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Research Article

Cytotoxicity of Polypyrrole and Polyaniline Matrixes for Biosensors

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Abstract

Introduction: An emerging alternative for low-cost friendly diagnostic devices is the development of biosensors. Biocompatible polymer semiconductors platforms are promising system for the immobilization of biomolecules ligands.

Objectives: The purpose of the present work was to synthesize polypyrrole (PPy) and polyaniline (PAni) films in order to evaluate the electrical conductivity and biocompatibility.

Methods: Polypyrrole and polyaniline films supported on polyethylene terephthalate (PET) substrate were prepared by aqueous in situ chemical oxidative polymerization of pyrrole and aniline with an oxidizing agent at 0-5°C. The films were cleaned with distilled water under ultrasound and the measurements of the electrical conductivity and the thickness of the films were performed. Biocompatibility test was carried out using NIH-3T3 mouse embryonic fibroblast-like cell line. Cells were placed in culture plates and incubated during 24 or 48 h in presence and absence of polymer films. Polymers were analyzed using the reductase enzymatic activity test by transformation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide into a colored reduced form (MTT).

Results: The best polypyrrole and polyaniline polymer films had electrical conductivity and thickness of 10-2 S/cm; 170 ± 36 nm and 102 S/cm; 318 ± 48 nm, respectively. The viability cell assay indicated that both polypyrrole and polyaniline are non-cytotoxic at the evaluated times.

Conclusions: There were synthesized two new kind of biocompatible polymer films for possible application in the manufacture of conductive matrixes for biosensors platforms.

Keywords: Polypyrrole; Polyaniline; Biocompatibility; NIH-3T3 Cells

Introduction

A biosensor is an analytical device that allows obtaining information in real-time and specific on a measurable biological system (molecules, structures, microorganisms, enzyme, antibody, cells, etc.) through the translating a biochemical interaction at the probe surface into a quantifiable and an amplifier [1]. Many of these biosensors are based on redox reactions detected by molecules in conjunction with an electrode; the reaction is detected electrochemically measuring loss or formation of substrates or products, by carrier electron mediator species. Direct routing between the redox zone of biomolecules and the electrode is difficult to achieve, currently working in the biomolecular surface modifications or implemented on new conductive materials for electrodes, seeking functional biointerfaces design. New organic transducers have been developed that prove to be stable and related to generate new biosensors. Conducting polymers have redox states that allow them to change their shape, conductivity, doping level, color, etc. Some of the more stables conductive polymers are polyaniline (PAni) and polypyrrole (PPy), implicated in many applications [1,2] such as tissue engineering [3,4].

The PAni has been used as base material for sensors and biosensors interfaces; it is effective mediator of electron transfer and enzymatic redox reactions. It can function as a matrix for immobilization of biomolecules due to its electronegativity. PAni is a flexible polymer, highly conductive and of low cost, excellent candidate for biosensors matrices, it also can be deposited directly on electrode surfaces in addition to controlling thickness and conductivity [4]. Besides having a large surface area and thermal stability (depending on synthesis and environment where their do) [2]. However, PAni is limited to a certain extent by its insolubility in aqueous media with poor soluble even in organic solvents. The possible solutions can usually found in copolymerization [5].

The PPy is another very popular conducting polymer because it has a high conductivity, stability and simple synthesis routes, both chemically and electrochemically. There are many studies about its application in sensors, actuators, etc [2,6]. With respect to biosensors, it can be stated that polypyrrole was one of the first conductive polymers most studied for its effect on mammalian cells (endothelial cells, neurons, keratinocytes, fibroblasts, etc.) and mesenchymal stem cells [4]. Different routes of synthesis of PPy have shown that the covalent or non-covalent state optimizes the interactions with specific cell types, improving PPy applications *in vivo* and *in vitro*, in compatibility with cell cultures and in tissue growth supports [4,6].

Both polymers PPy and PAni have been widely used and are therefore carried out all kind of biocompatibility tests on them and their different synthesis routes, showing their strengths as carrier material or matrix for adhesion, growth and cell proliferation [3]. Conductive polymers are considered an emerging group of materials for tissue engineering, for example, skeletal muscle; also provide mechanical and electrical stimuli to the cells. Since the polymers can be modified by various bioactive molecules, can changes its functionality incorporating proteins, peptides, etc.; making them ideal candidates for the growth of neuronal and muscle cells via electrical stimulation. There have been studies the application of pulsed electrical fields stimulation on mouse osteoblast resulted in a significant increase of DNA [7] synthesis whereas a uniform electrical fields influenced the clustering and distribution of proteins on the cell by the surface interface, and was able to control the locomotion in two fibroblast cell lines [8,9]. Biocompatibility of PPy for cell adhesion and proliferation with direct electrical stimulation of neuronal PC-12 lines, primary neuronal tissue, endothelial cells, fibroblasts and keratinocytes [3]. In studies of muscle cell, was measured using the compatibility test LDH (Lactate Dehydrogenase) to quantify the number of cells adhering to the films of PPy doped with different components (HA, DBS, PMAS, etc.) and with different thicknesses. The results of this study showed that some combinations of PPy showed increased adhesion and cell proliferation that others, but on the other hand, cell differentiation was lower, most were acceptable for cell growth [10]. However, the cell line 13S124, belonging to neurons of mouse, designed for cell adhesion on PPy films showed only 20.5% of adherence, when the films were synthesized by electrodeposition [11].

With respect to the PAni, have been studied samples of PAni due to their excellent response to H_2O_2, cholesterol and glucose. Test for PAni Scaffolds have conferred the role of electroactive polymer, candidate for biomedical applications in tissue engineering to of cardiac and neuronal systems [4]. *In vivo* studies showed that PAni in emeraldine base does not produce systemic inflammatory response in rodent models, which shows acceptable tolerance and bio-histocompatibility. There are composites scaffolds of polyurethane (PU), these composites have been added with PAni nanoparticles (PU+PA), polyurethane composites synthesized with PAni and further added with silver nanoparticles (AgNps) (PU+PA+AgNps). The tests were performed on adipocytes of mouse and measured cytotoxicity by MTT assay, where was found that the polyurethane showed cell viability below 60% compared to the control group, the PU+PA improved in 23% and PU+PA+AgNps increased by 42% viability [12].

Within the field of matrices for cell growth, have composites of collagen with poly(aniline) nanofibers, which retain their electric conductivity depending on the amount or PAni that was developed and in cytotoxicity on porcine skeletal muscle cells showed be suitable as a support cell. The tests were conducted in both composites, collagen alone and composites with conducting polymer nanofibers [13]. Moreover, in PAni films synthesized via polymerization of surface non electroless and over substrates of silicon (Si), have shown adhesion cellular for PC-12 cell (pheochromocytoma cells from rat adrenal medulla) greater than the proliferation obtained for substrates Si substrate without polymer. It is considered that the PC-12 cell prefers to attach to the rough surface of PAni, which promotes itself as the conducting polymer to build biosensors and tissue engineering science.[14] The purpose of the study was to synthesize PPyy and polyaniline (PAni) films evaluating the electrical conductivity and the thickness of

the films, and biocompatibility of NHI-3T3 mouse embryonic fibroblast-like cell line by viability cell number with MTT colorimetric method were performed.

Materials and Methods

Synthesis of conducting polymer thin films

Polypyrrole thin films (PPy) supported on polyethylene terephtalate (PET) substrates were obtained by *in situ* chemical oxidative polymerization of pyrrole at 5°C, according to the similar procedure previously reported [15]. A beaker contains a cold aqueous solution (80 mL) with 5 mmol of pyrrole monomer (Py) was kept under magnetic stirring while 5 mmol of an oxidizing agent ($FeCl_3$ (iron (III) chloride, reagent grade, 97%) or APS (ammonium persulfate reagent grade, 98%) was slowly added by dripping (4 drops/min) into the cold mixture. Then, PET substrates were vertically immersed into the beaker about 1 h in order to obtain the PPy films. Finally, the films were washed with deionized water under ultrasound and dried at room temperature. Additionally, other PPy films were prepared with similar procedure above described, but with a mixture of both oxidizing agents ($FeCl_3$-APS) in a molar ratio of 2.5:2.5 mmol with and without sodium dodecyl sulfate (for molecular biology, 10% in 18 megohm water). The obtained films were depicted as PPy-$FeCl_3$, PPy-APS, PPy- $FeCl_3$-APS and PPy- $FeCl_3$-APS-SDS.

Polyaniline films (PAni-HCl) were obtained at 5°C from a beaker containing 37 mL of doping agent (HCl, 0.75 mol L^{-1}M) and aniline monomer reagent grade, Sigma-Aldrich (1.9×10^{-3} mol). After 30 min of magnetic stirring, oxidizing agent APS (1.9×10^{-3} mol) dissolved in 3 mL of distilled water was slowly added by dripping into the cold mixture for 30 min. Subsequently, clean PET substrates were immersed vertically into the beaker when the color of the solution turns from transparent to green and they were kept for 30 min. Finally, after the deposition time, the conducting thin films were removed from the synthesis solution, they washed with deionized water under ultrasound and dried at room temperature.

Characterization of conducting polymer thin films

Square electrical resistance of the films was measured by using the four-probe method with a Loresta GP MCP-T600 resistivity meter. At least six zones of the films were measured to obtain a mean of the electrical resistance value.

Cell culture and treatment

NIH-3T3 mouse embryonic fibroblasts (ATCC CLR-1658) were culture in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin (Life Technologies, Gibco, Carlsbad, CA, USA). Cells were cultures in standard cell culture dishes (100 x 20 mm) and incubated at 37°C with 5% CO_2. The expected cells grow proliferation; they were harvested by trypsinizing the cell with 1 mL trypsin/EDTA and incubated at 37°C for 5 min to obtain the complete cell detachment for each experiment.

MTT assay-Viable cell number

The cell density of 2 x 10^4 cells/mL was culture into the wells of 24-well plate and incubated for 24 h for complete cell attachment and proliferation. The control group was considered as a 100% of cell viability. Cells previously cultured were incubated with new supplemented medium and the samples were placed floating over DMEM's surface into each well; ensuring that the conducting side of the polymer thin film was in direct contact with the medium. The viable cell number was evaluated by MTT salts (0.2 mg/ mL) dissolved in PBS and incubated for further 4 h, at 37°C with 5% CO_2, for the reduction of MTT salts to formazan form into the mitochondria of viable cells. Then, culture media was removed and it was added stabilizing solution (acidified isopropanol) to dissolve formazan crystals. Finally, aliquots were taken to measure optical density in a microplate spectrophotometer (Bio-Rad, Philadelphia, USA) at 655 nm. The MTT assays were realized at 24 and 48 h incubation time whit the samples. The obtained data were analyzed from three samples of three independent experiments.

BrdU assay

Cells cultured at 2 x 10^4 cells/mL were incubated for 24 h with new supplemented media and at the same time, samples were placed floating over DMEM's surface into each well and the BrdU assay was performed according to the manufacturer's instructions (Roche, Indianapolis, IN). Labeling Solution (bromodeoxyuridine, thymidine analogue), was added for the incorporation in new DNA synthesis. After the time of exposition, 24 h, the samples were removed and the cells were fixed at room temperature; then, it was added working solution that contains a mouse monoclonal antibody against BrdU and the plates there was incubated again. Two washed were performed and it was added Substrate Solution to develop color. Finally, absorbance for each group was measured in a microplate reader at a wavelength of 415 nm.

Statistic

The mean, standard deviation and percentages was calculated. The data was subject to Kolmogorov-Smirnov (Lilliefors) normality test and one-way ANOVA (Post-hoc) Tukey test. The significance was considered at $p<0.05$ with interval confidence of 95%.

Results

Electrical resistance of polymer thin films

The electrical resistance of the films changes according to the doping or oxidizing agents used in the synthesis. PAni doped with HCl had the lowest value in comparison to the PPy films. On the other hand, PPy films synthetized with $FeCl_3$ shown a high value of the electrical resistance and PPy-APS films are less resistive. The mixture of $FeCl_3$ and APS decrease the resistance of the PPy films, however it is increased slightly when SDS was added. It is clear that the type-oxidizing agent influence in the electrical resistivity of the PPy films (Figure 1).

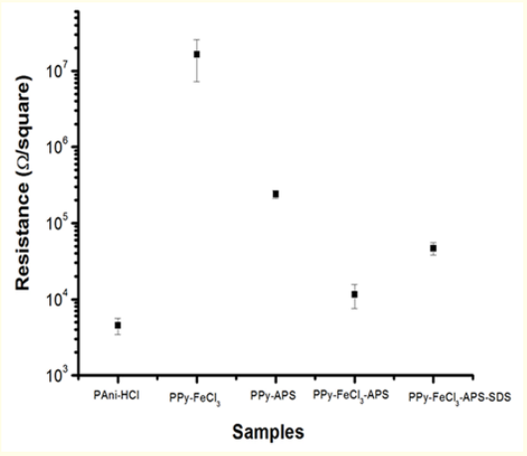

Figure 1: Square electrical resistance of the polypyrrole and polyaniline films. The bars represent mean standard deviation of at least six independent measurements on different zones of the films.

MTT assay-Viable cell number

The viable cell number at 24 h of exposure time with the different samples slightly reduced the viability. The samples exhibit the cytotoxicity as follow from most to less toxic: PET<PPy-$FeCl_3$-APS<PAni-HCl<PPy-APS<PPy-$FeCl_3$-APS-SDS<PPy-$FeCl_3$. ANOVA (post-hoc) Tukey test revealed that there were not significant differences ($p>0.05$) between groups at 24 h (Figure 2a). Thus, the contact of samples with cells at 48 h of incubation displayed as next: PET<PPy-APS=PPy-$FeCl_3$=PAni-HCl<PPy-$FeCl_3$-APS-SDS<PPy-$FeCl_3$-APS there were a significant difference ($p<0.05$) between PET and PPy-$FeCl_3$-APS, nevertheless the other group comparisons did not indicate significant differences as showed in Figure 2b.

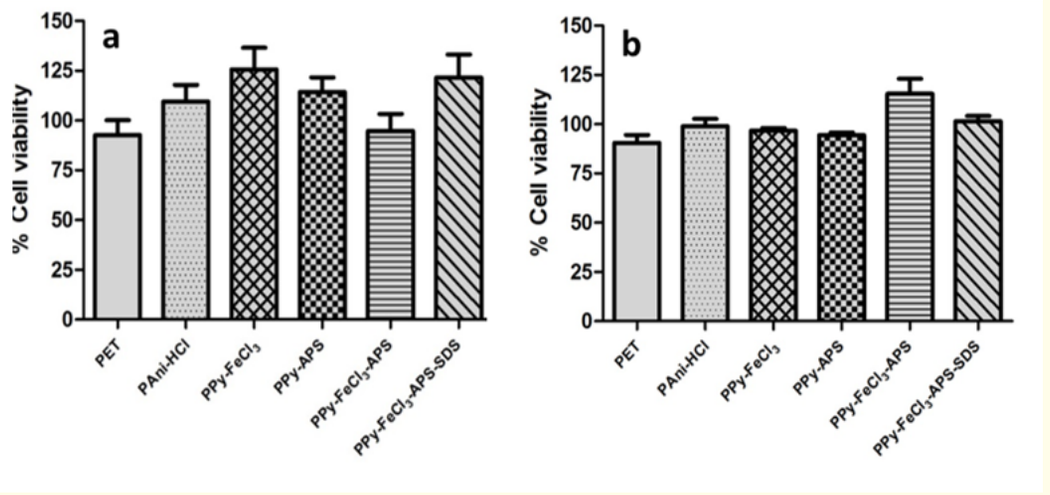

Figure 2: Cell viability of NIH 3TE.

Mouse cells were subculture with DMEM+10% FBS for 24 h (a) and 48 h (b) with the respective samples floating in the culture medium. The viable cell number was detected by MTT method (0.2m/ml of PBS) and the formazan was dissolved with isopropanol and analyzed by 655 nm. The bars represent mean±standard deviation of three independent experiments for reproducible data. PET=Polyethylene terephthalate, PAni-HCl=Polyaniline-chloride acid, PPy-$FeCl_3$=Polypyrrole with iron chloride, PPy-APS=Polypyrrole with ammonium persulfate, PPy-$FeCl_3$-APS= Polypyrrole with iron chloride and ammonium persulfate, PPy-$FeCl_3$-APS-SDS=Polypyrrole with last two oxidants and sodium dodecylsulfate.

BrdU assay

The viable cell number significantly reduced by the presence of PAni-HCl and significant different ($p<0.05$) when compare with PPy-$FeCl_3$-APS groups and PAni-HCl and PPy-APS groups resulted of ANOVA (Post-hoc) Tukey. The PAni-HCl values were the lowest along the assays, around 75% of cell viability. The graph with the results is showed in Figure 3.

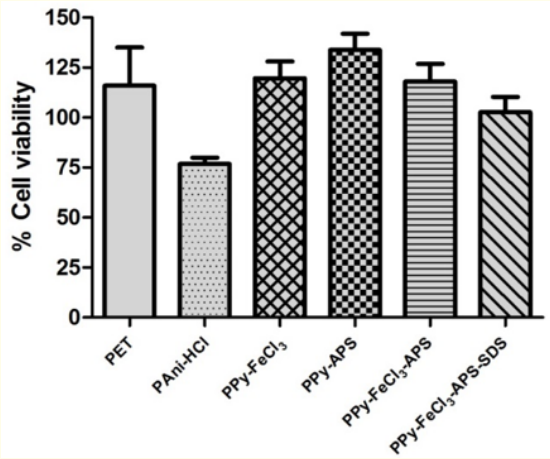

Figure 3: BrdU assay on NIH-3T3 cell viability. NIH-3T3 mouse cells were subculture with DMEM+10% FBS for 24 h with the respective samples floating in the culture medium for 24 h. Labeling Solution (Bromodeoxyuridine, thymidine analogue) was added for the incorporation in new DNA synthesis. The cells were fixed and added working solution (mouse monoclonal antibody against BrdU), two washed were performed and analyzed at 415 nm.

Discussion

Electroactive biomaterials are part of a new generation of "smart" materials that stimulate the cells electromechanical and electrochemical. The material includes conductive materials, polymers, electrets, piezoelectric materials, photovoltaic materials and electroconductive hydrogel [16]. The creation of stimulus-responsive biomaterials is very important for the tissue engineering. The desirable mechanical properties can easily be optimized for a specific application through binding biologically important molecules in to polymers, tissue engineering, cell affinity, biocompatibility [3]. Biosensors in recent years, there has been a growing interest in application of novel biosensors in cell culture and tissue engineering by real-time detection of small molecules such as glucose, lactose, and H_2O_2 as well as serum proteins of large molecular size, such as albumin and alpha-fetoprotein, and inflammatory cytokines, such as IFN-g and TNF-α [17].

Some studies have been identified that the PPy possess many excellent qualities and stimulus-responsive, good biocompatibility *in vivo* and *in vitro* [18,19], good chemical stability and reasonably conductivity under physiological conditions [19,20]. The electrical stimulation has some beneficial effects in enhanced regenerative nerve. Human body responds to electrical fields and the key component of neural communication in the body is the action potential generated at the synapse, conductive polymers lend themselves as excellent novel scaffolds for more efficient delivery of this stimulus type. Several theories have been suggested to explain the effect of electric stimulation on nerve regeneration, the three possible ways by which electrical stimulation could act directly on a neuron, including the redistribution of cytoplasmic materials, the activation of growth-controlling transport processes across the plasma membrane due to change in cell membrane potential, and the electrophoretic accumulation of surface molecules responsible for neurite growth or cell-substratum adhesion [4].

The best polypyrrole films (PPy-$FeCl_3$-APS) had an electrical conductivity about 10^{-2} S/cm and a thickness of 170 ± 36 nm, while polyaniline (PAni-HCl) is about 10^2 S/cm and 318 ± 48 nm, respectively. The electrical conductivity of the conducting polymers depends on the redox potential of the oxidizing agent, so that the variation of the electrical resistance of the PPy films could be associated to redox potential of the $FeCl_3$ and APS. In fact, the films less resistive (PAni-HCl and PPy-$FeCl_3$-APS) correspond to those that were synthesized with APS; it is stronger oxidizing potential than $FeCl_3$.

Here we found that the PAni-HCl, PPy-$FeCl_3$, PPy-APS, PPy-$FeCl_3$-APS, PPy-$FeCl_3$-APS-SDS have adequate biocompatibility reflected by the maintenance of viable cell number by MTT and BrdU assay after the direct contact with the medium for 24 and 48 hours. In case of PAni-HCl externs 75% viable cell reduction number and it is necessary further investigations. Previously reports state that

polyaniline is the second most important and studied biomaterial well-known as aniline black. PAni has many advantages in order to easy synthesis, low cost, good environmental stability, and good electrical ability [21,22]. Awkwardly, their biological use is limit due to PAni has been noted to induce inflammation once is implanted [22-24], probably related to their non-biodegradability. The polymers can conduct charge thanks to their electrons jump within and between the chains. The polymers possess a conjugated backbone, meaning that is formed by series of alternating single and double bonds [25]. The conductivity of PPy and PAni have been investigated and the values have been reported as follow 10^2 - 7.5 × 10^3 S/cm and 30-200 × 10^3 S/cm, respectively [2,26]. It has been reported when used ternary nanocomposite system, composed of polypropylene, redoped PAni nanofibers, and reduced graphene oxide for use in high energy density capacitor resulting in higher conductivity that can enhanced their properties for adequate biomedical applications [27].

The biocompatibility is a very important property of biomaterials. The good cellular response is essential for biomedical applications, PPy and PAni have been shown to support the adhesion and growth proliferation and differentiation of a large variety of cell types such as cardiac myoblasts [28], human osteosarcoma [29], rat glia cell [20,30], cerebral cortical cells [31,32], human neuroblastoma [30], rat pheochromacytoma [19], endothelial cells [33], human keratocytes [34], mesenchymal stem cells [35] showed no sing or externs of acute toxicity, mutagenesis, pyretogen, hemolysis or allergic responses. The previous results are comparable with our resulted here reported where the PPy together with APS, $FeCl_3$-APS, $FeCl_3$-APS-SDS did not induce significantly the cell viability after be in contact with NIH-3T3 mouse cells at 24 h and 48 h. Otherwise, the interaction of PPy with animal models has good biocompatibility without significant long-term effect *in vivo* with minimal tissue response [36,37]. In representative study, the implantation of PPy films into the cerebral cortex of rats, the films were well tolerated and allowed the formation of neural networks [38]. Neither the inoculation of PPy in mice no cytotoxic or allergic response were observed in spleen, liver and kidney [37].

In case of PAni some studies have evaluated the cell adhesion, proliferation y biocompatibility of PC-12 cells [14] concluded that the with the acceptable biological impact in culture. Other reports stated the support of neural cell growth, provided acceptable proliferation and adhesion without externs of significant pro-inflammatory effects [39-41]. Similarly, to our results, the culture of NIH 3T3 mouse fibroblast culture on films of PAni poly (2-acrylamido-2-methylpropanesulfonic acid) (PAni-AMPSA) [42] maintained growth habits similarly to those cultured in control surfaces and biocompatibility demonstrated by cell viability and BrdU assay. The contact of PAni variants; emeraldine base and emeraldine salts did not result cytotoxic with Hpc2 cardiac myoblast and inflammation in animal model with rodent [28,43] and rats [44]. However, different results have reported *in vitro* by cytotoxic effect in immortalized keratinocytes and hepatocellular carcinoma cell lines [41], similarly, the poor cell adhesion and growth [45], tissue incompatibility and fibrous tissue presence in animal rat models [46]. The incompatibility can be associated of the presence of dopants at the low molecular weight by the product leaking of the outer layer of polymers [47]. In contrast, the antibacterial effect of colloidal PAni against gram-positive and gram-negative bacteria was most pronounced for *Bacillus cereus* and *Escherichia coli*, with a minimum inhibitory concentration of 3500 µg/mL [40].

The lack of biocompatibility is theorized to be due to the different preparation protocols used in the experiments, if the PPy, PAni in presence of other compounds are prepared appropriately with repeated steps of rinsing, pretreatment, aging and extraction, the polymer should be completely compatible *in vitro* and *in vivo* [48].

Future research should be focused to evaluate the electrical stimulation delivered, electromechanical effects, biodegradability, the cell proliferation and the biological impact in animal models. In case of PAni-HCl externs 35% viable cell reduction number and it is necessary further investigations such as detection of pro-inflammatory cytokines to implemented therapeutic strategies.

Conclusion

Between the limitations of the study it is transcendent to mention that the studies focused on biocompatibility of PPy and PAni with fibroblast is limited. Here we reported that PAni-HCl, PPy-$FeCl_3$, PPy-APS, PPy-$FeCl_3$-APS, PPy-$FeCl_3$-APS-SDS are promising biomaterials for possible application in the manufacture of conductive matrixes for biosensors platforms.

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