The Bioactivity and Stability Evaluation of the PPy/Ca-P Hybrid Films on Titanium Alloy Implant

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This study has been carried out to investigate a new method to improve metallic implant bioactivity for osseointegration by producing a calcium phosphate (Ca–P) surface layer using an immersion process in the Simulated Body Fluid (SBF) of Polypyrrole (PPy) and Polypyrrole/poly(sodium-4styrensulfonate) (NaPSS) conductive hybrid polymers electrochemically synthesized on Ti6Al7Nb alloy. As formed PPy/Ca-P and PPy/PSS$^-$/Ca-P films were characterized electrochemically by Cyclic Voltammetry (CV), Tafel plots and spectroscopically using FT-IR technique. Also, in vitro cytotoxicity of these new organic/inorganic hybrids films was evaluated by yellow 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay after cultivation in a primary culture of osteoblast cells. The PPy/Ca-P hybrid film showed the best electrochemical stability and the highest value of cell viability.

Keywords: Implant alloy, Conductive polymer coating, Electrochemical tests, In vitro biocompatibility

The titanium and its alloys are bioinert materials used for orthopedic and dental applications [1, 2]. During implantation, a fibrous tissue is formed around titanium, leading to the implant isolation from the surrounding bone [3, 4]. For a good integration of the metallic implant into the bone, the bioactivity is a desirable property. The bioactivity of the metallic implants could be improved by generating of bioactive ceramics [5-7] and a calcium phosphate (Ca–P) layer on the metallic surface [8-11]. The Ca-P layers could create apatite after implantation without the formation of fibrous tissue, acting as an intermediate layer between the new bone and implant. This coating of Ca–P can be created on a titanium surface in an aqueous solution, at an ambient temperature, in the presence of a Simulated Body Fluid (SBF) which has the ions concentration nearly equal with the human blood plasma [12].

Furthermore, in order to increase the biomimetic deposition, the metallic implants were subjected to different pre-treatments [2, 13-15]. Recently a new method was developed based on conducting polymers, which presents important advantages for various applications, including medical field [16, 17]. Previous works demonstrate that the polypyrrole (PPy) has high electrical conductivity, good environmental stability and easy electrochemical synthesis in either aqueous or non-aqueous solution directly onto metallic substrates [18-20]. Moreover, in our previous studies was established that the PPy do not liberate toxic components which can inhibit growth and cellular proliferation, and also, this polymeric film has antibacterial effect [21]. As well, the biocompatibility of PPy coating was increased by adding different surfactants during polymerization process [22]. Also, the presence of the surfactant improves the conductivity of the polymer, wettability, compactness, the adherence of the polymer film on metallic substrate [22-24], and also, the electropolymerization can be made at lower potential [25].

In this study, in order to improve the titanium implant bioactivity for orthopedic and dental applications, the polypyrrole/surfactant hybrid films were electrodeposited on Ti6Al7Nb surface and then immersed in SBF solution, obtaining polymer/Ca-P active layers.

The electrochemical stability evaluation of the new polymer/Ca-P hybrid films deposited onto titanium alloy implant was investigated. Also, the *in vitro* cytotoxicity was evaluated in a primary culture of osteoblast cells.

Experimental part

Sample preparation

Ti6Al7Nb samples were elaborated as working electrodes with the following chemical composition: 0.1% C, 5.88% Al, 6.65% Nb, 0.3% Fe, 0.05% N, 0.2% O, rest Ti. Their surface was polished with two kinds of silica carbide paper from 320 to 4000 type. All impurities were ultrasonically removed by immersing in distilled water, ethyl alcohol and in acetone at room temperature for 10 min in each solution before polymer electrodeposition.

The electrochemical synthesis of PPy and PPy-PSS films was made according to our previous works [18, 20, 22].

In order to develop organic/inorganic hybrid films, Ti6Al7Nb/PPy and Ti6Al7Nb/PPy-PSS electrodes were immersed for 28 days in Simulated Body Fluid (SBF) [26] with the composition (g/L): 8.035 NaCl, 0.355 $NaHCO_3$, 0.225 KCl, 0.231 K_2HPO_4 x $3H_2O$, 0.311 $MgCl_2$ x $6H_2O$, 0.292 $CaCl_2$, 0.072 Na_2SO_4, and 0.160 mL HCl 1mol x L^{-1}, *p*H = 7.40.

Samples characterization

Infrared Analysis

FT-IR analysis for PPy/Ca-P and PPy/PSS$^-$/Ca-P hybrid complex prepared films was conducted with Spectrum 100 PerkinElmer equipment using diamond ATR technique. The scanning was ranged from 4000 cm^{-1} to 600 cm^{-1}.

Wettabilitty

The contact angle measurements were performed in order to establish the wettability behaviour of these active polymeric films, which is an important parameter that influences the osteoblast cells proliferation. Contact angle measurements toward distilled water have been performed with Contact Angle Meter KSV Instruments CAM

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100. Each measurement was made according to literature [21, 22].

Electrochemical tests

The electrochemical stability of PPy/Ca-P and PPy/PSS$^-$/Ca-P hybrid films was evaluated from Tafel procedure, Electrochemical Impedance Spectroscopy (EIS) and Cyclic Voltammetry (CV) in SBF. All measurements were performed using a one compartment cell with three electrodes: Ti6Al7Nb/PPy/Ca-P and Ti6Al7Nb/PPy/PSS$^-$/Ca-P as working electrodes, a platinum counter electrode and an Ag/AgCl, KCl reference electrode, connected to Autolab PGSTAT 302N potentiostat with NOVA software.

The EIS measurements were performed in frequencies domain between 100 kHz and 100 mHz, and the amplitude was ± 10 mV. The EIS results are discussed in term of Nyquist diagrams. EIS spectra obtained were fitted with Nova 1.8 software.

The Tafel regions of cathodic and anodic polarization curves were extrapolated in order to prove the anti-corrosion properties of PPy/Ca-P and PPy/PSS$^-$/Ca-P hybrid structures. Tafel plots were obtained by polarization with ± 150 mV *vs.* Ag/AgCl toward electrode potential in anodic direction with a scan rate of 2 mV/s. The main electrochemical parameters were estimated based on the Tafel plots: i_{cor} (corrosion current density), R_p (polarization resistance), E_{cor} (corrosion potential), and v_{cor} (corrosion rate).

In order to sustain the electrochemical stability of the obtained hybrid films, the polarization curves were performed *via* 10 cycles in the potential domain -0.5 ¸ 0.5 V *vs.* Ag/AgCl with a scanning rate of 0.05 V/s.

In vitro cytotoxicity test

Cell isolation and cultivation

Osteoblast cells were isolated from calvaria bones of newborn rats by enzymatic digestion method, as previously described [27], complying the ethics guidelines for animal experiments. Briefly, bone fragments (1-2 mm^3) were extensively washed in phosphate buffered saline (PBS), *p*H 7.4 and digested 4 times with 2 mg/mL collagenase IA (EC 3.4.24.3, Sigma-Aldrich) and 0.25% trypsin (EC 3.4.21.4, Sigma-Aldrich) for 20, 40, 60 and 90 min, respectively. Isolated cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) and 1% antibiotic mixture (Sigma-Aldrich). The culture was maintained in an incubator with humid atmosphere of 5% CO_2 and 95% air, at 37°C [28]. For cytotoxicity experiments, cells were harvested from subconfluent cultures using 0.25% trypsin-EDTA solution, resuspended in fresh serum supplemented medium and plated.

Cytotoxicity test

In order to investigate the cytotoxicity of titan composite materials, samples of Ti6Al7Nb/PPy/Ca-P and Ti6Al7Nb/PPy/PSS$^-$/Ca-P, as well as Ti6Al7Nb/Ca-P (control material) were placed into 24-well culture plates and were exposed to UV radiation, for 48h, in a sterilization cabinet (Scie-Plas, UK). Cell suspension was seeded on the composites, at a density of 1×10^5 cells/well and cultured in DMEM containing 10% FBS. The culture plates were incubated in standard conditions of cultivation, in 5% CO_2 atmosphere, at 37 °C, for

48 h. The effect of composite material samples on cell viability, in particular on the mitochondrial succinate dehydrogenases activity was evaluated by MTT assay, as previously described [29]. This method is based on the cleavage of yellow 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to insoluble purple crystals of formazan by the action of mitochondrial succinate dehydrogenases from viable cells. Briefly, the culture medium from each well was replaced with fresh medium containing 0.25 mg/mL MTT solution, in a 10:1 (v/v) ratio and the plates were incubated at 37°C, for 3 h. Then, 500μL isopropanol was added to each well to dissolve the formazan crystals by gently shaken on a platform, for 1 h. The colored solution was transferred to a 96-well culture plate and the optical density (OD) was read at 570 nm using a microplate reader (Sunrise Tecan). The measured OD is directly proportional to cell viability and the results were calculated using the following equation:

$$\text{Cell viability (\%)} = \text{OD sample / OD control x } 100 \qquad (1)$$

Three separate experiments were conducted and the results were expressed as mean ± SD.

Statistical analysis

Data were expressed as mean value ± SD for three independent samples (n = 3). Statistical analysis of the cytotoxicity data was performed using one-tailed paired Student's *t*-test on each pair of interest. Differences were considered statistically significant at $p < 0.05$, as a minimal level of significance.

Results and discussions

Surface and structure characterization of organic/inorganic hybrid complex films

Structure characterization of the hybrid complex coatings

FT-IR spectra of hybrid films in the region from 4000 to 600 cm^{-1} are presented in figure 1, in order to put in evidence if the NaPSS surfactant presence in polymerisation process influences the Ca–P coating on biomimetic PPy electrodeposition.

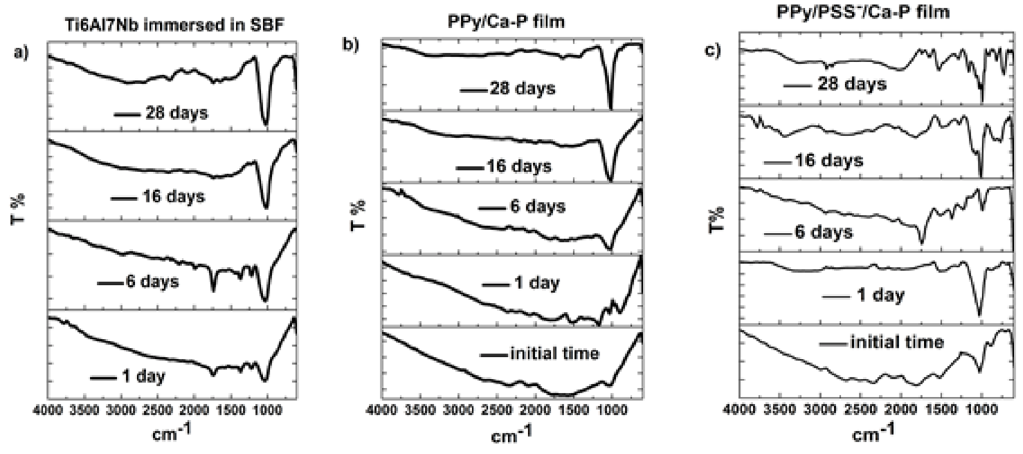

Fig. 1. FT-IR spectra for a) Ti6Al7Nb, b) PPy and c) PPy/PSS$^-$ immersed at different time in SBF

In all FT-IR spectra, after one day of immersion in SBF, the carbonate ions present peaks in the region of 1420 ÷ 1460 cm^{-1} due to ν_3 vibration mode of carbonate ion. Phosphate ions present a single ν_3 band at 1034 cm^{-1}, more intense in case of PPy/PSS^- film, which means that the apatite covered entire surface in all cases [30]. After 6 days of immersion in SBF solution of the Ti6Al7Nb/PPy/PSS^- electrode, the absorption of SO^{3-} ion peak in the NaPSS group is more intense at 1226 cm^{-1}, than in case of Ti6Al7Nb/PPy electrode, probably because of more possible interactions between PSS^- and calcium ions, Ca^{2+}. The peak at 1757 cm^{-1} is matching the C=C bond. Also, after 16 and 28 days of immersion in SBF the intensity of the peaks corresponding to phosphate, and carbonate ions is higher for coating with NaPSS surfactant presence than in case of PPy without dopant, which means that the Ca-P coating obtained on this polymer coating is increased.

On Ti6Al7Nb alloy and PPy film the apatite formation has almost the same behaviour.

Based on the FTIR spectral profiles, the formation of organic/inorganic hybrid composites during immersion process in SBF solution was confirmed.

Contact angle determinations

Contact angle (CA) determinations were done at different immersion times in SBF solution in order to study the influence on Ca-P bond formation of wettability behaviour. The contact angle evolution of electrodes with SBF immersion time is illustrated in figure 2.

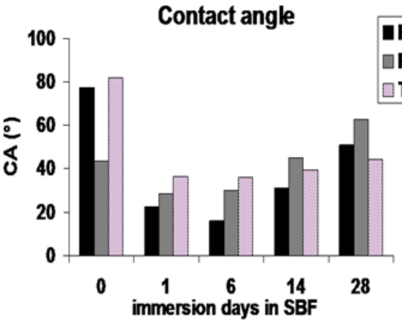

Figure 2. The contact angle variation of Ti6Al7Nb and Ti6Al7Nb/PPy and Ti6Al7Nb/PPy/PSS^- electrodes *vs.* immersion time in SBF

At initial time, the NaPSS surfactant presence in the polymer film decreases the contact angle value due to the presence of hydrophilic sulphonic groups. Also, as was found in the previous work [22], the roughness of polymer surface in the presence of surfactant is lower, 68 nm for PPy/PSS compared with 189 nm for PPy.

After 24 h of immersion in SBF the contact angle values are lower for all tested surfaces, indicating surfaces changes due to Ca-P bond formations, as was observed in FTIR analysis. Then, after 6 days of immersion in SBF solution the CA values slowly increase, probably because of the surface roughness modification due to the Ca-P formations which grow irregular on specific nucleation regions.

Electrochemical stability of PPy/Ca-P and PPy/PSS^-/Ca-P hybrid complex coatings

Cyclic voltammograms

In figure 3, the cyclic voltammetric curves for PPy/Ca-P and PPy/PSS^-/Ca-P systems are presented.

Both films obtained after 28 days of immersion in SBF presented a capacitive behaviour. Also, the good electrochemical stability was proved by the successive ten cycles which are practically overlaid.

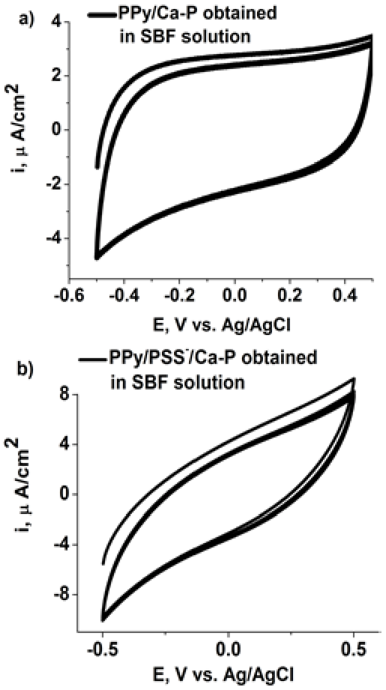

Fig. 3. Cyclic voltammograms for PPy/Ca-P and PPy/PSS^-/Ca-P hybrid films – -0.5 ÷ 0.5V, 0.05 V/sec, 10 cycles, in SBF solution

The electrochemical behaviour of PPy/Ca-P and PPy/PSS^-/Ca-P hybrid films suggest that the both films remain adherent on the titanium alloy during immersion in SBF solution for almost one month.

Electrochemical Impedance Spectroscopy

Figure 4 presents the Nyquist spectra for PPy/Ca-P and PPy/PSS^-/Ca-P electrodes after 28 days after immersion in SBF solution. In all cases EIS analyses were achieved at open circuit potential in SBF solution.

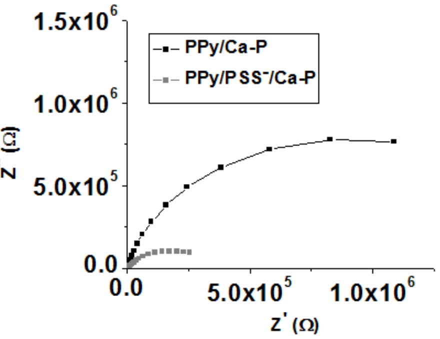

Fig. 4. Nyquist spectra for PPy/Ca-P and PPy/PSS^-/Ca-P in SBF solution

The electrical circuit (fig. 5) used to fit the EIS data contains two time constants, with next parameters: R_s - solution resistance; R_{coat} - the charge transfer resistance of the coating layer formed from polymer and apatite with a double layer capacitance (CFE_2); and R_{ct} - charge transfer resistance of the inner layer of the metal/electrolyte interface with a constant phase element (CFE_1).

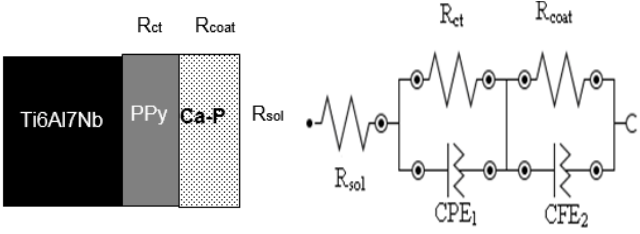

Fig. 5. The electrical circuit used for fitting EIS data

Table 1
ELECTROCHEMICAL PARAMETERS OBTAINED FROM FITTING OF EIS DATA

Coating layer	R_{sol} (Ω cm^2)	CPE_1 (F cm^{-2})	n_1	R_{ct} (Ω cm^2)	CFE_2 (F cm^{-2})	n_2	R_{coat} (Ω cm^2)
PPy/Ca-P	223	$4.51 \cdot 10^{-6}$	0.871	$1.94 \cdot 10^{+6}$	$9.36 \cdot 10^{-6}$	0.749	$9.36 \cdot 10^{+3}$
PPy/PSS^-/Ca-P	196	$8.67 \cdot 10^{-6}$	0.724	$3.25 \cdot 10^{+5}$	$3.62 \cdot 10^{-6}$	0.899	$5.32 \cdot 10^{+3}$

The results from table 1 reveal that the PPy/Ca-P film has a better polarization resistance than the PPy/PSS$^-$/Ca-P, and all PPy-based coatings displayed a pseudo-capacitive behaviour, as resulted from n$_2$ values comprised between 0.7 and 1.

Tafel diagrams

The corrosion behaviour of coated PPy/Ca-P and PPy/PSS$^-$/Ca-P hybrid films was estimated by Tafel plots (fig. 6) after 28 days immersion in SBF solution.

The corrosion parameters computed with Nova software from Tafel plots are presented in table 2.

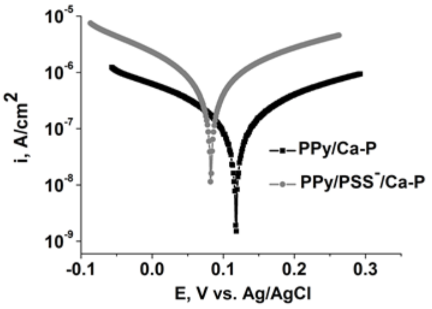

Fig. 6. The polarization curves for PPy/Ca-P and PPy/PSS$^-$/Ca-P hybrid complex coatings

Table 2

CORROSION PARAMETERS AND POLARISATION RESISTANCE OBTAINED FROM TAFEL DIAGRAMS

Coating layer	$i_{cor} \times 10^{-6}$ (A/cm^2)	$R_p \times 10^{+5}$ (Ω/cm^2)	E_{cor} (V)	$V_{cor} \times 10^{-2}$ (mm/year)
PPy/Ca-P	0.432	16.470	0.117	0.36
PPy/PSS$^-$/Ca-P	1.815	3.342	0.081	1.55

The corrosion current for PPy/PSS$^-$/Ca-P coating after 28 days of immersion in SBF is almost one order of magnitude higher than the value corresponding to PPy/Ca-P film. Taking into account that the corrosion current values obtained at initial time of immersion are much closed, 1.526.10^{-6} A/cm^2 for PPy film and 1.701.10^{-6} A/cm^2 for PPy/PSS$^-$ [22], indicate important changes in the polymer film structure during immersion and Ca-P film formation.

Although, the PPy/PSS$^-$ is known to be more compact and stable compared with PPy film [31], after immersion in SBF, the possible interaction between PSS$^-$, acting as dopant for PPy$^+$, and calcium ions, Ca^{2+}, could lead to the changes in the polymeric film conformation. Thus, the interaction between Ca-P compounds and PPy/PSS$^-$ film is improved, as was observed from FTIR analysis, but the polymeric film stability is affected.

The PPy/PSS$^-$ film stability fall off during immersion in SBF was also sustain by EIS measurements which indicate a lower charge transfer resistance, 3.25·10^{+5} Ω .cm^2, compared with 1.94·10^{+6} Ω.cm^2 for PPy/Ca-P film.

In vitro cytotoxicity testing of composite materials

In order to estimate the *in vitro* cytotoxicity of PPy/Ca-P and PPy/PSS$^-$/Ca-P coating films, we have evaluated the cell viability of a primary culture of rat osteoblasts cultured in the presence of composite materials samples for 48 h. Cell viability was quantified by MTT test, an indicator of living cells metabolism, in particular, of the mitochondrial enzymes activity. We have compared the cytotoxicity of Ti6Al7Nb/PPy/Ca-P and Ti6Al7Nb/PPy/PSS$^-$/Ca-P to that of Ti6Al7Nb/Ca-P cultivated in the same conditions.

Our results, presented in figure 7, showed that the percentage of viable cells varied between 168.5% for PPy/Ca-P and 148.1% for PPy/PSS$^-$/Ca-P samples, compared to 100% for Ti6Al7Nb/Ca-P control sample. These values indicated that all tested samples were not cytotoxic (grade 0), according to the International Standard ISO 10993-5 for evaluation of medical device cytotoxicity. Moreover, the values were significantly higher ($p < 0.05$) than that of control sample, indicating the ability of these composite materials to stimulate osteoblast cells metabolism.

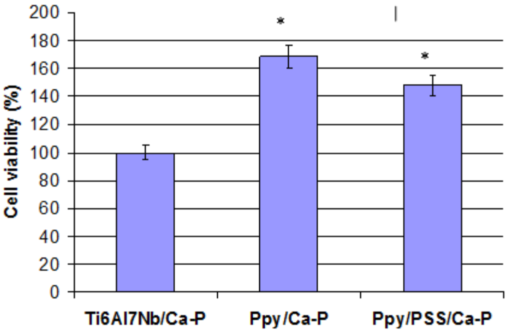

Fig. 7. Cell viability of rat osteoblasts cultivated on PPy/Ca-P and PPy/PSS$^-$/Ca-P samples, assessed by MTT assay. *$p < 0.05$, compared to Ti6Al7Nb/Ca-P (control sample)

Statistic examination of 48 h records for each sample-control pair showed that the osteoblast cells cultivated on PPy/Ca-P coating film presented higher values of cell viability than cells cultivated on PPy/PSS$^-$/Ca-P composite film. Both polymer coating films have significantly ($p < 0.05$) improved osteoblast viability, compared to Ti6Al7Nb alloy uncovered with polymeric film. The lower value of cell viability for PPy/PSS-/Ca-P film might be due to sulfonic group presence with higher hydrophilic character, which could induce cell apoptosis by reducing the protein adsorption, which mediates the cell adhesion on the biomaterial [32]. Also, other studies reported that an insertion of positive ions, such as Na$^+$ from the culture medium have affected the ion flux across the cell membrane, which was critical for the cell cycle progression [33].

The biomimetic PPy/PSS$^-$/Ca-P composite film has induced a better cell viability, compared to Ti6Al7Nb/Ca-P material, due to the presence of NaPSS surfactant that increased the amount of Ca-P on coating film, as demonstrated by FT-IR analysis results.

Taken together, these results suggested that the analyzed structure of PPy/Ca-P samples favored osteoblast cell proliferation that could be also a consequence of their higher electrochemical stability and wettability, two main factors influencing the cell adhesion onto the material surface [34-36].

Conclusions

In this investigation, PPy/Ca-P and PPy/PSS$^-$/Ca-P biomimetic coating films were successfully prepared by electrochemical deposition of PPy and PPy/PSS films onto titanium alloy and then immersed in SBF solution for 24 h.

The NaPSS surfactant presence in PPy film enhanced the Ca-P deposition on PPy based coating films, according to FTIR analysis. However, electrochemical investigation showed that the corrosion resistance of PPy/PSS$^-$/Ca-P coating was affected by structural changes of polymeric film due to the possible interactions between PSS$^-$ and Ca^{2+}.

In vitro study showed that the viability of osteoblast cells was promoted on PPy/Ca-P film and the osteoblast metabolism depends on the amount of Ca-P deposited on the coating film, which was influenced by the surfactant molecules entrapped into PPy film.

All designed titan-based composite materials allowed osteoblast proliferation and provides good candidates for bone tissue engineering.

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