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Faculty of Technology

Doctoral Thesis

IN VITRO TESTING OF POLYANILINE BIOLOGICAL PROPERTIES

In vitro testování biologických vlastností polyanilinu

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„Nothing can be more incorrect than the assumption one sometimes meets with, that physics has one method, chemistry another, and biology a third.“

Thomas Henry Huxley

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ABSTRACT

Nowadays, interest in conducting polymers continues to increase. Polyaniline, due to its unique properties, possesses an important place among conducting polymers. The fact that several cell types and tissues are responsive to electrical fields and stimuli has made polyaniline attractive for a number of biological and medical applications. Any material used in biomedicine must have excellent properties in bulk as well as on the surface, because the surface comes into contact with living tissues first. Materials suitable for biomedical applications are generally those with surfaces that promote intended cell adhesion, proliferation, migration, and differentiation. Although polyaniline has been studied for many years, there has been only limited investigation of its biological properties.

This work is therefore focused on a description of the biological properties of various polyaniline forms and their modifications. The cytotoxicity, hemocompatibility, and antibacterial activity of polyaniline are studied and summarized.

Keywords: polyaniline, biocompatibility, antibacterial activity, hemocompatibility.

ABSTRAKT

Vlastnosti elektricky vodivých polymerů (elektrická vodivost, snadná zpracovatelnost a modifikovatelnost) v kombinaci se skutečností, že řada buněčných typů reaguje na elektrické stimuly, umožnuje jejich aplikace v oblasti biomedicíny. Příkladem vodivých polymerů je polyanilin, který je předmětem této práce. Každý materiál vstupující do kontaktu s živým organismem musí být biokompatibilní. Biokompatibilita je přitom podstatná nejen v objemu, ale především na povrchu, který jako první přichází do kontaktu s tělními tekutinami a buňkami. Materiály vhodné pro biomedicínské aplikace by tedy měly, mimo jiné, umožnovat cílenou interakci v kontextu buněčné adhese, proliferace, migrace a diferenciace. Ačkoliv je polyanilin studován již po desetiletí, jsou informace o jeho biologických vlastnostech stále nedostatečné. Tato práce se zaměřuje právě na stanovení biologických vlastností polyanilinu a to v různých formách a modifikacích. Konkrétně pak byla studována jeho cytotoxicita, hemokompatibilita a antimikrobiální aktivita. Informace prezentované v této práci významně přispívají k poznání interakcepolyanilinu s buňkami, jež jsou nezbytným předpokladem pro jeho využití v biomedicínských oborech.

Klíčová slova: polyanilin, biokompatibilita, antibakteriální aktivita, hemokompatibilita.

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INTRODUCTION

Introduction of a novel biocompatible, non-cytotoxic and stable material for biomedical application remains always challenging. Biomaterials research is extremely multidisciplinary, including areas such as the clinical sciences, laboratory medicine, anatomy, immunology, cell biology, molecular biology, mechanical engineering, materials science, chemistry, and physics. Suitable materials for biomedical applications have surfaces that provide desired cell adhesion, proliferation and growth. Surface properties significantly influence the biological response and biocompatibility of a material. The critical properties for biomedical applications include hydrophobicity, surface charge, texture (roughness), presence of functional groups etc. Moreover, one suitable and unusual property of materials is electrical conductivity. The fact that several tissues are responsive to electrical fields and stimuli has made conducting polymers attractive for a number of biological and medical applications, especially tissue engineering. One of the well characterized conducting polymers is polyaniline. Even if polyaniline has been studied for many years, investigation of their biological properties has been neglected.

As a consequence from above mentioned facts, this thesis is focused multidisciplinary. Thus, some parts of the theoretical background of the thesis is not described in details. The thesis deals with the key properties of polyaniline and shortly describes the biological properties determined within this work. The effort is also to bring the insights to known facts about the biocompatibility and antimicrobial activity of polyaniline.

1. POLYANILINE

Since the discovery of conducting polymers (CP), interest in these polymers has continued to increase. They have already found various applications in many fields, such as anticorrosion coatings, supercapacitors, analytical electrodes, and solar cells (Tallman et al., 2002). More recently, researchers have applied the knowledge acquired on CP to the area of biomedical applications - for example, in areas of tissue engineering such as nerve regeneration (Liu et al., 2010), biosensors (Ahuja et al., 2007), and neural probes and interfaces (Green, 2009; Runge, 2010). Recently, applications in cell adhesion and proliferation (Bidez et al., 2006; Hu et al., 2007), cell differentiation (Genovese et al., 2008; Gilmore et al., 2009, Jun et al., 2009; Kaur et al., 2010), neuronal growth (Göbbels et al., 2010) and neurite outgrowth (Song et al., 2006; Zeng et al., 2013) have been published.

Polyaniline (PANI) is one of the most intensively studied and best characterised conducting polymers (Riede et al., 2002). Polyaniline is known for its electrical and optical properties, easy synthesis, and environmental stability (Shi et al., 2006; Bendrea et al., 2011; Zhang et al., 2012). It can easily be obtained by chemical or electrochemical polymerisation in aqueous solutions and can be produced as a powder, thin films, fibers, and colloidal suspension (Blinova et al., 2005; Zhao et al., 2009; Tokarský et al., 2013). Its pH-dependent conductivity, which depends on the degree of its oxidative state, and the fact that its interaction with living organisms is not fully understood are the major disadvantages of this conducting polymer in the context of biomedical applications.

1.1 Historical background

Polyaniline is probably the earliest synthetic polymer: the first information about polyaniline comes from 1862 and was recorded by Letheby, a professor of chemistry. At that time, polyaniline was known as aniline black. Later, in 1910, the British chemists Green and Woodhead discovered interesting characteristics of polyaniline and gave names to its individual oxidation states that are commonly used today – leucoemeraldine, emeraldine and pernigraniline. In 1968, the synthesis of polyaniline was described by Honzl (1968), and, in the same year, Surville reported the possible application of polyaniline in battery electrodes. Renewed interest in the polymer was provoked by the fundamental discovery of unusual electric properties of polyacetylene in 1977 by the Nobel Prize laureates Alan J. Heeger, Alan G. MacDiarmid and Hideki Shirakawa (Shirakawa et al., 1977).

1.2 Chemical synthesis of polyaniline

Polyaniline can be prepared by the chemical or electrochemical oxidation of aniline under acidic conditions. Selection of the polymerization method depends on the intended polymer application. The chemical synthesis of polyaniline is the simplest method of polymerization and also the oldest and most common (Vivekanandan et al., 2011).

Optimization of this method has been developed for two decades. The efficient polymerization of aniline is achieved only in an acidic medium, where aniline monomer forms the anilinium ion. The chemical oxidative synthesis of polyaniline involves the use of various inorganic or organic acids (i. e. HCl, H_2SO_4, $HClO_4$) in the presence of an oxidizing agent. Also, different inorganic oxidants can be used for the synthesis – $FeCl_3$, K_2CrO_4, K_2CrO_7, $Ce(SO_4)_2$, and $(NH_4)_2S_2O_8$ (Sapurina et Stejskal, 2012). The standard preparation of polyaniline is described in the IUPAC technical report (Stejskal et Gilbert, 2002).

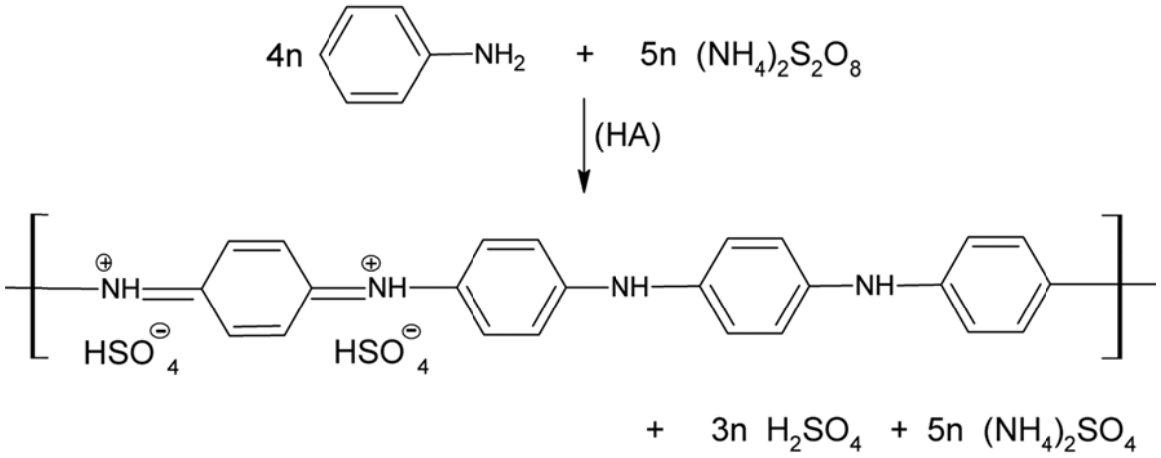

Fig. 1. *Scheme of aniline oxidation.*

The mechanisms of polymerization are as follows. (1) The initial step of the synthesis begins with the oxidation of aniline to a radical cation. (2) In the next step, the coupling of N- and para-radical cations takes place with the consecutive process of the re-aromatization of the divalent cation, yielding an intermediate para-aminodiphenylamine. (3) During the oxidation process of the biradical divalent cation, the initial product is the fully-oxidized pernigraniline salt form of polyaniline resulting from the high oxidizing power of the oxidant. (4) After exhaustion of all the oxidant, the pernigraniline salt is reduced by unreacted aniline and the green emeraldine salt is produced (Sapurina et Shishov, 2012).

Thus, emeraldine in a protonated state is obtained after the synthesis. To obtain the emeraldine base, the emeraldine salt is converted by deprotonation with an alkaline solution (mainly 0,1M ammonia aqueous solution). The emeraldine base can be further transformed to oxidized pernigraniline or reduced leucomeraldine (Ratheesh et Viswanathan, 2013).

1.3 Oxidation forms of polyaniline

Polyaniline can easily be doped by protonic acids. It can exist in three oxidation states – namely, as 1) leucoemeraldine, 2) emeraldine, and 3) pernigraniline (Yoon et al., 2011). Leucomeraldine as a fully reduced form and fully oxidised pernigraniline are environmentally unstable. The stable form, which is extensively studied, is the semi-oxidized form of polyaniline – emeraldine. Moreover, the emeraldine salt exhibits high electrical conductivity, while after deprotonation it loses electroactivity and conductivity (Boyer et al., 1998).

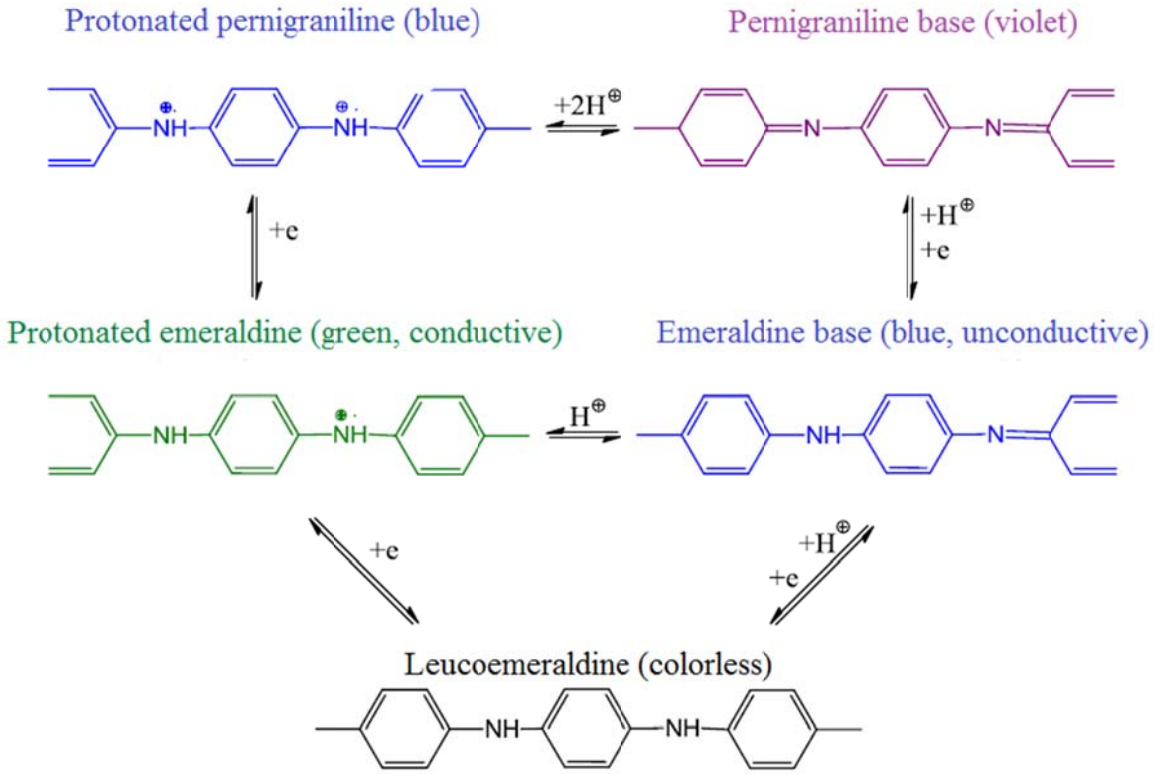

Fig. 2. *Oxidation forms of polyaniline*

1.4 Morphology of various polyaniline forms and modifications

The morphology of polyaniline not only depends on its oxidation form; it is also highly influenced by the polymerization conditions. While various polyaniline forms exist, in this work only chosen forms which were studied for the purpose of this thesis are described.

1.4.1 Powders

Standard oxidative polymerization leads to the granular morphology of polyaniline. However, if the polymerization of aniline is conducted in the acidic conditions of acetic acid or in water, nanotubes are formed (Fig. 3). The size of particles is influenced by the polymerization conditions as well (Stejskal, 2006).

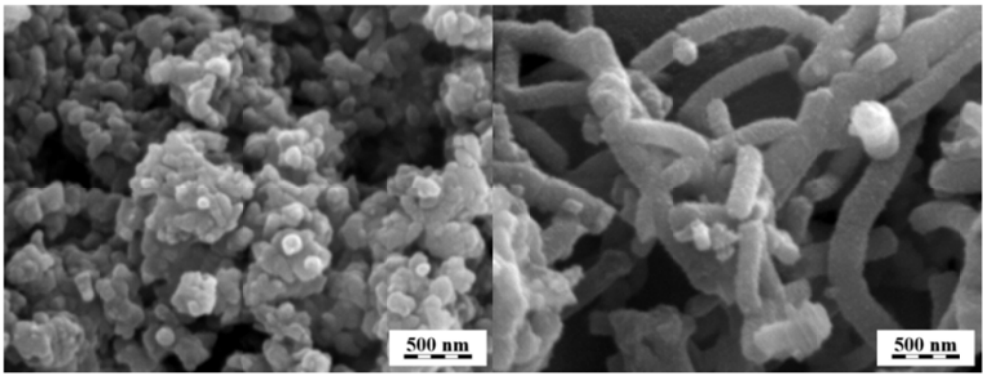

Fig. 3. *Morphology of polyaniline. On the left, granular morphology is visible; on the right, nanotubes have been formed (Stejskal, 2006).*

1.4.2 Films

During the oxidative polymerization of aniline, any substrate present in the reaction mixture is covered with a thin film of polyaniline of nanometre thickness. It is smooth at the beginning (Fig. 4 a), but as the polymerization continues and the film grows, the surface becomes rough and the morphology is globular in appearance (Fig. 4 b) (Sapurina et al., 2001; Riede et al., 2002). The average thickness of the film depends only slightly on the conditions during polymerization; a polyaniline film prepared in an aqueous medium has a thickness of 125 ± 9 nm, as determined by Stejskal and Sapurina (2005). In the presence of 1 mol.L^{-1} HCl during polymerization, the final film has a thickness of 109 ± 10 nm (Stejskal et Sapurina, 2005).

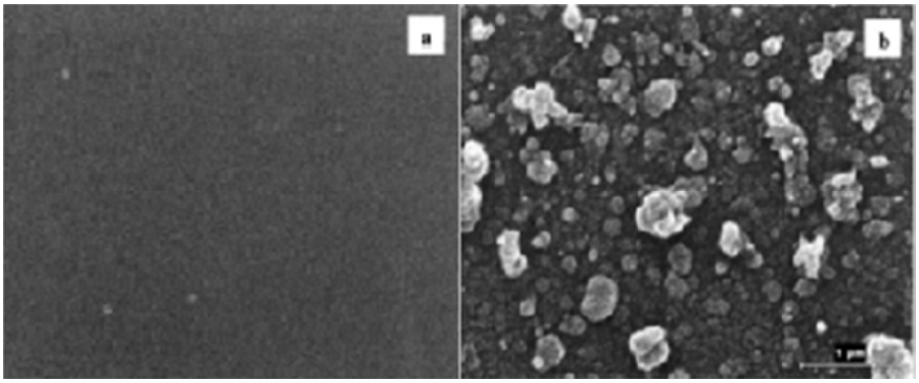

Fig. 4. *Morphology of polyaniline films surface. a) At the beginning and b) advanced stages of polymerization. (Sapurina et al., 2001)*

1.4.3 Colloid

A similar model to the one described in the case of polyaniline film is applicable to the formation of colloidal particles. Colloidal polyaniline is produced by the polymerization of aniline in an aqueous medium containing a suitable water-soluble polymer (stabilizer). The morphology of colloidal polyaniline particles is irregular with rice-grain particles and worm-like and coral-like nanowires (Stejskal et Sapurina, 2004). The size of dispersion particles depends on the concentration of the stabilizer as well as on the concentration of aniline monomer. However, according to Stejskal and Sapurina (2005) the average size of particles, after the standard preparation of colloidal polyaniline, was 241 ± 50 nm. According to Blinova (2005), where the ageing of colloidal dispersions in different environment were studied, the particle size of PANI colloid prepared under the same conditions was 170-230 nm. In the work of Kucekova et al. (2014), where colloidal polyaniline was prepared according to the standard method of preparation (Stejskal et Sapurina 2004), the particle size was 226.5 ± 0.5 nm. All sizes of particles were measured using photon correlation spectroscopy.

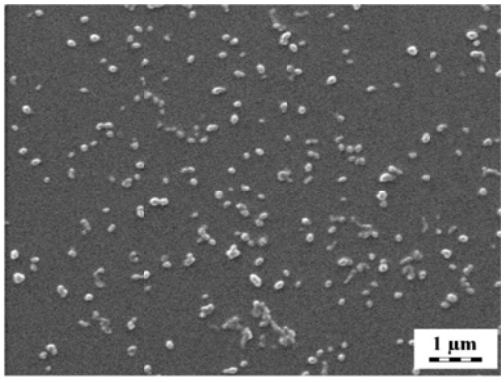

Fig. 5. *Morphology of colloidal polyaniline particles. (Stejskal et Sapurina, 2005)*

1.4.4 Polyaniline modified by polymeric acids

Various polymer modification techniques are used to improve polyaniline properties. One interesting polyaniline-based material is the intramolecular complex of PANI with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA). It was reported that this complex possesses high redox activity and exhibits improve biocompatibility (Nekrasov et al., 2008; Bayer et al., 2010). In the study by Gribkova (2011), PANI-PAMPSA was prepared in the molar ratio 1:2 (monomer of PANI:monomer of PAMPSA). An AFM image of this complex shows uniform morphology with globules (Fig. 6). PANI-PAMPSA produces globules ranging from 100 to 200 nm in size (Gribkova et al., 2011).

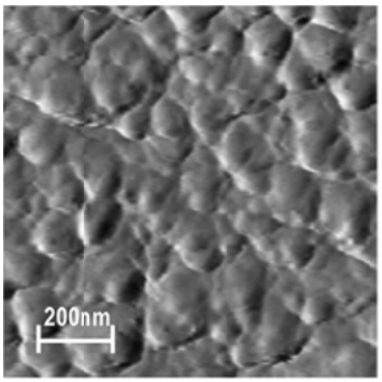

Fig. 6. *Morphology of the interpolymer complexes PANI-PAMPSA(Gribkova et al., 2011)*

1.5 Polyaniline conductivity

The conductivity of polyaniline is dependent on its degree of oxidation or protonation (Bessiere et al., 2004; Hu et al., 2007). Polyaniline becomes conducting in the protonated form of emeraldine salt, while the emeraldine base is unconducting. This was confirmed by the study of Stejskal and Gilbert (2002), in which aniline hydrochloride, oxidized with ammonium peroxydisulfate in aqueous medium, showed an electrical conductivity of 4.4 ± 1.7 S.cm^{-1}. In contrast, the conductivity of polyaniline base prepared thereof was only $1.4 \times 10–8$ S.cm^{-1}. In the form of a film, polyaniline hydrochloride reaches a similar level of conductivity to the powdered form. If polymerization occurs in an aqueous medium, the film conductivity is 2.6 ± 0.7 S.cm^{-1}. If the oxidation is carried out in an acidic medium (1 mol.L^{-1} HCl), the conductivity increases to 18.8 ± 7.1 S.cm^{-1} (IUPAC). The conductivity of polyaniline, after the incorporation of poly (amidosulfonic acid) – PAMPSA, was decreased compared to PANI hydrochloride. A value of 1.5×10^{-2} S.cm^{-1} was determined (Gribkova et al., 2011).

Concerning the conductivity of polyaniline colloid, its determination is far from simple. With regard to the fact that the overall conductivity of polyaniline dispersion is affected by the ionic conduction of the medium, the results for conductivity are not strictly accurate. The conductivity of polyaniline colloid was estimated only by Sulimenko (2001). In this study, the conductivity of dispersion and also its components after centrifugation were measured before and after dialysis. In spite of the fact that the measured data refer mainly to the conductivity of the dispersion medium, the behaviour of both dispersion and dry particles was changed. After thorough work, Sulimenko (2001) determined the conductivity of the dispersion dialysed against 0.01 M HCl to be 6.46 mS.cm^{-1}, and the conductivity of the supernatant liquid to be 7.18 mS.cm^{-1}. The conductivity of sedimented particles was much higher (58 mS.cm^{-1}).

1.6 Polyaniline solubility

Emeraldine salt is insoluble in aqueous solutions and in most common organic solvents because, in this state, polyaniline has cationic charges present in the polymer backbone. However, emeraldine base can be solubilised in various organic solvents, such as dimethyl sulfoxide, m-cresol, chloroform, n-methyl pyrrolidinone, and tetrahydrofuran (Avlyanov et al., 1995; Hodgson et al., 1994; Etchenique et Brudny, 1999). During deprotonation, the cationic charges upon the backbone are removed; hence, the emeraldine base is more accessible to dissolution. The solubility of polyaniline can also be affected by doping and can be improved, for example, by using sulfonic acids as dopants (Masdarolomoor et al., 2008; Mu et al., 1998).

2. BIOCOMPATIBILITY

Even though the aim of this thesis is to determine the biological properties of polyaniline, the following section will be focused on the biological properties of material generally. Biocompatibility is a key properties required for any material used in contact with tissues; it expresses the relationship between organisms and materials without production of undesirable effects (Kirkpatrick et al., 1998; Chen et al., 2008). In 1987, biocompatibility was defined as "the ability of a material to perform with an appropriate host response in a specific situation" (Williams, 1987.). In 2008, this basic definition was re-defined by Williams, who defined biocompatibility as "the ability of a material to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy" (Williams, 2008). Nowadays, this definition is most commonly used.

Biocompatibility is affected by many properties of a material, such as chemical composition (Wang et al., 2004), the leaching of toxic substances that may cause harm locally or systemically or otherwise negatively interfere with the cells (Laluppa et al., 1997), mechanical properties (Zhang et al., 2002; Engelmayr et al., 2008), and physical properties (Vladkova, 2010; Junkar et al., 2011). The series of standards ISO 10993, issued by the International Organisation for Standardization, states the basic biocompatibility requirements for a biomaterial: it should not be toxic, carcinogenic, antigenic, or mutagenic.

Since interactions between materials and biological systems occur in a wide range of applications and molecular processes, there is, in fact, no precise or ideal template for the accurate measurement of biocompatibility. Nevertheless, the criterion of a material's biocompatibility can be its acceptance by living organisms or tissue (Murray et al., 2007). However, considering legal aspects, before any material can be classed as biocompatible, it must be exposed to various studies ranging from *in vitro* tests, through animal experiments, to clinical trials (Wataha, 2012). The biosafety issues of testing are addressed by various guidelines. For example, the legal requirements for materials that come into contact with the human body are described in the ISO standards, namely in the 10993 series entitled "Biological evaluation of medical devices". It describes the requirements, parameters, conditions, and procedures adopted for biological evaluation and consists of 20 separate parts (see table 1). It should also be mentioned that with the growing interest in nanotechnology and the application of nanomaterials in different areas of industry, the question of their biological safety has become an important issue of research. Indeed, even if ISO 10993 defines the testing of a wide range of materials (i.e. powders, tubes, membranes), the determination of nanomaterial biocompatibility is not

described. In fact, testing of the biological properties of nanomaterials is still not exactly defined by any of international authority.

Tab. 1: The series ISO 10993 and its individual parts.

Title	Number/year
Evaluation and testing in the risk management process	ISO 10993-1:2009
Animal welfare requirements	ISO 10993-2:2006
Tests for genotoxicity, carcinogenicity and reproductive toxicity	ISO 10993-3:2003
Selection of tests for interactions with blood	ISO 10993-4:2002/Amd 1:2006
Tests for *in vitro* cytotoxicity	ISO 10993-5:2009
Tests for local effects after implantation	ISO 10993-6:2007
Ethylene oxide sterilization residuals	ISO 10993-7:2008
Selection of reference materials	ISO 10993-8:2001
Framework for identification and quantification of potential degradation products	ISO 10993-9:1999
Tests for irritation and delayed-type hypersensitivity	ISO 10993-10:2010
Tests for systemic toxicity	ISO 10993-11:2006
Sample preparation and reference materials	ISO 10993-12:2012
Identification and quantification of degradation products from polymeric medical devices	ISO 10993-13:1998
Identification and quantification of degradation products from ceramics	ISO 10993-14:2001
Identification and quantification of degradation products from metals and alloys	ISO 10993-15:2000
Toxicokinetic study design for degradation products and leachables	ISO 10993-16:1997
Establishment of allowable limits for leachable substances	ISO 10993-17:2002
Chemical characterization of materials	ISO 10993-18:2005
Physico-chemical, morphological and topographical characterization of materials	ISO/TS 10993-19:2006
Principles and methods for immunotoxicology testing of medical devices	ISO/TS 10993-20:2006

2.1 *In vitro* testing

Investigation of the initial interactions between a biological system and a material requires the use of an *in vitro* model (Frazier et Goldberg, 1989; Council, 2007). According to the definition, *in vitro* tests occur outside living organisms. For these tests, depending on their nature, microorganisms as well as cell cultures can be used (White et al., 1996). *In vitro* assays are the most fundamental way of testing biological responses because of their following advantages: 1) They are less expensive than *in vivo* testing (Dahl, 2005); 2) During *in vitro* tests, the environment of cells as well as their interface with a material can be controlled. Thanks to this, cell response can be measured in detail and with precision. The results therefore provide a better understanding of toxicological reactions to a material. 3) *In vitro* tests reduce the time of testing. 4) Smaller quantities of tested compounds are employed in these tests compared to other test arrangements (Soldatow et al., 2013). On the other hand, *in vitro* testing has some weaknesses. For example, *in vivo* cytotoxicity is a complex phenomenon involving cellular damage caused by physiological, inflammatory or other systemic effects (Bhola et al., 2010) which cannot be fully detected using *in vitro* testing.

In vitro tests are commonly used for screening purposes and for generating complex toxicological profiles. An *in vitro* system also provides additional value as "hazard identification" (Eisenbrand et al., 2002). Hence, *in vitro* testing cannot be adequately undertaken without consideration of other elements of the risk assessment paradigm. Considering the abovementioned ISO standard 10 993, most of the tests are carried out *in vitro*.

2.1.1 Cytotoxicity

One of the elementary tests enabling a better understanding of biocompatibility is the cytotoxicity test (Van Kooten et al., 1997; Dimitrievska et al., 2008). There is no precise definition of cytotoxicity; it depends on the nature of the study. In general, the cytotoxicity of any material expresses the potential of the material to induce adverse effect on cells (Boraldi et al., 2009). Exposing cells to cytotoxic material can lead to different results. Cytotoxic effects can be associated with cell death or the alteration of cell metabolisms (Eisenbrand et al., 2002). Cell viability may be decreased by the inhibiting halting of cell growth and division; cells may undergo necrosis leading to the loss of membrane integrity, or the genetic program of cell death (apoptosis) may be activated.

2.1.1.1 Cytotoxicity testing

Nowadays, numerous *in vitro* models to evaluate the cytotoxic effects of materials exist. The selection of a particular (i.e. the most suitable) method of cytotoxicity testing depends on the expected application and nature of the tested material. Cytotoxicity testing includes the monitoring of morphological changes in cells, the assessment of cell membrane integrity, and the measurement of cell viability and specific aspects of cell metabolisms (Laluppa et al., 1997).

Specific types of cytotoxicity testing are defined in the fifth part of ISO standard 10933-5. There are three types of cytotoxic assays: 1) extract tests, in which a material is extracted in specific conditions according to ISO 10993-12 (Tab.2) and the extract is exposed to cells; 2) direct contact tests, in which a material is placed in direct contact with cells without any barriers being present; and 3) indirect contact tests, in which contact between a material and the cell culture is achieved through diffusion, mediated, for example, by an agar layer or a Millipore filter. Indirect tests can be evaluated only by qualitative assessment.

Tab. 2: Standard surface areas and volumes of extraction liquids needed for cytotoxicity testing according to ISO 10993-12.

Thickness [mm]	Extraction ratio*	Examples of material forms
<0.5	6 cm^2. mL^{-1}	Film, sheet, tubing wall
0.5 – 1.0	3 cm^2. mL^{-1}	Tubing wall, slab, small moulded items
>1.0	1.25 cm^2. mL^{-1}	Larger moulded items
Irregularly shaped solid devices	0.2 g. mL^{-1}	Powder, pellets, foam, non-absorbent moulded items
Irregularly shaped porous devices (low-density materials)	0.1 g. mL^{-1}	Membranes, textiles

* Surface area or mass/volume ± 10%

In addition, the following parameters must be considered during cytotoxicity testing (Freshney, 2005): 1) the concentration of the extract/material – a wide range of sample or extract concentrations should be evaluated; 2) cell density – the use of confluent cells in most tests is not recommended (subconfluent cells

with approximately 80% confluency are commonly used at the start of the test; The recommended cell density depends mostly on the used cell type); 3) the medium – in some cases, the serum contained in the medium can mask the toxicity of a sample, which can lead to a false assessment of cytotoxicity; 4) exposure time – according ISO 10993 part 5, the time of exposure is set at 24 hrs; some samples act rapidly (in a few minutes), whereas others act more slowly (over several hours); in some cases, the evaluation of various times of exposure is recommended; 5) sterility – all used equipment, as well as the tested samples, must be sterile and handled aseptically throughout the test. If a non-sterile test material sample is used, it should be checked for contamination. Contamination could negatively affect the test and provide incorrect results for cytotoxicity; 6) replicates – a minimum of three replicates should be analysed for test samples and controls; 7) solvents – if a sample with limited solubility in water is tested, the used solvents (e. g. ethanol, dimethyl sulfoxide, propylene glycol) may be cytotoxic; therefore, a minimum concentration of solvent should be used and dilution should be achieved by varying the original extraction ratio of the test sample to the extraction medium. In addition, the solvent control must be included.

2.1.2 Cell death

Cell death is a fundamental part of life (Chaabane et al., 2013). It is linked to cell survival and cell proliferation at molecular levels (Maddika et al., 2007). Cell death can proceed in three ways: 1) by apoptosis, 2) by necrosis, and 3) by autophagy. Close connections between these types of cell death have, in fact, recently been discovered, but the abovementioned distinctions are still widely recognised. Apoptosis is the fastest, while necrosis and autophagy become visible when apoptosis is inhibited (Martinet et al., 2006; Smith et Yellon, 2011). Cell death plays a very important role in the development of an organism and in the maintenance of tissue integrity due to the elimination of abundant or damaged cells (Degterev et Yuan, 2008). However, cell death also participates in a variety of pathological processes such as cancer (Ghavami et al., 2009), autoimmune diseases (Ortone et al., 2008), and neurodegenerative disorders (Jellinger, 2001).

2.1.2.1 Apoptosis

Apoptosis, also known as programmed cell death, is a naturally occurring process (Alberts et al., 2002). Approximately 10 million cells per day undergo apoptosis in a healthy adult human (Curtin et Cotter, 2003). The term apoptosis has been used since 1972, when Kerr proposed this concept for mechanisms of controlled cell deletion. Apoptosis is connected with specific morphological and biochemical changes. Early observable markers are cytoplasmic shrinkage and

nuclear condensation caused by the action of caspases, as well as the release of anti-inflammatory cytokine (Hacker, 2000; Bortner et Cidlowski, 2002).

A typical property of the apoptosis mechanism is membrane blebbing, shown in fig. 7. Blebs are the progenitors of apoptic bodies in the plasma membrane, which contain small cytoplasmic fragments (Elmore, 2007). These apoptic bodies contain, on the outer side of the cytoplasmatic membrane, phosphatidylserine, which acts as a signal molecule for macrophages, and they are subsequently rapidly phagocytosed without any signs of inflammation. Therefore, this type of cell death is often difficult to observe *in vivo* (Kurosaka et al., 2003; Lauber et al., 2004).The change of phosphatidylserine location is also used for the detection of apoptosis by flow cytometry.

2.1.2.2 Necrosis

Compared to apoptosis, necrosis has different morphological features. During necrosis, blebbing does not occur.Necrosis leads to rapid death due to cell lysis accompanied by rapid permeabilization of the plasma membrane (Brown, 2008). The first step in necrosis is the swelling of cells and their organelles, which is followed by rupture of the plasma membrane and the subsequent loss of intracellular contents. The final step is rapid cell lysis (Kitanaka et Kuchino, 1999; Edinger et Thompson, 2004).

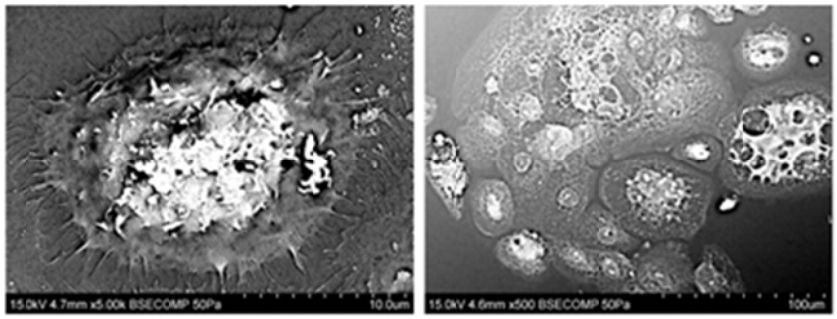

Fig. 7. *SEM images of H357 cells morphology after treatment different doses of erythrosine and the occurrence of apoptosis (left) and necrosis (right). (Garg et al., 2012)*

The release of cell contents through the damaged plasma membrane into the environment is responsible for the inflammatory response. This response is caused by the release of cytosolic constituents into intercellular space. When the immune system tries to eliminate these constituents, inflammation occurs. This response can trigger injury to surrounding cells and, in this way, the inflammatory reaction can be further extended (Chaabane et al., 2013).

Necrosis can be induced by various stimuli. Frequently, necrosis is active as a consequence of chemical or physical injury (Raffray et Cohen, 1997).

Moreover, bacterial toxins, peritoneal macrophages, or natural killer cells can participate in the induction of necrosis. Even if necrosis is commonly designated as non-programmed cell death, sometimes necrotic cell death can be controlled and programmed; it can occur when the cell is unable to undergo apoptosis – for example, due to the low level of adenosine triphosphate (ATP) (Los et al., 2002).

2.2 Hemocompatibility

As all materials used in the human body will come into contact with blood at some point, hemocompatibility is one of the major biocompatibility parameters. It is characterized by the critical interaction of blood with a foreign surface (Szebeni, 2012). Blood is one of the most complex biochemical systems in living organisms. Its various components play integral roles in several life functions, including the transport of oxygen, the destruction of invading pathogens by the production of antibodies, and the repair of damaged tissues by the blood clotting (Ganong, 2003). Basic hemocompatibility testing includes the detection of thrombosis, coagulation, platelet adhesion, and hemolysis, as well as immunology testing, and is summarized in part 4 of ISO standard 10993-4.

As already mentioned, blood contains various components of the immune system, as well as numerous types of cells with different functions (Hoffbrand et al., 2006). Leukocytes are involved in defending against infections and in some cases they phagocytose and digest debris. They can be classified into various cell types such as granulocytes (neutrophils, basophils, eosinophils), monocytes, lymphocytes, and macrophages. The most common class of granulocytes are neutrophils, which have a key role in innate immunity to bacterial infection.

In addition, blood contains small cell fragments called platelets or thrombocytes, derived from the cortical cytoplasm of large cells known as megakaryocytes. Platelets are involved in homeostasis and are responsible for blood clotting. Through their ability to adhere to the endothelial cell lining of damaged blood vessels, they help to repair and to stop the bleeding of the interrupted endothelium (Bauer et al., 1996; Alberts et al., 2002; Ganong, 2003; Martini et al, 2005).

Despite extensive research in the fields of biomaterials and medical devices, the complicated reactions between materials and blood are not fully understood. The first step in the contact of a foreign material with blood is the adsorption of plasma proteins. Next, platelet adhesion and the activation of coagulation pathways occur and the process is terminated with thrombus formation. The poor hemocompatibility of a material can cause thrombosis and inflammatory or immunological reactions (Horbett, 2004; Braune et al., 2013; Zhao et al., 2013).

In fact, the contact of any material with blood induces multiple defensive mechanisms, such as the activation of coagulation cascade, platelet adhesion, the

triggering of complementary systems, and others. These interfacial interactions are connected with various surface properties of a material – for instance, chemical composition, morphology, hydrophilicity/hydrophobicity, and electric charge. For example, surfaces with a positive charge are probably responsible for the formation of primary platelet clots (Aiping et Tian, 2006; Bagheri-Khoulenjani et Mirzadeh, 2012). Further, the coagulation response is also caused by the hydrophobicity of a surface due to the high absorption of plasma proteins onto such a surface (Kovach et al., 2014). In this connection, the water-soluble polymer polyethylene glycol (PEG) is an attractive material, thanks to its resistance to protein adsorption. This polymer was incorporated into various materials in numerous studies to increase the hydrophilicity of the surface and, in this way, to protect them against platelet adhesion and thrombus formation and thereby improve their hemocompatibility (Hansson et al., 2005; Christophis et al., 2010; Pei et al., 2011; Kovach et al., 2014).

The testing of interactions with blood is described by ISO standard 10993-4:2002/Amd 1:2006. According to the nature of the tested material and its intended application, this standard distinguishes between testing in the following categories: thrombosis, coagulation, platelets, haematology, and the complement system. Moreover, the tests can be carried out in three ways: in *in vitro*, *ex vivo* and *in vivo* arrangements.

2.2.1 Blood coagulation

According the ISO standard the coagulation is defined as the phenomenon that results from activation of the clotting factor cascade. Blood coagulation results from a series of proteolytic reactions and during the coagulation the insoluble fibrin is formed from the soluble plasma protein fibrinogen under the action of thrombin through the effect of coagulation cascade. The cascade is activated after exposition of subendothelial tissue factor to the blood flow following either the damage or activation of the endothelium (Heemskerk, 2002; Rumbaut et Thiagarajan, 2010).

Moreover, to avoid a generalized activation of the system and massive fibrin deposition important place takes the regulation of blood coagulation. The regulatory pathways involve anticoagulant proteins and cofactors which are able to inhibit and neutralize activated coagulation factors and limit their period of activity (Norris, 2003). The coagulation factors and anticoagulants involved in blood coagulations functions are as follows:

Factor I (fibrinogen) – is soluble complex glycoprotein synthesized in liver. The activation of this factor is caused by the disruption of fibrinogen arginine-glycine bonds which are hydrolysed through the thrombin action. The fibrin monomers are formed and the factor XIII is activated (Allford et Machin, 2004)

Factor II (prothrombin) – is a glycoprotein in blood plasma which plays essential role in the blood-clotting mechanism. Prothrombin is through the action of the Factor X transformed into a thrombin. Besides the factor I activation, thrombin activates factors V, VIII, IX, protein C or platelets (Rumbaut et Thiagarajan, 2010).

Factor III (tissue factor) – is glycoprotein receptor located in vascularized organs such as the brain, lungs, kidney, placenta, heart etc. Tissue factor initiates the extrinsic blood coagulation and catalyzes the conversion of the factors VII, X and II intio the active serine proteases factors VIIa, Xa and IIa (Chu, 2011).

Factor IV (calcium ions) – naturally occurs in blood plasma and platelets. The factor IV ensures the binding of coagulation factors to phospholipid (Ohkubo et Tajkhorshid, 2008).

Factor V (proaccelerin or labile factor) – it is not enzymatically active cofactor. This cofactor is in the presence of anionic phospholipids converted to Va. It can be also activated by thrombin. Factor V forms the prothrombinase complex with factor X. Mutations of this factors predispose for thrombosis, while deficiency of this factors is connected with hemorrhage (Norris, 2003; Huang et Koerper, 2008).

Factor VII (proconvertin) – it is inactivated, vitamin K dependent, plasma protein which is synthesized in the liver. 1% of this factor occurs also in active serine protease (factor VIIa). Proconvertin forms complex together with the tissue factor and this complex activate factors IX and X to factors IXa and Xa, respectively. The increased amount of this factor can lead to thrombotic disease (Jin et al., 2001; Norris, 2003).

Factor VIII (Antihemophilic factor A) – is a glycoprotein produced in liver as a single polypeptide chain precursor. This factor makes complex with the von Willebrand factor. It is activated by thrombin to VIIIa which plays role as a cofactor of factor IXa. The deficiency of anthihemophilic factor A invokes inherited coagulation disorders (McMullen et al., 1995; Fay, 2004).

Factor IX (Christmas factor) – is vitamin K dependent factor synthesized in the liver. This factor can be activated by either the intrinsic (by factor XI in the presence of Ca^{2+}) or extrinsic pathway (by VIIa-tissue factor- Ca^{2+} complex) to IXa factor. The activated form IXa is a serine proteinase which makes complex with the factor VIIIa and phospholipids in the presence of Ca^{2+}. This complex activates the factor X. Deficiency of factor IX in human plasma causes disease known as hemophilia B (Taran, 1997; Norris, 2003).

Factor X (Stuart-Prower factor) – is a vitamin K dependent serine protease produced in liver. This factor is activated by the complex of the tissue factor and VIIa or by the tenase complex (factors VIII-IX). Stuart-Prower factor makes complex with the factor V in the presence of Ca^{2+} whose function is in the conversion of prothrombin to thrombin. The deficiency of Stuart-Prower factor

leads to one of the rarest of the congenital coagulation disorders (Norris, 2003; Auerswald, 2006; Brown et Kouides, 2008).

Factor XI (Plasma thromboplastin antecedent) – is a plasma serine protease circulating in plasma as a disulfide linked homodimer. Its subunit is able to react with the thrombin and factor XIIa and generate factor XIa. Factor XI convertes the factor IX to its activate form IXa. The deficiency of this factor, known as hemophilia C, cause spontaneous bleding, but the deficiency is rare (Azad et al., 2011; Giannakopoulos et al., 2012; Goto et al., 2012).

Factor XII (Hageman factor) – is zymogen circulating in inactive form and produced by the liver.This factor consists of two chains – heavy and light connected by a disulfide bond. The activation of factor XII is done through the autoactivation by binding of the factor to an artificial or negatively cgarged surface or by the activation by kallikrein. Deficiency of this factor is not connected with bleeding (Schousboe, 2008; Stavrou et Schmaier, 2010).

Factor XIII (fibrin-stabilizing factor) – circulates in plasma as a tetrameric molecule consisting of two catalytic A-subunits and two carrier B-subunits connected together with non-covalent interactions. The A-subunit is synthesized by hepatocytes, monocytes, and megakaryocytes while B-subunit is synthesized by the liver. The activation to XIIIa is carried out through the thrombin in the presence of Ca^{2+}. The active form stabilizes the fibrin clot by crosslinkage (Ariëns et al., 2002; Dodt et al., 2013)

Protein C – is vitamin K dependent protein which circulates in plasma as an inactive zymogen. The pathway of protein C takes very important place in the prevention of thrombosis. The activation of protein C occurs via its binding to a tansmembrane receptor expressed on the endothelium called the endothelial protein C receptor (EPCR) in the presence of thrombin. First, free thrombin binds thrombomodulin and this complex activates protein C through a conformational change in the thrombin enzyme. The activated protein C dissociates form EPCR and bind to protein S. This complex is able to inactivate factors Va and VIIIa and thus prevents blood clotting (Esmon, 2003; Norris, 2003).

Protein S – is vitamin K dependent plasma glycoprotein synthesized in the endothelium. Protein S acts as a cofactor for activated protein C. The deficiency of this protein leads to increased risk of thrombosis (Norris, 2003; Rezende et al., 2004).

Antithrombin – is serine protease inhibitor which has essential role in the control of the process of coagulation. It is able to control the blood coagulation through the function of inhibition of many activated coagulation factors. It can inhibit almost all of the serine proteases involved in the coagulation cascade. Antithrombin functions are supported by its cofactor heparin. It inhibits important coagulation factors such as factors IXa, Xa, the complex of tissue

factor and factor VIIa or thrombin. The deficiency of antithrombin can cause a clinical problems associated with thrombosis (Norris, 2003; Quinsey et al., 2004).

Tissue factor pathway inhibitor (TFPI) – is a transmembrane glycoprotein produced by the endothelial cells. TFPI is a multidomain inhibitor which binds to factor Xa. TFPI/Xa complex then inhibits the complex TF/VIIa thus this complex function consists in the inhibition of extrinsic activation of the cascade (Panteleev et al., 2002; Price et al., 2004).

2.2.1.1 Blood coagulation pathway

As mentioned the coagulation cascade starts with the damage or activation of the endothelium what results to expression of the tissue factor to the blood flow. The coagulation cascade involves two distinct pathways – the extrinsic (tissue factor pathway) and intrinsic (contact activation pathway) pathways (Fig. 8). While the intrinsic pathway is activated after the deposition of blood to a negatively charged surface, the extrinsic pathway starts with the releasing of tissue thromboplastin (Butenas et Mann, 2001; Norris, 2003; Eyre et Gamlin, 2010).

Extrinsic (tissue factor) pathway – The extrinsic pathway is initiated when the tissue factor is realesed after vascular injury and comes into contact with plasma where binds to factor VII. The complex of tissue factor and activated factor VIIa then activates factors IX and X to factors IXa and Xa, respectively. The tissue factor pathway takes place in the presence of Ca^{2+} (Butenas et Mann, 2001; Gentry, 2004).

Intrinsic (contact activation) pathway – The contact activation pathway begins by the autoactivation of factor XII to XIIa after binding of factor XII to an artificial or negatively charged surface. The activation of factor XII is followed by activation of factors XI to XIa, prekallikrein to kallikrein and finally leads to conversion of factor IX to IXa (Norris, 2003; Eyre et Gamlin, 2010).

Both of these two pathways flow into activation of factor IX, which subsequently forms complex with factor VIIIa in the presence of phospholipids and calcium. The complex IX/VIIa is known as a tenase complex and converts factor X to Xa. Factor XA in the presence of the cofactor Va catalyse the conversion of prothrombin to thrombin. The final step of the coagulation process is the conversion of fibrinogen to fibrin through the action of thrombin (Allford et Machin, 2004; Gailani et Renné, 2007).

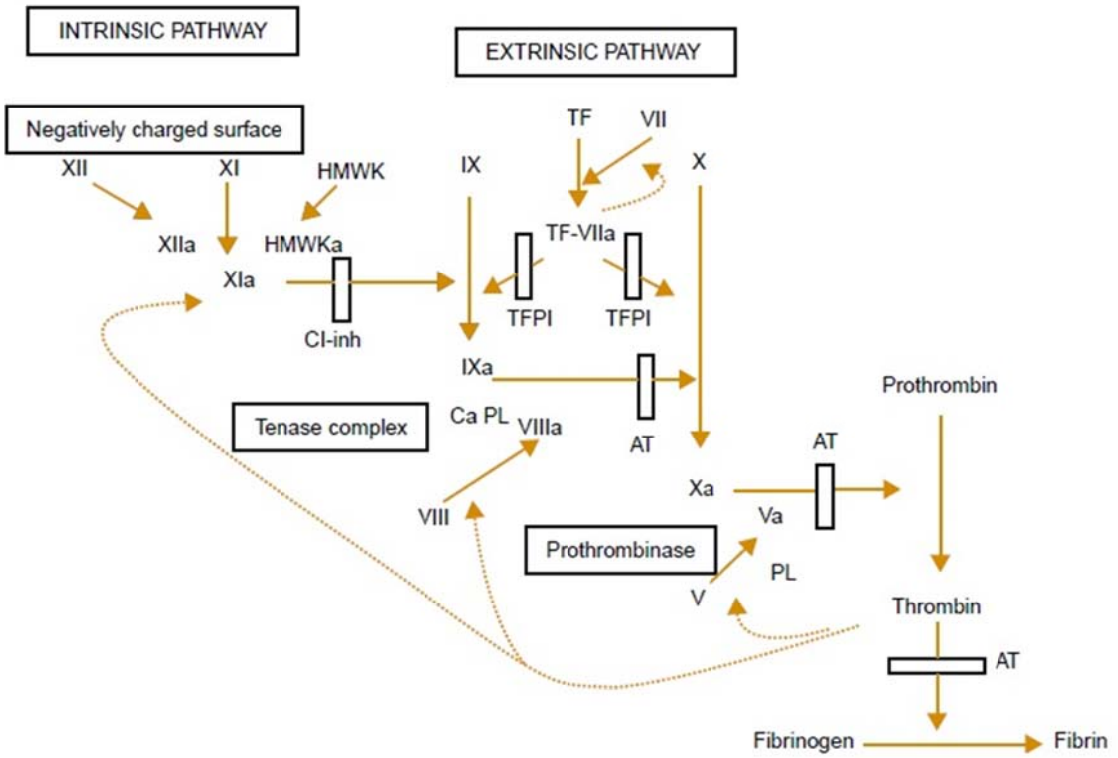

Fig. 8. *The extrinsic and intrinsic pathways of blood coagulation (Norris, 2003).*

3. ANTIBACTERIAL ACTIVITY

The application of materials in medicine brings about a problem related to the occurrence of nosocomial infections; therefore, the antibacterial activity of the substances should not be neglected. There is a huge range of agents which are known to exhibit antibacterial activity (Vasilev et al., 2009). The right choice of antibacterial agent depends on many factors, including the method of application and the site of possible infection. Antibacterial agents can act in a bacteriostatic or bactericidal way. While bacteriostatic agents are those which only inhibit the growth of microorganisms, bactericidal agents destroy them (Levison, 2004).

Antibacterial activity can be achieved through the incorporation of an antibacterial agent into a material. This can be achieved in different ways, such as 1) by coating or adsorption of the agent onto the material surface, 2) by immobilisation via ionic or covalent bonding, or 3) by direct incorporation during synthesis of the material (Radheshkumar et Münstedt, 2005; Guerra et al., 2005; Green et al., 2011).

3.1 Determination of antibacterial activity

Similarly as in cytotoxicity testing, antibacterial activity could be determined by various *in vitro* methods, depending on the nature of the tested material. Antibacterial activity testing of the surfaces of plastics is described in ISO 22196 Plastics - Measurement of Antibacterial Activity on Plastic Surfaces. This standard specifies not only methods for evaluating the antibacterial activity of plastic material, but also methods for other non-porous surfaces. The testing of material without antibacterial treatment is described in ISO 846 Plastics – Evaluation of the Action of Microorganisms.

For the purposes of ISO 22196, four main terms are defined: 1) antibacterial – the term describing conditions in which the growth of bacteria on surfaces is suppressed, or describing the effect of an agent which suppresses the growth of bacteria on the surfaces of the tested products; 2) antibacterial agent – an agent that inhibits the growth of bacteria on the surfaces of products by means of an antibacterial surface treatment or a compounded agent; 3) antibacterial activity – the difference between the logarithm of the viable cell count for an antibacterially-treated product and that for an untreated product after inoculation with bacteria and its incubation; 4) antibacterial effectiveness – the ability of an antibacterial agent to inhibit the growth of bacteria on the surface of a plastic treated with the agent, as determined by the value of the antibacterial activity.

ISO 22196 allows the use of two main bacterial species for evaluating antibacterial activity. *Staphylococcus aureus* and *Escherichia coli* should be used in every test. Of course, additional species can also be used, if required.

4. POLYANILINE BIOCOMPATIBILITY – STATE OF ART

As mentioned the information about the polyaniline biocompatibility and cell comptatibility are not plentiful. Polyaniline was studied mainly *in vivo* in terms of implantability and post-implant evaluation of the changes both of the polymer and tissue surrounding implant (Wang et al., 1999; Kamalesh et al., 2000; Mattioli-Belmonte et al., 2003); the second, prevailing group of test methods, is focused on assessment of in vitro proliferation and/or differentiation of cells on PANi surfaces (Bidez et al., 2006; Wang et al, 2008; Liu et al., 2010). Different variations of PANI (emeraldine, nigranidine and leucomeraldine) were evaluated after 19 to 90 weeks implantation in rats (Wang et al., 1999). Using XPS analysis, surfaces of implanted films were characterized and compared to spectra of virgin ones recorded before implantation. Comparison of spectra proved changes attributed to hydrolysis of PANI films due to exposure to the aqueous medium, reported also by (Kamalesh et al., 2000). Though a thin layer of fibrous tissue and dense matrix of reticular collagen fibres in the implanted region were observed, histological assessment has not revealed any significant inflammation near the implant (Wang et al., 1999). Histological examination and XPS analyses of PANI film prior and post implantation were also reported by (Kamalesh et al., 2000). The study confirmed that implanted emeraldine as well as nigraniline films did not show any abnormalities and did not trigger any inflammatory reaction in the subcutaneous tissue. With regard to surface characteristics changes as measured by XPS, similar results on hydrolytic degradation of films as showed in (Wang et al., 1999) were reported in this paper.

An in vivo and in vitro parallel testing of series of non-resorbable and resorbable polymers including PANI was reported in (Mattioli-Belmonte et al., 2003) in terms of tissue tolerance and cellular interactions. The results showed that PANI together with other nonresorbable polymers is a potential in vivo elicitor for adverse reactions of various types such as inflammation, fibrotic reactions. Simultaneously, after implantation of emeraldine PANI film in rats for 4 weeks, number of inflammatory cells was reduced compared to other tested polymers.

Adhesion and proliferation of H9c2 cardiac myoblasts on a polyaniline substrate were followed by Bidez (2006) and PANI was reported to allow for cell attachment and proliferation. Irrespective of the fact that the initial adhesion of H9c2 cells on the conductive surfaces was somewhat reduced, the overall rate of cell proliferation was similar to a tissue-culture-treated polystyrene control and after six days, the cells formed uniform, homogenous monolayer. Polyaniline films prepared by direct polymerization deposition, with four

different acids and by a casting on the surface of a polytetrafluoroethylene substrate were studied by Wang (2008). These polyaniline films were tested for proliferation and adhesion on PC-12 pheochromocytoma cells. It was found that PANI layers enable cell attachment and proliferation. Simultaneously, it was found out that the directly polymerized films showed much better ability for cell to adhere on the surface compared to the casting films (Wang et al. 2008). PC-12 cells derived from pheochromocytoma of the rat adrenal medulla were used in the publication (Liu et al., 2010) for investigation of biocompatibility of polyaniline films prepared by electroless surface polymerization. PC-12 cells were cultured onto PANI film coated Si wafer and bare Si wafer for one and two days, respectively. After one day of culture it was obvious that PANI coated surface allows for good cell adhesion and proliferation compared to Si surface without coating. Cells persisted to proliferate on the PANI surface also after two days culture and fluorescence microscopy images demonstrated that number of cells was increasing. Cell survival rate, relatively to control (tissue culture polystyrene plates), was also determined. It was found out that the survival rate of PC-12 cells on polystyrene plates was after two days higher compared to PANI coated surface and bare Si wafer. However, after four days, the survival rate of PC-12 cells on PANI film was the highest of the testing samples. Enhanced proliferation of the PC-12 cells on PANI compared to reference authors related to the outstanding biocompatibility of PANI surface.

5. POLYANILINE ANTIMICROBIAL ACTIVITY – STATE OF ART

As was mentioned, any material used in biomedical application is connected with the potential infections. Therefore, the antimicrobial activity of a material should be considered. The antimicrobial activity of polyaniline, mainly of its copolymers and composites was determined in few studies. The antibacterial activity of pristine polyaniline and nanocomposites of polyaniline with Au and Pd against *E. coli*, *Staphylococcus sp*., *Streptococcus sp*., *Klebsiella sp*. was observed by Boomi and Prabu (2013). The antibacterial effect against *E. coli* and S. aureus was confirmed also by Shi et al (2006) whose reported that films of poly(vinylalcohol) and polyethylene containing PANI significantly inhibited the growth of these bacteria. In the study of Gizdavic-Nikolaidis (2011) the antibacterial activity of emeraldine salt and base is reported. The results shows that conductive emeraldine base possess higher antibacterial activity than the base against wild-type *E. coli*, *S. aureus* and *P. aeruginosa.* Furthermore, the antibacterial activity of polyaniline was increased by copolymerization with aminobenzoic acids. The reduction in the attachment of *P. aeruginosa* and *B. subtilis* was observed by Prabhakar (2011) after the coating of polyurethane with polyaniline.

Moreover, Pal and Alocilja (2009) described electrically active polyaniline coated magnetic nanoparticles as a biosensor able to detect *Bacillus anthracis* endospores in contaminated food samples. Another study found out sensing of *E. coli* using polyaniline based impedimetric biosensor. The biosensor is based on antibody–antigen binding method on a polyaniline film surface linked with glutaraldehyde (Chowdhury et al., 2012).

In addition, also antifungal activity of polyaniline was demonstrated. Seshardi and Bhat (2004) observed high reduction in CFU of *Candida albicans* after incorporation of polyaniline to cotton. The nanocomposites poly(ester amide)/polyaniline nanofiber modified montmorillonite showed significant antifungal activity against *Aspergillus niger, Fusarium oxysporum* and *Coleotricum capcii* (Pramanik et al., 2014). In another study, polyaniline has been reported to be protecting against fungi *Alternaria solani* and *Fusarium oseyspurum* (Chauhan et al., 2010).

6. AIMS OF THE DOCTORAL STUDY

The study was focused on the determination, description, and explanation of the basic biological properties of polyaniline in the context of its biocompatibility. The main goal was to enhance the understanding of interactions between various polyaniline forms and cells. During the study, different polyaniline forms were synthesized, subsequently modified and characterized in terms of its material and biological properties.

7. SUMMARRY OF RESULTS

Thesis is presented in a form of summary of published works supplemented by discussion modules based on three published articles. One submitted and two articles prepared for submission into the journal are also discussed. The thesis focused on the following problems: **1)** Determination of polyaniline impurity profile and cytotoxicity of polyaniline precursors and oligomers. **2)** The purification of polyaniline powders leading to improvement in their cytotoxicity. **3)** Determination of biological properties of colloidal polyaniline. **4)** Testing of cell/polyaniline films interactions. **5)** Polyaniline hemocompatibility. **6)** Polyaniline antimicrobial properties.

1) Determination of polyaniline impurity profile and cytotoxicity of polyaniline precursors and oligomers. Polyaniline is one of the most intensively studied and best characterised conducting polymer in terms of its material properties, but considering its biological characterization the information are not plentiful. Thus the cell/polyaniline interaction was the main topic of the study. Polyaniline can be prepared in various forms differing in their chemical and physical properties, of which a conducting, green polyaniline salt and a less conducting polyaniline base are widely used. The preparation of polyaniline salt involves an oxidative polymerization of aniline hydrochloride (AH) in the presence of ammonium persulfate (APS). In order to obtain polyaniline base, this step is followed by deprotonation of the polyaniline hydrochloride with ammonia. The brief description of the reaction course implies that substances with potential cytotoxicity, present in polyaniline, can be residual precursors and oligomers involved in the reaction. Regarding precursors, they were widely studied in context of their impact on human health. Briefly listed, their acute toxicity (AH - Jenkins et al., 1972; APS - Signorin et al., 2001), carcinogenicity (AH - Ma et al., 2008; APS - Kurokawa et al., 1984), teratogenity (AH - Matsumoto et al., 2001), genotoxicity (AH - Sekihashi et al., 2002), mutagenicity (AH - Martinez et al., 2000; APS - Ishidate et al., 1984), the Ames assay (Brennan and Schiestl, 1997) and chromosomal aberrations (AH - Chung et al., 1995; APS - Ishidate 1988) have been tested and reported. Also lower oligomers, such as dimmers, trimers and tetramers may cause problems and recently are considered as more important impurities. The cytotoxicity and cellular response of aniline oligomers has already been subject of study Zhang et al., (2012) suggesting that of the tested oligomers, aniline trimer showed the highest cytotoxicity to fibroblasts (NIH/3T3) and epithelial cells (A549). However, for the practical application of polyaniline in biomedicine, it is also important to know the combined cytotoxicity effect of both the precursors and oligomers as simultaneously leaching impurities which can, in practice, notably influence the biocompatibility of this polymer. Moreover both precursors are

water soluble and their residues can be therefore easily released from otherwise insoluble polyaniline when subjected to physiological conditions in body. Nevertheless, this information about the combined cytotoxicity has not been so far provided. This topic was addressed in the first part of the research and polyaniline impurity profile and cytotoxicity of its components were investigated. The impurity profile was determined using HPLC. The preliminary results of combined precursors cytotoxicity was published in Plasty and kaučuk (**APPENDIX I**) and will be the part of publication dealing with impurity profile of polyaniline (the manuscript is prepared for publication). The results show that concentrations of aniline hydrochloride lower than 1 $mg.mL^{-1}$ do not provoke cytotoxic effect on NIH/3T3. In case of the oxidation agent, ammonium persulfate, the critical cytotoxic concentration is lower. Concentrations 0.25 and 0.1 $mg.mL^{-1}$ reaches 77% (mild cytotoxicity) and 95% (no cytotoxicity) of cell viability compared to reference. However, the combination of both precursors in their nontoxic concentrations possesses significant cytotoxic effect. The preliminary results show, that polyaniline cytotoxicity is rather connected to the oligomers than to residual precursors. This is important information which can lead to better polyaniline purification.

2) The purification of polyaniline powders leading to improvement in their cytotoxicity. The cytotoxicity of polyaniline in powder form has been previously published in work of Humpolíček et al. (2012). There has been observed that extracts of polyaniline salt and base possesses significant cytotoxicity. The polyaniline salt showed severe cytotoxicity, with cell viability below 40% in extracts with concentrations higher than 25% applied on HaCaT. The extract concentrations higher than 10% possess moderate cytotoxicity on cell line HepG2. Only the extract concentration 1% was without cytotoxic effect on both cell lines. The polyaniline base showed lower cytotoxicity compared to the salt. The cytotoxic effect on both cell lines was absent at around extract concentration of 25%. In context of these results, two purification procedures have already been published. The purification by re/deprotonation was published in the same work (Humpolíček et al., 2012), where significant reduction of cytotoxicity was achieved through deprotonation and reprotonation procedure. The purification of polyaniline powder by reprecipitation was published (Stejskal et al., 2014). During the study, the third method, namely purification through the Soxhlet extraction was also examined. Here, the polyaniline powder was treated with six solvents: methanol; 1,2-dichlorethan; acetone; hexane; 0.2 M hydrochloric acid and ethyl acetate. The results suggest that the most effective agents for purifications are methanol and 0.2 M hydrochloric acid which significantly decrease the cytotoxicity level compare to results published

by Humpolíček et al. (2012). For more information see table 3. The manuscript is already prepared for submission into the journal.

3) Determination of biological properties of colloidal polyaniline. Polyaniline can exist in different forms and the biological properties of all of them are worth studying. The biological characteristics of colloidal polyaniline were presented by "Kuceková Z., Humpolíček P., Kašpárková V., Perečko T., Lehocký M, Hauerlandová I., Sáha P., Stejskal J. in article Colloidal polyaniline dispersions: antibacterial activity, cytotoxicity and neutrophil oxidative burst. *Colloids and Surfaces B: Biointerfaces.* 2014, 116: 411-417" for the first time (**ARTICLE I**). The results indicate that the cell viability decreased with increasing polyaniline concentration in the cultivation media. While colloidal polyaniline exhibited threshold of cytotoxicity on HaCaT cells at a concentration about 345 $\mu g.mL^{-1}$, in the case of NIH/3T3 cells this concentration was lower (around 105 $\mu g.mL^{-1}$). According to the annexin/propidium assay, the cytotoxicity threshold for NIH/3T3 cells seems to be around 150 $\mu g.mL^{-1}$, which is higher than the limit of 105 $\mu g.mL^{-1}$ determined by the MTT assay. Below this concentration, the number of healthy cells increases and the number of apoptotic cells dramatically decreases. Due to the fact that MTT assay does not distinguish early apoptic and healthy cells, the concentration of 150 $\mu g.mL^{-1}$ seems to be more relevant. This concentration is also an un-provoking limit for neutrophil activity, measured through the detection of reactive oxygen species. The determination of antibacterial activity was also included in this study and the results are mentioned below.

4) Testing of cell/polyaniline films interaction. The application of polyaniline in biomedicine is mostly connected with the polyaniline films, not powders. The interaction of cells with surfaces depends mainly on used cell line and surface properties. For example it is known, that the best cell adhesion is expected to the surface with total surface energy similar to the cells (Amaral et al., 2006). It is also well known that electrical stimulation facilitate nerve and muscle cell regeneration (Peckham and Knutson, 2005), wound healing of skin (Kloth, 2005) or bone repair (Ciombor and Aaron, 2005). More recently, the impact of conductivity on cell differentiation and molecular cell parameters were discovered. In work of Hsiao et al. (2013) it is for example described the application of aligned composite nanofibers of polyaniline and poly(lactic-co-glycolic acid), as an electrically active scaffold for coordinating the beatings of the cultured cardiomyocytes synchronously. Another exciting use of conducting polymer in electrical stimulation is induction of pre-commitment of fibroblasts into cardiomyocytes (Genovese et al., 2008).

As it was already mentioned, the polyaniline films were studied on proliferation and/or differentiation of variety of cells (Bidez et al., 2006, Wang et al., 2008, Liu et al., 2010; Wang et al., 1999; Kamalesh et al., 2000; Mattioli-Belmonte et al., 2003). The information about the cell compatibility on pristine polyaniline salt and base was nevertheless limited, as the so far published studies were mainly focused on polyaniline copolymers. The preliminary work focusing on cell proliferation on pristine polyaniline films was published in 2012 in "Humpolíček P., Kašpárková V., Stejskal J., Kuceková Z., Ševčíková P., Proliferace buněk na vodivém polymeru, polyanilinu. *Chemické listy.* 2012, 106(5): 380-383" (**ARTICLE II**). Both forms of polyaniline film (base and salt) were studied. It was demonstrated that cell of HepG2 cell line is able to adhere to, and proliferate on all tested polyaniline surfaces. Higher attachment and proliferation were observed on the polyaniline base compared with the polyaniline salt.

Based on the results presented in ARTICLE II it was decided to study the cell/polyaniline films interaction in more details. The following study with preliminary title: "Cell compatibility on polyaniline salt, base and polyaniline with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA)" reveal the surface properties of studied polyaniline (AFM, Surface energy) and its interaction with NIH/3T3 cells. It is focused on cell adhesion and proliferation on pristine and modified polyaniline. The polyaniline surface was modified *via* incorporation of polymeric acid PAMPSA by two ways – by standard polymerization with PAMPSA in a reaction mixture (PANI-PAMPSA-R) or by deposition of the acid onto the polyaniline surface (PANI-PAMPSA-D). The determination of surface properties using contact angle measurement and atomic force microscopy was also used to explore the main important parameters influencing the cell/polyaniline interaction. Most important results are summed in figure 9. It was observed that the fibroblasts are able to attach on all studied surfaces, except of PANI-PAMPSA-D. The best fibroblasts proliferation was observed on standard, unmodified polyaniline salt and base. Compare to this standard polyaniline, cells proliferated on PANI-PAMPSA-R slower. Better cell growth was observed on the surface which contains lower amount of PAMPSA (PANI-PAMPSA-R2:1 *vs* PANI-PAMPSA-R1:1). The results also clearly show, that the most important surface parameter influencing cell adhesion on polyaniline surfaces is surface energy, while topography and conductivity have only limited impact. This study is a preliminary part of the following work which will concentrate on polyaniline interactions with stem cells.

5) Polyaniline hemocompatibility. Important part of material biocompatibility is its hemocompatibility, which has never been studied on polyaniline. Therefore, platelet adhesion and interaction with coagulation factors

were investigated and manuscript dealing with this topic is already submitted to Materials Science and Engineering C "Kuceková Z., Humpolíček P., Kašpárková V., Pelková J., Modic M., Junkar I., Sáha P., Stejskal J. Hemocompatibility of polyaniline" (**MANUSCRIPT I**). The hemocompatibility was determined on pristine as well as on modified polyaniline films. The results show that surfaces of the standard polyaniline base and salt induced blood coagulation and platelet adhesion. The coating with PAMPSA notably improved surface properties of polyaniline in terms of blood coagulation, which was hindered by interaction of PANI with coagulation factors X, V and II.

Another problem connected with application of polyaniline films concerns pH of the transition from conducting salt to non-conducting base. In case of standard polyaniline it namely occurs at pH 4.5 which means that polymer loses its conductivity under physiological pH. One of the possibilities to increase the pH of this transition is doping with polymeric acids. Therefore, the pH dependent transition of PAMPSA modified polyaniline was also studied. It was demonstrated that PANI-PAMPSA-D showed improved pH stability with the transition from conducting salt to non-conducting base occurring at pH of 6. This opens a new possibility for polyaniline applications in biomedical field.

6) Antimicrobial properties of polyaniline. Application of polymers in biomedicine brings about a problem related to occurrence of nosocomial infections. Thus, the antibacterial activity is another important attribute of biomaterials. As the nanosilver is one of the most frequently studied antibacterial agents, its combination with polyaniline can form promising antibacterial material. In the study "Kuceková Z., KašpárkováV., Humpolíček P., Ševčíková P., Stejskal J. Antibacterial properties of polyaniline–silver films. *Chemical Papers.* 2013, 67(8): 1103-1108." (**ARTICLE III**) the antibacterial activity of polyaniline films (salt and base) both in the pristine and nanosilver - modified form was studied. The gram-negative *E. coli* and gram-positive *S. aureus* were used as typical pathogens. The results obtained in this study showed that polyaniline salt completely reduced bacterial growth compared with the reference polystyrene surface. In contrast to polyaniline salt, polyaniline base showed no effect on bacterial growth and its deposition with silver suppressed the growth of gram-positive *S. aureus* only. The antibacterial activity of colloidal polyaniline was also determined (**ARTICLE I**). The antibacterial effect of colloidal polyaniline was most pronounced against *B. cereus* and *E. coli*, with a minimum inhibitory concentration (MIC) of 3500 $\mu g.mL^{-1}$. The behavior of *S. aureus* was analogous to *P. aeruginosa*, with MIC detected at 8500 $\mu g.mL^{-1}$.

Except of papers relating to the particular topics of the study, four manuscripts were published using the methods for determination of cell viability established through the Ph.D. study. These are following:

1) Ševčíková P., Kašpárková V., Hauerlandová I., Humpolíček P., **Kuceková Z**., Buňková L. Formulation, antibacterial activity and cytotoxicity of 1-monoacylglycerol microemulsions. *Eur J Lipid Sci Technol*. 2014, 116(4): 448-457.

2) López-García J., **Kuceková Z**., Humpolíček P., Mlček J., Sáha P. Polyphenolic Extracts of Edible Flowers Incorporated onto Atelocollagen Matrices and Their Effect on Cell Viability. *Molecules*. 2013, 18(11): 13435-13445.

3) Kuceková Z., Mlček J., Humpolíček P., Rop O. Edible flowers – antioxidant activity and impact on cell viability. *Cent Eur J Biol*. 2013, 8(10): 1023-1031.

4) Kuceková Z., Mlček J., Humpolíček P., Rop O., Valášek P., Sáha P. Phenolic Compounds from Allium schoenoprasum, Tragopogon pratensis and Rumex acetosa and Their Antiproliferative Effects. *Molecules*. 2011, 16(11): 9207-9217.

Tab. 3: Cytotoxicity of PANI extracts of various concentrations presented as average absorbance ± SD (Abs) and as relative value compared to reference (RV) according to ISO 10 993-5 standard [a].

Solvent		100%	75%	50%	25%	10%	1%
Methanol	Abs	0.4007 ± 0.0303	0.3830 ± 0.0144	0.3811 ± 0.0170	0.8212 ± 0.1447	0.9158 ± 0.1293	0.9573 ± 0.0550
	RV	0.37 D	0.36 D	0.35 D	0.76 B	0.85 A	0.89 A
1,2-dichlorethan	Abs	0.3928 ± 0.0184	0.3999 ± 0.0184	0.6209 ± 0.0680	0.4031 ± 0.0271	0.3651 ± 0.0040	0.9164 ± 0.1367
	RV	0.36 D	0.37 D	0.58 C	0.37 D	0.34 D	0.85 A
Aceton	Abs	0.3776 ± 0.0126	0.3807 ± 0.0307	0.4603 ± 0.0328	0.3942 ± 0.0114	0.3650 ± 0.0115	1.1649 ± 0.0613
	RV	0.35 D	0.35 D	0.43 C	0.37 D	0.34 D	1.08 A
Ethylester kys. Octovej	Abs	0.3756 ± 0.0352	0.4225 ± 0.0218	0.5160 ± 0.1132	0.4144 ± 0.0150	0.3776 ± 0.0173	1.2131 ± 0.0632
	RV	0.35 D	0.39 D	0.48 C	0.38 D	0.35 D	1.12 A
Hexan	Abs	0.3951 ± 0.0481	0.4482 ± 0.0159	0.5687 ± 0.0629	0.4133 ± 0.0279	0.3791 ± 0.0289	1.1803 ± 0.1067
	RV	0.37 D	0.42 C	0.53 C	0.38 D	0.35 D	1.09 A
0,2M HCl	Abs	0.3428 ± 0.0280	0.3466 ± 0.0231	0.3492 ± 0.0074	0.7886 ± 0.0949	0.8711 ± 0.0561	0.8842 ± 0.1037
	RV	0.32 D	0.32 D	0.32 D	0.73 B	0.81 A	0.82 A

[a] Cytotoxicity in relative values equal to 1 corresponds to 100 % cell survival compared to reference. Values >0.8 are assigned to no cytotoxicity (A), 0.6–0.8 mild cytotoxicity (B), 0.4–0.6 moderate cytotoxicity (C), and <0.4 severe cytotoxicity (D). Reference absorbance was 1.0784±0.0951 = 100%.

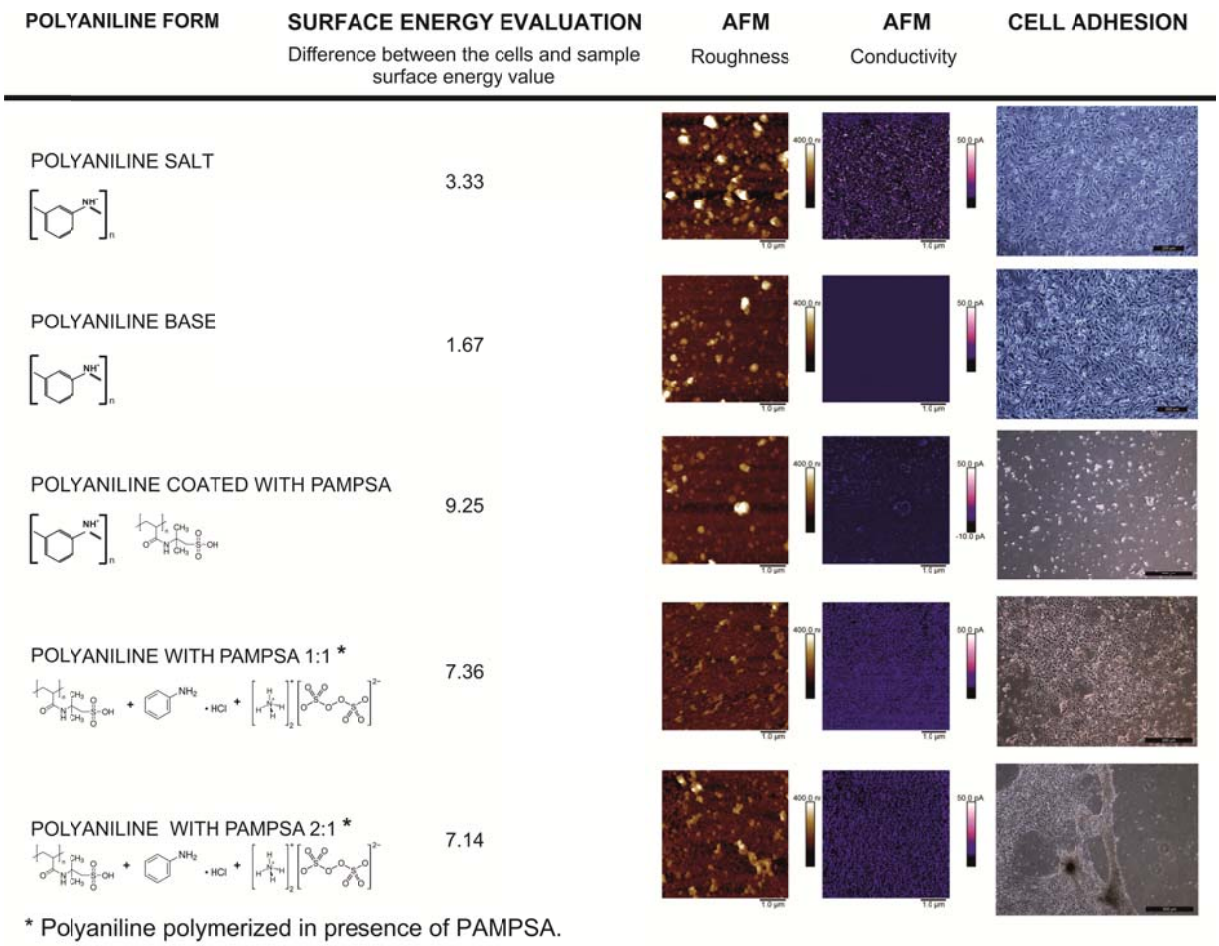

Fig. 9. *The surface properties and cell adhesion of polyaniline films and its modifications.*

Tab. 4: The tested biological properties of different polyaniline forms.

Polyaniline forms	Tested properties	Publication of results
Colloidal PANI	Cytotoxicity Antibacterial activity Neutrophil oxidative burst	ARTICLE I: **Kuceková Z.**, Humpolíček P., Kašpárková V., Perečko T., Lehocký M, Hauerlandová I., Sáha P., Stejskal J. Colloidal polyaniline dispersions: Antibacterial activity, cytotoxicity and neutrophil oxidative burst. *Colloids and Surfaces B: Biointerfa*ces. 2014, 116: 411-417.
PANI-salt/base film	Cell attachment	ARTICLE II: Humpolíček P., Kašpárková V., Stejskal J., **Kuceková Z**., Ševčíková P. Cell Proliferation on a Conducting Polymer (Polyaniline). *Chemické listy*. 2012, 106(5): 380-383.
PANI films with Ag	Antibacterial activity	ARTICLE III: **Kuceková Z.**, Kašpárková V., Humpolíček P., Ševčíková P., Stejskal J. Antibacterial properties of polyaniline–silver films. *Chemical Papers*. 2013, 67(8): 1103-1108.
PANI-salt/base film	Cell proliferation Cell migration Impact on blood coagulation Platelets adhesion	Under preparation: Cell compatibility on polyaniline salt, base and polyaniline with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) MANUSCRIPT I: **Kuceková Z**., Humpolíček P., Kašpárková V., Pelková J., Modic M., Junkar I., Sáha P., Stejskal J. Hemocompatibility of polyaniline.
PANI-PAMPSA-D PANI-PAMPSA_R1:1 PANI-PAMPSA_R2:1	Cell attachment Cell proliferation Cell migration Impact on blood coagulation Platelets adhesion	Under preparation: Cell compatibility on polyaniline salt, base and polyaniline with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) MANUSCRIPT I: **Kuceková Z**., Humpolíček P., Kašpárková V., Pelková J., Modic M., Junkar I., Sáha P., Stejskal J. Hemocompatibility of polyaniline.
Precursors – AH and APS	Cytotoxicity	Under preparation

8. CONTRIBUTION TO THE SCIENCE

The PhD study focused on understanding of the interactions between various forms of polyaniline and eukaryotic as well as prokaryotic cells. During the study, different forms of polyaniline were synthesized, modified and characterised by variety of relevant methods. Considering biological properties, the cytotoxicity, cell attachment and migration, hemocompatibility and antimicrobial activity of this polymer were determined. Important part of the study relied also in introduction and validation of the flow cytometry technique at Tomas Bata Univerzity in Zlín as a method suitable for determination of cell/polymer interactions.

The following topics can be considered as the most important contributions of the PhD work to the science and practice:

1) Determination of polyaniline impurity profile and cytotoxicity of polyaniline precursors and oligomers. The identification of most important impurities and their cytotoxicity was performed and results are prepared for publication.

2) The purification of polyaniline powders using Soxhlet extraction with the aim to improve their cytotoxicity. This study shows significant improvement of polyaniline powder cytotoxicity after extraction and allows for better understanding of polyaniline impact on the cells. The results are already prepared for publication.

3) Determination of biological properties of colloidal polyaniline in terms of its cytotoxicity, antibacterial activity and basic immune response has already been published. The results provide missing information about the biological properties of this interesting polyaniline form.

4) Cell/polyaniline films interactions determined on different cell lines provide remarkable information which are important for future work focused on stem cells interaction with polyaniline films. The first part of this topic has already been published and the second one is prepared for publication.

5) Polyaniline hemocompatibility was studied for the first time and the results of this work were submitted for publication. The study provides information necessary for application of polyaniline in blood contacting devices. The modification to improve polyaniline hemocompatibility was also published.

6) Antimicrobial properties of polyaniline in its pristine form and in combination with nanosilver were clarified and the study has already been published.

Of the above mentioned topics in summary significantly improved the knowledge about the biological properties of polyaniline. Understanding the interactions between polyaniline and different cell types may be useful in the biomedical applications involving polyaniline, mainly in tissue engineering.

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LIST OF ABBREVIATIONS

A549	Human lung adenocarcinoma epithelial cell line
AFM	Atomic force microscopy
AH	Aniline hydrochloride
APS	Ammonium persulfate
AT	Antithrombin
ATP	Adenosine triphosphate
CFU	Colony forming units
Ci-inh	CI-inhibitor
CP	Conducting polymer
H9c2	Rat cardiac myoblast cell line
HaCaT	Human immortalized non-tumorigenic keratinocyte cell line (CLS 300493)
HepG2	Human hepatocellular carcinoma cell line (ATCC HB-8065)
HMWK	High molecular-weight kininogen
HPLC	High performance liquid chromatography
MIC	Minimal inhibitory concentration
NIH/3T3	Mouse embryonic fibroblast cell line (ATCC CRL-1658)
PAMPSA	Poly(2-acrylamido-2-methyl-1-propanesulfonic acid)
PANI	Polyaniline
PC-12	Rat adrenal pheochromocytoma cell line
PL	Phospholipids
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
XPS	X-ray photoelectron spectroscopy

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List of publications

Ševčíková P., Kašpárková V., Hauerlandová I., Humpolíček P., **Kuceková Z**., Buňková L. Formulation, antibacterial activity and cytotoxicity of 1-monoacylglycerol microemulsions. *Eur J Lipid Sci Technol*. 2014, 116(4): 448-457.

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Colloidal polyaniline dispersions: Antibacterial activity, cytotoxicity and neutrophil oxidative burst

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ABSTRACT

Polyaniline colloids rank among promising application forms of this conducting polymer. Cytotoxicity, antibacterial activity, and neutrophil oxidative burst tests were performed on cells treated with colloidal polyaniline dispersions. The antibacterial effect of colloidal polyaniline against gram-positive and gram-negative bacteria was most pronounced for *Bacillus cereus* and *Escherichia coli*, with a minimum inhibitory concentration of 3500 $\mu g\,mL^{-1}$. The data recorded on human keratinocyte (HaCaT) and a mouse embryonic fibroblast (NIH/3T3) cell lines using an MTT assay and flow cytometry indicated a concentration-dependent cytotoxicity of colloid, with the absence of cytotoxic effect at around 150 $\mu g\,mL^{-1}$. The neutrophil oxidative burst test then showed that colloidal polyaniline, in concentrations <150 $\mu g\,mL^{-1}$, was not able to stimulate the production of reactive oxygen species in neutrophils and whole human blood. However, it worked efficiently as a scavenger of those already formed.

1. Introduction

Ever increasing numbers of papers dealing with the biocompatibility of conducting polymers are evidence of the growing interest in these materials. Whereas polypyrrole is better described and more intensively studied in the context of biological properties, information about polyaniline uses in biosciences is less frequent. So far, conductivity and electrochemical behavior were the main focuses of attention. The interesting properties of polyaniline have recently led to an investigation of its possible uses in biomedical applications. This concerns especially the objects that are associated with electrical properties, such as brain, cardiac or neural tissues and cells. Namely, applications in cardiomyocyte synchronization [1], myoblast differentiation [2], neuronal lineage differentiation [3], skeletal muscle [4] or cardiac tissue engineering [5] have been reported. Also, the cytotoxicity, irritation and sensitization potential [6], cell proliferation [7] and antibacterial properties [8–10] of polyaniline powder prepared according to a procedure provided by IUPAC [11] have already been described. Other studies, dealing with biological properties, such as the *in-vivo* tissue response of polyaniline, are mostly based on the testing of polyaniline films cast on various carrier surfaces [12], polyaniline composites [13] or electrospun blends [14].

Although the biological applications of polyaniline are on the rise, they are limited to a certain extent by its insolubility in aqueous media. It is commonly known that conducting polyaniline is poorly soluble even in organic solvents, which strongly influences its processability. Hence, a considerable effort has been devoted to the preparation of processable forms of this polymer. Possible solutions to this challenge can be found in copolymerization [15] or protonation with acids containing relatively long alkyl side chains, which may enhance the solubility in solvents [16]. The preparation of conducting polymer colloids is another approach how to cope with this problem.

Colloidal polyaniline dispersions are prepared when aniline is oxidized in an aqueous medium containing a suitable water-soluble polymer acting as a steric stabilizer. Various polymers have been tested as stabilizers, including poly(vinyl alcohol-*co*-vinyl acetate) [17], poly(vinyl alcohol) [18], poly(methyl vinyl ether) [19], poly(ethylene oxide) [20] or cellulose ethers [21–23]. Also poly(*N*-vinylpyrrolidone) turned out to be an efficient stabilizer of polyaniline colloidal particles and has been successfully employed [24–26]. In light of the numerous applications of colloidal

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polyaniline that have been proposed [27–29], it seems worthwhile to examine the basic biological properties of this promising material.

In the present study, a colloidal polyaniline dispersion, employing poly(*N*-vinylpyrrolidone) as the stabilizer, was prepared and characterized. The main target of the study was to determine the influence of the colloid on prokaryotic and eukaryotic cells via assessing the cytotoxicity on two cell lines and determining the antibacterial properties on representatives of gram-positive and gram-negative bacteria. In addition, the generation of human reactive oxygen species by neutrophils has also been studied. The biological properties of colloidal polyaniline are being reported here for the first time.

2. Materials and methods

2.1. Preparation of colloidal polyaniline dispersion

Aniline hydrochloride (0.2 M) was oxidized with ammonium peroxydisulfate (0.25 M) [30] in the presence of a stabilizer, 2 wt% poly (*N*-vinylpyrrolidone) (PVP; Fluka, type K90, molecular weight 360,000). Aniline hydrochloride (259 mg) was dissolved in an aqueous solution of PVP (4 wt%) to 5 mL of solution. The polymerization of aniline was started at room temperature, close to 20 °C, by adding 5 mL of aqueous solution containing 571 mg ammonium peroxydisulfate. The mixture was briefly stirred and left at rest for 2 h. The resulting dark green dispersion of polyaniline hydrochloride was transferred into a membrane tubing (Spectra/Por 1, Spectrum Medical Instruments, USA; molecular weight cut-off 7,000) and exhaustively dialyzed against 0.2 M hydrochloric acid to remove residual monomers and by-products, such as ammonium sulfate.

2.2. Particle size

The size and distribution of the colloidal particles were determined by dynamic light scattering using a Zetasizer Nano ZS instrument (Malvern Instruments, UK). Measurements of the hydrodynamic radii of colloidal particles, expressed as z-average particle diameters, were performed at 25 °C. The intensity of scattered light ($\lambda = 633$ nm) was observed at a scattering angle of 173°. The polydispersity index (PDI) was evaluated by assuming log-normal distribution of particle sizes. Prior to measurements, the performance of the instrument was verified by using polystyrene latex nanoparticles with the nominal size of 92 ± 3 nm (Thermo Scientific, Germany).

2.3. Polyaniline concentration

Optical spectra of polyaniline colloids were recorded in the wavelength range of 200–800 nm with a Photo Lab 6600 UV-vis spectrometer (WTW, Germany) after defined dilution with 1 M hydrochloric acid. The concentration of polyaniline in colloidal dispersion was calculated from the absorbance at wavelength of 395 nm by using the Lambert–Beer law, $A = \varepsilon cl$, where $\varepsilon = 31{,}500 \pm 1{,}700\ \mathrm{cm^2\ g^{-1}\ cm^{-1}}$ is absorption coefficient [18], c is the concentration of polyaniline and $l = 1$ cm is the optical path.

2.4. Antibacterial testing

The testing of antibacterial properties of polyaniline was conducted with representatives of gram-negative and gram-positive bacterial strains. *Bacillus cereus* (CCM 2010), *Staphylococcus aureus* (CCM 3953), *Escherichia coli* (CCM 3954) and *Pseudomonas aeruginosa* (CCCM 3955) were employed in the test. All strains were obtained from the Czech Collection of Microorganisms (CCM, Czech Republic). Bacteria were grown on nutrient agar ($5\ \mathrm{g\ L^{-1}}$ peptone, $3\ \mathrm{g\ L^{-1}}$ beef extract, $15\ \mathrm{g\ L^{-1}}$ agar; Hi-Media Laboratories, India) and $5\ \mathrm{g\ L^{-1}}$ sodium chloride (Lach-Ner, Czech Republic) at 37 °C. Initial inocula of the microorganisms were prepared from the 24 h cultures, and bacterial suspensions were adjusted to contain 10^6 CFU $\mathrm{mL^{-1}}$ by diluting them with a nutrient broth containing $5\ \mathrm{g\ L^{-1}}$ peptone, $3\ \mathrm{g\ L^{-1}}$ beef extract and $5\ \mathrm{g\ L^{-1}}$ sodium chloride.

For antibacterial testing, the colloidal polyaniline dispersion was diluted with a nutrient broth to obtain polyaniline concentrations ranging from 2,000 to 8,500 $\mu\mathrm{g\ mL^{-1}}$ and inoculated with 200 μL of bacterial suspension. After a 24 h incubation, 100 μL of decimal dilutions were spread over agar plate surfaces and incubated for 24 h at 37 °C. Colonies were counted and minimum inhibitory concentration (MIC) was calculated. Each experiment was repeated four times.

The pH determination of the bacterial suspension in the absence and the presence of a polyaniline colloid was carried out by using a Spear pH meter tester (Eutech) before and after cultivation in order to monitor the pH changes occurring in the suspension during bacterial growth.

2.5. Test of cytotoxicity

Prior to *in-vitro* cytotoxicity testing, the samples were disinfected by dry heat at 120 °C for 40 min. Cytotoxicity testing was performed with a human immortalized keratinocyte cell line (HaCaT, Cell Lines Service, Catalog No. 300493, Germany) [31] and a mouse embryonic fibroblast cell line (ATCC CRL-1658 NIH/3T3, USA). The HaCaT cells were cultivated using Dulbecco's Modified Eagle Medium – high glucose, with added 10% fetal bovine serum and penicillin/streptomycin, $100\ \mathrm{U\ mL^{-1}}$ (PAA Laboratories GmbH, Austria). The ATCC-formulated Dulbecco's Modified Eagle's Medium (catalog No. 30-2002), with added bovine calf serum to a final concentration of 10% and penicillin/streptomycin, $100\ \mathrm{U\ mL^{-1}}$, was used as the culture medium in case of NIH/3T3 cells.

The tested samples were diluted to concentrations of 520, 345, 171, 155, 130, 105, 70, 35, and 20 $\mu\mathrm{g\ mL^{-1}}$ in the culture medium. Cytotoxicity testing was conducted according to the EN ISO 10993-5 standard procedure, with modification. Cells were pre-cultivated for 24 h, and the culture medium was subsequently replaced with dilutions of polyaniline colloid. As a reference, cultivation in a pure medium without colloid was used. To assess cytotoxic effect, a MTT assay (Invitrogen Corporation, USA) was performed after one-day cell cultivation in the presence of colloidal polyaniline. All the tests were performed in quadruplicates. The absorption was measured at 570 nm with an Infinite M200 Pro NanoQuant (Tecan, Switzerland). Dixon's Q test was used to remove outlying values, and mean values were calculated. The cell viability is presented in two ways in order to provide a comprehensive view of the results: (1) as a percentage of cells present in the respective extract relative to cells cultivated in a pure extraction medium without colloidal polyaniline (100% viability), and (2) by using the *t*-test expressing the statistical differences between the averages of individual dilutions compared to the reference.

The morphology of the cells was assessed after 24 h of cultivation in the presence of a colloid. The changes in the cell morphology were observed with an Olympus inverted fluorescent microscope (Olympus, CKX 41, Japan). The fluorescent staining of DNA was performed using the Hoechst 33258 dye (Invitrogen Corporation, USA).

2.6. Apoptosis versus necrosis rate

To distinguish healthy, apoptotic and necrotic cells after the contact of the cell cultures with colloidal polyaniline, staining with annexin V/propidium iodide (BD Biosciences, Canada) was

used. The method of cell cultivation, sample preparation and precultivation was the same as in the cytotoxicity test. After that, the colloidal dispersion was removed, and the remaining adherent cells were rinsed with a phosphate buffered saline (PBS), treated with trypsin and added to a previously removed, non-adherent cell population from the same treatment. Cells were re-suspended in a buffer and stained by annexin V–FITC in a concentration of 2.5 $\mu g\,mL^{-1}$ and by propidium iodide in a concentration of 5 $\mu g\,mL^{-1}$. After 15 min in the dark, cells were analyzed by a BD FACSCanto flow cytometer (BD Biosciences, Canada).

2.7. Oxidative burst in human neutrophils and whole blood

The effect of polyaniline on (1) the activation of neutrophils, determined by a generation of reactive oxygen species (ROS) and (2) the scavenging of already formed ROS, where neutrophil ROS production was initiated by the addition of phorbol 12-myristate 13-acetate (PMA), was tested. The following tests were, therefore, performed: (a) measurements of chemiluminescence in whole human blood; (b) measurements of chemiluminescence in isolated neutrophils with discrimination of the effects on extra- and intracellular ROS production; and (c) cell-free assay as a reference.

The neutrophils were isolated from the human blood of healthy donors with written informed consent. Blood was collected by venous puncture. The study was performed in accordance with the Declaration of Helsinki. The blood was gently mixed with dextran from *Leuconostoc mesenteroides*, M_r 425,000–575,000 (Sigma Aldrich, D1037, USA) (2:1) and left at room temperature for 20 min. A buffy coat was collected and centrifuged at 190 g for 10 min at 4 °C. Red blood cells were lysed with hypotonic cold hemolysis and overlaid on Ficoll (GE Healthcare, Sweden). After centrifugation at 390 *g* for 30 min at 4 °C, the neutrophils were washed in cold PBS and used for the experiment. The neutrophil viability was over 95%, as verified with a CASY cytometer (Roche, Switzerland). The statistical differences between reference and individual studied samples were evaluated using a *t*-test.

2.8. Chemiluminescence

The chemiluminescence of human neutrophils in the whole blood was measured in a 96-well microplate luminometer (Luminometer LM-01T, Immunotech, Czech Republic) at 37 °C. Aliquots of polyaniline and luminol (250 $\mu mol\,L^{-1}$) were mixed. To ensure a sufficient concentration of extracellular peroxidase in PMA-stimulated cells, the mixture was supplemented with horseradish peroxidase (HRP; Sigma Aldrich, USA) with the final concentration of 8 $U\,L^{-1}$. Finally, 50-times diluted blood was added and the reaction started via the addition of PMA (0.05 $\mu mol\,L^{-1}$, Sigma Aldrich, USA). The chemiluminescence of the samples was recorded for 1 h, and a quantitative determination of the signals was performed by integrating the chemiluminescence signal over the entire measuring period [32]. Activated samples without polyaniline served as a positive reference. Spontaneous chemiluminescence (without activators) was also measured. The colloidal polyaniline dispersions with concentrations of 171, 17, 1.7, 0.17, 0.017, and 0.0017 $\mu g\,mL^{-1}$ were tested.

The extracellular chemiluminescence in isolated neutrophils was determined by using isoluminol (Sigma Aldrich) and HRP; in the case of intracellular chemiluminescence, luminol was applied. For elimination of extracellular ROS, catalase (2000 $U\,L^{-1}$) (Sigma Aldrich, USA) and superoxide dismutase (100 $U\,L^{-1}$) (Worthington Biochemical Corporation, USA) were added. The samples were measured for 30 min in a 96-well microplate luminometer (Luminometer LM-01T, Immunotech, Czech Republic) at 37 °C [32].

In a cell-free assay, the chemiluminescence was initiated in a culture medium containing polyaniline with the addition of hydrogen peroxide (100 $\mu g\,mL^{-1}$) in the presence of horse radish peroxidase (2 $U\,mL^{-1}$) and luminol (10 $\mu g\,mL^{-1}$). The experiment was conducted in triplicates and each of the samples was measured for 30 min [33]. Correspondingly, as in the previous test dealing with ROS formation, the statistical differences between the reference and individual samples were determined using a *t*-test.

3. Results and discussion

3.1. Characterization of colloidal dispersion

The size of the dispersion particles is one of the crucial parameters influencing the performance of colloids in biological applications. As reported earlier [30], polyaniline particles in dispersion have a typical average size of hundreds of nanometers and are therefore rated as colloids. Dynamic light scattering measurements showed that the particle size of the tested dispersion, expressed as a z-average diameter, was of 226.5 ± 0.5 nm, with an average polydispersity index of 0.145 ± 0.004. These data indicate that colloidal polyaniline is a homogeneous dispersion with a nearly uniform, single population of particles in the expected size range. However, it must be remembered that the size of particles, determined by dynamic light scattering, is z-averaged according to the scattering intensity of each particle fraction. In practice relevant to biological applications, volume and even number averages are more appropriate, and they are smaller than *z*-averages.

The UV–vis spectra of the dispersion diluted with 1 M hydrochloric acid showed a pattern slightly different from that reported by Stejskal and Sapurina [30], with a broad absorption maxima at about 310–395 nm and increasing absorbance toward the measuring limit of the instrument, 800 nm. Stejskal and Sapurina reported an additional local maximum at the wavelength of 854 nm [30]. The above characteristics confirm that the tested sample can be considered as a representative of the standard colloid prepared using procedure recommended by IUPAC, with a poly-(*N*-vinylpyrrolidone) as a stabilizer.

3.2. Antibacterial properties

The susceptibility of bacteria against colloidal polyaniline seems to be species dependent (Table 1). The results showed that the growth of gram-negative *E. coli* was insignificantly reduced from 8.6 to 8.4 log CFU mL^{-1} by 2,000 $\mu g\,mL^{-1}$ of colloidal polyaniline, and full inhibition was observed at a concentration of 3,500 $\mu g\,mL^{-1}$. *P. aeruginosa* was the most resistant bacteria, and colloidal polyaniline with a concentration of 3,500 $\mu g\,mL^{-1}$ only caused a decrease by two orders of magnitude (from 8.9 to 6.9 log CFU mL^{-1}). An antibacterial effect was first observed at the highest tested concentration of 8,500 $\mu g\,mL^{-1}$. The behavior of gram-positive *S. aureus* was analogous to *P. aeruginosa*, with MIC detected at 8,500 $\mu g\,mL^{-1}$. However, a lower colloid concentration of 3,500 $\mu g\,mL^{-1}$ inhibited the growth of *S. aureus* slightly better compared to *P. aeruginosa*, with a reduction of three orders of magnitude (from 8.6 to 5.7 log CFU mL^{-1}) (Table 1). The second tested gram-positive bacteria, *B. cereus*, was completely eliminated after a colloidal polyaniline concentration of 3,500 $\mu g\,mL^{-1}$ was applied, as similarly observed for *E. coli*.

The antibacterial activity observed in the current study can be compared with that determined on polyaniline powder published by Gizdavic-Nikolaidis et al. [34]. Although their paper is primarily devoted to antibacterial properties of functionalized polyanilines and polyaniline copolymers with aminobenzoic acids, standard polyaniline (emeraldine) salt was used as a reference. This comparison can be also performed, thanks to the similarity of the methods used for antibacterial testing. Correspondingly to current

Table 1
Values of minimum inhibitory concentration (MIC) and log CFU for tested bacteria.

Polyaniline [μg mL^{-1}]	*Staphylococcus aureus*	*Bacillus cereus*	*Escherichia coli*	*Pseudomonas aeruginosa*
0	8.6	7.2	8.6	8.9
2000	7.6	7.2	8.4	8.7
3500	5.7	MIC	MIC	6.9
8500	MIC	N/A	N/A	MIC

N/A, not applicable.

work employing a polyaniline colloid, Gizdavic-Nikolaidis et al. [34] prepared suspensions of standard polyaniline powder in a nutrient broth. A comparison of results on polyaniline colloid with those recovered from re-suspended polyaniline powder revealed that colloid seems to possess higher antibacterial activity. Tests performed on powdered polyaniline showed that concentrations higher than 10,000 μg mL^{-1} are efficient against *P. aeruginosa* and *S. aureus*. *E. coli* was inhibited exactly by 10,000 μg mL^{-1}. Hence, all the concentrations here were higher compared with those determined for a colloidal form.

An interesting observation was made on bacterial suspension-added polyaniline at low concentrations, where bacterial growth was observed. Here, the suspension changed its color from originally being green to gray/colorless, except for the suspension containing *P. aeruginosa*, which turned out to be red due to naturally red color of these bacteria. The reason for the bleaching was found in the change of the pH which, thanks to the bacterial growth, increased, thus causing a gradual de-protonation of the polyaniline and resulting in the change of color. As an example, for *E. coli* and *S. aureus*, the pH increased from 6.9 to 8.3 and from 6.6 to 7.5, respectively.

3.3. Cytotoxicity

The results of *in-vitro* cytotoxicity testing are reported in two ways: (1) as mean values and standard deviations of individual measurements with the statistical differences compared to the reference, determined by a *t*-test; and (2) according to the procedure given in the international standard ISO 10993-5, where a cytotoxicity equal to 1 corresponds to 100% cell survival, values of >0.8 are assigned to the absence of cytotoxicity, 0.6–0.8 to mild, 0.4–0.6 to moderate and <0.4 to severe cytotoxicity (Table 2). The results show that with an increasing concentration of colloidal polyaniline in the cultivation media, the viability of cells decreased. The threshold polyaniline concentration for cytotoxicity is about 345 and 105 μg mL^{-1} in the case of HaCaT and NIH/3T3 cells, respectively. Below these concentrations, the colloidal polyaniline does not negatively influence the cells viability. Analogous results were published in the work of Oh et al. [35], who tested the cytotoxicity of polyaniline in the form of nanoparticles with poly(*N*-vinylpyrrolidone) as a stabilizer, and a non-spherical shape differing in aspect ratio. The authors did not observe any notable impact on cell viability below the polyaniline concentration of 25 μg mL^{-1}, and significant decreasing in cell viability was first detected above the concentration of 100 μg mL^{-1}. From the current results recorded on both cell lines, it can be concluded that the HaCaT cell line is more resistant toward the cytotoxic action of colloidal polyaniline and even above the threshold concentration, the cytotoxic effect is only mild in regard to the ISO standard scale. More sensitive NIH/3T3 cells, which are widely used for cytotoxicity evaluation, were damaged to a greater extent at higher concentrations of colloidal polyaniline in the cultivation medium, and the cytotoxic effect, at the two highest concentrations of 345 and 520 μg mL^{-1} upon this cell line, can be described as severe.

Microscopic observations of HaCaT cells (Fig. 1) support the previously discussed results demonstrating the changes in cell quantity and morphology induced by polyaniline dispersion. At the concentration of 70 μg mL^{-1}, only rare and negligible cell damage can be observed, which is visualized in red circles. An increase of polyaniline concentration to 345 μg mL^{-1} undoubtedly influenced cell viability via decreasing cell numbers and simultaneous changes to their morphology. This is especially notable after DNA staining.

Interesting conclusions can be drawn from a comparison of the current results with the report of Vaitkuviene et al. [36], who studied the effect of polypyrrole nanoparticles on cell viability. Primarily mouse embryonic fibroblasts (MEF), mouse hepatoma (MH-22A) cells, and human Jurkat T lymphocytes were used in their work. Although polypyrrole is generally considered to be a biocompatible polymer, the level of cytotoxicity was of 77.6 μg mL^{-1}, and in the case of one of the studied cell lines, Jurkat cells, even of 19.4 μg mL^{-1}. The authors also described the dose- and cell line-dependent cytotoxic effects. The average diameter of polypyrrole particles was of 28 nm. This might explain these surprising differences because the size of the particles, considered as one of the most important factors influencing the biological properties of materials, lies well below the limit of 100 nm, which is

Table 2
Cytotoxicity and apoptotic/necrotic rates of various concentrations of colloidal polyaniline reported as means ± standard deviations (SD) ($n = 4$) according to requirements of ISO 10993-5.

Polyaniline [μg mL^{-1}]	HaCaT		NIH/3T3		Apoptotic/necrotic rate on NIH/3T3		
	Mean ± SD	ISO	Mean ± SD	ISO	Necrotic (%)	Apoptotic (%)	Healthy (%)
520	0.409 ± 0.032**	0.61	0.227 ± 0.008*	0.36	24	54	22
345	0.446 ± 0.045**	0.67	0.220 ± 0.005*	0.34	19	57	24
170	0.579 ± 0.056	0.86	0.428 ± 0.012*	0.67	12	36	52
155	0.632 ± 0.043	0.94	0.374 ± 0.022*	0.58	11	16	73
130	0.651 ± 0.037	0.97	0.413 ± 0.012*	0.65	13	19	68
105	0.663 ± 0.029	0.99	0.499 ± 0.006*	0.78	12	8	80
35	0.660 ± 0.049	0.99	0.529 ± 0.008*	0.83	12	21	67
20	0.672 ± 0.015	1.00	0.528 ± 0.006*	0.83	16	14	70
Reference	0.666 ± 0.061	1.00	0.639 ± 0.025	1.00	9	16	75

According to requirements of ISO 10993-5: cytotoxicity equal to 1 corresponds to 100% cell survival, values of >0.8 are assigned to no cytotoxicity, 0.6–0.8 mild cytotoxicity, 0.4–0.6 moderate toxicity, and <0.4 severe cytotoxicity.

* $P < 0.05$, statistical difference between the reference and individual concentration of polyaniline.

** $P \leq 0.01$, statistical difference between the reference and individual concentration of polyaniline.

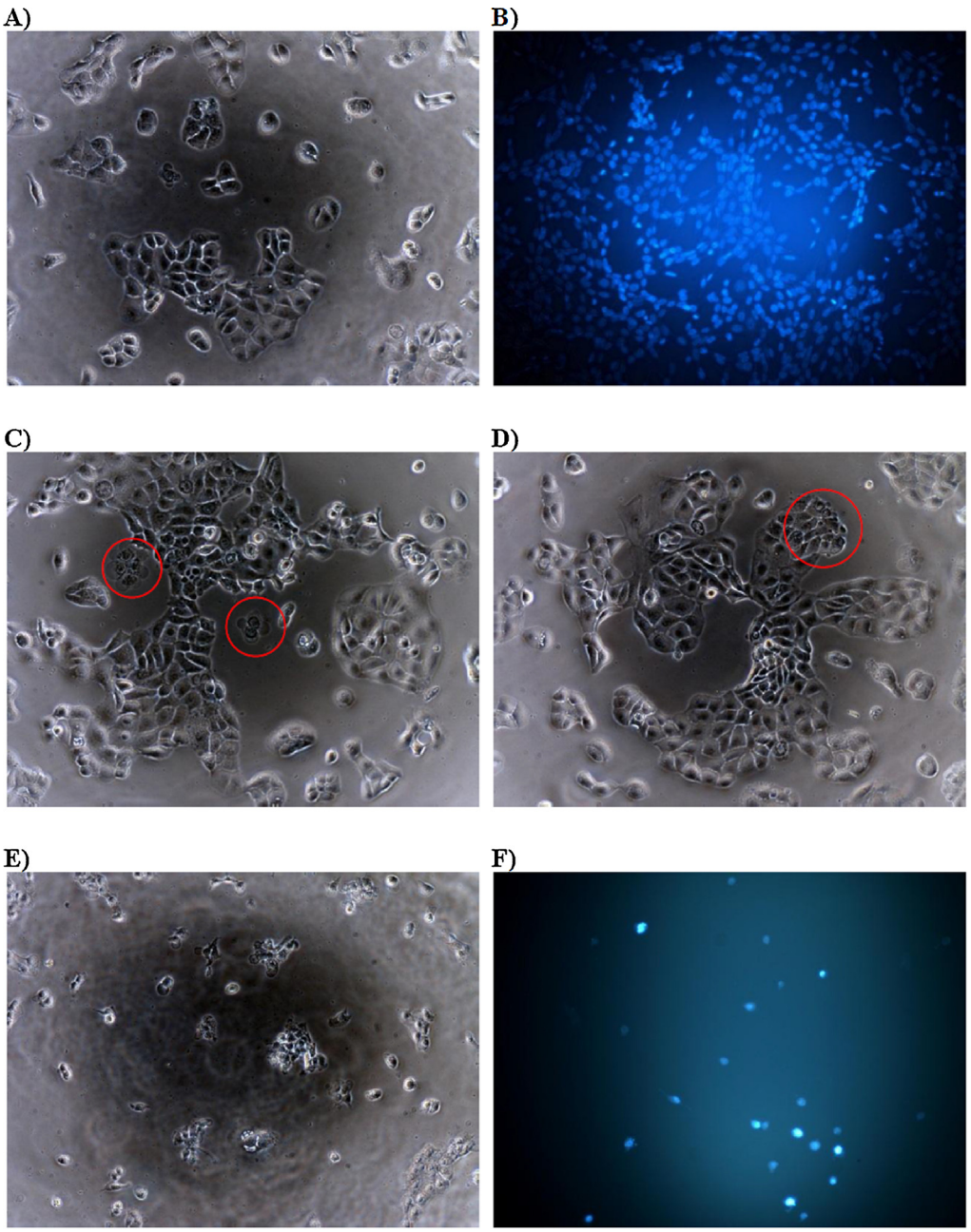

Fig. 1. Microphotographs of HaCaT cell line after the cytotoxicity test: (A) reference; (B) reference – DNA stained by Hoechst; cells treated with (C) 70 μg mL^{-1} colloidal polyaniline; (D) 130 μg mL^{-1} colloidal polyaniline; (E) 345 μg mL^{-1} colloidal polyaniline; (F) 345 μg mL^{-1} colloidal polyaniline – DNA stained by Hoechst. Magnification – 100×.

considered critical for entering the particular material into the cells.

Unfortunately, the cytotoxicity data recorded on colloidal polyaniline are incomparable to the cytotoxicity of standard polyaniline powder, as the testing methodologies are completely different. The cytotoxicity testing of polyaniline in powder form was performed only by Humpolicek et al. [6], who tested an extract (0.2 g of polyaniline in 1 mL of cultivation medium) according to ISO 10993-5. Here, the polyaniline polymer was absent, and the cytotoxicity was mainly assigned to the presence of residual monomers and low-molar mass oligomers present in the extract. Thanks to purification through dialysis, the colloidal dispersion should be free of these impurities, and its cytotoxicity should likely be a result of the polyaniline polymer as such, and not the low-molecular-weight substances.

3.4. Apoptosis versus necrosis rate

The cytotoxicity determined by MTT assay represents the total number of viable cells surviving contact with the cytotoxic substance. It does not reveal, however, the reason why the viability is decreasing. Moreover, as the MTT assay is based on the mitochondrial metabolism, which is also expressed by early apoptotic cells, the test does not distinguish between early apoptotic and healthy cells. Thus, the combination of MTT assay with the annexin/propidium iodide assay can provide a more informative view on the impact of tested materials on the cells. In the case of colloidal polyaniline, the number of healthy, necrotic and apoptotic cells was observed to be dose dependent, and the impact of colloidal polyaniline on each of the groups can be clearly seen (Table 2). According to the annexin/propidium iodide assay, the

Table 3
Neutrophils activation measured as ROS production.

Polyaniline [μg mL^{-1}]	0.00171	0.0171	0.171	1.71	17.1	171
Blood	99.97 ± 3.16	100.71 ± 0.01	98.93 ± 0.94	98.17 ± 1.04	96.10 ± 0.27	98.05 ± 3.58
Neutrophils extracellular	89.14 ± 6.12	89.38 ± 8.52	82.29 ± 11.56	65.02 ± 9.06*	62.22 ± 10.50*	61.07 ± 9.09*
Neutrophils intracellular	101.84 ± 1.10	101.90 ± 0.49	100.37 ± 3.22	97.13 ± 0.44	95.99 ± 4.46	95.66 ± 2.23

The results are shown as % of spontaneously formed ROS = 100% (reference). The values are expressed as mean value ± standard deviation, $n = 3$.
* $P < 0.05$.

Table 4
Polyaniline scavenging of ROS (where neutrophils ROS production was initiated by PMA).

Polyaniline [μg mL^{-1}]	0.00171	0.0171	0.171	1.71	17.1	171
Cell-free assay	96.0 ± 3.4	94.1 ± 5.4	95.6 ± 2.5	75.9 ± 1.5*	6.4 ± 1.6*	0.0 ± 0.00*
Blood	104.4 ± 4.1	105.5 ± 1.3	95.6 ± 6.2	66.7 ± 4.2*	2.8 ± 1.0*	0.0 ± 0.0*
Neutrophils extracellular	108.2 ± 5.1	107.8 ± 4.5	99.4 ± 6.3	52.1 ± 4.2*	0.0 ± 0.0*	0.0 ± 0.0*
Neutrophils intracellular	114.3 ± 12.7	117.7 ± 10.3	113.0 ± 13.1	96.6 ± 3.8	23.2 ± 5.3*	0.0 ± 0.0*

The effects of colloid on ROS production in a cell-free assay as well as in blood and isolated neutrophils. The results are shown as a % of stimulated control where H_2O_2 and PMA stimulated samples = 100% (reference). The values are expressed as mean value ± standard deviation, $n = 3$.
* $P < 0.01$.

cytotoxicity threshold is of around 150 μg mL^{-1} and is higher than the limit of 105 μg mL^{-1} determined according to MTT. Below this concentration, the number of healthy cells increases and the number of apoptotic cells dramatically decreases. Due to the aforementioned differences between the MTT and annexin/propidium assays, the concentration of 150 μg mL^{-1} seems to be more relevant for potential applications of colloidal polyaniline in biomedicine.

3.5. Suppression of oxidative burst in human neutrophils

Neutrophil leucocytes represent professional phagocytic cells. When appropriately stimulated, they undergo dramatic physiological and biochemical changes resulting in phagocytosis, chemotaxis and degranulation with the activation of ROS production, known as the respiratory burst [37]. Neutrophils work as professional killers and instructors of the immune system in the context of infection and inflammatory diseases. Thus, the investigation of their reactions is an important part of biocompatibility studies.

At first, the effect of colloidal polyaniline on the activation of neutrophils was measured by monitoring ROS generation. The amount of formed ROS was then compared with the spontaneous (not induced) chemiluminescence of neutrophils. The results revealed that ROS were absent, and therefore colloidal polyaniline alone did not cause the neutrophil activation (Table 3). However, the measurements of extracellular chemiluminescence indicated that colloidal polyaniline, at concentrations of 171, 17 and 1.71 μg mL^{-1}, was able to work as an ROS scavenger. The cell-free assay also showed that the scavenging effect of polyaniline colloid toward ROS, generated in a cell-free system of luminol–hydrogen peroxide–peroxidase, is concentration dependent. Namely, the concentrations of 171, 17 and 1.7 μg mL^{-1} significantly decreased the ROS content, while the lower tested concentrations were inefficient. Based on these results, a second test was performed on whole blood and isolated neutrophils, in which ROS formation was stimulated with PMA prior to colloidal polyaniline addition (Table 4). Correspondingly to the cell-free assay, the significant scavenging effect of polyaniline colloid toward ROS generated by PMA-stimulated neutrophils was observed in concentrations of 171 and 17 μg mL^{-1}.

To disclose whether the polyaniline colloid can influence intra or extracellular ROS production, an additional experiment was conducted resulting in the conclusion that in isolated neutrophils stimulated with PMA, colloidal polyaniline inhibited both extracellular and intracellular ROS production. Polyaniline was, however, more efficient in the inhibition of extracellular ROS. The inhibition of both extra- and intracellular ROS production initiated with polyaniline correlated with its effects on ROS, determined in the cell-free assay. It can be therefore suggested that the influence of colloidal polyaniline on ROS occurrence in whole blood or isolated neutrophils arise due to the ROS scavenging properties. In the context of the cytotoxicity results recorded on an annexin/propidium assay, the critical concentration of colloidal polyaniline for biologically safe application is 150 μg mL^{-1}, which is also an un-provoking concentration for the neutrophil activity.

4. Conclusions

Aqueous dispersions of colloidal polyaniline can be included among the promising conducting and electroactive systems, possessing advantageous properties for biomedical applications. The information on its fundamental biological characteristics is presented here for the first time. The summary results from the study show that polyaniline colloid demonstrates a low antibacterial activity with the most susceptible bacterial species being *B. cereus* and *E. coli*, which were both inhibited with a colloid concentration of 3500 μg mL^{-1}. In the context of the cytotoxicity results recorded on an MTT assay, the toxic effect of this system was found to be dose- and cell-line dependent; more sensitive NIH/3T3 cells were damaged to a higher extent compared with HaCaT cells. Flow-cytometry data recorded on an annexin/propidium assay, capable of distinguishing healthy, apoptotic and necrotic cells after polyaniline treatment, indicated that the critical colloid concentration for biologically safe applications is of 150 μg mL^{-1}. This is also an un-provoking concentration limit for neutrophil activity, measured through the detection of reactive oxygen species. The combination and mutual assessment of obtained results suggests that the conducting polyaniline colloid possesses low cytotoxicity and the absence of an oxidative burst, however a weak antibacterial performance should be considered a known limitation.

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ARTICLE II

Humpolíček P., Kašpárková V., Stejskal J., **Kuceková Z**., Ševčíková P. Cell Proliferation on a Conducting Polymer (Polyaniline). *Chem listy*. 2012, 106(5): 380-383.

LABORATORNÍ PŘÍSTROJE A POSTUPY

PROLIFERACE BUNĚK NA VODIVÉM POLYMERU, POLYANILINU

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Klíčová slova: polyanilin, proliferace, lidské jaterní buňky

Úvod

V posledním desetiletí došlo k výraznému rozvoji znalostí o vodivých polymerech a tím i k jejich rozšíření do praktických aplikací. Elektrická vodivost těchto polymerů se pohybuje v rozmezí 0,01–30 S cm^{-1} a je srovnatelná s vodivostí anorganických polovodivých materiálů. Mezi nejčastěji studované vodivé polymery patří polypyrrol, polyacetylen a polyanilin. Polyanilin může běžně existovat v několika formách, které se navzájem liší stupněm oxidace nebo protonace[1]. Nejběžnějšími formami jsou zeleně zbarvená vodivá polyanilinová (emeraldinová) sůl a nevodivá modrá báze (Schéma 1). K přechodu mezi těmito dvěma formami dochází při pH 5–6 a vodivost polyanilinu tímto přechodem klesá z jednotek S cm^{-1} na 10^{-9} S cm^{-1}. Obě uvedené formy se liší nejen z hlediska elektrických vlastností, ale odlišují se i svým chováním při kontaktu s živými organismy, tkáněmi či jednotlivými buňkami. Polyanilin je jedním z vodivých polymerů, pro něž se hledalo využití především s ohledem na jejich schopnost reagovat změnou elektrické vodivosti na vnější stimuly. Tato schopnost vedla k jeho aplikacím v oblasti senzorů[2], antikorozních nátěrů[3], katalýzy organických reakcí[4] či palivových článků[5].

Velmi zajímavé a slibné je využití polyanilinu v biomedicínských aplikacích. V této oblasti se výzkum a vývoj soustřeďuje především na jeho uplatnění v regeneraci srdeční[6] či nervové tkáně[7]. Nevýhodou dosud publikovaných prací zabývajících se biomedicínskými aplikacemi polyanilinu je skutečnost, že se většinou zaměřují na sledování vlastností polyanilinových kompozitů a nikoliv polyanilinu samotného. To je výhodné z hlediska konkrétních aplikací či výrobků, ale zároveň je tím výsledek práce omezen vždy jen na daný, konkrétní systém bez možnosti zobecnění výsledků pro ostatní aplikace. Nejčastěji studovanými materiály jsou kompozity polyanilinu s poly-L-lysinem[6], s poly(L-laktidem-*co*-ε-kaprolaktonem)[8] nebo želatinou[9].

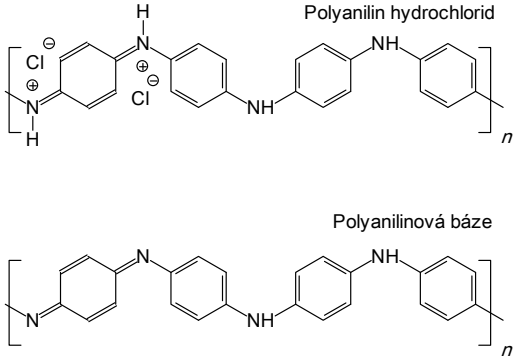


Schéma 1. **Vodivá polyanilinová sůl a nevodivá polyanilinová báze**

Cílem této práce bylo studium metodiky testování vazby a proliferace lidských buněk na povrchy filmů připravených z „čistého“ polyanilinu jak z jeho vodivé, tak i nevodivé varianty.

Experimentální část

Příprava polyanilinových filmů

Při přípravě polyanilinových filmů pro testování proliferace buněk bylo využito schopnosti vodné reakční směsi používané pro oxidaci anilinu vytvářet film o submikrometrové tloušťce na površích substrátů, které jsou do této reakční směsi ponořeny[10]. Tvorba filmu probíhala *in situ* přímo v polystyrenových kultivačních miskách (TPP, Switzerland). Anilin hydrochlorid byl rozpuštěn ve vodě a smíchán se stejným objemem vodného roztoku peroxodisíranu amonného; koncentrace obou látek ve výsledné směsi byla 0,2 mol l^{-1} a 0,25 mol l^{-1} (cit.[10]). Vzniklá směs byla nalita do kultivačních misek. Reakce probíhala při laboratorní teplotě a byla ukončena

po 10 min, kdy na polystyrenovém povrchu narostl zelený film vodivé polyanilinové soli. Jednotlivé misky byly vypláchnuty 0,2 mol l^{-1} roztokem kyseliny chlorovodíkové k odstranění sraženiny polyanilinu na povrchu filmu, poté methanolem a následně byly vysušeny na vzduchu. Výsledný film má globulární morfologii[10] a tloušťku kolem 100 nm. Vodivý film polyanilinové soli (hydrochloridu), byl v 50 % experimentů převeden na modrou, nevodivou polyanilinovou bázi působením 1 M roztoku hydroxidu amonného.

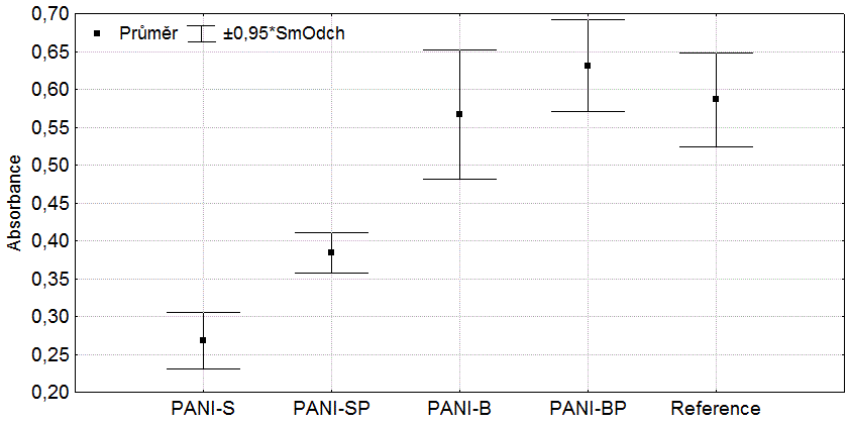
Studium růstu buněk

V této práci byla použita buněčná linie HepG2 od firmy ATCC (HB-8065). Jedná se o linii lidských hepatocelulárních buněk pocházejících z karcinomu jater. Kultivační podmínky byly následující: jako kultivační médium bylo použito Eagle's Minimum Essential Medium od firmy ATCC doplněné fetálním bovinním sérem v poměru 1:10. Dále bylo do 550 ml média přidáno 5 ml 2 mM L-glutaminu a 2 ml gentamycinu o koncentraci 50 μ g ml^{-1} (PAA Laboratories GmbH, Austria). Buňky byly kultivovány v inkubátoru HeraCell 150i (Thermo Scientific, USA) při teplotě 37 °C v 5% atmosféře CO_2. Práce byly prováděny v laminárním boxu II. třídy HERAsafe KSP (Thermo Electron LED Gmbh, Německo).

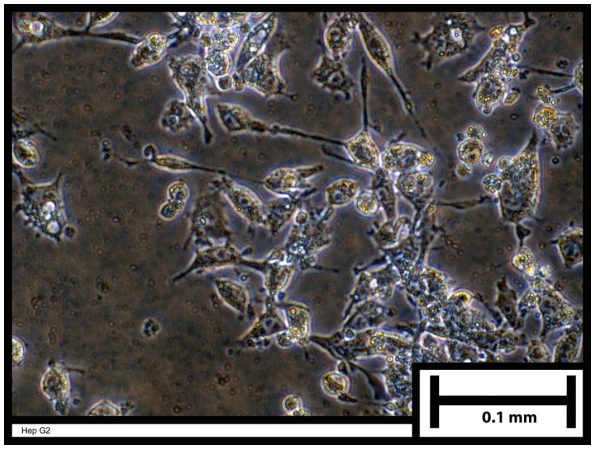
Vzorky filmů byly před vlastním testem rozděleny do 4 skupin. První skupinu tvořily polyanilinové filmy solí polyanilinu (PANI-S), druhou skupinu představovaly filmy polyanilinových bází (PANI-B), třetí a čtvrtá skupina odpovídaly filmům polyanilinové soli a báze, které byly před začátkem testu po dobu 30 min ponořeny do „ultračisté“ vody (Simplicity, Millipore, USA). Tyto vzorky jsou dále označované jako PANI-SP a PANI-BP. Buňky byly na připravené povrchy naneseny v koncentraci $1 \cdot 10^5$ buněk v 1 ml a následně kultivovány po dobu 3 dnů. Schopnost buněk přichytit se na povrch daného filmu a jejich morfologické charakteristiky byly stanoveny pomocí invertovaného mikroskopu s fázovým kontrastem CKX41 (Olympus, Japan). Ke stanovení počtu buněk, které narostly za dobu tří dnů, byla využita kolorimetrická metoda MTT, vyhodnocená na přístroji Sunrise Elisa Reader (Tecan, Switzerland). Statistická průkaznost rozdílů mezi počtem buněk uchycených na jednotlivých typech polyanilinových filmů byla stanovena *t*-testem.

Výsledky a diskuse

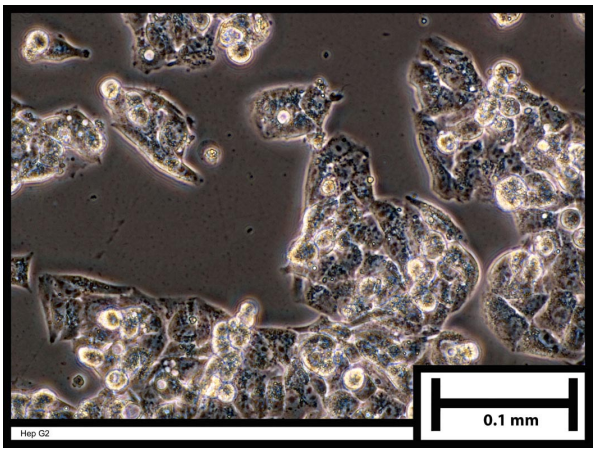
Z obr. 1 je patrné, že na všech čtyřech typech sledovaných povrchů PANI vzorků se buňky dokázaly bez obtíží „přichytit“ a následně růst. Tento výsledek je v souladu s dříve publikovanými pracemi[11], které prokázaly, že povrchy pokryté filmy polyanilinu umožňují bezproblémovou adhezi buněk. Z obr. 2 je však také zřejmé, že ve srovnání s kontrolním vzorkem (obr. 3), kde buňky rostly na tkáňovém polystyrenu běžně užívaném pro kultivace buněk, došlo ke změně jejich morfologie. Ze srovnání je patrné, že buňky ztrácely svůj typický tvar, ohraničení buněk bylo méně jasné a zároveň vznikalo více ektoplazmatických výběžků, pseudopodií. Schopnost buněk přichytit se a růst na polymerních površích je dána především hydrofilitou či hydrofobicitou povrchu, která souvisí s volnou povrchovou energií a charakteristickými funkčními skupinami na povrchu filmů. Pozorovaná vazba a růst buněk na povrchu polyanilinu jsou tedy podmíněny jeho hydrofilitou[12]. I když je polyanilin hydrochlorid podstatně hydrofilnější než polyanilinová báze, jak potvrzují měření kontaktních úhlů[13], zjištěný rozdíl adhezi buněk na sledované povrchy neovlivnil. Kromě hydrofility povrchu je proces buněčné adheze rovněž ovlivněn fyzikálními, povrchovými vlastnostmi, např. zrnitostí, topografií nebo rigiditou. V případě sledovaných polyanilinových filmů však podstatné rozdíly v kvalitě



Obr. 1. **Průměrná absorbance kvantifikující hustotu buněk na sledovaných filmech připravených ze vzorků polyanilinu a její směrodatná odchylka;** PANI-S – polyanilinová sůl, PANI-SP – upravená polyanilinová sůl, PANI-B – polyanilinová báze, PANI-BP – upravená polyanilinové báze, reference – tkáňový polystyren



Obr. 2. **Morfologie buněk rostoucích na filmu tvořeném polyanilinovou bází**



Obr. 3. **Morfologie buněk referenčního vzorku – růst na tkáňovém polystyrenu**

povrchu pozorovány nebyly.

Zatímco výsledky získané studiem vazby buněk na povrchy připravené z polyanilinu jsou v souladu s očekáváním i s výsledky publikovanými jinými autory, výsledky studie zaměřené na sledování proliferace buněk na studovaných filmech jsou nové a překvapivé. Oproti očekávání byly detegovány rozdíly v proliferaci buněk na jednotlivých typech filmů: ochota buněk růst na daném filmu se významně lišila a závisela na tom, zda se jednalo o film připravený z polyanilinové soli, báze či o filmy, které byly upraveny opláchnutím „ultračistou“ vodou.

Film připravený z vodivé polyanilinové soli (PANI-S), která má nejvyšší potenciál pro praktické použití, vykazuje ze všech testovaných filmů nejnižší schopnost proliferace buněk. Naopak, proliferace zaznamenaná na povrchu pokrytém polyanilinovou bází (PANI-B) byla statisticky významně vyšší. Nejvyšší proliferace byla pozorována na povrchu upraveného, ultračistou vodou opláchnutého filmu polyanilinové báze (PANI-BP). Také v případě povrchu tvořeného upravenou polyanilinovou solí (PANI-SP) byla pozorována ve srovnání s původním, neupraveným filmem vyšší proliferace buněk. To lze vysvětlit tím, že vlivem působení ultračisté vody dochází nejen k odstranění nečistot, ale i k postupné deprotonaci tenkého filmu polyanilinové soli naneseného na nosiči a jeho přeměně na film tvořený převážně polyanilinovou bází. Výsledná proliferace na povrchu upraveného PANI-SP je tedy jen velmi obtížně srovnatelná s proliferací detegovanou na původním povrchu PANI-P. Jednotlivé výsledky vyjadřující schopnost buněk růst na sledovaných površích jsou patrné z obr. 1, který znázorňuje hodnoty absorbance MTT. Výše uvedené rozdíly byly potvrzeny také matematicko-statistickým zpracováním. Mezi schopností povrchů PANI-S a PANI-SP stimulovat proliferaci buněk byl detegován průkazný rozdíl. Statisticky průkazný byl pak i rozdíl mezi PANI-S a PANI-SP oproti PANI-B, PANI-BP a kontrolnímu vzorku. Mezi PANI-B a PANI-BP pak nebyly detegovány žádné statisticky významné

Tabulka I
Hodnoty proliferace dle MTT testu stanovené na jednotlivých površích a jejich statisticky průkazné rozdíly

Povrch [a]	Průměr	Směrodatná chyba průměru	Směrodatná odchylka	Minimum	Maximum
PANI-S [A]	0,2678	0,0065	0,0389	0,1780	0,3240
PANI-SP [B]	0,3839	0,0047	0,0283	0,3020	0,4310
PANI-B [C]	0,5673	0,0150	0,0899	0,4270	0,7410
PANI-BP [C]	0,6314	0,0107	0,0640	0,5250	0,7340
Reference [C]	0,5862	0,0132	0,0649	0,4360	0,7680

[a] Statisticky průkazné rozdíly jsou vyznačeny pomocí indexů. Rozdílné indexy ve sloupci 1 značí statisticky průkazný rozdíl $P \leq 0{,}05$ mezi příslušnými vzorky. PANI-S – polyanilinová sůl, PANI-SP - upravená polyanilinová sůl, PANI-B – polyanilinová báze, PANI-BP – upravená polyanilinová báze; Reference – tkáňový polystyren

rozdíly, stejně jako mezi těmito dvěma vzorky a vzorkem referenčním. Výsledky ukázaly, že povrch filmu z PANI-BP vykázal v průměru vyšší proliferaci buněk, než referenční polystyrenový povrch. Tento rozdíl je však statisticky neprůkazný.

Podrobné výsledky statistického zpracování jsou uvedeny v tab. I. Z prezentovaných výsledků vyplývá, že vodivá polyanilinová sůl stimuluje proliferaci buněk méně, než polyanilinová báze. Procesem úpravy pak dochází ke zlepšení proliferace. To může být způsobeno jednak odstraněním potenciálně toxických nečistot a v případě filmu polyanilinové soli také její částečnou deprotonací a přeměnou na polyanilinovou bázi, která vykazuje „příznivější" biologické vlastnosti. V průběhu konverze polyanilinu hydrochloridu na polyanilinovou bázi dochází též k odstranění kyseliny chlorovodíkové, resp. chloridových protiontů (Schéma I), což rovněž může vést ke zlepšení atraktivity polyanilinových povrchů pro buňky. Použití různých polyanilinových solí, např. síranu, dusičnanu nebo fosforečnanu, by mohlo poskytnout odpověď na otázku, do jaké míry jednotlivé protionty odpovídají za interakci buněk s povrchem substrátu.

Závěr

Výsledky studie zaměřené na sledování schopnosti lidských jaterních buněk přichytit se na povrchy připravené z vodivé polyanilinové soli i nevodivé polyanilinové báze potvrdily, že se sledované buňky váží na oba studované filmy. Následná proliferace buněk na sledovaných filmech ukázala statisticky průkazné rozdíly ve schopnosti buněk růst v závislosti na typu polyanilinového filmu a jeho následné úpravě. Z provedených testů je zřejmé, že vodivá polyanilinová sůl je pro růst buněk nejméně příznivá, což limituje její praktické biomedicínské využití bez dalšího studia procesu přečištění, případně dalších úprav. Navazující výzkum bude orientován na sledování elektrické vodivosti polyanilinu při interakci s živou hmotou.

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P. Humpolíček[a,b], V. Kašpárková[a,c], J. Stejskal[d], Z. Kuceková[a,b], and P. Ševčíková[c] *([a] Centre of Polymer Systems, [b] Polymer Centre, [c] Department of Fat, Surfactans and Cosmetics Technologies, Faculty of Technology, Tomas Bata University, Zlín, [d] Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Prague):* **Cell Proliferation on a Conducting Polymer (Polyaniline)**

Polyaniline (PANI) belongs to a group of conducting polymers that show numerous properties useful in biomedical applications. Although PANI has long been studied in terms of interaction with human tissue, the published studies are mostly focused on composites of polyaniline with other polymers, not allowing for generalization of the obtained results. The present study is focused on the adhesion of human liver cells to a conducting and non-conducting polyaniline films. The ability of human liver cells to attach to both types of the tested surfaces was confirmed. Cell proliferation on the PANI surfaces was monitored in relation to material biocompatibility and to its practical applications. The results showed statistically significant differences in cell proliferation depending on the type of PANI film. The study thus points out the need to test materials in their neat forms, which allow for better generalization of the test results leading to their broader applications.

ARTICLE III

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ORIGINAL PAPER

Antibacterial properties of polyaniline–silver films

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In situ polymerised thin polyaniline (PANI) films produced on polystyrene dishes were tested for their antibacterial activity with respect to *Escherichia coli* and *Staphylococcus aureus*, representing both gram-positive and gram-negative bacteria. PANI films were subsequently used for the reduction of silver ions to metallic Ag. PANI salt and base in original forms and after the deposition of Ag were studied. PANI salt showed a significant antibacterial effect against both bacteria strains while the efficacy of neat PANI base was only marginal. After the Ag deposition, the PANI base exhibited different levels of antibacterial effect depending on the type of the bacterial strain; the growth of gram-positive *Staphylococcus aureus* was inhibited depending on the Ag concentration on the film, while *Escherichia coli* remained uninfluenced. Efficacy of the PANI salt with deposited Ag against both bacteria strains was comparable with that of PANI alone and was not affected by the Ag concentration. The results show that Ag deposition can be a suitable method for the preparation of PANI base films with improved antibacterial properties.

Keywords: polyaniline, silver, antibacterial properties, *Escherichia coli*, *Staphylococcus aureus*

Introduction

Besides all its advantages, application of polymers in medicine brings also a problem related to the occurrence of nosocomial infections. Therefore, a considerable effort has been exerted to develop polymers or composites with efficient antibacterial properties. In addition to polymer materials which possess an intrinsic antibacterial activity, these properties can be also achieved through (*i*) coating or adsorption of an antibacterial agent onto the polymer surface; (*ii*) immobilisation of an antibacterial agent in the polymer via ionic or covalent bonding or (*iii*) by direct incorporation of an antibacterial agent into the polymer during its synthesis (Radheshkumar & Münstedt, 2005; Bílek et al., 2011). Recently published studies indicate that among the polymers inherently showing antibacterial properties are also conducting polymers such as polyaniline (PANI) (Stejskal et al., 2010), which has been the subject of considerable attention due to its potential in biomedical and other promising applications (Pelíšková et al., 2007). Its efficacy against gram-positive and gram-negative bacteria and against fungi was first reported by Seshadri and Bhat (2005) and Shi et al. (2006). Seshadri and Bhat (2005) prepared cotton fabrics coated with an in situ polymerised PANI salt. They observed significant reduction of the colony-forming units (CFU) of gram-positive *Staphylococcus aureus* (*S. aureus*, 95 %), gram-negative *Escherichia coli* (*E. coli*, 85 %) and *Candida albicans* fungi (92 %),

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which was explained by the activity of ions contained in PANI against the bacterial cell-wall. Also the following study of Shi et al. (2006) reported total reduction of *E. coli* and *S. aureus* after 24 h of incubation on composite PANI films. In this case, possible explanation of the observed antibacterial effect was based on the FTIR measurements revealing the possibility of the change in molecular structure of the PANI composite after its interaction with the bacterial species. These papers offered also two different explanations of the mechanism of the PANI antibacterial effect, namely (*i*) possible reaction of acidic dopants on the polymer chains with the bacteria (Seshadri & Bhat, 2005) and (*ii*) electrostatic adherence between the PANI macromolecules and the bacteria (Shi et al., 2006). Antibacterial properties of functionalised and standard PANIs were subject of interest in the thorough work of Gizdavic-Nikolaidis et al. (2011). Though all tested substances showed antibacterial activity, functionalised polymers were more efficient compared to the standard ones. Not only the PANI polymer alone but also its acetone extracted oligomers were reported to possess antibacterial properties (Gizdavic-Nikolaidis et al., 2012). Also Humpolicek et al. (2012) reported a notable cytotoxicity of the PANI emeraldine salt on human cells. Though the last mentioned study was conducted using eukaryotic, not bacterial cells, it also confirms the negative influence of PANI on cellular viability.

Silver has been known as one of the most powerful disinfectants (Sharma et al., 2009) possessing a wide range of activities against bacteria, moulds, and yeasts (Lansdown, 2002). Numerous papers confirm the enhancement of antibacterial properties of polymers after the inclusion of Ag, whether in form of nanoparticles, salts or oxides. In addition, the incorporation of Ag into the matrix of conducting polymers can provide a composite material with excellent properties combining the activities of Ag as an enhancer of conductivity and, simultaneously, the antibacterial protection of the prepared composite material (Kelly et al., 2007; Nesher et al., 2011; Prabhakar et al., 2011; Jia et al., 2012; Shi et al., 2012; Tamboli et al., 2012; Zhao et al., 2012). The synergistic antibacterial effect of PANI and Ag against *E. coli* and *S. aureus* has recently been demonstrated by Jia et al. (2012) who studied PANI–silver nanocomposites containing PANI nanofibres and Ag nanoparticles. The PANI–Cu-doped ZnO nanocomposite (Liang et al., 2012) and the PANI–sulfosilicate composite (Nabi et al., 2011) can be presented as other examples of PANI composites with antibacterial activity.

In the present study, PANI salt and base films containing Ag nanoparticles were successfully prepared through a two-step synthesis, the in situ deposition of PANI films (Stejskal & Sapurina, 2005) followed by the reduction of silver nitrate to Ag (Stejskal et al., 2008, 2009). Their antibacterial activity was tested on gram-negative *E. coli* and gram-positive *S. aureus* strains and compared with the activity of PANI and a polystyrene reference surface. For this purpose, the international standard ISO 22196 "Plastics – measurement of antibacterial activity on plastic surfaces" was used.

Experimental

Polyaniline films

Thin PANI films were deposited in situ directly on polystyrene culture plates during the chemical oxidation of aniline (Stejskal & Sapurina, 2005). Aniline hydrochloride (Fluka, Switzerland) and ammonium peroxydisulfate (Lach-Ner, Czech Republic) were used as delivered. Both aniline hydrochloride (purum; 2.59 g, 20 mmol) and ammonium peroxydisulfate (purum, 5.71 g, 25 mmol) were separately dissolved in water to 50 mL solutions. The solutions were mixed and the resulting mixture was immediately poured into culture plates. The reaction proceeded at ambient temperature for 1 h during which a PANI film was formed on the polystyrene surface. The reaction mixture containing the PANI precipitate was then separated, and the excess of PANI on the polystyrene surface was removed by rinsing the plates with 0.2 M hydrochloric acid and with methanol, and dried in air. Half of the plates with the film of green PANI salt (PANI-S) were transferred to a blue PANI base (PANI-SB) through a deprotonation in a 1 M ammonium hydroxide solution. The film thickness was ≈ 125 nm (Stejskal & Sapurina, 2005).

Silver nitrate (Lach-Ner, Czech Republic) was dissolved in 0.1 M nitric acid or 1 M ammonium hydroxide to form 10^{-3}–10^{-7} M solutions. These were poured over the films of PANI-S and PANI-SB, respectively, and left for 24 h to interact. Under such conditions, the emeraldine form of PANI was converted to pernigraniline, and silver ions were reduced to silver metal (Stejskal et al., 2009). The samples were denoted as PANI-S-Ag-10^{-x} and PANI-SB-Ag-10^{-x}, where x = 3–7, for PANI-S and PANI-SB, respectively.

Antibacterial properties

Prior to testing, the samples were disinfected by an exposure to an UV-radiation source (258 nm) emitted from a low-pressure Hg lamp (UV-C Long Life 30 W/G30TB, Phillips, The Netherlands). Polyaniline is stable under such treatment (Blinova et al., 2005). As model microorganisms, the gram-negative *E. coli* (ATCC 8739) and the gram-positive *S. aureus* strains (ATCC 6538) were used. The test was performed according to ISO 22196 with a modification. Nutrient broth with 1 % peptone (M244) and nutrient agar No. 2 (M1269) were used in the test (HiMedia Laboratories, India).

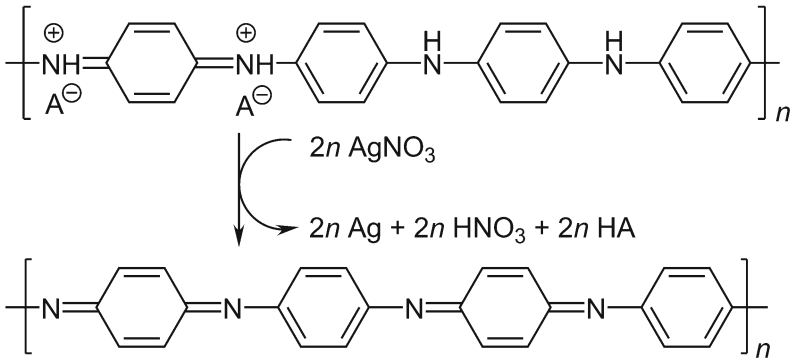

Fig. 1. PANI salt reduces silver nitrate to metallic Ag. Emeraldine form of PANI is converted to pernigraniline (Stejskal et al., 2008, 2009).

E. coli and *S. aureus* were inoculated in two concentrations, namely 4.5×10^8 CFU mL^{-1} and 2.1×10^6 CFU mL^{-1} (CFU means colony-forming unit); 0.25 mL of the bacterial suspension was pipetted onto the PANI and PANI–Ag coated culture plates. Incubation was carried out at 35 °C and 100 % humidity for 24 h. After the incubation, the bacterial suspension was removed, the PANI surfaces were rinsed with 6.25 mL of soyabean casein digest medium (M011, HiMedia Laboratories, India) followed by the addition of lecithin and Tween 80 (HiMedia Laboratories, India) and then homogenised using a vortex. The dilutions were inoculated into Petri dishes with agar (M091, HiMedia Laboratories, India) and cultivated for 24–48 h. The results are expressed as the reduction of bacterial growth in the logarithmic scale at the corresponding dilution relative to the positive control, bacterial growth on the surface of neat polystyrene Petri dishes. All tests were performed in quadruplicate.

Results and discussion

Although PANI is considered as a promising conducting polymer for the application in biomedicine or more generally in biotechnology, only a few studies dealing with the antibacterial properties of this polymer have been published (Shi et al., 2006; Seshadri & Bhat, 2005; Gizdavic-Nikolaidis et al., 2011). Moreover, the majority of the studies is focused on the conducting PANI salt and only limited attention has been devoted to non-conducting PANI base (Gizdavic-Nikolaidis et al., 2012).

The published papers dealing with antibacterial activity of PANI and PANI based composites can be, for the purpose of the present study, divided into two groups: those concerning the activity of bare PANI and those concerning the properties of PANI containing Ag. In the current study, Ag nanoparticles are deposited on the PANI surface by the immersion of naked PANI films in a silver nitrate solution. The reaction follows the mechanism presented in Fig. 1.

Table 1. Antibacterial effect of PANI and PANI–Ag films

PANI-type	Antibacterial activity[a]	
	E. coli	*S. aureus*
PANI-S	> 5.6	> 5.7
PANI-S-Ag-10^{-3}	> 5.6	> 5.7
PANI-S-Ag-10^{-4}	> 5.6	> 5.7
PANI-S-Ag-10^{-5}	> 5.6	> 5.7
PANI-S-Ag-10^{-6}	> 5.6	> 5.7
PANI-S-Ag-10^{-7}	> 5.6	> 5.7
PANI-SB	0	0.98
PANI-SB-Ag-10^{-3}	0	> 5.7
PANI-SB-Ag-10^{-4}	0	5.7
PANI-SB-Ag-10^{-5}	0	3.7
PANI-SB-Ag-10^{-6}	0	0.72
PANI-SB-Ag-10^{-7}	0	0.17
Polystyrene[b]	–	–

a) Reduction of colony-forming units (CFU) in logarithmic scale; *b*) reference surface.

Polyaniline

Conclusions from the previously published papers dealing with antibacterial activity of PANI (Seshadri & Bhat, 2005; Shi et al., 2006) are in agreement with the results from the current work demonstrating significant impact of PANI-S on *E. coli*. From Table 1 it is obvious that PANI-S completely reduces bacterial growth compared with the reference polystyrene surface. Contrary to this finding, the films of PANI-SB were inefficient against *E. coli* and exhibited only negligible effect against *S. aureus*. This corresponds to the findings of a comprehensive study performed by Gizdavic-Nikolaidis et al. (2012) who found out that PANI inhibited bacterial growth more efficiently in its conducting form compared to the non-conducting one. Although these authors studied mainly the effect of functionalised PANI, some of their conclusions can be adopted for the discussion of the current results. Shi et al. (2006) suggested that the antibacterial effect of PANI consists in the cell death caused by the destruc-

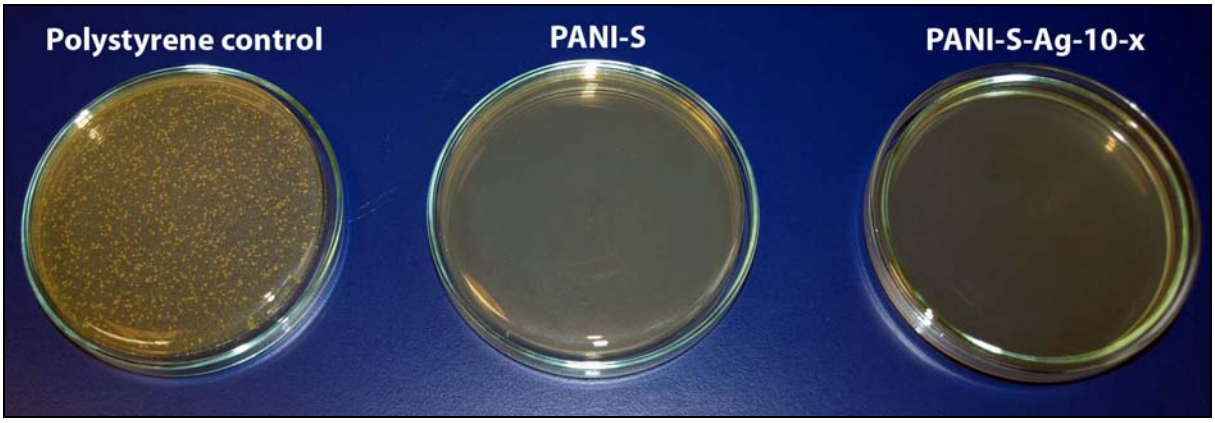

Fig. 2. Antibacterial activity of PANI-S-Ag and PANI-S films against *S. aureus* in comparison with polystyrene reference.

tion of the bacterial cell-wall via electrostatic contact. Moreover, Gizdavic-Nikolaidis et al. (2012) concluded that antibacterial activity of the functionalised PANI is significantly enhanced at lower concentrations compared to that of standard PANI; however, the reduced conductivity of PANI derivatives limits their possible applications. Considering the absence of the antibacterial effect of PANI-SB against *E. coli* and its weak antibacterial effect against *S. aureus* detected in the current study, both explanations suggested by Seshadri & Bhat (2005) and Shi et al. (2006) and Gizdavic-Nikolaidis et al. (2012) seem to be possible. However, the results of a recent study conducted by Humpolicek et al. (2012) show that the impact of acidic dopant is a more appropriate explanation of detected antibacterial activity.

Due to several fundamental differences between the properties of PANI-S and PANI-SB, exact determination of the decisive property for the antibacterial action is difficult. The conducting PANI-S exhibits surface acidity, it is hydrophilic, and it is a polycation. The chemical nature of the dopant acid may also be of importance. Non-conducting PANI-SB, on the contrary, shows basic character, it is electrically neutral and hydrophobic, i.e. possibly more similar in properties to ordinary organic compounds. PANI-S differs from PANI-SB also in colour (green versus blue). Though this does not seem to be important, the fact that many antibacterial agents are organic dyes may be of some inspiration.

Polyaniline–silver (PANI–Ag)

The key problem in the evaluation of the studies dealing with the antibacterial effect of PANI combined with Ag is their inconsistency in terms of different methods of PANI preparation, results evaluation or the lack of information about the concentration and form of Ag. A pioneering study published by Prabhakar et al. (2011) presented the reduction of biofilm formation (*P. aeruginosa* and *B. subtilis*) on polyurethane coated with a PANI–Ag nanoparticle composite. Tamboli et al. (2012) found that in situ polymerised powder of PANI–Ag possesses antibacterial activity against *B. subtilis* superior to that of Ag nanoparticles as such. Using the disc diffusion method, which cannot be directly correlated with our test procedure, the minimum bactericidal concentration of PANI–Ag was determined to be 25 μg mL^{-1}. Analogous procedure for PANI–Ag composite preparation was used by Jia et al. (2012) who reported enhanced antibacterial activity of the composite against bacteria and yeast in comparison with both PANI and naked Ag particles, respectively.

An improvement of the antibacterial activity of PANI after the Ag deposition onto the polymer surface was observed also in the current work. While all PANI-S-Ag^{-x} samples showed full antibacterial effect against both strains independent on the Ag concentration and no antibacterial effect of Ag deposited PANI-SB-Ag^{-x} against *E. coli* was observed, the behaviour of PANI-SB and PANI-SB-Ag^{-x} with respect to the gram-positive *S. aureus* was different (Table 1). In this case, the antibacterial effect corresponded to the concentration of Ag on the PANI base films. Apparently, PANI-SB, PANI-SB-Ag-10^{-7} and PANI-SB-Ag-10^{-6} show similar and only marginal antibacterial activity, as they reduce the bacterial growth only by about one logarithmic order of magnitude. An increase in the concentration of the deposited Ag enhanced the antibacterial properties of the composite and improved the efficacy of PANI-SB-Ag-10^{-5} and PANI-SB-Ag-10^{-4} by three and five logarithmic orders, respectively. Finally, at the highest concentration of Ag (PANI-SB-Ag-10^{-3}), the growth of gram-positive bacteria was completely reduced. In Fig. 2, Petri dishes with the colony-forming units of *S. aureus* for PANI-S-Ag^{-x}, PANI-S, and polystyrene reference surfaces are depicted, illustrating the reduction of the bacterial colonies number in the order: PANI-S-Ag^{-x} = PANI-S, polystyrene.

Estimation of Ag concentration on PANI film

The film formation and Ag deposition on PANI films were carried out in polystyrene culture cells having the cylindrical compartments of 1.1 cm in diameter and 2.2 cm in depth. Based on the study of Stejskal and Sapurina (2005) it can be assumed that the thickness of the PANI film is of about 125 nm. Considering the cell geometry, the mass of the deposited PANI film is 0.316 mg when using the density of PANI hydrochloride of 1.33 g cm^{-3} (Stejskal & Gilbert, 2002). From the stoichiometry of the reaction shown in Fig. 1, 1 g of PANI hydrochloride is able to reduce 4.6 mmol of silver nitrate and produce 0.496 g of metallic Ag; the resulting composite PANI (pernigraniline)–Ag film thus contains 37.6 mass % of Ag. The PANI hydrochloride film present in the culture cell has thus the capacity to reduce 1.45 μmol of silver nitrate.

The calculated volume of the cell in the culture plate is 8.36 mL. When completely filled with a 10^{-3} M silver nitrate solution, it contains 8.36 μmol of silver nitrate, which is in mole excess with respect to the PANI film deposited on the cell surface. When the concentration of silver nitrate decreases to 10^{-4} M or lower, the content of Ag in the composite film also decreases because PANI are able to convert all the silver nitrate to Ag without its capacity being fully consumed. It should be kept in mind, however, that the amount of Ag need not be the decisive parameter in the antibacterial performance. The size, shape and spatial distribution of Ag nanoparticles within the PANI film, which are probably affected by silver nitrate concentration during the Ag deposition, may also have considerable influence on the antibacterial performance. This is the subject investigated in the forthcoming study.

Conclusions

Although PANI has been intensively studied for decades, the reports dealing with its antibacterial activity are scarce and the literature data suffer from inconsistency in the ways of polymer preparation, tested forms of PANI (base/salt, film/powder, polymer–silver composite), and methods of antibacterial testing. Irrespective of these differences it can be undoubtedly concluded that both the PANI salts as well as its composites with Ag show significant antibacterial activity. The antibacterial effect against gram-negative bacteria *E. coli* and gram-positive *S. aureus* was also confirmed by the results of the current study performed on PANI salt films (PANI-S) and on the corresponding composites with Ag (PANI-S-Ag). Moreover, the presented results, in combination with the conclusions of an earlier study (Humpolicek et al., 2012) proving the absence of skin irritation and sensitisation effects of PANI, offer promising possibility of using these materials in biomedical applications.

Contrary to the PANI salt, the PANI base shows no effect on the bacterial growth and its deposition with silver suppresses the growth of gram-positive *S. aureus* only. The enrichment of PANI with Ag can hence improve the antibacterial properties of the PANI base, which generally has a less-negative impact on living biological systems.

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Abstract: Thin films of pristine conducting polyaniline hydrochloride (PANI-S) and non-conducting polyaniline base (PANI-B) have been studied for their hemocompatibility for the first time. It was demonstrated that surfaces of these standard polymers induced blood coagulation and platelet adhesion. The polyaniline modification via incorporation of poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA), either by standard polymerization with PAMPSA in a reaction mixture (PANI-PAMPSA-R) or by deposition of the acid onto the polyaniline surface (PANI-PAMPSA-D), notably improved surface properties of these polymers in terms of blood compatibility. Of the tested films, PANI-PAMPSA-D significantly influenced homeostasis and completely hindered coagulation. The interaction with coagulation factors X, V and II was found to be the main reason for this behaviour. No interaction with factor I or fibrinogen was detected. The film surface also affected blood platelet adhesion causing its significant decrease. Moreover, PANI-PAMPSA-D showed improved pH stability with the transition from conducting salt to non-conducting base occurring at pH of 6. Interestingly, PANI-PAMPSA-R had an impact on coagulation but not on platelet adhesion. The combined conductivity, anticoagulation activity, low platelet adhesion and improved pH stability of PANI-PAMPSA-D open up new application possibilities for this polymer, not only as a biomaterial but also in blood-contacting devices, such as catheters or blood vessel grafts.

Hemocompatibility of polyaniline

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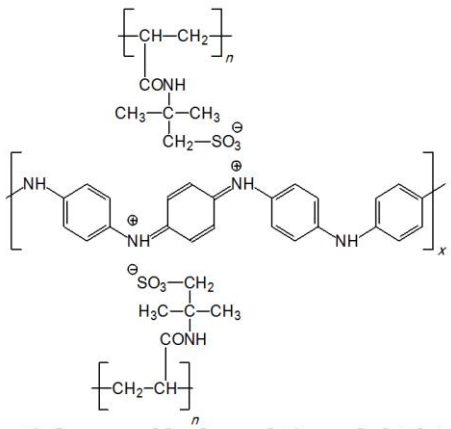
ABSTRACT

Thin films of pristine conducting polyaniline hydrochloride (PANI-S) and non-conducting polyaniline base (PANI-B) have been studied for their hemocompatibility for the first time. It was demonstrated that surfaces of these standard polymers induced blood coagulation and platelet adhesion. The polyaniline modification *via* incorporation of poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA), either by standard polymerization with PAMPSA in a reaction mixture (PANI-PAMPSA-R) or by deposition of the acid onto the polyaniline

surface (PANI-PAMPSA-D), notably improved surface properties of these polymers in terms of blood compatibility. Of the tested films, PANI-PAMPSA-D significantly influenced homeostasis and completely hindered coagulation. The interaction with coagulation factors X, V and II was found to be the main reason for this behaviour. No interaction with factor I or fibrinogen was detected. The film surface also affected blood platelet adhesion causing its significant decrease. Moreover, PANI-PAMPSA-D showed improved pH stability with the transition from conducting salt to non-conducting base occurring at pH of 6. Interestingly, PANI-PAMPSA-R had an impact on coagulation but not on platelet adhesion. The combined conductivity, anticoagulation activity, low platelet adhesion and improved pH stability of PANI-PAMPSA-D open up new application possibilities for this polymer, not only as a biomaterial but also in blood-contacting devices, such as catheters or blood vessel grafts.

Keywords: Polyaniline; Poly(2-acrylamido-2-methyl-1-propanesulfonic acid); Hemocompatibility; Blood coagulation; Platelet adhesion

GRAPHICAL ABSTRACT



Significant influence on blood coagulation and platelet adhesion.

HIGHLIGHTS

- Hemocompatibility of polyaniline (PANI) have been studied for the first time.
- Standard polyaniline salt and base induced blood coagulation and platelet adhesion.
- Polyaniline modified with polymeric acid PAMPSA hindered coagulation.
- PANI-PAMPSA interacts with coagulation factors X, V and II but not I or fibrinogen.
- PANI-PAMPSA showed improved pH stability.

1. Introduction

Despite remarkable progress in understanding the blood coagulation system, as well as progress in the development of blood-compatible biomaterials and blood-contacting devices, the problem of foreign-surface-induced thrombosis still remains unsolved. In fact, the contact of any material with blood induces multiple defensive mechanisms, such as the activation of coagulation cascade, platelet adhesion, the triggering of complementary systems, and others. The most common compound with known anticoagulant activity is heparin, and its efficacy is mainly ascribed to the presence of sulfate, sulfamic, and carboxylic groups and their arrangement along the polysaccharide backbone of this polymer (Fig. 1a). It has already been reported that synthetic polymers and copolymers with heparin-like activity might be applicable to medical devices or surfaces coming into contact with blood.

Polyaniline (PANI), as a conducting polymer, has immense potential with regard to practical applications in the biomedical field. In particular, cardiomyocyte synchronization [5], myoblast differentiation [6], neuronal lineage differentiation, and cardiac tissue engineering [7] have been highlighted with respect to the use of conducting polymers. Commonly, the standard polyaniline, emeraldine salt is prepared *via* oxidative polymerization of aniline hydrochloride in the presence of ammonium persulfate. Considering the biological properties of this standard PANI, the number of papers is surprisingly limited. Humpolicek et al. [8] studied pristine PANI powders for their cytotoxicity, irritation, and sensitization potential. Also cell proliferation on PANI films [9] and implantability in tissues was investigated by Kamalesh et al. [10] and Mattioli-Belmonte et al. [11]. To the best of author's knowledge, no information about PANI hemocompatibility has previously been published.
Considerable advantages of PANI consist also in its ability to easily form thin films, which can be further modified using various dopant acids with the aim to change surface properties of this polymer. It opens new possibilities for targeted surface modification of PANI by acids, or generally substances, showing desired properties. Among such substances, synthetic polyanions have been observed to exhibit notable anticoagulant effects. Methacrylic copolymers containing, similarly to heparin, the abovementioned functional groups can be listed as examples [1]. Namely, poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPSA; Fig. 1b) was shown to act against blood clotting in a similar way to heparin, either alone [2] or incorporated in copolymers [3]. Setoyama et al. [4] also reported that PAMPSA is capable of inhibiting the activation of serum complement activity, which is another important factor with respect to the applicability of materials in biomedical engineering.

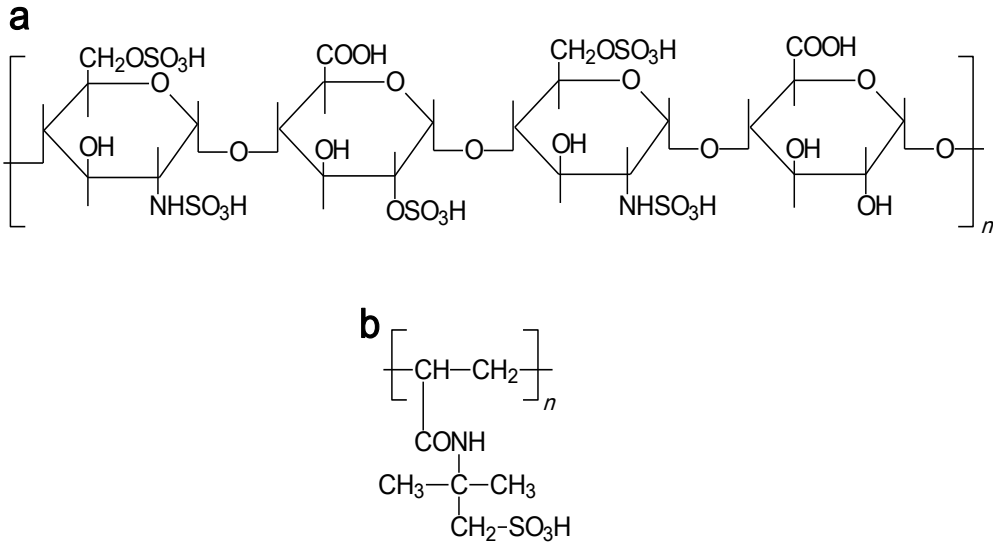


Fig. 1. Formulae of (a) heparin and (b) poly(2-acrylamido-2-methyl-1-propanesulfonic acid).

In the present study, PANI has a dual role. The first is represented by easy modification of surfaces by deposition of thin PANI films of submicrometre thickness. This is done *in situ* on substrates immersed in the reaction mixture used for the oxidation of aniline [12]. The second role then consists in the subsequent immobilization of PAMPSA onto polyaniline films by the formation of a mutual salt (Fig. 2). The feasibility of a polyanion–polycation complex formation between PANI and PAMPSA has been demonstrated [13, 14] and the resulting material was used in the electrodes of energy-storage devices [15, 16]. PANI-PAMPSA films have also been tested for their biocompatibility [17], and normal cell adhesion, proliferation, and low cytotoxicity were observed.

This paper is focused on the changes in blood coagulation and platelet adhesion induced by the surfaces of different PANI films, namely PANI hydrochloride (PANI-S), PANI base (PANI-B), and PANI salts with PAMPSA (PANI-PAMPSA). The PANI-PAMPSA films were prepared using two procedures. In the first, reprotonation of PANI-B using PAMPSA solution was performed giving rise to films with acid deposited onto the PANI surface (PANI-PAMPSA-D). In the second procedure, a standard oxidative polymerization of aniline was conducted, with PAMPSA added directly into the reaction mixture of aniline hydrochloride and ammonium peroxydisulfate (PANI-PAMPSA-R).

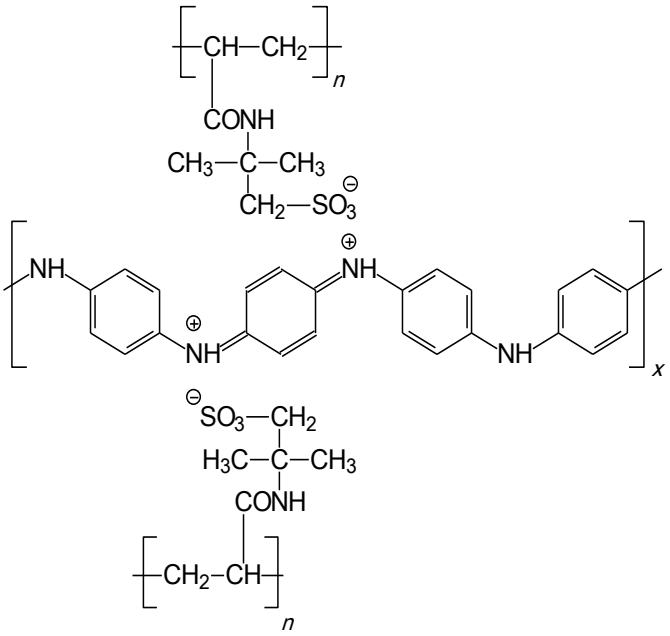


Fig. 2. Polyaniline salt with poly(2-acrylamido-2-methyl-1-propanesulfonic acid).

2. Materials and methods

2.1 Deposition of polyaniline films

The surfaces of blood collection tubes (Vacuette, Austria) were coated with conducting films of polyaniline salt (PANI-S) prepared *via* the oxidation of aniline. Aniline hydrochloride (2.59 g; Sigma-Aldrich) was dissolved in 50 mL water; ammonium peroxydisulfate (5.71 g; Sigma-Aldrich) was similarly dissolved to 50 mL of solution. Subsequently, both solutions were mixed at room temperature and immediately poured into tubes [12]. The concentrations of reactants were thus 0.2 M aniline hydrochloride and 0.25 M ammonium peroxydisulfate [18]. After 1 h, the tubes were emptied and the films of green conducting PANI-S deposited on the walls were rinsed with 0.2 M hydrochloric acid, followed by methanol, and left to dry in air.

Some films were deprotonated by immersion in 1 M ammonium hydroxide for 12 h and thus converted to blue, non-conducting films of polyaniline base (PANI-B). In order to prepare PANI films with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PANI-PAMPSA-D), the reprotonation of PANI-B with a 7.5 % v/v aqueous solution of PAMPSA (typical molecular weight M = 2×10^6 g mol^{-1}; Sigma-Aldrich) was performed by exposing the PANI film surface to the PAMPSA solution. The neutralization reaction was left to proceed for 24 h; then the residual PAMPSA solution was poured out and the film was rinsed with methanol and left to dry in air.

The second type of PANI-PAMPSA film was prepared with PAMPSA present in the reaction mixture of aniline hydrochloride and ammonium peroxydisulfate. For this purpose, modified procedures published by Stejskal et al. [19], Yoo et al. [20], and Bayer et al. [17] were employed. First, an aqueous solution of PAMPSA was prepared with a target concentration corresponding to 0.028 mol (5.8 g) of its constitutional units, (2-acrylamido-2-methyl-1-propanesulfonic acid). In practice, 38.5 ml of 15 % PAMPSA solution was diluted by water to volume of 375 ml. Aniline hydrochloride (0.028 mol, 3.6 g) was then added to the PAMPSA solution and stirred at room temperature for 1 h. The molar ratio of aniline hydrochloride to PAMPSA was adjusted to 1:1 (see above) and similarly to 2:1 and the corresponding samples were assigned as PANI-PAMPSA-R1:1 and PANI-PAMPSA-R2:1, respectively. The oxidant, ammonium peroxydisulfate (0.025 mol, 5.8 g), at a 1 : 0.9 aniline hydrochloride to oxidant mole ratio, was dissolved separately in 25 ml water and added to the PAMPSA–aniline hydrochloride solution. The polymerization was completed within 60 min. The films were rinsed with water to remove the adhering PANI-PAMPSA precipitate and left to dry in air. In the contrast to PANI-PAMPSA-D, the PANI-PAMPSA-R films contain also sulfate or hydrogen sulfate counter-ions produced by the decomposition of peroxydisulfate in addition to PAMPSA. For that reason, the molecular structure of PANI-PAMPSA-R may be structurally closer to PANI-S films than to PANI-PAMPSA-D.

2.2 UV–Vis spectra

The UV–Vis spectra of the PANI-PAMPSA-D films were recorded in the presence of aqueous solutions with pH ranging from 4 to 7.5 using the UV/VIS Spectrometer (PerkinElmer, USA). The inner surfaces of standard polystyrene cuvettes were covered by PANI-PAMPSA-D films according to procedure described above. The individual solutions with varying pH were subsequently added into cuvettes to initialize de-protonation. The cuvettes were inserted into spectrophotometer and the spectra were recorded within the wavelength range of 300 to 1100 nm immediately and after 1 h at each pH.

2.3 Anticoagulation test

In all tests, venous blood was collected from healthy donors by venous puncture after obtaining informed consent. All tests were conducted in accordance with the Helsinki Declaration. The following coagulation parameters in human blood plasma treated with citric acid were studied: (1) thrombin clotting time (TCT); (2) activated partial thromboplastin time (aPTT), and (3) prothrombin time (PT). The tests were performed using a SYSMEX CA-1500

(Siemens, Germany). Each of the samples was assessed three times. To determine the interaction of PANI films on the surface with blood plasma, the coagulation factors factor X (Stuart-Prower factor), factor V (proaccelerin), factor II (prothrombin), and factor I (fibrinogen) were selected and the following methods were employed for their determination. The Clauss assay was used to detect the impact on Factor I using a SYSMEX CA-1500 (Siemens, Germany). The activities of factors X, V and II in plasma were determined by performing a modified prothrombin time test using an ACL ELITE Pro (IL-Instruments, Italy). Plasma was diluted and added to commercial plasma, deficient always in the tested factors (HemosIL™, Instrumentation Laboratory, USA).

2.4 Platelet adhesion

Additional information about the interaction of PANI films with human blood was obtained by the platelet adhesion test. For this type of experiment, the PANI films were deposited on discs of diameter 10 mm. The discs were subsequently incubated in the presence of 1 mL of human blood. Sample incubation was performed in a 24-well microtiter plate at 37 °C under shaking at 200 rpm. After incubation, the samples were washed with phosphate buffered saline in order to remove the remaining blood and fixed with 1 ml 50 % trichloroacetic acid (Sigma-Aldrich) at 4 °C. After 1 h, the platelet number was quantified using sulforhodamine B (SRB) assay (Sigma-Aldrich, USA). Absorbance was measured with a Lambda 1050 UV/VIS/NIR spectrophotometer (Perkin Elmer, USA) at a wavelength of 565 nm. The assay was performed in triplicates.

3. Results and discussion

3.1. Blood plasma coagulation

Standard films of PANI-S, PANI-B and PANI-PAMPSA-R did not have any significant impact on the coagulation parameters (Table 1). Coagulation on the PANI-PAMPSA-D surface, however, was prevented. Taking into consideration that PANI-PAMPSA-D is composed of PANI and PAMPSA, which is a polymeric acid anchored on the PANI surface, and the fact that neither standard PANI-S nor PANI-B induce any changes in blood clotting, it can be suggested that the observed anticoagulant activity of PANI-PAMPSA-D is a surface effect of the used polymeric acid and is not inherently caused by PANI as such. This conclusion can be supported by the fact that PANI-PAMPSA-R, in which the PAMPSA is incorporated directly under polymerization into the PANI film and not

attached on its surface, does not show the above-described anticoagulation effect. Some synergy between both components, however, cannot be ruled out and will be tested later.

Theoretically, three important variables influencing coagulation of blood in the contact with foreign matter can be identified (1) the acidity (pH), (2) the surface charge, and (3) the interaction with coagulation factors. As acidity is one of the above mentioned factors, the pH was measured on freshly taken blood and after blood addition to Vacuette tubes coated with the tested PANI films. The measurements showed that the pH of blood was not notably influenced by any of the tested samples and remained within the range of values for which no effect on blood clotting is expected (PANI-S: pH = 7.02±0.01; PANI-B: pH = 7.06±0.02; PANI-PAMPSA-D: pH = 6.95±0.01). This is understandable because the specific mass of PANI films is low, of the order of 10 μg cm^{-2}, and such small quantities cannot significantly affect the bulk acidity of the blood in the tube. It is generally accepted that a significant reduction in thrombus formation starts at a pH under 6.8 and thus coagulation is reduced [21]. As to the second factor, blood coagulation on foreign materials is influenced by negative charge present on the surface [22]. Hence, clotting on foreign material is reported to be activated by contact with a hydrophilic, negatively charged surface. Considering the surface properties of all the tested samples, it can be concluded that their behavior with respect to coagulation is not influenced by surface charge, because negative charges on PAMPSA are balanced by the positively charged PANI backbone. The fact that the PANI-PAMPSA-D film is conducting, and thus antistatic, may play some role. The conductivity alone, however, cannot be the reason for this effect. Typical conductivities of PANI-S and PANI-B are of the orders 10^{0} and 10^{-9}–10^{-11} S cm^{-1}, respectively [18]. Irrespective of these significantly different values, the coagulation properties of both films are comparable. A conductivity of $\approx 10^{-2}$ S cm^{-1} was reported for PANI-PAMPSA-D [13], which is a value within the above interval. Also the conductivity of PANI-PAMPSA-R with anticoagulation activity absent does not deviate from the values typically reported for conducting PANI and is about 1.7 S cm^{-1} [17].

Table 1

Impact of polyaniline surfaces on selected coagulation parameters expressed as times (s) to the coagulation start.

	Reference	PANI-B	PANI-S	PANI-PAMPSA-D	PANI-PAMPSA-R-1:1	PANI-PAMPSA-R-2:1
PT	12.1 ± 0.1	11.9 ± 0.2	12.2 ± 0.1	NC	12.0 ± 0.1	12.0 ± 0.1
aPPT	26.4 ± 0.1	25.6 ± 1.1	26.4 ± 0.0	NC	29.9 ± 0.2	29.2 ± 0.1
TCT	18.6 ± 0.1	16.7 ± 0.1	17.56 ± 0.2	NC	18.8 ± 0.1	18.2 ± 0.1

Note: NC = No coagulation; Reference – not coated surfaces of blood collection tube. Normal range for a healthy person is: PT 11.0–13.5, aPTT 25–32, TCT>20

Although it is known that both extrinsic (tissue factor) and intrinsic (contact activation) coagulation pathways are interconnected *in vivo* [23], the plasma-coagulation cascade is usually divided into the two abovementioned pathways for the convenience of discussion and coagulopathy testing. It is beyond the scope of this article to provide a comprehensive explanation of coagulation cascade and platelet adhesion in its complexity; this can be found, for example, in Vogler and Siedlecki [24]. Generally, PT detects the defects or deficits in extrinsic and "common coagulation pathways", aPTT detects the defects or deficits in intrinsic and common coagulation pathways, and TCT is used for discriminating between problems in thrombin generation (normal TCT) and the inhibition of thrombin activity (abnormal TCT). Considering the fact that PANI-PAMPSA-D influences all studied coagulation variables (Table 2), it can be concluded that it affects the common pathway as a consequence of interference with coagulation factors. Thus, the effect of PANI on the main factors of the common pathway, factor X, factor V, factor II, and factor I, was studied in more detail (Table 2). The results clearly show that, in contrast to all other tested films, PANI-PAMPSA-D interacts with factor X, factor V and factor II, but not with factor I. Such

interaction with the tested coagulation factors might be regarded as parallel to the effect of heparin sulfate, which binds to the enzyme inhibitor antithrombin III (AT), thus causing its activation. The activated AT then inactivates thrombin and other proteases involved in blood clotting, most notably factor X formation found in the common pathway. PANI-PAMPSA-D, therefore, might follow a similar pathway, as it further influences factors in a "common way", with the exception of factor I.

Table 2

Impact of polyaniline surfaces on selected coagulation factors

	Reference	PANI-B	PANI-S	PANI-PAMPSA-D	PANI-PAMPSA-R-1:1	PANI-PAMPSA-R-2:1
X (%)	100	100	98.4	25.8	94.5	96.4
V (%)	100	89.8	70.5	0.7	86.2	88.0
II (%)	100	92.7	86.7	0.4	83.6	84.9
I (g/L)	2-4[a]	3.7	3.5	2.3	3.3	3.4

Note: [a] Normal range for a healthy person. Reference: uncoated surfaces of blood collection tube. The factors X, V and II are expressed as percentage of values determined for reference.

The above given conclusion indicate that heparin and PAMPSA exhibit some common features, which probably play a role in their anticoagulation activity. Both species are polymers, although the molecular weight of heparin is much lower compared to PAMPSA, and the polysaccharide backbone of heparin is rigid in contrast to the flexible chain observed in PAMPSA [14]. Both polymers are polyanions and both contain nitrogen groups and sulfur-contaning ionizable groups. This is illustrated by the covalent bond $–NH–SO_3^- H^+$ in heparin (Fig. 1a) and the ionic bond $–NH^+–SO_3^-$ in PANI-PAMPSA salt (Fig. 2). It has still to be determined which combination of factors is responsible for PAMPSA anticoagulation activity. The above results indicate that the role of PANI is limited to the immobilization of PAMPSA at its surface.

3.2. Platelet adhesion on polyaniline surfaces

Using sulforhodamine B colorimetric assay, the total mass of platelets adhered to the surface was determined, after the staining of their intracellular proteins with sulforhodamine B. The results clearly show that platelet adhesion is significantly decreased on all surfaces modified with PAMPSA irrespective of whether the PAMPSA was deposited or added to the polymerization mixture. Of all the PANI-PAMPSA samples, the PANI-PAMPSA-D surface exhibited the lowest level of platelet adhesion compared to the polystyrene reference, PANI-S, and PANI-B (Table 3). Remarkably, platelet adhesion was reduced more by PANI-PAMPSA-R 2:1 than by PANI-PAMPSA-R 1:1, although the amount of PAMPSA in the former film was lower. The behaviour of samples with PAMSA anchored to the surface or incorporated directly into the polymer is hence similar in respect to platelet adhesion, but dissimilar with respect to the behaviour of these samples during the blood plasma coagulation.

Table 3

Platelets adhesion on polyaniline surfaces

	Reference[a]	PANI-B	PANI-S	PANI-PAMPSA-D	PANI-PAMPSA-R-1:1	PANI-PAMPSA-R-2:1
Platelet adhesion	2.5 ± 0.2	2.0 ± 0.3	2.5 ± 0.5	0.4 ± 0.0	1.0 ± 0.0	0.6 ± 0.1

[a] A polystyrene was used as a reference.

3.3 pH stability of PANI-PAMPSA-D films

The UV–VIS spectra of the PANI-PAMPSA-D films under different pH were recorded with the aim to evaluate the pH of the transition between conducting PANI-PAMSA-D salt and its non-conducting, deprotonated base. From the spectra (Fig. 3) it is seen that at pH 4 and 4.5 PANI-PAMPSA-D has two absorption maxima typical for the conducting polyaniline form, namely at 410 nm and at approximately 800 nm, which are assigned to the π–π* transition of the benzenoid rings and the polaron band transitions, respectively [25]. The figure also illustrates that at pH higher than 4.5 the conversion from the conducting salt to non-conducting base occurs. The evidence of this transition is a gradual shift of the spectrum maxima from 800 nm towards lower values. At the same time the shift of the maximum is not

abrupt but gradual and first at pH of 7 and 7.5 the maxima are of 650 nm, which corresponds to the n–π* transition of quinonoid rings. Under these conditions the film is fully deprotonated and base is formed. The results obtained indicate that surface deposition of polyaniline with PAMPSA improves the pH stability of this film, which retains at least part of its conductivity under pH of 6. In comparison with standard polyaniline, emeraldine, with transition from salt to base occurring at pH > 4 [26] it is clear improvement. The enhanced stability under physiological conditions might be of importance for PANI applications in biomedicine.

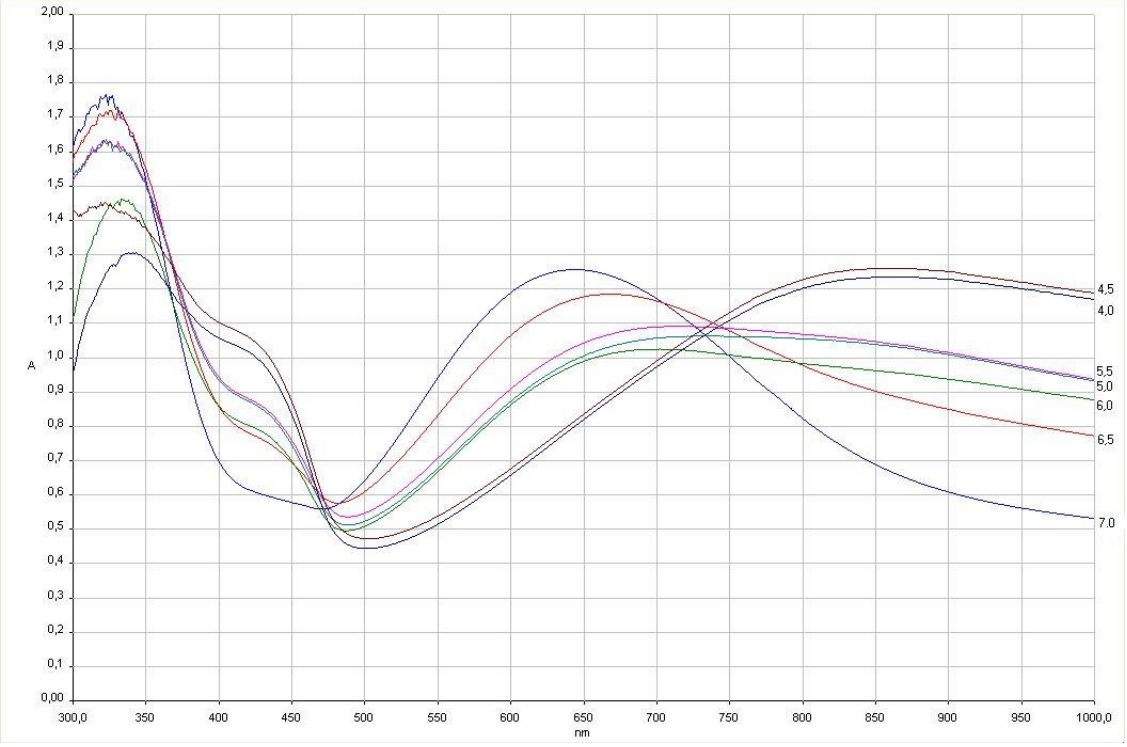


Fig. 3. UV–vis spectra of the PANI–PAMPSA-D films recorded at pH between pH 4–7.

4. Conclusions

The results of the study show that standard polyaniline salt and base are not suitable as blood contacting materials. It was clearly demonstrated that PANI surfaces allow for platelet adhesion and induce coagulation when in contact with blood. The coating with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) has a significant impact on blood coagulation, which is hindered by the interaction with three coagulation factors, X, V and II. This modified polymer film also significantly reduced platelet adhesion, when compared to the other tested polyaniline films. Moreover, PANI-PAMPSA-D showed improved pH stability with the transition from conducting salt to non-conducting base occurring at pH of 6. This is an increase when comparing this value with standard PANI-S showing pH transition already at

pH > 4. The mentioned fact highlights the significant potential of this surface modification with respect to practical applications. The combination of conductivity, anticoagulation activity, low platelet adhesion capacity as well as improved pH stability of polyaniline surfaces coated with poly(2-acrylamido-2-methyl-1-propanesulfonic acid) open up new application possibilities for this polymer, not only as a biomaterial but also in blood-contacting devices, such as catheters or blood vessel grafts.

Acknowledgements

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APPENDIX I

Kuceková Z., Humpolíček P., Kašpárková V., Stejskal J. Cytotoxicity of polyaniline precursors. *Plasty a kaučuk – special*. 2012, 49: 15-17.

Cytotoxicity of polyaniline precursors

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Synthetic polymers are commonly used in medical applications, mainly thanks to their advantages such as homogeneity of individual sources or broad spectrum of applicable technologies. Polypyrrole and polyaniline are conducting polymers which are currently most studied in terms of bio-applications. The study deals with the cytotoxicity of ammonium persulfate and aniline hydrochloride, monomers used for polyaniline polymerization. The cytotoxicity test was performed using MTT assay. Two cell lines (HaCaT and HepG2) were employed and the cytotoxicity of different concentrations of ammonium persulfate and aniline hydrochloride was determined. The in vitro tests were based on the requirements of the standard EN ISO 10993: Biological Evaluation of Medical Devices, Part 5: Tests for Cytotoxicity.

1. Introduction

Nowadays, synthetic polymers and composites are commonly used in various biomedical applications such as therapeutics or antimicrobial agents, transfection vectors or fluorescent labels (Madden et al., 2000; Hutchison et al., 2000). One of them, polyaniline has been for example used for biosensing (Guimard et al., 2007), and tissue engineering (Mawad D. et al, 2011) due to unique electrochemical (Chang et al., 2007), chemical and physical properties (Langer et al., 2004), electrical conductivity and good environmental stability (Ben-Valid et al., 2010). However, to be useful in tissue engineering and biomedicine, its biological properties, mainly biocompatibility and cytotoxicity, are very important (Saikia et al., 2010). Cytotoxicity is a critical factor to consider when evaluating potential use of any polymer for biological applications. It means that polymer cannot leach toxic substances that may cause harm locally or systemically or otherwise interfere with the physiological responses of the cells.

It can be found several forms of polyaniline with different chemical and physical properties, of which a green polyaniline (emeraldine) salt and a polyaniline base are widely used (Stejskal et al., 2002). The preparation of polyaniline salt involves an oxidative polymerization of aniline hydrochloride in the presence of ammonium persulfate. In order to obtain polyaniline base, this step is followed by deprotonation of the polyaniline hydrochloride with ammonia. The study was hence focused on determination of the cytotoxicity of individual precursors of polyaniline, namely ammonium persulfate and aniline hydrochloride.

2. Materials and Methods

2.1. Cell cultivation

Cytotoxicity testing was performed using two different cell lines. In the study, human immortalized non-tumorogenic keratinocyte cell line (HaCaT) (Boukamp et al., 1988) supplied by Cell Lines Service (300493, Germany) and Human hepatocellular carcinoma cell line (HepG2) from ATCC (HB-8065, USA) were used. Dulbecco's Modified Eagle Medium – high glucose, added 10 % fetal bovine serum and Penicillin/Streptomycin, 100 U/ml (100 g/ml), respectively (PAA Laboratories GmbH, Austria) was used as the culture medium for HaCaT. HepG2 cells were cultivated in ATCC-formulated Eagle's Minimum Essential Medium added 10 % fetal bovine serum, 2 mM l-glutamine and 50 g/ml gentamycin (PAA Laboratories GmbH, Austria).

2.2. Test of cytotoxicity

Aniline hydrochloride (APS, Fluka, Switzerland) and ammonium persulfate (AH, Lach-Ner, Czech Republic) were used as delivered. APS and AH were dissolved in culture medium for individual cell line to obtain the highest used concentration, which was 0.25 mg/ml and 25 mg/ml in case of AH and APS, respectively. Cytotoxicity testing was conducted in accordance with EN ISO 10993-5. Cells were precultivated for 24 h at concentration of 1.10^5/ml and the culture medium was subsequently replaced with AH and APS solutions in following concentrations: for AH 0.25; 0.19; 0.15; 0.10; 0.05 and 0.03 mg/ml; for APS: 25; 19.2; 15; 10; 5; 1.1; 1 and 0.5 mg/ml. Also, two combinations of APS and AH were assessed: 0.19 mg/ml of AH with 19.2 mg/ml of APS and 0.03 mg/ml of AH with 1.1 mg/ml of APS. As a control experiment, pure medium without monomers was used. To assess cytotoxic effect, the MTT assay (Invitrogen Corporation, USA) was performed after one-day cell cultivation in solutions. All the tests were performed in quadruplicates. The absorbance was measured at 570 nm by Sunrise microplate absorbance reader (Tecan, Switzerland). Dixon's Q test was used to remove outlying values and mean values were calculated. The cell viability, expressed as percentage of cells present in respective solution relatively to cells cultivated in pure medium without monomers (100% viability), was determined. The morphology of cells was observed by an inverted Olympus phase contrast microscope (Olympus, CKX41).

3. Results and Discussion

The biological properties of materials, including cytotoxicity, and their behaviour in contact with tissues are very important for their application in medicine and pharmacy. Polyaniline is promising polymer for this application but the studies dealing with its toxicity are scarce. Hence, this study is dealing with the cytotoxicity of monomers AH and APS, which are commonly used in PANI preparation.

According to EN ISO 10993-5 standard, the cytotoxicity is assessed via scaling of cell viability after application of test solutions for 24 h. Providing that cell viability of control sample is 100 %, viability higher than 80 % is attributed to absence of cytotoxicity, viability between 80 and 60 % to mild cytotoxicity, viability between 60 and 40 % to moder-

ate cytotoxicity and if a cell survival is lower than 40 %, the sample cytotoxicity is designated as severe.

Following the above mentioned ISO standard, the study revealed that AH does not express the cytotoxicity in either of concentrations on both cell lines used (Fig. 1 and 2). In case of HepG2 cells, the AH in concentration of 0.25 mg/ml even increased the cell viability in comparison with control. HaCaT cells in the presence of solution with concentration of 0.19 mg/ml reached viability of control sample. The remaining concentrations of AH induced cell viability between 90 % to 98 % in case of HaCaT cells and 88 % to 94 % in case of HepG2 cells, which means absence of cytotoxicity.

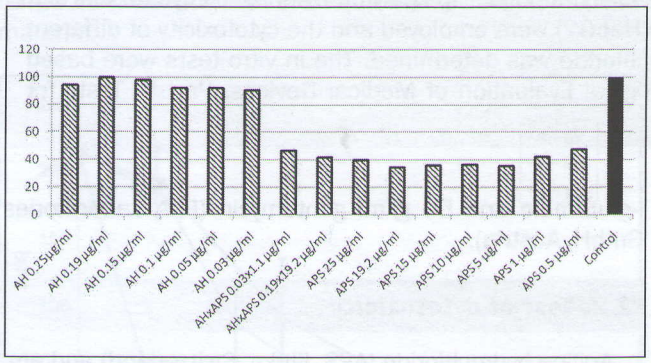


Fig. 1 *Viability of HaCaT cells cultivated in the presence of APS and AH solutions*

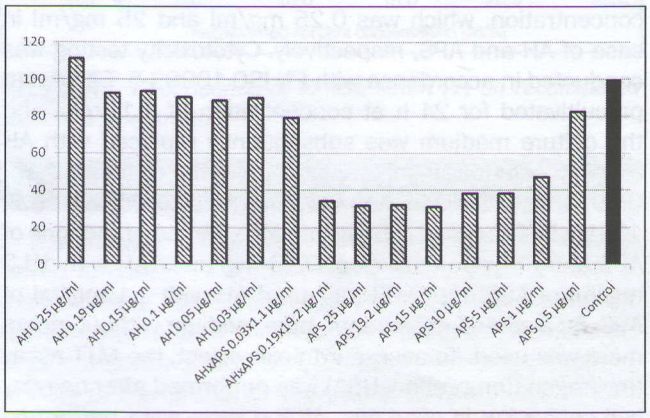


Fig. 2 *Viability of HepG2 cells cultivated with APS and AH solutions*

On the contrary, different concentrations of APS caused different levels of cytotoxicity and differences were also observed between both cell lines used. APS in concentration of 1.0 mg/ml showed moderate cytotoxicity in both cell lines. Commonly, the APS concentrations higher than 5 mg/ml gave rise to severe cytotoxicity in both cell lines except of the concentration of 25 mg/ml and HaCaT cells. At this concentration, the cell viability was of 40 % which responds to moderate cytotoxicity. Significant differences in cell viability were observed after treatment with 0.5 mg/ml APS solution. This solution decreased cell viability to 47 % when applied on HaCaT (moderate cytotoxicity), whilst 83 % of viability in HepG2 cells was reached. Hence, concentration 0.5 mg/ml of APS does not have cytotoxic effect on HepG2 cells.

Interesting fact is that, there is a significant difference between the effect of combinations of APS and AH on HaCaT and HepG2 cells. While the combination of AH and APS in concentrations of 0.03 and 1.1 mg/ml decreased viability of HepG2 cells by 21 %, in case of HaCaT cells it was by 54 %. The second used combination of AH and APS of monomers (0.19 and 19.2 mg/ml) caused decreasing of cell viability by 66 % and 68 % in HepG2 and HaCaT cells, respectively. Hence, both combinations of monomers exhibited moderate cytotoxicity in HaCaT cells, but in HepG2 cells the higher total monomer concentration induced severe cytotoxicity, whilst the lower one showed only mild cytotoxic effect.

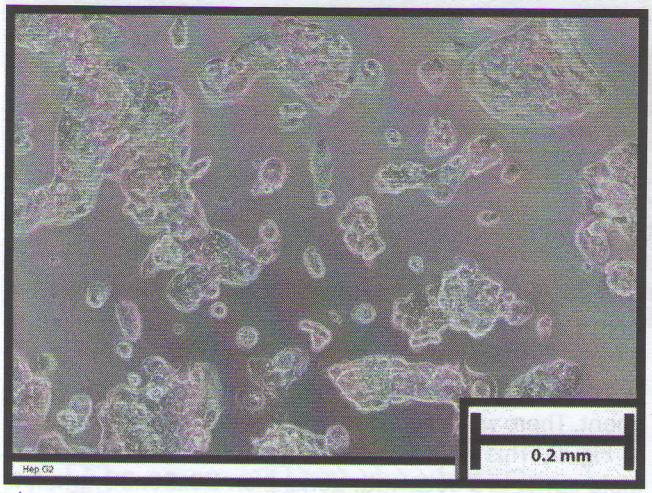
The influence of monomers on cell viability is also illustrated by photomicrographs (Fig. 3). Fig. 3a) shows HepG2 cells not treated with AH or APS solutions, where cell viability was of 100 %. Fig. 3b) to e) illustrate HepG2 cells treated with monomer solutions. In Fig. 3b) absence of cytotoxicic effect is presented, which is characteristic with unchanged cell morphology. In the Fig. c) to e), changes in cell morphology are clearly visible. HepG2 cells do not have typical shape and their boundaries are less clear.

This study finds out that aniline hydrochloride in concentrations up to 0.25 mg/ml show absence of cytotoxicity and can be safely used for materials in bio-applications. In contrast, the ammonium persulfate in tested concentrations is cytotoxic and should be avoided.

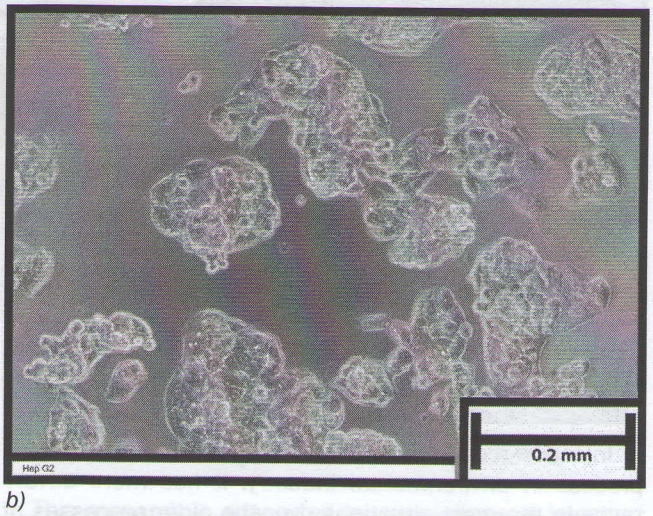
Table 1. *Cell viability in the presence of AH and APS solutions of various concentrations; HaCaT and HepG2 cells (average absorbance ± SD).*

HaCaT		HepG2	
Concentration (mg/ml)	**Absorbance**	**Concentration (mg/ml)**	**Absorbance**
AH 0.25	0.3406± 0.0166**	AH 0.25	0.4508 ± 0.0101**
AH 0.19	0.3611 ± 0.0221	AH 0.19	0.3749 ± 0.0090**
AH 0.15	0.3548 ± 0.0180	AH 0.15	0.3786 ± 0.0277**
AH 0.10	0.3340 ± 0.0136**	AH 0.10	0.3655 ± 0.0220**
AH 0.05	0.3339 ± 0.0182**	AH 0.05	0.3580 ± 0.0144**
AH 0.03	0.3282 ± 0.0107**	AH 0.03	0.3644 ± 0.0372**
AH/APSa	0.1686 ± 0.0142**	AH/APSa	0.3213 ± 0.0114**
AH/APSb	0.1525 ± 0.0122**	AH/APSb	0.1385 ± 0.0063**
APS 25.0	0.1457 ± 0.0084**	APS 25.0	0.1288 ± 0.0200**
APS 19.2	0.1256 ± 0.0099**	APS 19.2	0.1309 ± 0.0095**
APS 15.0	0.1310 ± 0.0099**	APS 15.0	0.1260 ± 0.0019**
APS 10.0	0.1323 ± 0.0169**	APS 10.0	0.1555 ± 0.0174**
APS 5.0	0.1295 ± 0.0083**	APS 5.0	0.1555 ± 0.0109**
APS 1.0	0.1536 ± 0.0151**	APS 1.0	0.1915 ± 0.0174**
APS 0.5	0.1724 ± 0.0154**	APS 0.5	0.3351 ± 0.0089**
Control	0.3623 ± 0.0310*	Control	0.4052 ± 0.0434*

Note: Values with different superscripts show significance level within column: P<0.05 (,**). a AH/APS in concentration 0.03/1.1 mg/ml. b AH/APS in concentration 0.19/19.2 mg/ml.*



a)



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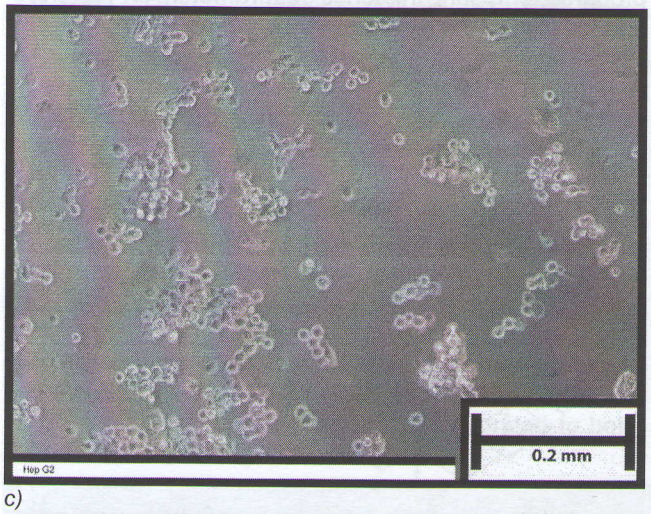
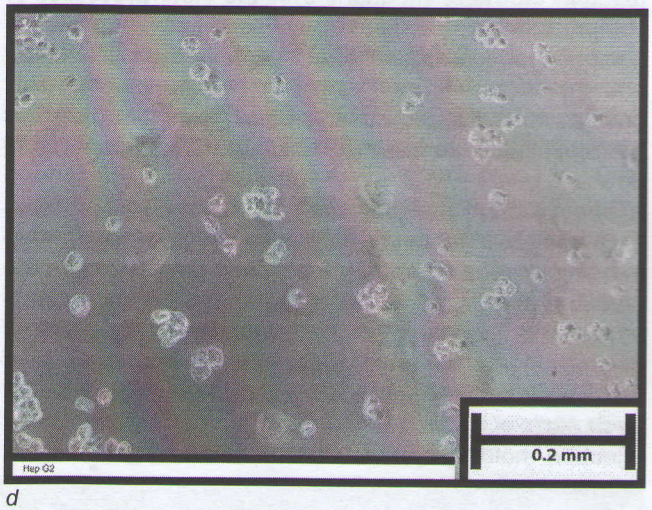
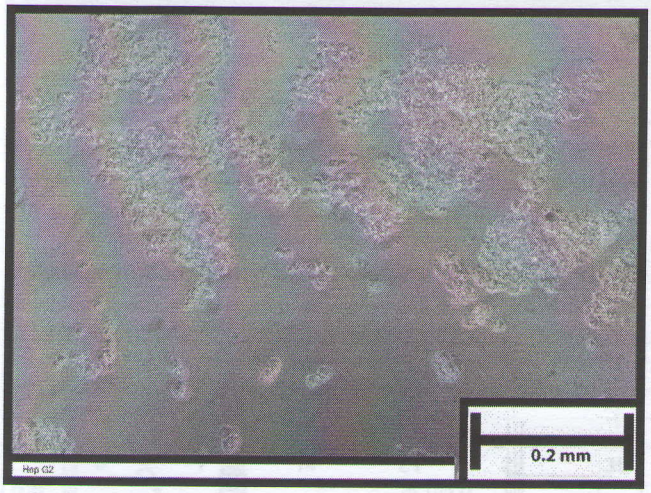


c)



d



e)

Fig. 3 *Photomicrographs of HepG2 cells a) untreated control, b) cells treated with 0.25 mg/ml AH (no cytotoxicity), c) combination of AH and APS (0.03/1.1 mg/ml) (mild cytotoxicity), d) combination of AH and APS (0.19/19.2 mg/ml) (severe cytotoxicity) and e) 15 mg/ml APS (severe cytotoxicity)*

4. Conclusion

Conducting polymers, including polyaniline, with acceptable biocompatibility can contribute to the development of advanced functional materials for biomedical applications. Crucial property of these materials is their cytotoxicity. Hence, the cytotoxicity of monomers employed for synthesis of polyaniline, aniline hydrochloride and ammonium persulfate, was determined in this study. The study demonstrated that aniline hydrochloride is not cytotoxic in used concentrations. On the other hand, ammonium persulfate shows high levels of cytotoxicity. Cytotoxicity was also detected on combinations of both tested monomers. The presented results can improve understanding of the polyaniline cytotoxicity and can be helpful in improvement of its preparation procedure and utilization for biomedical applications.

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