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The biocompatibility of polyaniline and polypyrrole: A comparative study of their cytotoxicity, embryotoxicity and impurity profile

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ABSTRACT

Conducting polymers (CP), namely polyaniline (PANI) and polypyrrole (PPy), are promising materials applicable for the use as biointerfaces as they intrinsically combine electronic and ionic conductivity. Although a number of works have employed PANI or PPy in the preparation of copolymers, composites, and blends with other polymers, there is no systematic study dealing with the comparison of their fundamental biological properties. The present study, therefore, compares the biocompatibility of PANI and PPy in terms of cytotoxicity (using NIH/3T3 fibroblasts and embryonic stem cells) and embryotoxicity (their impact on erythropoiesis and cardiomyogenesis within embryonic bodies). The novelty of the study lies not only in the fact that embryotoxicity is presented for the first time for both studied polymers, but also in the elimination of inter-laboratory variations within the testing, such variation making the comparison of previously published works difficult. The results clearly show that there is a bigger difference between the biocompatibility of the respective polymers in their salt and base forms than between PANI and PPy as such. PANI and PPy can, therefore, be similarly applied in biomedicine when solely their biological properties are considered. Impurity content detected by mass spectroscopy is presented. These results can change the generally accepted opinion of the scientific community on better biocompatibility of PPy in comparison with PANI.

1. Introduction

The impact of bioelectricity on physiological processes can be observed either on the level of individual cells, *e.g.,* the stem cell differentiation [1] and cell movement [2], or on the level of tissues, *e.g.*, the physiology of electro-sensitive tissues or wound healing [3]. In the preparation of a biocompatible biointerface, the combination of electronic and ionic conductivity is one of the key parameters; in this respect, conducting polymers (CP) are considered to be an excellent solution as they inherently combine both of these types of conductivities [4]. Though polyaniline (PANI) and polypyrrole (PPy) are the most studied conducting polymers, and there are a number of studies dealing with their preparation, characterization, and physico-chemical properties, the works which focus on their comparison are scarce as regards their chemistry [5,6] and especially their biological properties [7,8].

Both PANI and PPy have been used extensively for the preparation of composites with other materials and subsequently tested in terms of their biological properties, such as their *in vivo* capacities to cause reactions in tissues [9,10]. In spite of this, however, there are few studies describing the biological properties of pure PANI (*e.g.* in the form of powder [11]; colloids [12] or films [13,14]) or PPy [15]. This lack of knowledge is critical if biologically-oriented applications are to be considered.

The crucial characteristic of any material used in tissue engineering is its biocompatibility. It is very well known that biocompatibility is a complex characteristic combining a range of individual biological properties which are preferably tested using alternative *invitro* methods. Although *invitro* experiments can provide valuable information, its interpretation still presents a challenge, as any model (such as different cell lines) can behave differently and different conclusions can therefore be drawn. When comparing results published in literature, inter-laboratory variations in assay performance must also be considered, as the number of tests is not validated and each laboratory can apply a slightly different experimental set-up. For example, such variation and inconsistency can occur as a result of the procedures for handling embryonic stem cells in individual laboratories. The presented work is,

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therefore, aimed at performing and discussing a complex comparative study of the fundamental biological impacts of PANI and PPy on NIH/3T3 fibroblasts, embryonic stem cells (ESc), and embryoid bodies (EBs) observed under the same conditions. Fibroblasts were chosen as they are the most frequently used line for the determination of cytotoxicity; ESc are a lineage with considerable potential for application in biomedicine and tissue engineering thanks to their naive phenotype and ability to differentiate into a variety of cell lineages; and the choice of EBs was motivated by the fact that their interaction with materials can be used as a marker of embryotoxicity.

The motivation for this study emerged from long term experience with CP and because PPy is understood by the scientific community to be more biocompatible than PANI even though the supposed superiority of PPy is not based on any experimental data.

2. Material and methods

2.1. Preparation and characterization of polymers

Polyaniline salt (PANI-S) was prepared using a standard procedure [16]. Specifically, a 0.2 M aqueous solution of aniline hydrochloride (Penta, Czech Republic) was oxidized with 0.25 M ammonium peroxydisulfate (APS; Penta, Czech Republic). Polymerization was carried out at room temperature for 12 h. The resulting green solids of polyaniline salt were collected on a filter, rinsed with 0.2 M hydrochloric acid, and similarly with acetone, and dried at room temperature over silica gel.

Polypyrrole salt (PPy-S) was synthetized by oxidizing 0.2 M pyrrole (Sigma-Aldrich) with 0.5 M iron (III) chloride hexahydrate (Sigma-Aldrich) in an aqueous environment. The oxidant to pyrrole mole ratio was 2.5. The mixture was left to polymerize at room temperature for 12 h. The black solids were collected on a filter and, similarly to PANI-S, rinsed with 0.2 M hydrochloric acid followed by acetone, and dried at room temperature over silica gel.

A part of both, PANI-S and PPy-S were deprotonated to polyaniline base (PANI-B) and polypyrrole base (PPy-B) by immersing the solids in an excess of 1 M aqueous ammonium hydroxide. SEM photomicrographs were captured using a JEOL 6400 microscope.

2.2. Preparation of polymer extracts

Samples were extracted according to a modification of protocol ISO 10993-12. The modification of the standard procedure involved the ratio between the mass of the extracted samples and the volume of the extraction medium. The standard procedure employs 0.2 g polymer per 1 mL cultivation medium. As the PPy samples were very fluffy and extremely difficult to separate from the medium after extraction, the ratio of 0.05 g of powder per 1 mL of cultivation medium was used for all tested samples. In this way a sufficient volume of each polymer extract was obtained for testing. Extraction was performed in chemically inert closed containers using an aseptic technique at 37 ± 1 °C under stirring for 24 h. The extracts were separated from the powders by double centrifugation at 1000 g for 15 min. The parent extracts (100%) were then diluted in a complete medium to obtain a series of dilutions with concentrations of 1, 5, 10, 25, and 50%. All extracts were used within 24 h. Prior to *in-vitro* testing, the samples were disinfected by means of sterile filtration through a 0.22 μm syringe filter (Millipore). Each of the concentrations was tested in quadruplicates, in four separate sets of experiment.

For mass spectroscopy analyses, the polymers were extracted using the procedure described above, but with deionized water as the extraction medium.

2.3. Characterization of polymer extracts

Aqueous polymer extracts were analysed using a 1260 Series liquid chromatography system (Agilent Technologies, Santa Clara, CA) coupled to a 6520 Accurate-Mass Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with a dual-spray electrospray ionization source. Aliquots of 5 μL were injected as an infusion into the system with no column installed. Compounds were eluted at 30 °C with an isocratic flow rate of 0.3 mL min^{-1} of 1% (v/v) formic acid in water. The positive ion mode mass spectrometry conditions were as follows: gas temperature, 300 °C; fragmentor voltage, 75 V; capillary voltage, 3.000 V; nozzle voltage, 2000 V; scan range m/z 50 to 1700; 1 scan/s. The internal mass reference ions m/z 121.050873 and m/z 922.009798 were used to keep the mass axis calibration stable during the analysis.

2.4. Cell lines and media

2.4.1. Mouse embryonic fibroblast cell line NIH/3T3 (ATCC CRL-1658TM)

ATCC–formulated Dulbecco's Modified Eagle's Medium, catalogue no. 30-2002, with added calf serum (BioSera, France) to a final concentration of 10% and penicillin/streptomycin, 100 U mL^{-1} (GE Healthcare HyClone, UK) was used as the culture medium.

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 gelatine. Complete media containing Dulbecco's Modified Eagle's Medium (DMEM), 15% fetal calf serum, 100 U mL^{-1} penicillin, 0.1 mg mL^{-1} streptomycin, 1 × non-essential amino acids solution (all from Gibco-Invitrogen; USA), 0.05 mM 2-mercaptoethanol (Sigma–Aldrich; USA), and 1000 U mL^{-1} leukemia inhibitory factor (Chemicon; USA) were used for the cultivation [13].

2.4.2. Cytotoxicity on ES R1 and NIH/3T3 cell lines

Cytotoxicity testing was performed according to ISO protocol 10 993-5. Cells were pre-cultivated for 24 h and seeded at a density of 5000 cells per cm^2 in the case of ESc or 12,000 per cm^2 in the case of NIH/3T3. The extracts were applied onto cells for 48 h (ESc) or 24 h (NIH/3T3). To assess the cytotoxic effects of PANI and PPy extracts on ESs, the mass of viable cells was determined as the level of ATP using Cellular ATP Kit HTS (Biothema, Sweden). Samples were prepared and analysed as published [18]. Before lyses, the morphology of the cells was observed and documented using an inverted Olympus phase contrast microscope (Olympus IX51, Japan) fitted up with a digital camera (Olympus E-450, Japan). To assess the viability of NIH/3T3 cells, MTT assay was used [19]. As a reference giving 100% cell viability, cells cultivated in pure complete media were used. The results are presented in two different ways. In addition to the strict processing of data according to the requirements of ISO 10 993-5 standard, statistical evaluation was also conducted using one-way analysis of variance ANOVA. ATP and MTT assays were evaluated using a Luminometer Infinite m200pro (Tecan, Switzerland).

2.4.3. Embryotoxicity

Embryotoxicity was determined as the likelihood of the formation of beating foci (the impact on cardiomyogenesis) and erythroid (red) clusters/colonies (the impact on erythropoiesis) within spontaneous differentiating ESc after exposure to the studied extracts. ESc differentiation was induced through the formation of embryoid bodies (EBs) by hanging drop techniques (400 cells per one 35 μL drop) in leukemia-inhibitory-free complete media described in ([20]. After 5 days, EBs were transferred to a gelatinized 24-well plate (one EB per well) in serum-free medium for the next 15 days and the medium was replaced with fresh medium every three days. Serum-free medium contained DMEM-F12 medium (1:1), 100 U mL^{-1} penicillin, 0.1 g mL^{-1} streptomycin, and 1 × insulin-transferrin-selen (ITS) supplement (all from Gibco-Invitrogen; USA). Differentiating cells were observed and documented using an inverted Olympus phase contrast microscope (Olympus IX51, Japan) equipped with digital camera (Olympus E-450, Japan) [13].

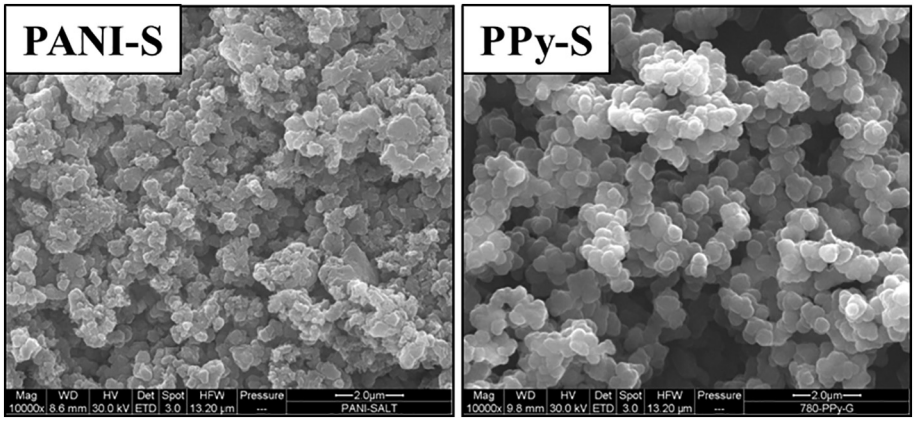

Fig. 1. The morphology of PANI-S and PPy-S visualized by SEM.

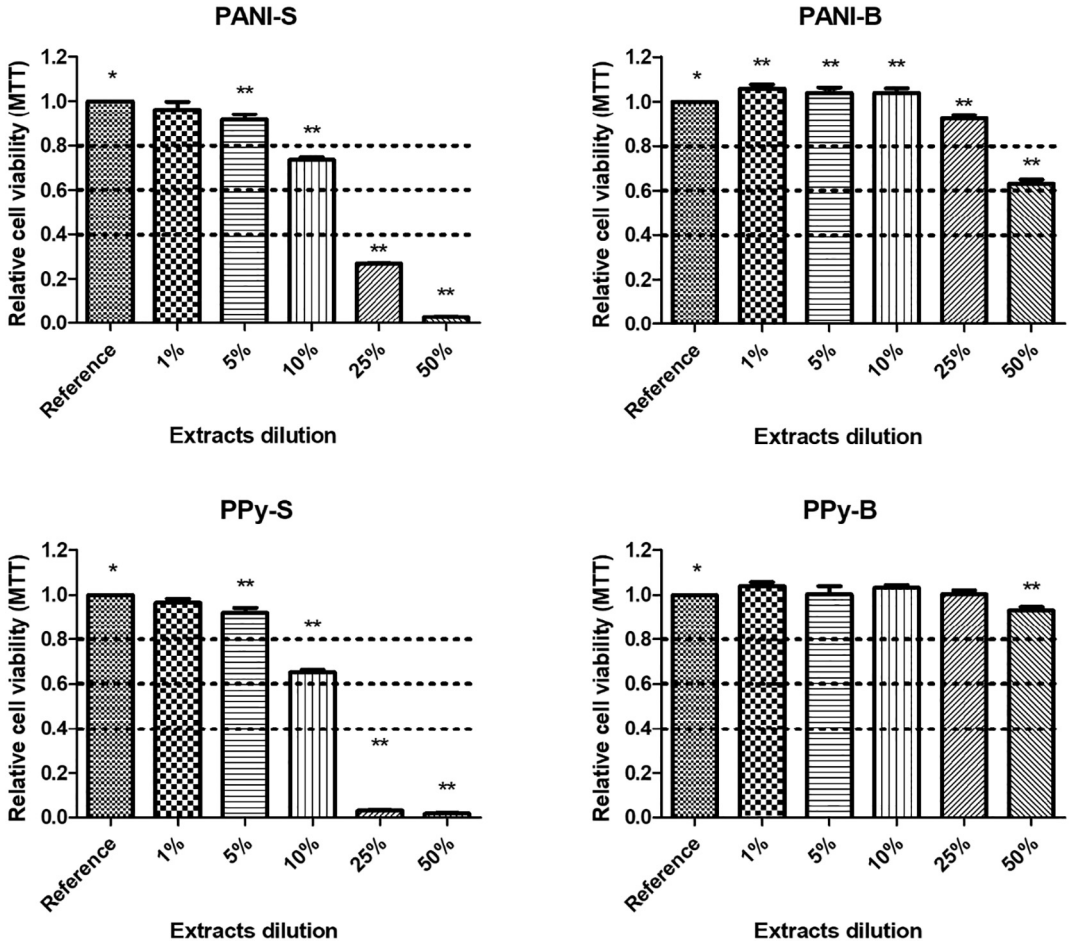

Fig. 2. Cytotoxicity of extracts of PANI and PPy towards NIH/3T3 cells determined by MTT assay. The different superscripts correspond to significant differences ($P \leq 0.05$) compared to the reference. The dashed lines highlight the limits of viability according to EN ISO 10993-5: viability > 0.8 corresponds to no cytotoxicity, > 0.6–0.8 mild cytotoxicity, > 0.4–0.6 moderate cytotoxicity and < 0.4 severe cytotoxicity.

3. Results and discussion

Each of the studied polymers was synthesized using the most common preparation procedure employed within the chemical oxidation routes. Therefore, PANI was synthetized using the oxidation of aniline hydrochloride with ammonium persulfate according to the respective IUPAC protocol [16], and PPy was prepared *via* the oxidation of pyrrole with iron(III) chloride, which is the oxidant of first choice in the preparation of this polymer (Fig. 1) [21,22].

There is a common opinion in the scientific community and literature that PPy is more biocompatible than PANI. This generally accepted opinion is, however, based on indirect comparisons of studies conducted on these polymers [23]. The present study is the first to compare the biological properties of these two most important CP determined under the same conditions, using the same cell lineages and methodology, and performed in the same laboratory. The results are therefore unique and difficult to compare meaningfully with those published in any previous works. Previous

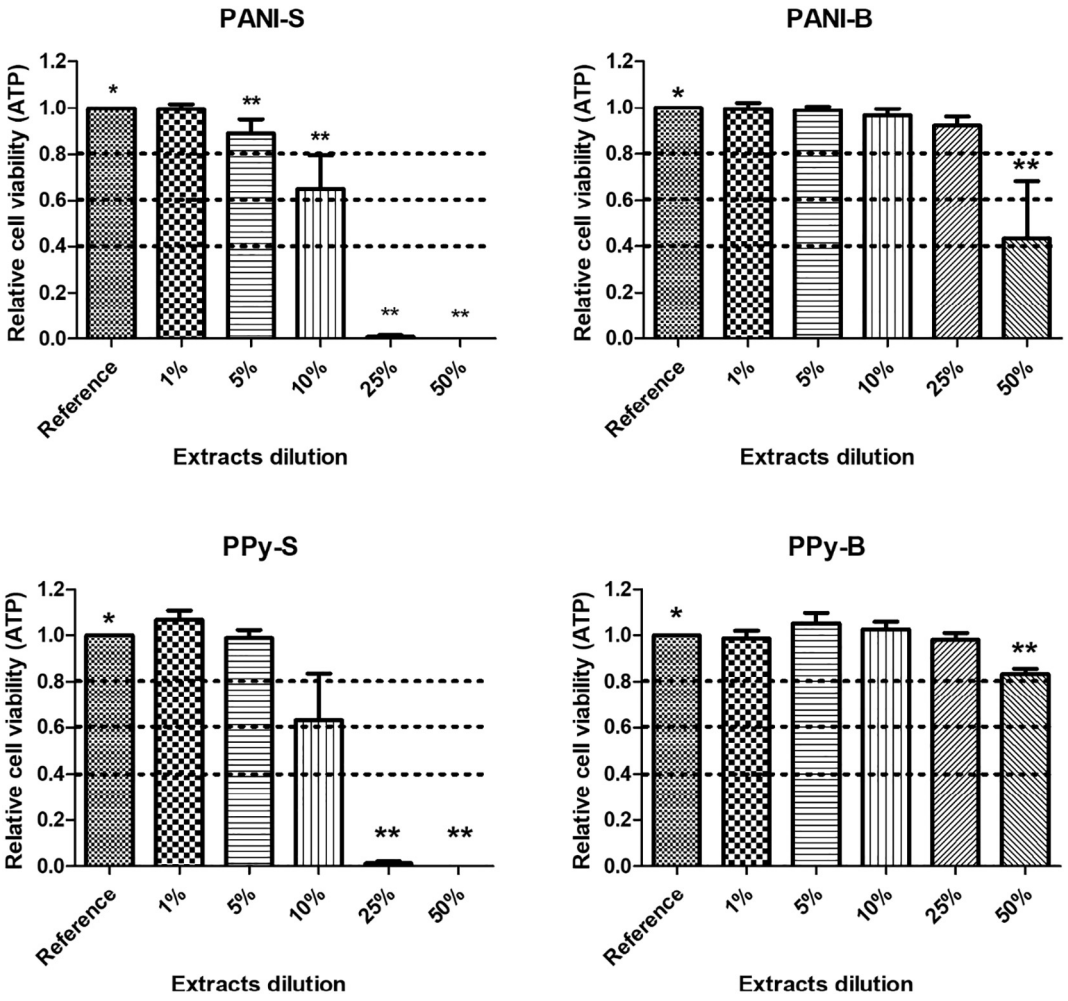

Fig. 3. Cytotoxicity of extracts of PANI and PPy on ESc determined by the relative level of ATP compared to the reference. The different superscripts correspond to significant differences ($P \leq 0.05$) compared to the reference. The dashed lines highlight the limits of viability according to EN ISO 10993-5: viability > 0.8 corresponds to no cytotoxicity, > 0.6–0.8 mild cytotoxicity, > 0.4–0.6 moderate cytotoxicity and < 0.4 severe cytotoxicity.

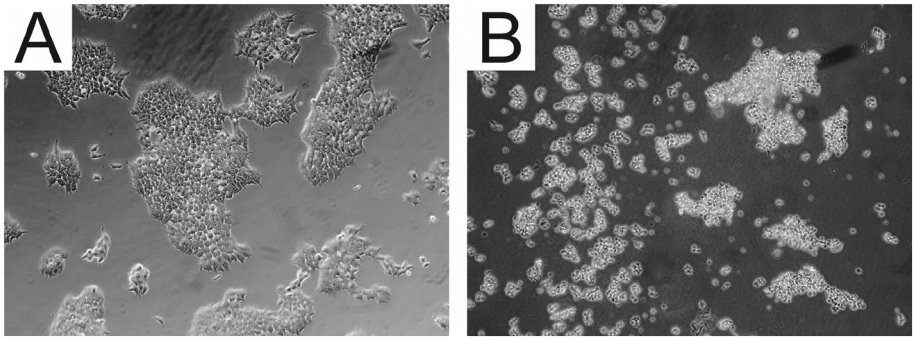

Fig. 4. The cytotoxicity of a tested sample towards ESc. A) reference, B) cytotoxic effect observed after the application of the 5% extract of PPy-S. Cell destruction after the application of PPy extract is obvious. Magnification: 100×.

studies mainly investigated these polymers separately, using different methodologies, test protocols, and ways of processing the results obtained. Moreover, most of these works do not examine these CP alone, but their composites or blends with other thermoplastic polymers [24,25].

Therefore, the motivation of this study was to provide a comprehensive view of the biological properties of PANI and PPy as a base line for additional advanced studies dealing with the exploitation of these promising CP in biomedicine, regenerative medicine, and biosensors in electro-sensitive tissues.

Cytotoxicity tests are the test of first choice when the biocompatibility of materials, including polymers, is evaluated. According to the EN ISO 10993 protocol, mouse fibroblasts are the cells most commonly used to determine the cytotoxicity of polymers after the application of their extracts. Due to advances in the biomaterial sciences, ESc are also frequently used to determine and evaluate the biocompatibility of materials and products. Thus, the cytotoxicities of PANI and PPy were determined not only using NIH/3T3 fibroblasts but also ESc. The results regarding NIH/3T3 cells are presented in Fig. 2 and illustrate that the extracts of both salts, PANI-S and PPy-S, lost their cytotoxicity at concentrations of 5% and below. In contrast, PPy-B did

Table 1
Cardiomyogenesis or erythropoiesis observed after contact of ES R1 ECs with PANI and PPy extracts. Results are expressed as a number of beating foci or erythroid clusters relative to the reference.

	Formed beating foci [%]			
Extract concentration [%]	PANI-S	PANI-B	PPy-S	PPy-B
Reference	100	100	100	100
1	100	100	100	100
5	50	100	75	100
25	0	100	0	100
	Formed erythroid clusters [%]			
Reference	100	100	100	100
1	100	100	75	100
5	13	100	50	100
25	0	100	0	100

not induce any cytotoxicity, even when NIH/3T3 cells were cultivated in the presence of 50% extract; in the case of PANI-B the corresponding effect was observed at an extract concentration of 25%. The results unambiguously illustrate that PANI and PPy in their base form are notably less cytotoxic than both polymers in the form of salts. With respect to ECs (Figs. 3 and 4), the results were very similar to those for NIH/3T3 cells. That is, both bases exhibited notably lower cytotoxicity in comparison with the corresponding salts.

The embryotoxicity of PANI and PPy was studied with respect to the spontaneous differentiation of ES R1 cells using two parameters, namely the formation of beating foci, which is a marker of cardiomyogenesis, and the formation of erythroid clusters as a marker of erythropoiesis.

The results of the embryotoxicity test summarised in Table 1 show only minor differences within the two sets of samples, namely when the PANI-S with PPy-S and PANI-B with PPy-B are compared. Both PANI-B and PPy-B performed equally and the levels of formation of beating foci and erythroid clusters even corresponded to the reference. Therefore, it was not possible to determine the threshold concentration of extract at which cardiomyogenesis or erythropoiesis were influenced by these polymer extracts. The impacts of the PANI-S and PPy-S were, however, different, and their extracts terminated cardiomyogenesis and erythropoiesis in all EBs at a concentration of 25% in cultivation medium. The example of erythroid clusters formation is shown on the Fig. 5. The most adverse effect on erythropoiesis was exhibited by the PPy-S, which was capable of terminating 25% EB at an extract concentration of 1%. Similarly to cytotoxicity, bigger differences were observed between the behaviours of the respective salts and bases than between the pure forms of PANI and PPy.

To the best the authors' knowledge, there is only one previously published comprehensive study focused on the cytotoxicity of PANI in its native globular form [11] and one study dealing with the cytotoxicity of PPy prepared by the standard oxidation method [10] including both *in-vitro* and *in-vivo* investigations. In both of these studies, biocompatibility was assessed using extracts of polymers in culture medium or saline. There are also other studies concerning the cytotoxicity of PPy, but focused on their nanoparticle form [26,27], which make their results non-comparable to ours as the methodology of preparation is different. It is also well known that cytotoxicity of nanoparticles is different from that of the polymer in globular form and, in addition to chemical composition, it depends also on size and shape of the nanoparticles.

As mentioned above when describing the methods used for investigating the biological properties of PANI and PPy, a ratio of 0.05 g to 1 mL^{-1} of cultivation medium instead of the ISO-defined ratio of 0.2 g to 1 mL^{-1} was used to prepare the extracts of all samples in the test. The reason for this modification was the difficulty of separating PPy from the supernatant (medium) after polymer extraction. For the sake of comparison, PANI extracts were prepared in the same manner. The different starting concentrations of extracts obtained in this work make meaningful comparison of our results with those in literature somewhat complicated. Nevertheless, the comparison was performed and the current results were correlated with those reported by Humpolíček et al. [11] who investigated cytotoxicity of standard extracts (0.2 g to mL^{-1}) of PANI salt and PANI base. For comparison purposes, it can be considered that concentrations of extracts from current work are one-fourth of the concentrations prepared by standard procedure. In the work of Humpolíček, the cytotoxicity towards HaCaT and HepG2 cells was determined, revealing non-cytotoxic threshold concentrations of 1% for PANI-salt and 10% for PANI-base. These results are in accord with those observed for NIH/3T3 and ESc in the current study, which showed an absence of cytotoxicity at extract concentrations of 5% for PANI-S and 25% for PANI-B. When considering the use of different cell lines in both studies and fact that correlation of cytotoxicity with concentrations of impurities in an extract may not always be strictly linear, it can be concluded that the cytotoxicities of PANI-S and PANI-B, as determined in the current study, are similar.

The biocompatibility of PPy was determined by Wang et al. [10] using PPy-salt extracted at a ratio of 1 g to 10 mL saline, according to ISO protocol 10,993. Their work demonstrated that the viability and proliferation rates of Schwann cells in the presence of PPy extract in culture medium at a concentration of 25% (in the original article, referred to as 100 μL of 50% extract and 100 μL of cultivation medium) even improved in comparison with the control sample containing a corresponding amount of saline solution in medium, and equalled the cell behaviour in plain medium. The authors hence concluded that the polymer extract exhibited the absence of cytotoxicity. The current study, however, showed that PPy-S samples were cytotoxic down to a concentration of 5% and that cytotoxicity was reduced by transferring PPy-S to its base form (PPy-B), which was non-cytotoxic in the presence of 50% extract. Though the polymers from these two studies were prepared by the oxidation of pyrrole with $FeCl_3$, different concentrations and ratios of individual monomers were used during syntheses; moreover, Wang et al. provided no information on the purification of the polymer used.

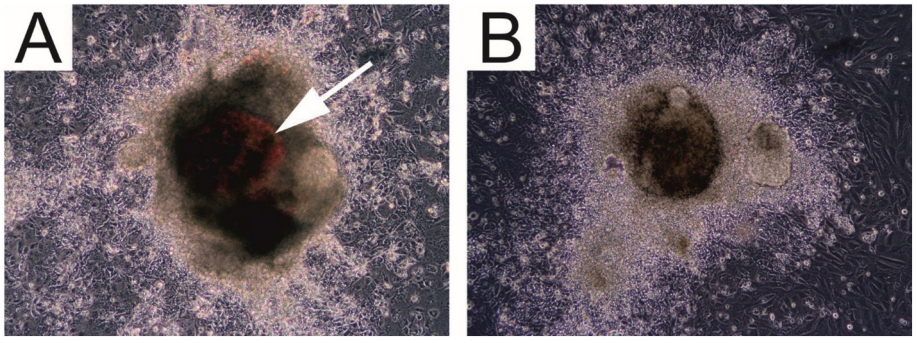

Fig. 5. The formation of erythroid clusters (red cluster marked with arrow) within the embryoid body. A) positive reference; B) absence of the red erythroid cluster after cultivation in the presence of 25% extracts of PANI-S. Magnification 100×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

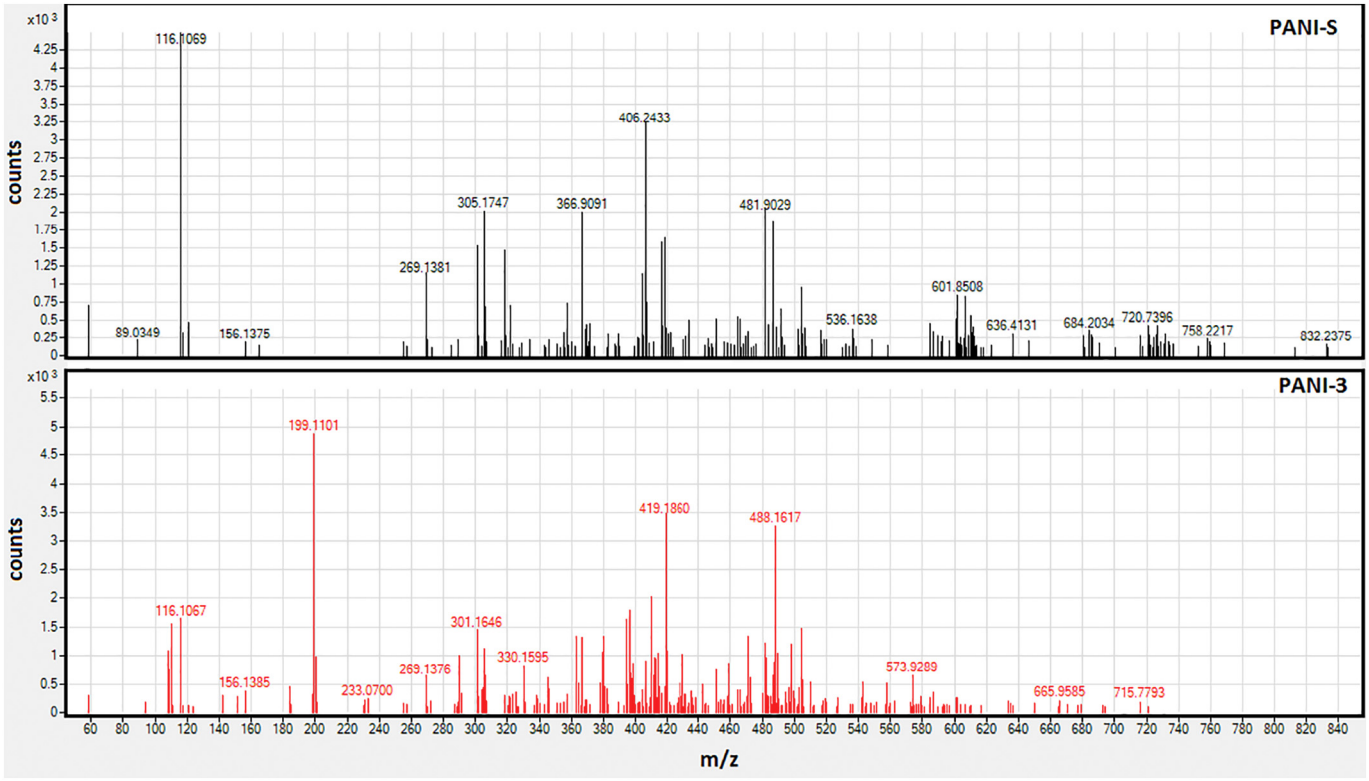

Fig. 6. Mass spectra of PANI-S and PANI-B samples.

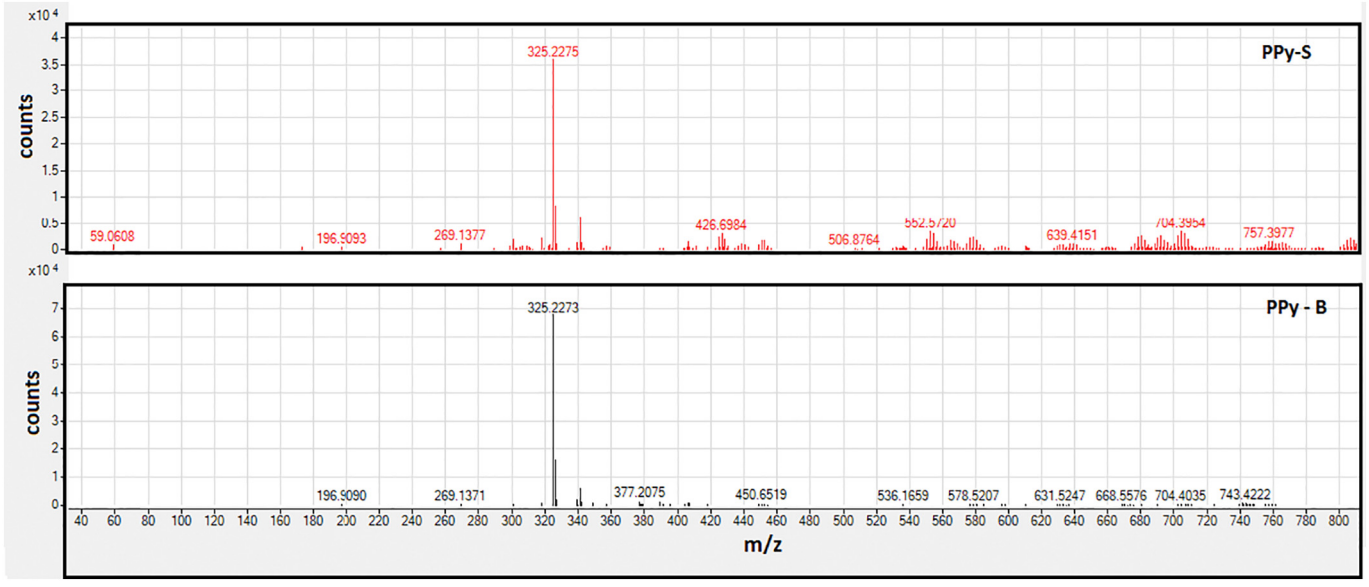

Fig. 7. Mass spectra of PPy-S and PPy-B samples.

It is, however, much more important that, according to the results in Figs. 2 and 3, the cytotoxicities of PANI and PPy in their respective forms are comparable. To be more specific, the cytotoxicity depends more on the form of PANI or PPy (salt *vs* base) than on the type of polymer (PANI *vs* PPy). It should, however, be stressed that the current work covers only the most commonly used ways of synthesizing these conducting polymers. Considering other possible oxidation agents, whether of chemical or biological origin, the situation with respect to cytotoxicity might be different.

The impact of PANI and PPy on erythropoiesis and cardiomyogenesis has never been studied before. Nevertheless, it can be concluded that the threshold concentrations at which embryogenesis processes are influenced correlate with the cytotoxicities of individual polymer extracts. Similarly to cytotoxicity, embryogenesis and cardiomyogenesis depends more on the form of polymer (salt *vs* base) than on its type (PANI *vs* PPy).

As already mentioned, systematic work was previously undertaken to determine the reasons for the cytotoxicity of PANI. Based on the work of Stejskal et al. [28] and Kašpárková et al. [29] it can be assumed that such cytotoxic effects are mainly connected with the presence of low-molecular-weight impurities. In order to improve our view of the correlation between biocompatibility and the contents of impurities,

mass spectroscopy analyses of the studied samples were conducted with the aim of obtaining an insight into the structures of impurities extracted from polymers that might be responsible for their cytotoxic effects. The mass spectra of PANI-S and PANI-B are presented in Fig. 6. As can be seen, the samples contain a relatively rich mass profile with several major molecular ions $(M^+H)^+$. By comparison, it can be noticed that the PANI-S sample comprises slightly more fractions with molecular weights higher than 700 g mol^{-1} than the PANI-B sample. On the other hand, PANI-B exhibits a more complicated mass profile especially in the region 300–550 *m/z*. Although many signals of various intensities are present in both samples, it is obvious that PANI-B contains masses not found in PANI-S, such as those in the low *m/z* region – for example, 199.1101 and 233.0700. Of all the masses present, only the following were identified in PANI-S: aniline (*m/z* = 94.0645), *p*-benzoquinone imine (*m/z* = 108.0682), and *p*-aminophenol (*m/z* = 110.0599). Also tetramer (*m/z* = 290.1297, [30]) and quinoneiminoid structure (*m/z* = 290.1297) proposed by Kříž et al. [31], and Stejskal and Trchová [32] were assigned as possible impurities present in the extract.

As shown in Fig. 7, both PPy extracts exhibited much simpler mass spectra than extracts of PANI. In PPy-S and PPy-B, one dominant ion, *m/z* = 325.2275, was found, while higher masses were detected only with low intensities, in particular in PPy-S extracts. The substance corresponding to this mass, however, is probably non-cytotoxic as it is present in both samples. An interesting question therefore arises as to which of the substances present in PPy-S are involved in cytotoxic effects.

After careful inspection of MS data, it can be concluded that it was not possible to detect linear oligomers as possible impurities, as they were probably absent from the samples. The exact masses calculated, however, indicate that the impurities extracted from the samples comprise oxygen and that, therefore, oxygen containing by-products are probably the most frequent impurities in the samples.

In spite of the limited success of MS in identifying impurities in PANI and PPy, these spectroscopic analyses showed that both PANI-B and PPy-B contain lower numbers and amounts of impurities and have relatively simple impurity profiles. The reduced contents of impurities logically reflect the process to which both polymers are subjected during the transformation from salt to base. The process of re-protonation can, therefore, be considered as an additional purification step removing substances with potentially cytotoxic effects.

4. Conclusion

It is generally accepted in the scientific community that polypyrrole shows more favourable biological properties than polyaniline, which is reflected by prevalence of publications dealing with the first mentioned polymer. Until now, however, no study provided direct comparison of these two polymers in terms of their biological properties recorded under the same conditions. Therefore, both polypyrrole and polyaniline were synthetized by the most common procedures and studied within one laboratory to eliminate the inter-laboratory differences. Two parameters of biocompatibility were studied; the first, basic one - cytotoxicity was investigated using common fibroblasts NIH/3T3 cell line and embryonic stem cells. The second, advanced parameter - embryotoxicity was studied in terms of the impact of each of the polymers on the erythropoiesis and cardiomyogenesis within the embryonic bodies. The direct comparison of both polymers using the same methodology showed that the form of the polymer (salt *vs* base) is more important than its type (polypyrrole *vs* polyaniline) when cytotoxicity and embryotoxicity are taken into consideration. Especially the polymers in the form of bases proved low cytotoxicity and embryotoxicity. To clarify the reasons for determined differences in biological properties of polyaniline and polypyrrole, the mass spectroscopy was used to determine their impurity profiles. The detected impurities can't, however, fully explain observed differences. For example, with respect to presence of major molecular ions, the extract of polypyrrole base exhibited simpler mass spectra in comparison with extract of polyaniline base. However, as it was already mentioned their biological properties were similar. Therefore, the presented results should mainly provoke the scientific community to compare also other biological properties of both conducting polymers as, according to currently presented results, they are more similar than previously assumed and their application potential can be, at least, comparable.

Acknowledgments

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