an S-O group. The peak at wavelength 2924 cm\(^{-1}\) also corresponds to the N-H group of the secondary amide.

Regarding the spectrum of PLA nanofibers, the carbonyl group (C=O) is present at the wavelength 1752 cm\(^{-1}\), the C-O-C group present at 1085 and 1182 cm\(^{-1}\), the C-C group at 866 cm\(^{-1}\), the CH\(_3\) group at 1452 cm\(^{-1}\), and the characteristic group C-H (CH\(_3\)) is at 2945 - 2995 cm\(^{-1}\) [9, 10]. Also, the PLA nanofibers encapsulated with GS, there are groups belonging to both PLA and GS. In addition, a band was observed at approximately 3251 cm\(^{-1}\), attributed to the N-H and OH-O groups.

Therefore, the presence of additional GS peaks in the PLA+GS spectrum shows that GS has been successfully incorporated into PLA nanofibers. Also, the presence of peaks attributed to PLA after the encapsulated of GS, but with lower intensities, could indicate a possible chemical interaction between GS groups and PLA groups.

Contact Angle
As can be seen from Figure 5, the contact angle of uncoated TiZr and coated with PLA nanofibers and GS drug embedded change the contact angle from hydrophilic to hydrophobic values, meaning from 45 value for (a) sample to 89.93 for (b) sample and finally to 109.20 for sample (c).

Atomic Force Microscopy (AFM)
The normalized median values for the adhesion force evaluated from the AFM measurements [20] are shown as F-Z height diagrams in Figure 6. The uncoated TiZr sample has the highest adhesion force of approx. 45 nm, extending 300 nm from the surface. This can be attributed to the hydrophilic character of the sample. Hydrophilic surfaces are covered with a thin water layer of a few nanometres in ambient conditions. The water layers present on the sample and on the tip can join when the tip and sample are in proximity, forming a capillary neck between them.

The forces values for TiZr+PLA and TiZr+PLA+GS are of similar values. The forces values are in the range 15 - 20 nm and extend to a height of around 450 nm. The low forces can be attributed to the hydrophobic character of the samples. The small slope of the graph, is representative of a soft surface, combined to the relative distance to which the forces are present we can infer a degree of elasticity of the samples attributed to the polymeric coating. The addition of gentamicin doesn’t affect in a significant way the properties of the sample. This comment sustains the antibacterial effect of coating.
Antibacterial tests
For the determination of the antibacterial effect, the selected positive bacteria was from the priority target group for study ESKAPE pathogens [7] comprising six highly virulent and antibiotic aggressive resistant bacterial pathogens including our choice, Staphylococcus aureus. The selected, Gram negative strain is Escherichia coli which was not formally recognized as part of the ESKAPE group of pathogens but being identified as a major cause of hospitals acquired infections is most used in antibacterial tests. The spectrophotometer was used, to read an optical density at 600 nm of infected media and having contact with the specimens (T0 at 0 h, T1 at 24 h) and also positive control, and infected media without any samples (C0 at 0 h, C1 at 24 h). These were used in the following equation, which was proposed by Jaiswal et al. [17], for the measurement of the 1%, the growth inhibition index:

\[ I% = \frac{(C1-C0) - (T1-T0)}{(C1-C0)} \times 100. \]

The obtained values for inhibition index are closed to each other being 30.29 for Staphylococcus aureus and 33.57 for Escherichia coli. Knowing from previous paper that uncoated TiZr [30] has no antibacterial effect, it is clearly that inhibition is due to complex coating. The interaction mechanism of bacteria with coating is based on a biofilm formation having as a first step bacterial adhesion. This process is by both properties of the substrate and by environmental factors meaning, bacterial and material properties, and activity of serum or tissue proteins. The substrate characteristics, such as material chemical composition surface charge, its hydrophobicity and roughness with specific protein are important as well [22]. Taking into account that according to the literature all bacteria could display a large number of cellular forms [39], being able to change shape during their cycle of life, may be the importance of various influence factors on antibacterial effect can be modified as well. It will be possible with such idea and more experiments to complete the aspects of interrelationship between metallic substrate with and without drug incorporated and antibacterial effect.

Conclusions
A TiZr surface coated with PLA nanofibers and embedded with gentamicin was elaborated and the drug release kinetics was established. The kinetic parameters for five mathematical models were evaluated and the best correlation coefficient \( R^2 \) was obtained for the Peppas-Korsmeyer model indicating the existence of a non-Fickian diffusion mechanism for nanofibers-encapsulated GS. The antibacterial inhibition index evaluated against a positive and a negative Gram bacteria such as Staphylococcus aureus and Escherichia coli respectively was found close to each other and some correlation between surface properties and environmental factors on biofilm formation were proposed.

Acknowledgement
This work was supported by the European Social Funds (FSE), POCU, through the project “The preparation of doctoral students and postdoctoral researchers in order to acquire applied research competence - SMART” (no. 13530/16.06.2022).

Conflict of interest
The authors declare no conflict of interest.

References


37. Wu Z, Chan B, Low J, Champ CJ, Dennis Hey HW, Microbial resistance to nanotechnologies: An
important but understudied consideration using antimicrobial nanotechnologies in orthopaedic implants. Bioact Mater., 2022; 16: 249-270.

