The biocompatibility of polyaniline and polypyrrole 2[1]: Doping with organic phosphonates

The biocompatibility of polyaniline and polypyrrole 21: Doping with organic phosphonates

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ABSTRACT

Conducting polymers (CP) can be used as pH- and/or electro-responsive components in various bioapplications, for example, in 4D smart scaffolds. The ability of CP to maintain conductivity under physiological conditions is, therefore, their crucial property. Unfortunately, the conductivity of the CP rapidly decreases in physiological environment, as their conducting salts convert to non-conducting bases. One of the promising solutions how to cope with this shortcoming is the use of alternative "doping" process that is not based on the protonation of CP with acids but on interactions relying in acidic hydrogen bonding. Therefore, the phosphonates (dimethyl phosphonate, diethyl phosphonate, dibutyl phosphonate, or diphenyl phosphonate) were used to re-dope two most common representatives of CP, polyaniline (PANI) and polypyrrole (PPy) bases. As a result, PANI doped with organic phosphonates proved to have significantly better stability of conductivity under different pH. It has also been shown that cytotoxicity of studied materials determined on embryonic stem cells and their embry-otoxicity, determined as the impact on cardiomyogenesis and erythropoiesis, depend both on the polymer and phosphonate types used. With the exception of PANI doped with dibutyl phosphonate, all PPy-based phos-phonates showed better biocompatibility than the phosphonates based on PANI.

Keywords: Polyaniline, Polypyrrole, Conducting polymers biokompatibility, Phosphonates, Embryonic stem cells

1. Introduction

Compared with metals that exhibit solely electronic conductivity, the combined electronic and ionic conductivity of CP is one of their most attractive properties when the applications in regenerative medicine, tissue engineering or bio-sensing are considered **[1,2]**. Ionic conductivity of CP is fundamental for the communication with biological objects, as it relies on ionic fluxes. PANI and PPy are two members of CP family, which are intensively studied for biological applications, **[3,4]**; however, their conductivity depends strongly on environmental conditions. Besides the impact of pH, which will be discussed below, the conductivity of PANI is notably influenced by temperature during polymerization **[5,6]**. The study of Bláha et al. [6] showed that the increase of temperature from -20 to 40 °C during PANI synthesis played the key role in controlling its molecular structure, morphology and crystallinity, and it strongly affects the conductivity. Similarly as in PANI, conductivity of PPy is also influenced by preparation temperature. Ready-synthetized PPy changes its conductivity with temperature variation **[7]** and different temperatures applied during PPy synthesis have impact on its resulting conductivity as well **[8]**. Influence of various doping agents, e.g., chlorine or dodecylsulfate anions **[9]** or p-toluenesulfonic, itaconic and fumaric acids on PPy conductivity cannot be ignored, either **[10].**

As mentioned above, the critical factor limiting the use of CP in a number of biomedical applications is the decrease of their conductivity under physiological pH, as the conducting salts are converted to nonconducting bases already below this pH region **[11]**. There have been outlined, and even tested, several approaches how to improve the pH stability of CPs including, for example, electrochemical polymerization or re-protonation with perfluorooctanesulfonic **[11]** or poly(2-acryla-mido-2-methyl-1-propanesulfonic acids) **[12].** The perspective approach to solve this limitation can be the use of alternative “doping” methods, which are not based on the classical protonation but on the application of acidic hydrogen atoms **[13].**

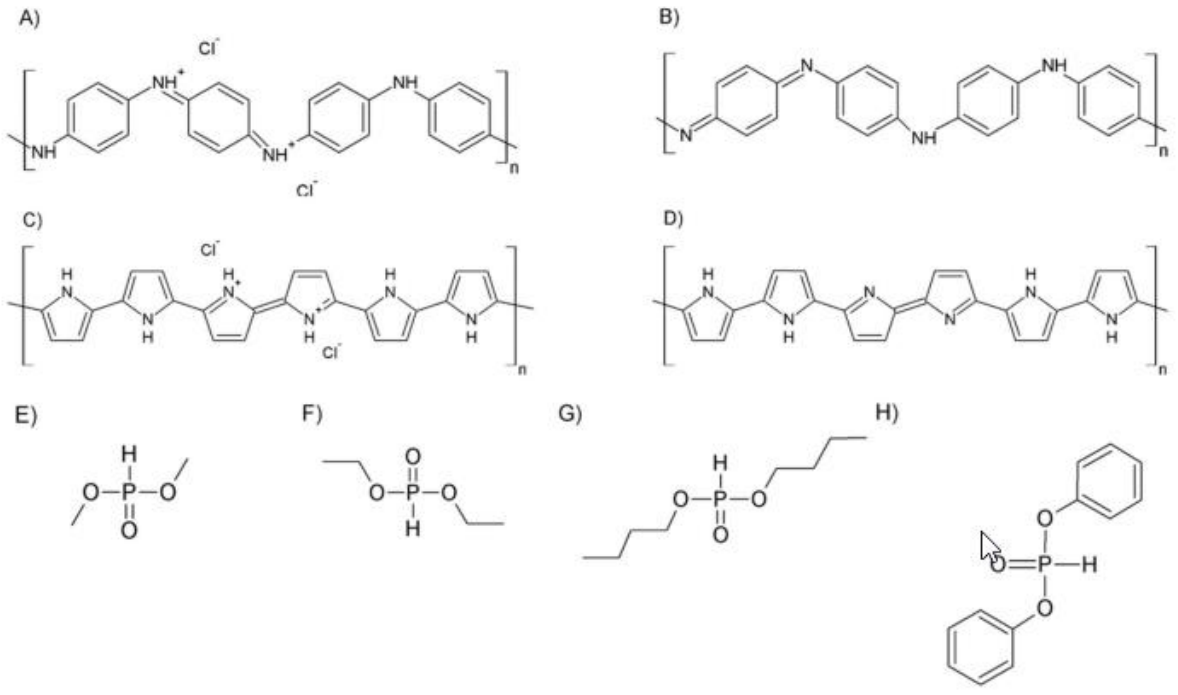

Fig. 1. Formulae of A) polyaniline salt (PANI-S); B) polyaniline base (PANI-B); C) polypyrrole salt (PPy-S); D) polypyrrole base (PPy-B); E) dimethyl phosphonate (DMPH); F) diethyl phosphonate (DEPH); G) dibutyl phosphonate (DBPH); H) diphenyl phosphonate (DPPH), the precursors of studied samples.

Several papers recently published show the possibility of this alternative doping to prepare the PANI with the organic phosphonates, which demonstrate interesting properties depending on the type of phosphonate used **[13-15]**. The present study extends the previous work to PPy and uses these organic phosphonates as a novel approach how to improve the stability of conductivity of PANI and PPy at various pH, mainly at the physiological pH. The work also discusses the biological properties of these new promising CP, primarily with respect to influence of phosphonate type used for CP modification.

2. Materials and methods

2.1. Sample preparation

Polyaniline, emeraldine salt **(Fig. 1A)**, was prepared by standard oxidation of 0.2 M aniline hydrochloride (Penta, Czech Republic) with 0.25 M ammonium peroxydisulfate (Lach-Ner, Czech Republic) in aqueous medium at room temperature **[16]**. Globular polypyrrole (**Fig. 1C**) was synthetized by the oxidation of 0.2 M pyrrole (Sigma-Aldrich) with 0.5 M iron(III) chloride hexahydrate (Sigma-Aldrich) in water. The respective mixtures were left to react at room temperature for 24 h. Then the solids were collected on a filter, rinsed with 0.2 M hydrochloric acid and ethanol and dried in air and over silica gel.

Both solids were subsequently converted to PANI and PPy bases in 1 M ammonium hydroxide (**Fig. 1B, D**), rinsed with ethanol and dried as above. PANI and PPy bases were suspended in dimethyl phosphonate (DMPH**, Fig. 1E**), diethyl phosphonate (DEPH**; Fig. 1F**), dibutyl phosphonate (DBPH; Fig. 1G), or diphenyl phosphonate (DPPH; **Fig. 1H**) (all from Sigma Aldrich) without using any diluent. After 3 days, the solids were collected on a filter, rinsed with ethanol, and dried in air and then over silica gel. The samples were denoted according to their composition, with CP and phosphonate components as follows: PANI-DMPH, PANI-DEPH, PANI-DBPH, PANI-DPBH, PPy-DMPH, PPy-DEPH, PPy-DBPH and PPy-DPBH.

2.2. Cell lines

The embryonic stem cell ES R1 line (ESC) **[17]** was propagated in an undifferentiated state by culturing on gelatinized tissue culture dishes in complete media. The gelatinization was performed using 0.1% porcine gelatin solution in water. Complete media containing Dulbecco's Modified Eagle's Medium (DMEM), 15% fetal calf serum, 100 UmL^{-1} penicillin, 0.1$mgmL^{-1}$ streptomycin, 1x non-essential amino acids solution (all from Gibco-Invitrogen; USA), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich; USA) and 1000 U mL^{-1} of leukemia inhibitory factor (Chemicon; USA) were used for the cultivation **[18].**

2.3. Preparation of extracts of PANI, PPy and their respective phosphonates

The testing of cytotoxicity was performed on polymer extracts obtained according to ISO 10993-5 protocol. Samples were extracted according to ISO 10993-12 with the following modification: the ratio 0.05 g polymer per 1 mL of cultivation medium was used instead of ISO defined 0.2 g polymer per 1 mL. Extraction was conducted in chemically inert closed containers using aseptic techniques at 37 ± 1 °C under stirring for 24 h. Subsequently, the extract was separated from the polymer powder by centrifugation at 1000 g for 15 min followed by second centrifugation of supernatant liquid under the same conditions. The parent extracts (100%) were then diluted in a complete medium to obtain a series of dilutions. All extracts were used within 24 h. Prior to in-vitro testing, the extracts were

sterilized by filtration through the 0.22 pm filter (Millipore, USA). All tests were performed in quadruplicates, in four separates sets.

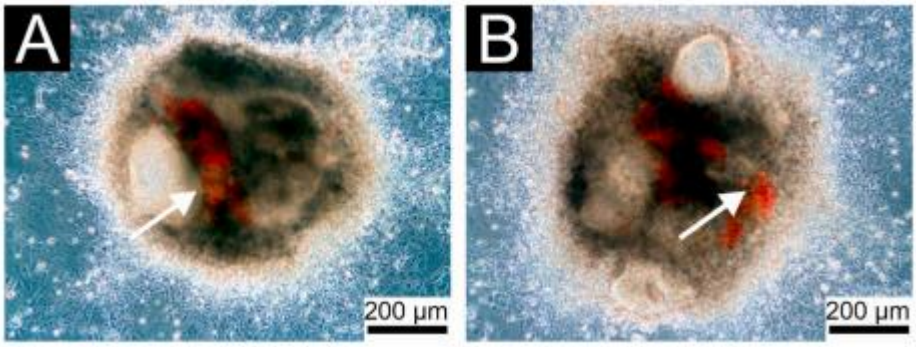

Fig. 2. The formation of erythroid clusters (red cluster marked with arrow) within the embryoid bodies. A) Positive reference, B) in the presence of 25% extracts of PPy-DBPH. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Cytotoxicity on ESC

The cells have been seeded at density of 5000 cells per cm2 24 h before treatment. As a reference giving 100% cell viability, the cells cultivated in the pure complete medium were used. The cells were treated by extracts for 48 h. To assess cytotoxic effects, mass of viable ESC was determined as a level of adenosine triphosphate (ATP) using Cellular ATP Kit HTS (BioThema, Sweden). Samples were prepared and analysed as published previously **[19]**. Before lyses, the morphology of the cells was observed and documented using an inverted Olympus phase contrast microscope (Olympus IX51, Japan) supplemented with digital camera (Olympus E-450, Japan).

2.5. Embryotoxicity

The embryotoxicity was determined as the ratio of the formation of embryoid bodies (EBs) with beating foci (impact on cardiomyogenesis) and erythroid (in **Fig. 2** shown in red) clusters/colonies (impact on erythropoiesis) within spontaneously differentiating ES R1 cells compared to reference. The ESC differentiation was induced through the formation of EBs by hanging drop techniques (400 cells per 35 µL drop) in leukemia inhibitory factor-free complete medium mentioned above, which was also used for sample extraction. After 5 days, EBs were transfer to gelatinized 24-wells plate (one EB per well) to serum-free media for next 15 days. Medium was replaced by fresh one each three days of cell culturing. Serum-free media contained DMEM-F12 media (1:1), 100 UmL^{-1} penicillin, 0.1$mgmL^{-1}$ streptomycin and 1x insulin-transferrin-selen (ITS) supplement (all from Gibco-Invitrogen; USA). Differentiating cells were observed as above.

2.6. Conductivity measurements

The DC conductivity was measured employing van der Pauw method on compressed polymer pellets having 13 mm diameter and thickness of 1.0 ± 0.2 mm. A Keithley 230 Programmable Voltage Source in serial connection with a Keithley 196 System DMM was used as current source and a Keithley 617

Programmable electrometer was used for the potential difference measurement. Measurements were carried out at stable ambient conditions at temperature 24 ± 1 °C and relative humidity 35 ± 5%. The conductivity was calculated from the linear part of the current-voltage characteristics, and is reported as an average value from measurements performed in two perpendicular directions conducted with the aim to reduce influence of the sample inhomogeneity.

3. Results and discussion

Recently, CP have received considerable attention due to their possible applications in regenerative medicine or tissue engineering of electro-sensitive tissues **[2,20-22]**. One of the studies conducted by Humpolíček et al. **[20]** compared the biocompatibility of PANI and PPy. The results demonstrated significant differences between biocompatibilities of the polymers in salt and base forms (**Fig. 1**). However, the differences between PANI and PPy in their respective forms (PPy-S vs PANI-S, PPy-B vs PANI-B) were negligible. In the present study, the PANI and PPy were prepared according to the procedure used in that study with a modification consisting in using phosphonates as dopants. The results obtained on the here-tested materials can be therefore compared and discussed with the results obtained on non-modified PANI and PPy in the mentioned study **[20]**.

Here, the cytotoxicity was determined in accordance to ISO 10993-5 with the modification consisting in the use of different cell type, namely ESC. In addition to the cytotoxicity, embryotoxicity was also determined. Commonly, the embryotoxicity can be tested by using two main groups of methods: in vivo and *in vitro*. Here, the in-vitro testing of ESC differentiation processes of cardiomyogenesis and erythropoiesis were performed. The *in-vitro* cardiomyogenesis from ESC illustrates processes in the developing of embryo and helps to elucidate the mechanisms of differentiation of the cardiac cells. In-vitro erythropoiesis is observed by the formation of EBs with erythroid clusters **(Fig. 2**). These two processes are among the first steps of determining embryonic development **[23]**.

One of the early studies dealing with cytotoxicity of PANI was published in 2012 by Humpolíček et al. **[24]** who used a standard protocol of ISO 10993-5 and NIH/3T3 cells. However, in the already-mentioned work of the same authors from 2018 **[20]**, cytotoxicities of PANI and PPy were determined using ESC (**Fig. 4B**) and are, therefore, comparable with a current study conducted on PANI-phosphonates (**Figs. 3 and 4A**). The cell morphology after exposition to the extracts is shown in the **Fig. 3.** It can be concluded that neither of the extracts influence the morphology of ESC. In the comparison with pristine PANI, the PANI-DPPH and PANI-DEPH show similar cytotoxicity towards ESC as polyaniline salt (PANI-S), while cytotoxicity of PANI-DBPH and PANI-DMPH are close to that of polyaniline base (PANI-B). Closer inspection of data revealed that the PANI-DPPH and PANI-DEPH were strongly cytotoxic even at very low extract concentrations (1%) in the cultivation medium. The embryotoxicity data expressed in percentage of formation of EBs with beating foci or erythroid clusters are presented in **Table 1**. The PANI-DPPH and PANI-DEPH exhibit embryotoxic effect in all tested concentrations and their embryotoxicities are even higher than that of PANI-S. On the other hand, PANI-DBPH and PANI-DMPH do not induce any embryotoxicity in any of the tested concentrations and induce the same, zero level, of cytotoxicity as PANI-B.

The cytotoxicity of all tested PPy phosphonates was low and, surprisingly, it was even lower than that of pristine polypyrrole salt (PPy-S) **[20]**. As in the case of PANI phosphonates, extracts of PPy phosphonates have no influence on cell morphology **(Fig. 5)**. The lowest cytotoxicity exhibited PPy-DBPH and especially PPy-DEPH. It is thus evident that the biological properties of PPy-S improved by the treatment with phosphonates **(Fig. 6).** The embryotoxicity of PPy modified with phosphonates were even lower than their cytotoxicities.

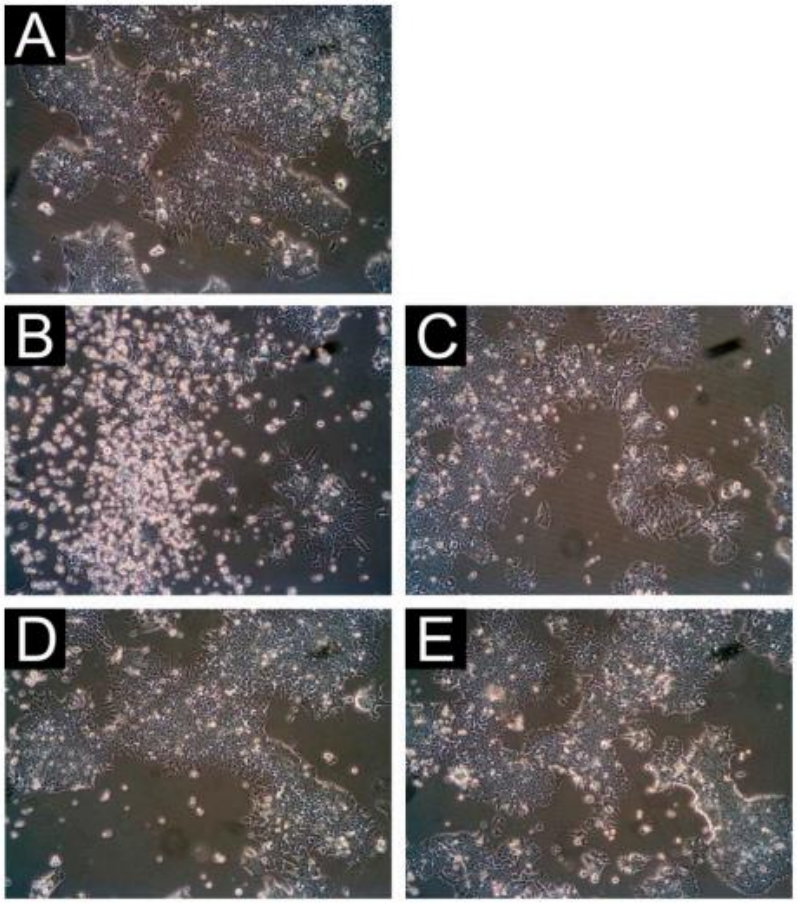

Fig. 3. The morphology of ESC after exposition to the extracts. A) Reference; B) PANI-DPPH 10%; C) PANI-DBPH 50%; D) PANI-DEPH 1%; E) PANI-DMPH 25%. Magnification 40 x.

In fact, mild decrease (to 88%) of formation of EBs with erythroid clusters was observed only for PPy-DMPH and only in case of the highest tested extract concentration (50%). Formation of EBs with beating foci was not affected by the studied extracts at all, and no decrease in the percentage of EBs with clusters was detected **(Table 2)**.

Cytotoxicity data obtained on ESC in current study obviously demonstrated that PPy-phosphonates exhibit much lower cytotoxicity than their corresponding PANI analogues. Interestingly, in the contrast to PPy where doping with DPPH and DEPH produced only negligible cytotoxic effect, the same phosphonates were significantly cytotoxic when combined with PANI. However, with exception of the PANI-DPPH and PANI-DEPH, all samples exhibited lower cytotoxicity than pristine polymer salts, PANI-S and PPy-S, and were comparable with cytotoxicity of both bases, PANI-B and PPy-B **[20].**

Based on the results from embryotoxicity testing, it can be concluded that all tested PPy-phosphonates and PANI-DBPH and PANI-DMPH do not show any harmful effect in terms of cardiomyogenesis and erythropoiesis. On the other hand, PANI-DPPH and PANI-DEPH significantly decreased the formation of EBs with beating foci or ery-throid clusters in comparison with the reference. In fact, only 50% of EBs with beating foci and clusters were formed after treatment with only 1% extract of PANI-DPPH, and no EBs with beating foci or clusters formed in contact with higher concentrations of the PANI-DPPH extracts, as well as with the whole range of extract concentrations prepared of PANI-DEPH. Here,

similarly as for cytotoxicity, all phos-phonate-doped samples, except the PANI-DPPH and PANI-DEPH, showed lower embryotoxicity than pristine PANI-S and PPy-S, and were absent of embryotoxic effect as the PANI-B and PPy-B samples **[20]**.

The most varying performance after being used as a dopant was observed in the case of DEPH. This phosphonate showed the highest cytotoxicity among all samples when used together with PANI; on the contrary, PPy with the same phosphonate had no cytotoxic effect in the whole range of extract concentrations tested. The corresponding effect was confirmed also by embryotoxicity testing, where PANI-DEPH exhibited the severe embryotoxicity whilst for PPy-DEPH the embryotoxic effect was absent.

The **Fig. 7** summarizes the results of the conductivity measurements of CP doped with organic phosphonates under study at various pH. These results provide further insight into relation of conductivity and pH in the interval of pH 3-9. It can be clearly seen that there was a decrease in the conductivity with the increasing pH value from 3 to 9 in both of the examined samples of PANI-S and PPy-S. While the conductivity decrease in PANI is dramatic and limits the use of this polymer under physiological conditions, the reduction of the conductivity of PPy is marginal. Several differences can be found between polymers doped with different phosphonates: (1) At low pH values, both polymers show the highest conductivity when doped with DPPH. Based on the combination of Raman and EPR spectroscopy the higher conductivity of PANI-DPPH compared to other samples has been explained by a higher polaron delocalization and mobility **[13]**.

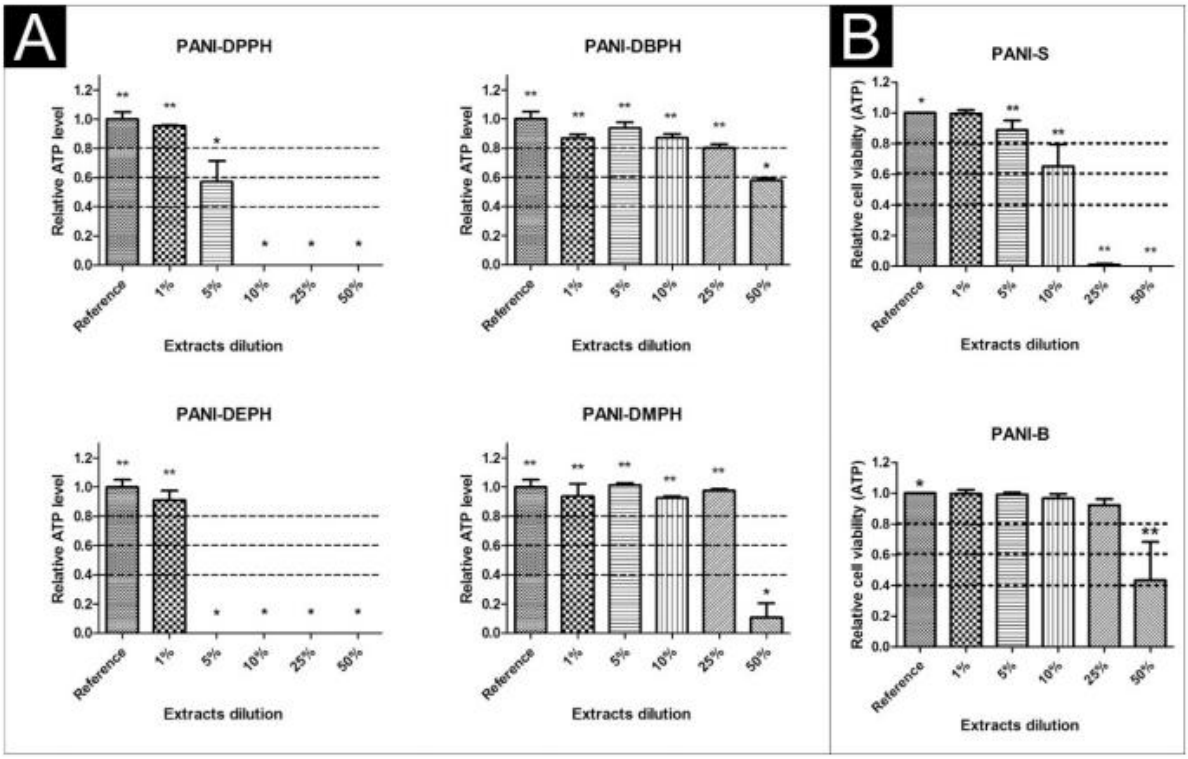

Fig. 4. Cytotoxicity of extracts of PANI on ESC. (A) PANI doped with phosphonates, (B) pristine PANI salt (PANI-S) and base (PANI-B) prepared according to IUPAC technical report **[16]**. Reproduced from **[20]**. The different superscripts correspond to significant differences ($P \leq 0.05$) compared to the reference. The dashed lines highlight the limits of viability according to EN ISO 10993-5 with modification: viability > 0.8 corresponds to no cytotoxicity, > 0.6-0.8 mild cytotoxicity, > 0.4-0.6 moderate cytotoxicity and < 0.4 severe cytotoxicity.

Particularly large increase of conductivity (4 orders of magnitude higher than other systems under study) was found for PANI-DPPH. PPy-DPPH had also higher conductivity compared to other systems at pH 3 but the difference was much smaller, less than one order of magnitude. (2) With increasing pH, the conductivity was found to decrease in all systems but for PANI-DPPH and PANI-DEPH the decrease was smaller for pH < 5. On the other hand, the behaviour of PPy-DPPH is different: there is a steep decrease of conductivity when going from pH 3 to 4.

The trends in conductivity development with pH agree well with data reported in previous study dealing with PANI doped with the same organophosphonate dopants **[13].** However, with the exception of PANI-DPPH the conductivities determined in current work are markedly lower in comparison with those reported in **[25]**. The reason could be fact that the measurements in our work started first at pH 3, compared to lower starting pH value used in the study of Bláha et al. **[13]** where the measurements were performed at more acidic state after doping with phosphonates. The increase in the conductivity of PANI with the doping using various phosphonates correlates well with their expected increasing acidity from DMPH through DEPH, DBPH and DPPH, as evaluated from the chemical shift in the ^{31}P NMR spectra **[26]**. The higher values of conductivity found for PANI-DPPH and PANI-DEPH correlate also well with the increased level of their cytotoxicity.

Table 1 The impact of extracts of PANI-phosphonates on cardiomyogenesis (expressed as percentage of EBs with beating foci) and erythropoiesis (expressed as percentage of EBs with erythroid clusters). Comparison with pristine PANI-S and PANI-B.

		EBs with formed foci or clusters [%]					
	Extract dilution [%]	DPPH	DBPH	DEPH	DMPH	PANI-S [a]	PANI-B [a]
EBs with beating foci	Reference	100	100	100	100	100	100
	1	50	100	0	100	100	100
	5	0	100	0	100	50	100
	25	0	100	0	100	0	100
EBs with erythroid clusters	Reference	100	100	100	100	100	100
	1	50	100	0	100	100	100
	5	0	100	0	100	13	100
	25	0	100	0	100	0	100

[a] *Reproduced from Humpolíček et al.* ***[20].***

Since the conductivity depends on the type and dopant content, both the higher cytotoxicity and higher conductivity might be explained by higher content of the phosphonate dopants **[8]**. It is, however important, that in case of PANI the doping by phosphonates, especially by DPPH improves the pH stability of PANI, including the stability in physiological pH region. In the case of PPy, both the PPy-S and phosphonate doped PPy systems shoved better stability in increased pH compared to the respective systems with PANI but no further improvement of the stability with the phosphonate dopants was observed.

Humpolíček et al. **[20]** proved in their work similar cytotoxicity for PANI-S and PPy-S extracts. It is, therefore worth recording that these polymers when re-doped with phosphonates exhibit notably lower cytotoxicities. In this respect it can be mentioned that pyrrole, a monomer used for polypyrrole synthesis, is an interesting bioactive molecule and has numerous applications in therapeutically active compounds including fungicides, antibiotics, anti-inflammatory drugs **[27]**, cholesterol reducing drugs **[28]** or antitumor agents. Therefore, the combination of this active molecule with phosphonates might be the reason for better biological activity of PPy samples in the comparison with polymers where the precursor of PANI, aniline, is employed, as it is known for its cytotoxicity.

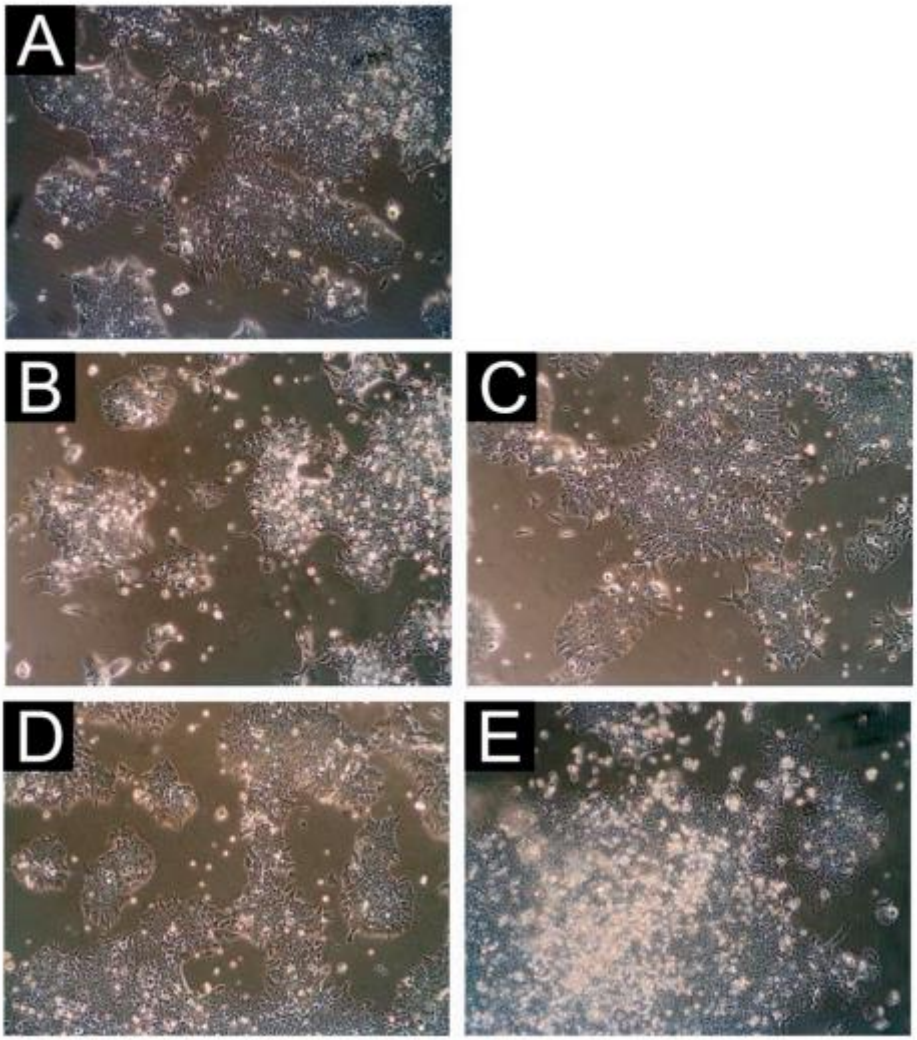

Fig. 5. The morphology of ESC after exposition to the extracts. A) Reference; B) PPy-DPPH 50%; C) PPy-DBPH 50%; D) PPy-DEPH 50%; E) PANI-DMPH 50%. Magnification 40x.

4. Conclusions

The results demonstrate that the major barrier of practical use of CP and mainly of PANI, the limited conductivity under physiological conditions, can be overcome by their doping with organic phosphonates. Though published studies report on several ways of improving pH stability of conductivity, none of the studies provided an insight into the biocompatibility of these polymers. The findings from this study suggest that PPy in combination of DPPH, DEPH, DBPH, DPPH and PANI in combination of DBPH, and partially with DMPH provide attractive properties for applications in biomedicine thanks to their low cytotoxicity and reasonable high level of conductivity at physiological pH. Regrettably, PANI-DPPH and PANI-DEPH exhibit rather high cytotoxicity but their conductivities were reported to be higher than those of other tested samples. There was a significant correlation between high cytotoxicity and high values of conductivity of PANI doped with DPPH and DEPH. An extended study with more focus on investigation of interactions between phosphonates and CP can be, therefore, of high interest. We conclude that especially PPy doped with phosphonates with improved properties could be considered as a novel biomaterial with added conductivity value.

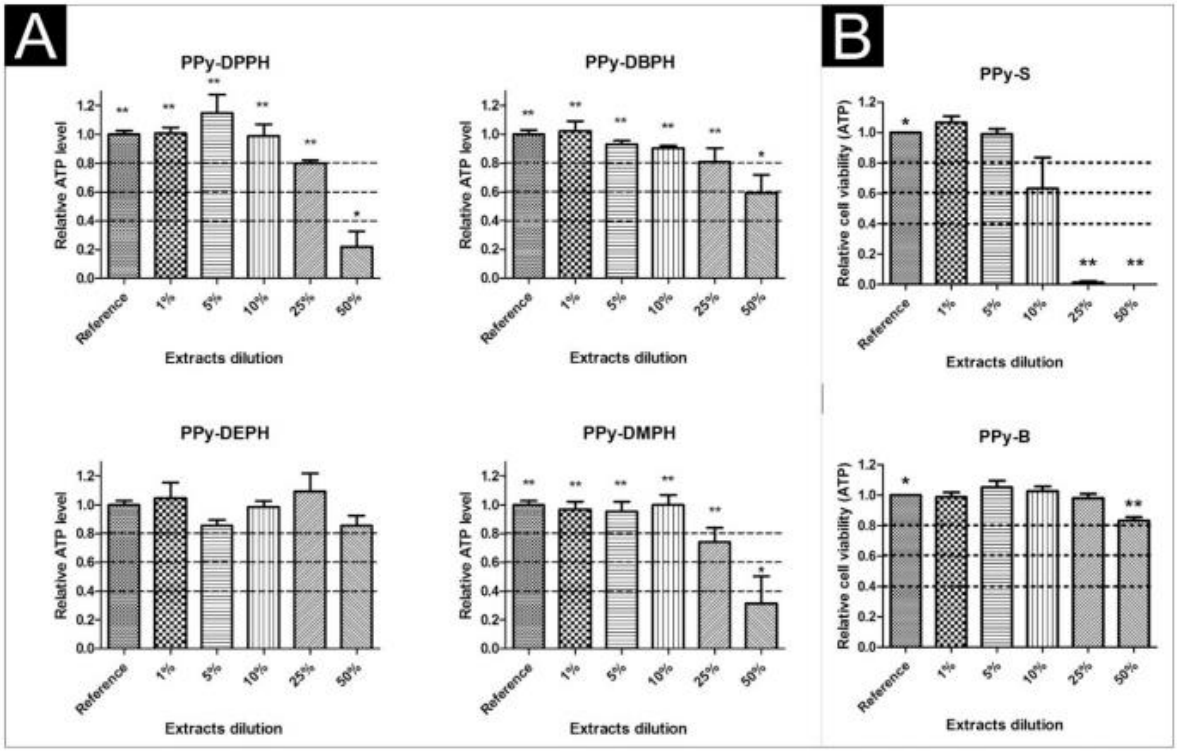

Fig. 6. Cytotoxicity of extracts of PPy determined on ESC. (A) PPy doped with phosphonates, (B) pristine polypyrrole salt (PPy-S) and base (PPy-B) prepared according to **[20]** and reproduced from the same study. The different superscripts correspond to significant differences ($P \leq 0.05$) compared to the reference. The dashed lines highlight the limits of viability according to EN ISO 10993-5 with modification: viability > 0.8 corresponds to no cytotoxicity, $>$ 0.6-0.8 mild cytotoxicity, $>$ 0.4-0.6 moderate cytotoxicity and < 0.4 severe cytotoxicity.

Table 2 The impact of extracts of PPy-phosphonates on cardiomyogenesis (percentage of EBs with beating foci) and erythropoiesis (percentage of EBs with erythroid clusters). Comparison with pristine PPy-S and PPy-B.

		EBs with formed foci or clusters [%]					
	Extract dilution [%]	DPPH	DBPH	DEPH	DMPH	PPy-S [a]	PPy-B [a]
EBs with beating foci	Reference	100	100	100	100	100	100
	1	100	100	100	100	100	100
	5	100	100	100	100	75	100
	25	100	100	100	100	0	100
EBs with erythroid clusters	Reference	100	100	100	100	100	100
	1	100	100	100	100	75	100
	5	100	100	100	100	50	100
	25	100	100	100	88	0	100

[a] *Reproduced from Humpolíček et al.* ***[20].***

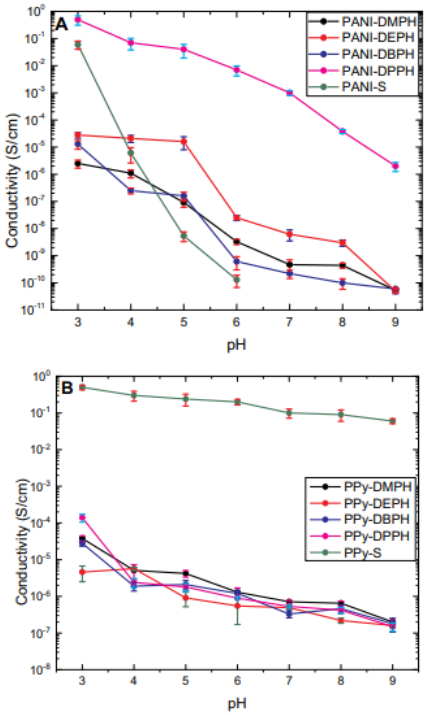

Fig. 7. The conductivity of A) PANI and B) polypyrrole doped with organic phosphonates under various pH and the comparison with pristine PANI-S and PPy-S.

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