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The effect of synthesis method and oxidizing agent on cytotoxicity and ecotoxicity of polyaniline

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[1] (Syntheses and physical characterisation).

[2] (Biological studies).

ABSTRACT

The unique properties of polyaniline (PANI), as representative of conducting polymers (CPs) predetermine their use in biomedical field. While the material properties of PANI prepared by chemical or microwave synthesis are well known, their biological properties have not been adequately addressed and compared. To fill this gap, PANI powders were prepared by either chemical or microwave synthesis using different oxidizing agent (ammonium persulfate, APS vs potassium iodate, KIO3) and studied. The effect on cytotoxicity and ecotoxicity were determined according to the ISO protocol and inhibition of bioluminescence in Photobacterium phosphoreum, respectively. The significant effect of both factors, the synthesis route and oxidizing agent, on biological performance was confirmed. The choice of optimum preparation condition for PANI synthesis highly improved its cytotoxicity and ecotoxicity compared to previously described PANI forms. Results of the here presented work are crucial for the further utilization of PANI in wide spectrum of biomedical applications.

Keywords: Polyaniline Conductive polymer Microwave synthesis Cytotoxicity Ecotoxicity

1. Introduction

Biomedical applications, from bioelectronics to regenerative medicine, require not only biocompatible materials, but also especially the materials, which can respond to external stimuli, such as electrical current. Conducting polymers (CPs) meet both mentioned criteria and are thus among the so-called "smart materials" that have a very promising future for biomedical applications [1-7]. CPs could even replace conventional metallic and electronic inorganic materials [2] due to their versatility and ability to be modified according to aimed application. The conjugated carbon-carbon double bond structure is responsible for the properties of CPs such as low ionization potential, high electron affinity or low-energy optical transmission [3]. CPs can be easily oxidized or reduced by doping to control their conductivity [4,7].

Furthermore, unlike metals, they exhibit both electron- and ion-based conductivity. This is especially important once we consider that electricity of materials is mostly electron-based while the bioelectricity is based exclusively on the ion exchange. The combined electron/ion based conductivity of CPs are thus especially advantage once the effective exchange of electrical-based information or stimulus between materials and living cells or tissues or organs are expected [5,6].

Polyaniline (PANI) is one of the well-known representatives of CPs, which is broadly used due its simple and cheap synthesis, excellent redox properties, good conductivity, environmental stability and biocompatibility [1-4,8]. Several forms of PANI can be prepared by relatively easy synthesis, such as powders [1], thin films [6], colloidal dispersions [8] or colloidal dispersion-based films [9]. All PANI forms can be further used for preparation of composites or blends, e.g. the preparation of colloidal dispersions as stimuli responsive component of hydrogels to create a 3D-scaffold [10,11]. Moreover, the PANI has biocompatibility comparable to other conducting polymers [11], is safe for topical application [12], and can be further functionalized to became bioactive, e.g. immunomodulatory [13]. Nowadays material must however meet even another criteria, especially to minimize the impact of their production / degradation on the environment. There is however not much studies concerning this aspects. Namely, Shokry et al. [14] tested the effect of a PANI)/2-acrylamido-2-methylpropanesulfonic acid composite material coated with silver nanoparticles and graphene oxide quantum dots on the aquatic environment. The mean EC50 values of the material after 48 h of exposure for freshwater Ostracods were 157.6 ± 6.4 mg/l and 476 ± 25.1 mg/l for the saltwater Artemia salina. The low toxicity of this material is attributed to the presence of a stable PANI layer, which reduces the release of Ag nanoparticles [14]. The low scientific interest in the ecotoxicity of PANI can be related to its stability and non-degradability. The environmental impact can be however related even to the low molecular impurities present in PANI [15]. Thus, the modification of preparation route or use of non-traditional oxidation agents is important [16].

PANI powders are commonly prepared by oxidation of aniline hydrochloride (AnH) with ammonium persulfate (APS) in an aqueous acidic medium [16]. However, the presence of residual APS oxidant is associated with the cytotoxicity of PANI powder [15,17]. Even there are different possibilities of additional PANI purification [18-20] the selection of different oxidizing agent seems to be more efficient way to improve the biocompatibility of PANI. For example, the use of potassium iodate (KIO_3) as oxidizing agent lead to PANI material which shows good biocompatibility [21-23]. KIO_3 also provides good quality PANI samples over a wide range of synthesis parameters [23-26]. In addition, unlike preparation of PANI using APS, the KIO_3 can be added in large quantities during polymer synthesis, which facilitates the large scale up PANI production for industrial applications [25,26]. As far as the bactericidal properties of PANI are also frequently concerned, they remain the same regardless of which oxidizing agent was used, APS or KIO_3 [23,27,28].

Except of use of different oxidizing agent, method of synthesis has also influence on PANI properties. For an example, classical chemical (CS) synthesis is most commonly used way for preparation of PANI [17, 23,29]. However, over the several decades, microwave (MW) synthesis approach is seen as eco-friendly alternative for preparation of polymer materials, although it was rarely used for preparations CPs [30]. The main reason for this was that the classical MW approach could not maintain a constant flow of microwave energy in reaction vessel as the MW system was switch off when the desirable temperature is reached [23]. Gizdavic-Nikolaidis et al. [23,31-35] were the first to apply fast and facile enhanced MW approach for the preparation of PANI nanomaterials for large scale-up production, where they were able to maintain constant temperature in the system, by externally cooling the reaction vessel while simultaneously applying microwaves. In comparison to samples prepared by CS (5 h), the enhanced MW synthesis produced similar yields of PANI in a shorter time (10 min) [31]. Another advantage of enhanced MW approach is possibility to optimize synthesis conditions by varying microwave power and obtain PANI products with different molecular weights [32]. It has already been verified that the pH of the reaction solution has a crucial effect on the morphology of the prepared PANI particles [23]. For instance, with decreasing pH, in the case of H_2SO_4-doped PANI, the particles changed from plate-like with few fibrils to porous structures composed of rod-like short formations [34], while HCl-doped PANI leads to the formation of elongated and compact PANI nanostructures [23,35]. Moreover, Gizdavic et al. [35] showed for HCl-doped PANI that the morphology of PANI products obtained at different MW power levels did not differ noticeably. The particle morphology is also influenced by the oxidizing agent used during PANI synthesis. Gizdavic-Nikolaidis et al. [32] compared morphology of PANI prepared using both APS and KIO_3 oxidizing agents. The authors showed that the use of higher power during MW synthesis (40 and 70 W) gives rise to a more compact and better-defined fiber-like morphology when using KIO_3 compared to APS. On the other hand, the APS-prepared PANI showed smaller rod-like morphologies intermixed with flat structures [32]. Morphology of PANI influences also biological properties, such as antioxidant activity, which increases with the surface area of the PANI nanofibers [33]. The importance of PANI particle morphology is also confirmed by the study of Oh et al. [36]. They prepared four versions of PANI nanomaterials that differ in aspect ratios (2.09 - 5.35 length to diameter, L/D) and investigated the effect of particle shape on cytotoxicity. The result showed that cell viability decreased when cells were cultivated with samples with a lower aspect ratio. The lowest cell viability was observed in the presence of a sample with an average aspect ratio of 2.09 L/D. The aspect ratio was also found to have an effect on the production of radical oxygen species (ROS), with PANI nanomaterials with a low aspect ratio inducing more ROS in the cells [36]. Maintaining PANI conductivity is important for its practical use in biomedicine, and MW synthesis does not adversely affect the conductivity. It also makes no difference, whether the APS or KIO_3 was used as the oxidizing agent. PANI samples prepared by MW synthesis at 40 W exhibited a conductivity of 3.62 S cm^{-1} when using APS and 3.41 S cm^{-1} when KIO_3 was used [32].

The presence of residual impurities originating from raw materials used for PANI synthesis often negatively affect cell viability [12]. Selecting the most suitable synthesis method, oxidizing agent, and purification method is thus crucial to developing a biocompatible PANI. Despite the fact that the material properties of PANI synthesized by MW and CS using different oxidation reagents (APS, KIO_3) have already been examined [32], the cytocompatibility and ecocompatibility of such prepared PANI samples have not been studied and compared. We therefore hypothesized that both the preparation method and used oxidizing agent have a significant impact on the cytocompatibility and ecocompatibility of PANI.

2. Materials and methods

2.1. Chemicals, cell media and bacterial strains

Aniline (ACS reagent > 99.5%), hydrochloric acid (ACS reagent, 37%) and ammonium persulphate (reagent grade 98%), potassium iodate (KIO_3), N-methyl-2-pyrrolidone (NMP) were purchased from Sigma-Aldrich. ATCC-formulated Dulbecco's Modified Eagle's Medium were purchased from PAA Laboratories GmbH, Austria, Penicillin/ Streptomycin were purchased from Healthcare HyClone, United Kingdom, and bovine calf serum from BioSera, France. Photobacterium phosphoreum strain used for ecotoxicity testing was from Czech collection of microorganisms (CCM 3421).

2.2. MW and CS Syntheses of PANI powders

PANI was prepared by aniline oxidation with potassium iodate (KIO_3), $PANI^{KIO3}$ or ammonium persulfate (APS), $PANI^{APS}$. KIO_3 (0.432 g) or APS (0.4606 g) was added to an aqueous solution of 1.25 M hydrochloric acid (12 mL), followed by addition of 0.480 mL of aniline. The mole ratio of oxidizing agent to aniline was 0.88 for KIO_3 and 0.94 for APS, both of which are below 1.25, as previously recommended in literature for aniline oxidation process using APS oxidizing agent [24, 37]. Because the polymerization process and PANI product prepared using KIO_3 as an oxidizing agent are not affected by the mole ratio KIO_3 to aniline, KIO_3 is more suitable for large-scale industrial production of PANI [25,26].

Both microwave (MW) syntheses and classical chemical (CS) syntheses were carried out for 10 min at room temperature. MW experimental parameters are set up as earlier reported by Gizdavic-Nikolaidis et al. [32]. An external cooling circuit maintained constant temperature of the reaction mixture and constant irradiation power. In comparison with CS approach (0 W), MW experiments were accomplished at 93 W power. The reaction mixture was filtered and washed thoroughly with distilled water for both MW-PANI and CS-PANI samples. The PANI product was dried in a vacuum oven at 40 °C overnight.

2.3. UV-Vis characterization

All PANI samples were dissolved in NMP (N-methyl-2-pyrrolidone) solvent with concentration of 0.01 $g.L^{-1}$. The UV-Vis spectra were recorded with Shimadzu UV-2102PC spectrophotometer over the wavelength range 250-900 nm at ambient temperature.

2.4. DTA-TGA characterization

Thermo gravimetric analysis (TGA) and differential thermal analysis (DTA) were studied using SDT 2960 Simultaneous TGA-DTA TA INSTRIMENTS heating from room temperature to 1000 °C with heating rate of 10 ° C/min under a helium flow rate of approximately 80 mL/min.

2.5. SEM analysis

Prior to purification: SEM was carried out using a Philips XL30S Field Emission Gun with a SiLi (lithium drifted) EDS detector with Super Ultrathin Window. The PANI samples were 10 mm in diameter, mounted on aluminum studs using adhesive graphite tape, and sputter coated using a Polaron SC7640

Sputter Coater at 5 — 10 mA and 1.1 kV for 5 min. After purification: The morphology of the particles was investigated using a scanning electron microscope (SEM; Nova NanoSEM 450, FEI, USA) operating at voltage 5 kV.

2.6. Cytotoxicity of extracts

The cytotoxicity was determined using a mouse embryonic fibroblast cell line (ATCC CRL-1658 NIH/3T3, USA). ATCC-formulated Dulbecco's Modified Eagle's Medium (PAA Laboratories GmbH, Austria) containing 10% bovine calf serum (BioSera, France) and 100 U mL^{-1} of Penicillin/ Streptomycin (GE Healthcare HyClone, United Kingdom) was used as the cultivation medium.

The cytotoxicity test was performed according to the ISO standard 10992-12 with modification. The standard procedure employs 0.2 g polymer per 1 mL cultivation medium. As the PANI samples were fluffy and extremely difficult to separate from the medium after extraction, the ratio of 0.1 g of powder per 1 mL of cultivation medium was used. Extraction was performed in chemically inert closed containers using aseptic techniques at 37 °C under stirring for 24 h. The extracts were filtered by MF-Millipore® Membrane Filter with 0.22 µm pore size. On day one, sample extracts were prepared and cells were seeded to preincubate in the microtitration test plates with a concentration of 1 x 10^5 cells per mL. The cells were incubated at 37 °C in 5% CO_2 in humidified air for 24 h. On the second day, the extracts were diluted with culture medium to obtain the following concentrations: 100%, 75%, 50%, 25%, 10% and 1% of parent extract. All assays were performed in quadruplicates. The medium was sucked from plates and replaced by individual extracts. The plates with extract were incubated for 24 h. Cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-diphe-nyltetrazolium (MTT cell proliferation assay kit, Duchefa Biochemie, Netherlands) assay. The absorbance was measured at 570 nm and the reference wavelength was fixed on 690 nm. The results are presented as the reduction of cell viability in percentage when compared to cells cultivated in medium without the extracts of the tested materials.

Since the samples showed high cytotoxicity in preliminary studies, it was necessary to remove the low-molecular impurities, which are intermediates or by-products of the aniline oxidation [18]. Therefore, PANI were tested in two series: 1) native samples; 2) purified samples. PANI was purified in 3 steps: The powders were first purified with 0.2 M HCl, with 50 mL of 0.2 M HCl added to each sample and the acid changed every day for 7 days. In the second step, the PANI powders were extensively rinsed with methanol. Similarly, 50 mL methanol was added, and changed every day for 5 days. In the last step, the powders were repeatedly rinsed with 50 mL acetone, and the acetone was exchanged daily for 5 days. For better purification efficiency, the samples were continuously shaken. Acetone was evaporated at room temperature. The purification procedure was inspired by the work of Kaspárková et al. [18] and IUPAC Technical Report by Stejskal [17].

2.7. Ecotoxicity - Inhibition of bioluminescence in Photobacterium phosphoreum

The method for evaluating the ecotoxicity of sample extracts was based on protocol of EN ISO 11348. Samples in powder form were extracted in phosphate-buffered saline (pH 7.2) at 1:10 ratio (w/w) for 24 h using a laboratory shaker. The liquid phase was separated by centrifugation and was used for the ecotoxicity testing. $ZnSO_4$ was used as a reference substance. A set of dilutions of the sample leachate was put into contact with the Photobacterium phosphoreum bacteria suspension and incubated for 30 min. The decrease in the luminescence of bacteria was measured and evaluated (GloMax® 20/20

Luminometer). The concertation of the leachate causing 50% inhibition of the luminescence (IC_{50}) was calculated as an endpoint parameter of the test.

3. Results and discussion

3.1. UV-Vis confirm the synthesis of PANI

The UV-Vis spectra showed typical PANI structure in all samples either synthesized conventionally (CS) or in the microwave (MW). The UV—Vis spectra have two characteristic peaks (Fig. 1). First peak at 325 nm is assigned to π — π* transition in the benzenoid ring (B), while the second peak at 630 nm is attributed to the transition of an electron from the highest occupied molecular orbital (HOMO, πb) of the benzenoid ring to the lowest unoccupied molecular orbital (LUMO, πq) of the quinoid ring (Q). The Q/B ratio for PANIKIO3 was 0.79 for CS-PANIKIO3 and 0.80 for MW-PANIKro3 sample, while for PANIaps was 0.76 for CS-PANIaps and for 0.77 for MW-PANIaps sample, respectively. These results showed that there is more benzenoid than quinonoid groups in the PANI structures, which is in an agreement with our previously published result [23,32].

The UV-Vis results suggest that the PANI structure was not significantly changed by exposure to different levels of microwave irradiation.

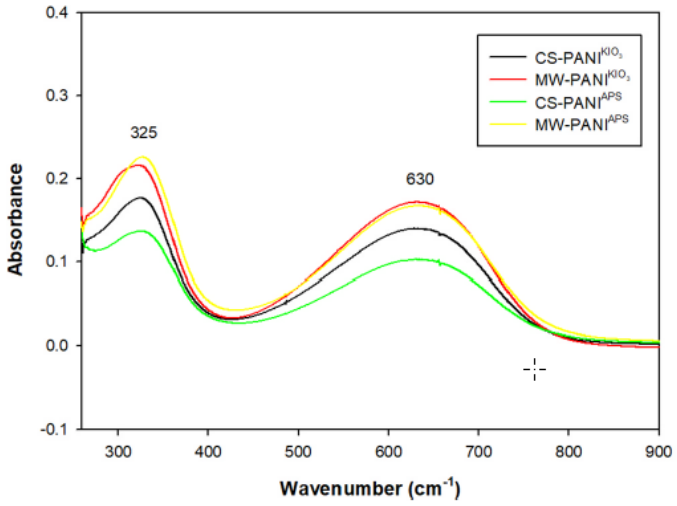

Fig. 1. UV-Vis spectra of MW and CS PANI samples synthetized using KIO_3 or APS as oxidizing agent.

3.2. DTA-TGA characterization

The DTA and TGA thermograms of CS-PANI and MW-PANI samples prepared using either APS or KIO_3 oxidizing agent recorded under a helium atmosphere are shown in Fig. 2.

More detailed examination of TGA results indicated degradation of both CS-PANI and MW-PANI samples proceeding in three steps (Fig. 3). First stage of PANI decomposition in all samples is around (94-95 °C), corresponding to water loss due to evaporation process [38]. While the water loss was greater for both $PANI^{APS}$ samples (10.6% for $CS\text{-}PANI^{APS}$ and 11.5% for $MW\text{-}PANI^{APS}$ sample), it was lower for $PANI^{KIO3}$ samples (6.5% for $CS\text{-}PANI^{KIO3}$ and 6.8% for $MW\text{-}PANI^{KIO3}$ sample). The second weight

loss corresponded to evolution of HCl dopant in CS-PANI samples (200-300 °C). While the dopant degradation proceeded similarly for both CS-PANI^{KIO3} and MW-PANI^{KIO3} samples, it was different for PAN-I^{APS} samples. Two distinct weight losses were observed for MW-PANI^{APS}, the first at 205.3 ° C due to loss of HCl dopant and the second at 297.3 ° C caused by smaller short-chain PANI fragments. For CS-PANI^{APS} only one weight loss at 245.6 °C was detected. At temperatures above 400 °C and over 700 °C, the break of polymer PANI chain occurs involving the following stages. In the first step, elimination of many small polymer fragments at 430 °C occurs (Figs. 3B and 3D) followed by elimination of aromatic fragments and decarboxylation of extended aromatic structures, with CO_2 evolution and formation of smoke at 550 °C. Finally, decomposition of the residue with elimination of substituted aromatic fragments and graphitization at temperatures over 655 °C takes place [38,39].

DTA curves of PANI samples showed an endotherm peak due to water loss at a temperature range 55-105 °C (Figs. 2A and 2B), and two characteristic exothermic peaks at (250-280 °C) and (400-550 °C) which can be associated with the loss of the dopant and the polymer PANI chain break into aromatic fragments, respectively [38]. These results correspond to observation recorded by TGA.

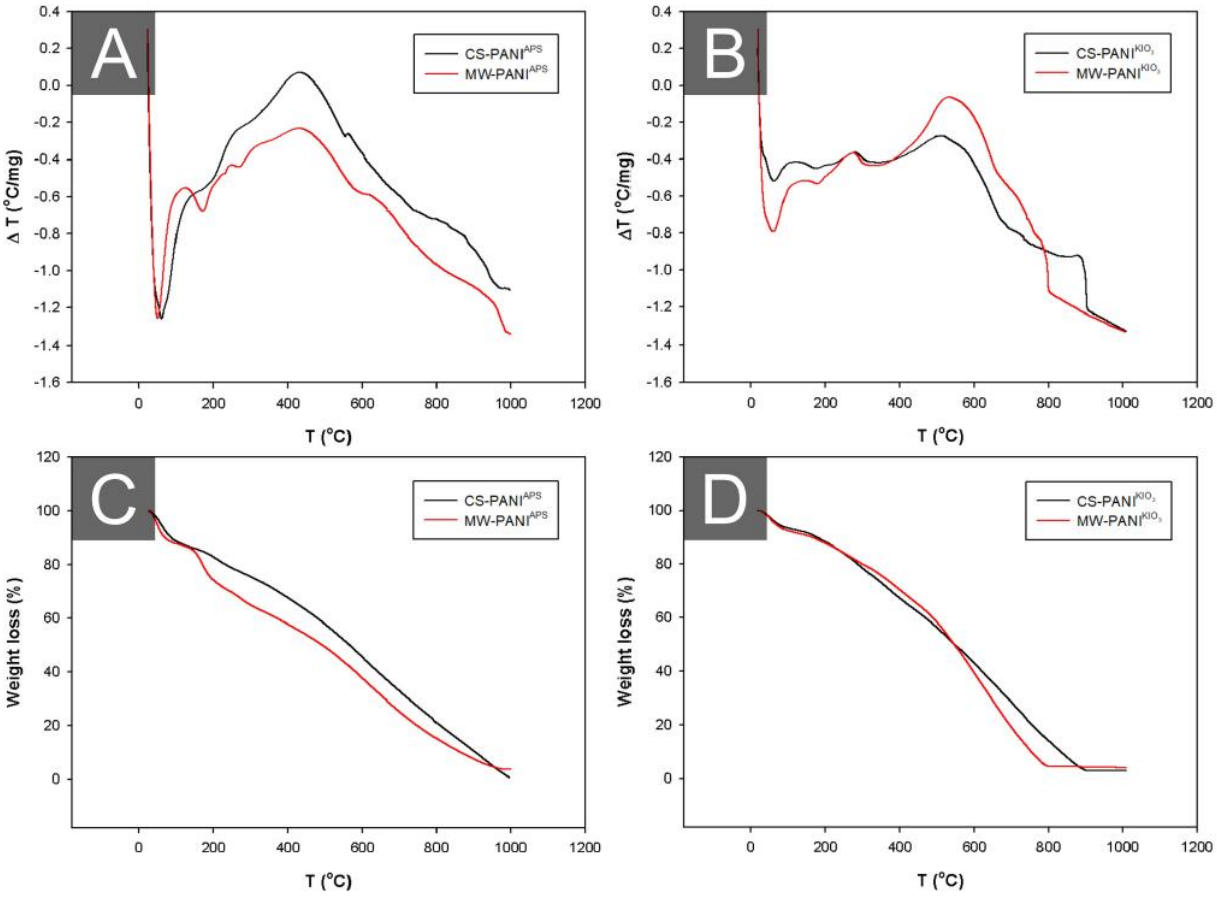

Fig. 2. DTA and TGA curves of PANI samples. A) DTA curves of CS-PANI^{APS} and MW-PANI^{APS} samples; B) DTA curves of CS-PANI^{KIO3} and MW- PANI^{KIO3}; C) TGA curves of CS-PANI^{APS} and MW-PANI^{APS}; and D) TGA curves of CS-PANI^{KIO3} and MW-PANI^{KIO3}.

3.3. Morphology of samples

The morphology of the studied samples was evaluated by SEM and is linked to the chosen preparation procedure. Though the morphology of CS and MW samples has already been published in Gizdavic-Nikolaidis et. al. [32], in current study the PANI samples subjected to.

thorough purification were studied. The morphologies of the samples before and after purification are compared in Fig. 4 showing minor differences between purified and non-purified samples. A subtle difference can be noticed in the CS-PANI^{APS} sample, where the unpurified sample shows a more mesh-like structure, whereas after purification the morphology is more globular. Furthermore, purified MW-PANI^{KIO3}, MW-PANI^{KIO3_P} is somewhat different from its non-purified counterpart and shows rods-like structure.

3.4. Cytotoxicity - MW-PANI^{KIO3} shows absence of cytotoxicity

The cytotoxicity of individual samples before purification was noticeably high (Fig. 5, A-D). Even a 1% extract of the samples in which APS was used as an oxidizing agent, reached the limit of cytotoxicity (threshold is 0.7). Samples with KIO_3 showed slightly better results, but still not satisfactory.

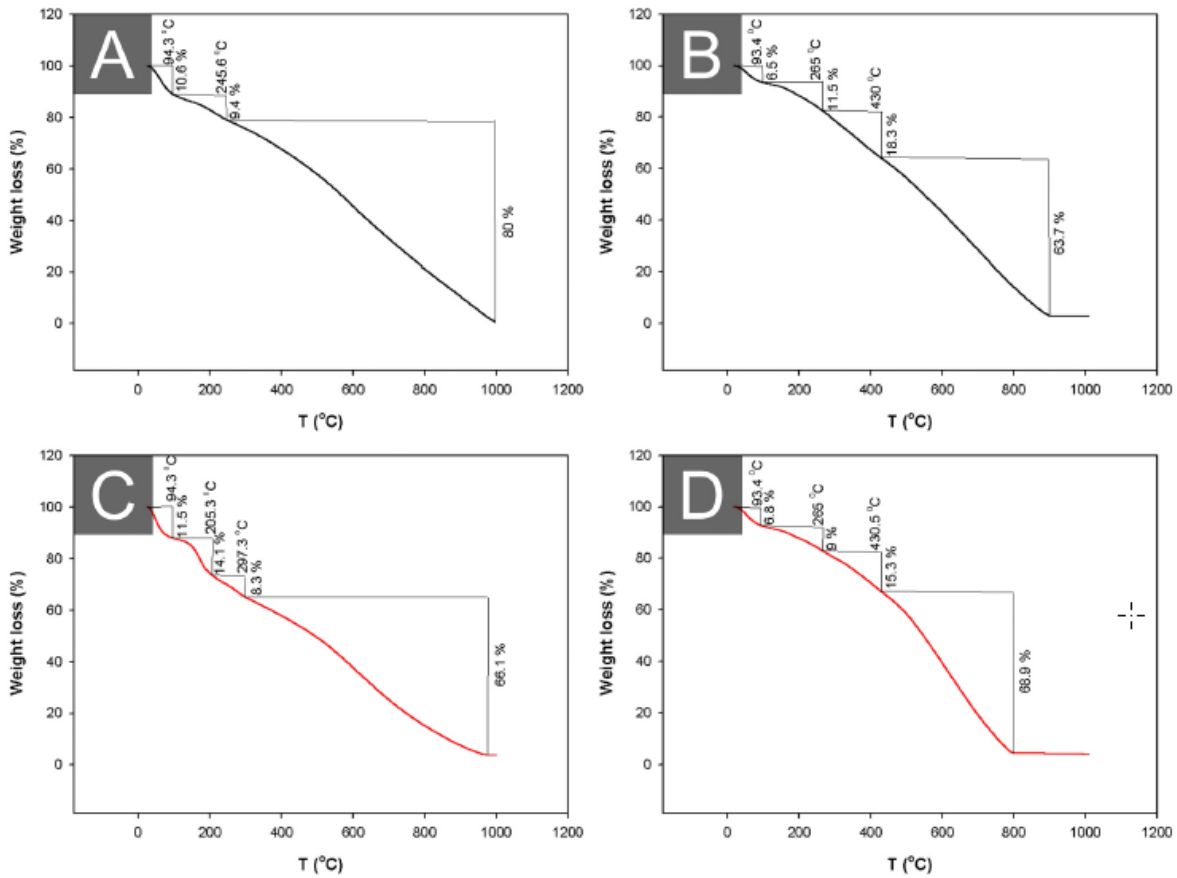

Fig. 3. TGA curves of A) CS-PANI^{APS}; B) CS-PANI^{KIO3}; C) MW-PANI^{APS} and D) MW- PANI^{KIO3}.

After purification consisting in three-step washing with HCl, acetone and methanol (Fig. 5, F-H), samples prepared with APS showed comparable cytotoxicity to those before purification. Improvement was only observed in the presence of 1% extract. However, purified PANI^{KIO3_P} showed significantly improved results for both MW and CS samples. Sample CS-PANI^{KIO3_P} lost its cytotoxicity at 25% extract concentration, and 50% extract was at the cytotoxicity threshold limit with viability close to 0.7. The best results were obtained for the MW-PANI^{KIO3_P}. This sample showed cytotoxic effect only in the presence of 100% extract. Because non-purified PANI exhibits significant cytotoxicity, these results confirm that for biological applications, PANI samples must be purified after synthesis [4].

The cytotoxicity of PANI is caused by the presence of reaction byproducts rather than PANI itself. Therefore, many studies dealing with the cytotoxicity reduction by purification of PANI samples can be found in scientific literature. Gizdavic-Nikolaidis et al. used acetone at the second purification step and found it efficient in this process [23,25,26, 32,40]. Purification of PANI in current study was also inspired by the work of Kaspárková et al. [18] and IUPAC Technical Report by Stejskal [17]. In addition, Stejskal et al. [19] used a re-precipitation approach to purify PANI prepared by standard aniline oxidation using APS. PANIs of different morphologies (globular and nanotubular) were dissolved in N-methylpyrrolidone (NMP) or concentrated sulfuric acid and precipitated in methanol. Sulphuric acid re-precipitation was found to have no significant effect on cytotoxicity. The use of NMP only slightly improved cytotoxicity. Furthermore, Kaspárkova et al. [18] performed purification of PANI^{APS} powders by Soxhlets extraction in polar and non-polar solvents (methanol, 1,2-dichloroethane, acetone, ethyl acetate, hexane, or 0.2 M aqueous hydrochloric acid). The low molecular weight products, which are mainly responsible for cytotoxicity, were best extracted using methanol and 0.2 M HCl. Cell viability reached more than 70% in contact with 1%, 5% and 25% extracts of such purified samples, in comparison, unpurified samples showed absence of cytotoxic effect only in the presence of 1% extract. Purification was less successful with acetone, ethyl acetate, dichloroethane and hexane, which was also reflected in the cytotoxicity results, where as low as 10% extract showed a significant toxic effect.

In addition to purification, the cytotoxicity of PANI is also influenced by the precursors used for synthesis. Kaspárkova et al. [15] synthesized PANI by oxidation of aniline with APS in aqueous solutions of various acids (sulfuric, nitric, phosphoric, hydrochloric and methanesulfonic) to investigate influence of dopant on cytotoxicity. Here, PANI synthesized in the presence of phosphoric acid was the most promising candidate in terms of cytotoxicity, with the extract showing no cytotoxicity up to 25%. This result also correlated with the content of impurities released from PANI determined by HPLC, with phosphoric acid having the lowest impurity level. Oxidizing agents play also a major role in the biological properties of PANI. The presence of residual oxidant APS, as well as preparation technique used, affect the cytotoxicity of PANI powder.

Fig. 5 shows that better cell viability results are achieved when KIO_3 is used as an oxidizing agent. Samples where APS was present as an oxidizing agent were already cytotoxic at 10% of the extract and neither the preparation procedure (CS or MW) nor the purification affected the cytotoxicity. The results for PANI^{KIO3} samples were different. Purification improved the biological properties of PANIs prepared with both types of synthesis. However, the best results were obtained for the MW- PANI^{KIO3} sample (Fig. 5, F). Gizdavic-Nikolaidis et al. showed that PANI product prepared with $KI0_3$ oxidizing agent can be successfully purified with acetone, resulting in no residual $KI0_3$ or toxic aniline oligomers in the final PANI product [23,31,40]. For sure, the short time required to prepare PANI sample and the lack of time for side reactions [23,32] when using the eco-friendly enhanced MW approach can also be the reason to the fact that MW-PANI^{KI03} sample is less toxic than CS-PA-Ni^{KI03} sample. We hypothesized that in case of PANI prepared with APS, this oxidizing agent could be adsorbed on the surface and/or embedded in the PANI structure making these samples more difficult to purify. Therefore, samples

where the APS was used during synthesis show higher toxicity because APS is a very strong oxidizing agent that creates free radicals that can cause cell damage [17].

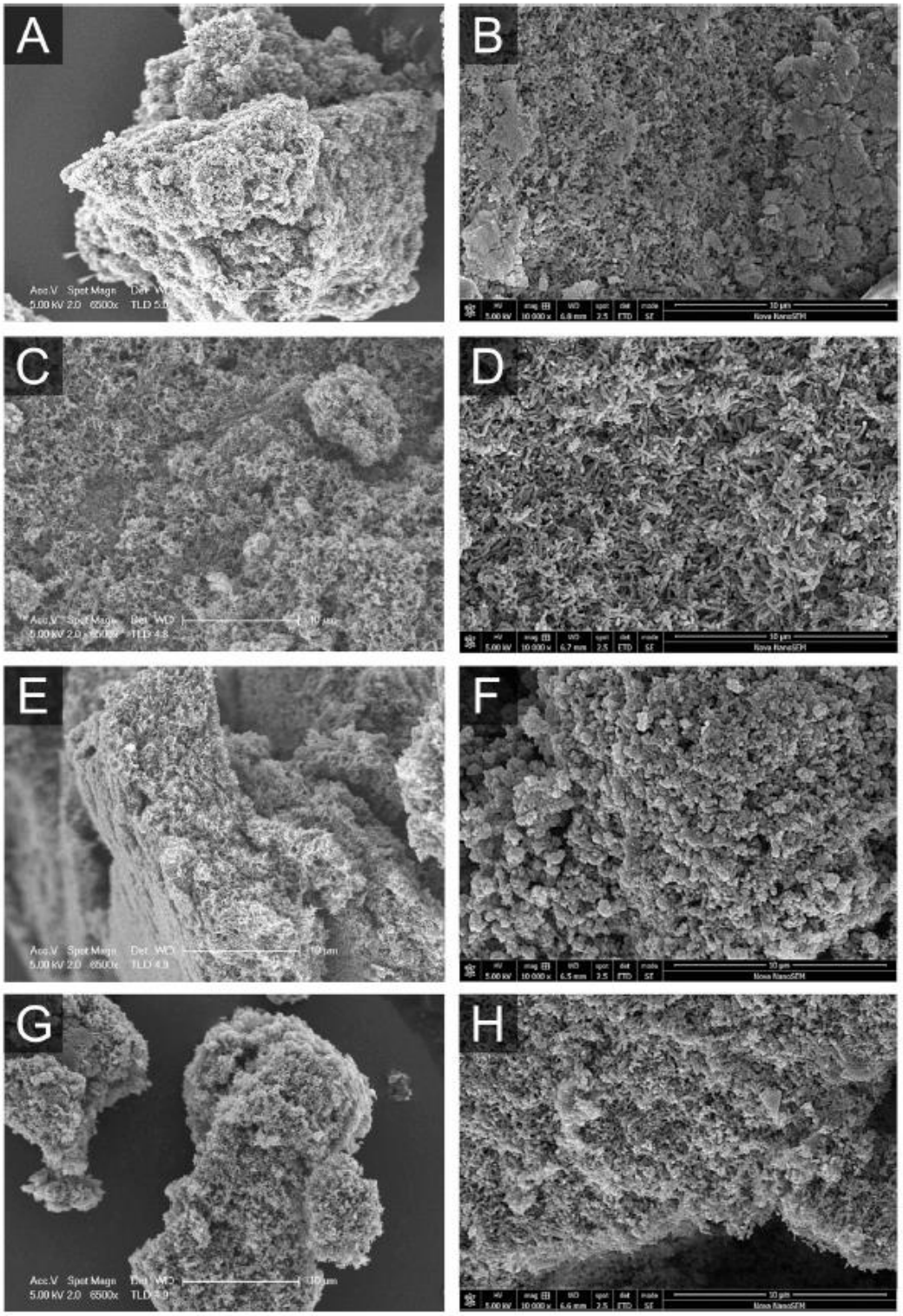

Fig. 4. SEM micrographs of A) CS-PANI^{KIO3}; B) CS-PANI^{KIO3}-p; C) MW- PANI^{KIO3}; D) MW- PANI^{KIO3_P}; E) CS-PANI^{APS}; F) CS-PANI^{APS_P}; G) MW-PANI^{APS}; H) MW-PANI^{APS_P}.

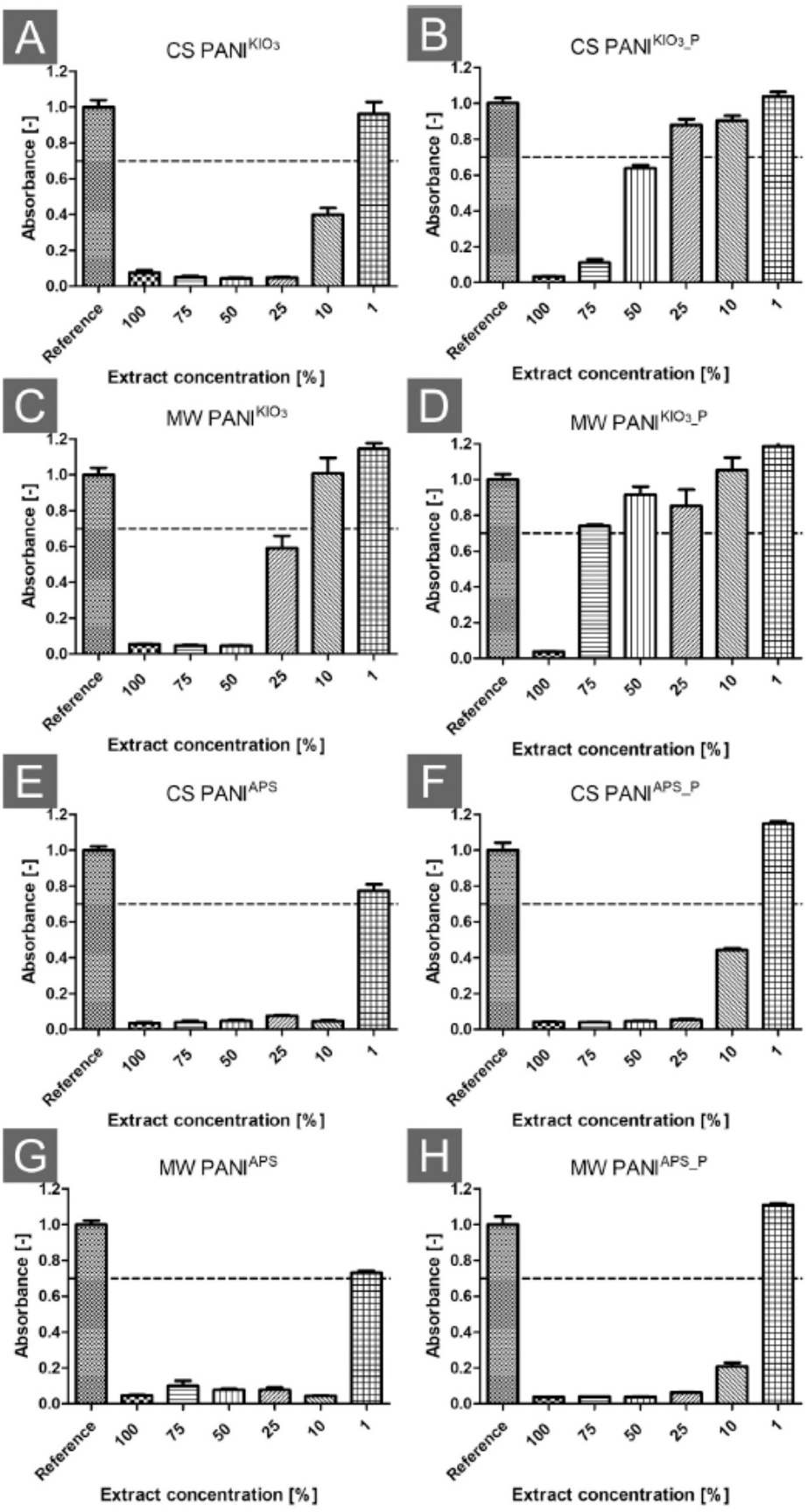

Fig. 5. Determination of cell viability for individual samples: A) CS-PANI^{KIO3}; B) CS-PANI^{KIO3-P}; C) MW- PANI^{KIO3}; D) MW-PANI^{KIO3_P}; E) CS-PANI^{APS}; F) CS-PANI^{APS_P}; G) MW-PANI^{APS}; H) MW-PANI^{APS_P}.

Increased ROS concentration during cell culture generated by APS is associated with damage to membrane proteins and leads to enhanced cell apoptosis. Increase intracellular ROS levels in lysosomes lead to progression of the epithelial-mesenchymal transition, which rises metastatic activity of cells [41]. For an example, KIO_3 is commonly used for the iodization of salts. Iodate IO_3^- is then reduced to I^- in the body and it is used by the thyroid gland. Nevertheless, the reduction process causes oxidative damage to the membrane [42]. Cao et al. [43] demonstrated that the KIO_3 inhibits the antioxidant activity of rat tissues. Considering the possible adverse effects of the oxidizing agents APS and KIO_3 on organisms, based on previously published results, KIO_3 seems to be a better candidate, which correlates with our findings.

By choosing an environmentally friendly technology and selecting a suitable oxidizing agent, KIO3, we were able to synthesize PANI, which exhibits very good cytotoxicity making such PANI a promising candidate for various biomedical applications such as wound dressings and tissue engineering, as previously reported by our research group [21-23]. However, despite its good biological properties, poor solubility and processability of PANI limit its practical use [44]. The solution is to create composites by combining PANI with e.g. biopolymers to obtain high viscosity solutions from which PANI/biopolymer nanofibers can be produced using, for example electrospinning [23-25]. In addition, PANI solubility can be further improved by copolymerizing aniline with substituted anilines that impart solubility to the resulting functionalized PANI copolymers (fPANIs) [23]. For an example, Gizdavic-Nikolaidis et al. showed that fPANI and poly(lactic acid) will give rise to biocompatible nanomaterial suitable for use as a scaffold in tissue engineering [21] or as an antimicrobial wound dressing [22]. The combination of PANI and silk fibroin can provide scaffolds suitable for skeletal muscle regeneration [45]. Xu et al. [46] showed that a hydrogel of cellulose and PANI can be used for nerve regeneration. In this case, PANI promoted the regeneration of peripheral nerves in rats [46]. Moreover, Zare at al. reported [47] that conductive PANI-based nanocomposites can be used in various biomedical fields, such as antimicrobial therapy, drug delivery, biosensors, nerve regeneration, and tissue engineering.

3.5. Ecotoxicity - depends on both, the synthesis method and the oxidizing agent used

Reducing the impact of technologies and products on the environment is one of the main challenges we are facing nowadays. The environmental impact of materials such as PANI is mostly related to the effect on the water ecosystem. To assess this risk a widely used ecotoxicity test of the materials was conducted. The test is based on the measurement of the bioluminescence produced by the bacteria Photobacterium phosphoreum. The toxic effect should be seen as the decrease in bioluminescence intensity upon the inhibition of the related enzyme systems and the overall energetic state of cells.

The concentration of the extract causing 50% inhibition (IC_{50}) was chosen as the endpoint value and the cut-off value below which the sample should generally be recognized as significantly ecotoxic is 1% of IC50. The results are shown in Table 1. For all PANI samples, the IC_{50} (%) values were slightly higher than 1%, which means, that the samples should not be regarded as ecotoxic, but the values are still very close to the ecotoxicological threshold. It is worth noting that the experiments were conducted on pristine PANI without any previous purification to show the ecotoxicity of the raw product of the given synthetic procedure. Some of the reactants are relatively toxic and their traces are present in the product, so it could be expected that the ecotoxicity could be further decreased by an additional purification of the products. Bioluminescence inhibition tests correlate well with results of cytotoxicity conducted also on non-purified PANI. The MW-PANI^{KIO3} sample had the lowest ecotoxicity potential but the differences between different PANI variants were small. The ecotoxicity or more generally the lifecycle assessment of PANI was not in the center of scientific effort until now, while the number of

studies concerning the antimicrobial activity are more common. For an example, the effect of various forms PANI on microorganisms has been previously determined, e.g. the PANI powder [48], coating [49], or modified PANI [50,51] showed antimicrobial activity properties.

Table 1 Inhibition of bioluminescence in Photobacteriumphosphoreum for PANI samples.

Sample	log (IC_{50})	IC_{50} (%)
Zn^{2+}	0.38	2.4×10^{-4}
MW-PANI^{APS}	0.15	1.4
CS-PANI^{APS}	0.16	1.5
MW-PANI^{KIO3}	0.61	4.1
CS-PANI^{KIO3}	0.03	1.1

Consequently, some ecotoxicological effects could be also expected. The scientific literature however do not address this aspect adequately. Most of the published studies focus on the catalytic activity [52] or pollutant remediation [53] by PANI and its composites. Some other studies focus on the green chemistry principles in the PANI preparation but the ecotoxic aspects itself are till now neglected. The here presented data should thus fill this gap in out knowledge and also emphasize the scientific community to focus on lifecycle of PANI.

4. Conclusion

In particular, due to their electrical properties and biocompatibility, CP are among the attractive candidates used in tissue engineering as biointerfaces. However, PANI often exhibits cytotoxic effects because of the presence of residual impurities such as oxidizing agents, etc. In this work, PANI was prepared using two different reagents, APS and KIO_3. Furthermore, the biological properties of PANI synthesized by two different methods were compared: by MW and CS syntheses. The aim was to find the most suitable synthesis method, oxidizing agent and purification method for the preparation of PANI with good biocompatible properties. It was found that the PANI^{APS} showed strong cytotoxicity already in the presence of 10% extract. Neither the method of synthesis nor the purification itself significantly affected cytotoxicity in this case. The effect of purification was observed for PANI^{KIO3}, when the cytotoxicity was considerably lower. The best results were obtained for PANI^{KIO3_P} prepared by MW synthesis, where the cytotoxic effect was observed only in the presence of 100% extract. The combination of ecofriendly technology and the oxidizing agent KIO_3 has resulted in the most promising PANI candidate for biomedical applications.

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