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Cysteine modified polyaniline films improve biocompatibility for two cell lines

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ABSTRACT

This work focuses on one of the most exciting application areas of conjugated conducting polymers, which is cell culture and tissue engineering. To improve the biocompatibility of conducting polymers we present an easy method that involves the modification of the polymer backbone using L-cysteine. In this publication, we show the synthesis of polyaniline (PANI) films supported onto Polyethylene terephthalate (PET) films, and modified using cysteine (PANI-Cys) in order to generate a biocompatible substrate for cell culture. The PANI-Cys films are characterized by Fourier Transform infrared and UV–visible spectroscopy. The changes in the hydrophilicity of the polymer films after and before the modification were tested using contact angle measurements. After modification the contact angle changes from 86° ± 1 to 90° ± 1, suggesting a more hydrophylic surface. The adhesion properties of LM2 and HaCaT cell lines on the surface of PANI-Cys films in comparison with tissue culture plastic (TCP) are studied. The PANI-Cys film shows better biocompatibility than PANI film for both cell lines. The cell morphologies on the TCP and PANI-Cys film were examined by florescence and Atomic Force Microscopy (AFM). Microscopic observations show normal cellular behavior when PANI-Cys is used as a substrate of both cell lines (HaCaT and LM2) as when they are cultured on TCP. The ability of these PANI-Cys films to support cell attachment and growth indicates their potential use as biocompatible surfaces and in tissue engineering.

1. Introduction

The use of polymers in biomedical applications has increased considerably in the past decades [1,2]. Polymers conductors (CPs) were also shown to tune cellular activities through electrical stimulation such as cell growth [3,4] and cell migration [5] and this led to a considerable interest in conducting polymers and their derivatives for tissue engineering applications [6–8]. CPs have electrical and optical properties similar to those of metals and inorganic semiconductors, but they also possess attractive properties similar to those of common polymers, such as ease of synthesis and good processability compared to metals [9–11]. Polyaniline (PANI) is one of the most promising candidates moreover; many studies have been devoted to this polymer. The PANI is one of the best characterized conducting polymers, it shows diversity of structural forms, high environmental stability, and it can transport charges in an ease way [12,13]. The first researchers who demonstrated that this polymer is biocompatible in vitro and in vivo were Mattioli-Belmonte et al. [14]. The published studies dealing with PANI biocompatibility can be divided into two main groups. The first group is focused on in-vivo testing [15,16]. The second, prevailing group is dealing with assessment of in vitro proliferation and/or differentiation of cells on PANI surfaces [17–19].

Toward efficient development of biomaterials with higher biocompatibility, it is of critical importance to understand and control the physicochemical and biological interactions that occur at the interface between materials and cells. Cell adhesion is a key factor for the regulation of adherent cell growth, cell migration and differentiation [20,21]. Cell behavior in culture is generally influenced by the properties of the biomaterials surfaces such as the surface charge [22], chemistry [23], roughness and surface free energy [24]. On the other hand contact angle measurement depends on the surface hydrophilicity (or hydrophobicity), roughness, porosity, pore size and its distribution. In other words, this technique analyses the physical and chemical properties of the surface. This technique is useful due to the hydrophobic/hydrophilic balance plays a major role in the attachment cells to biomaterials [25].

There are many works dealing with the biocompatibility of PANI; however, there is few information about the modified PANI to improve biocompatibility. For this reason we present here an easy method that involves the modification of the polymer using cysteine amino acid. Some studies dealing with cysteine dissolved in the growth media, Walker et al. have demonstrated that L-cysteine improves growth of human peritoneal mesothelial cells (hPMC) in vitro [26]. Other researchers use the cysteine to functionalized surfaces in order to use it as a linker for molecules; such as Zhang et al. that they have modified

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surfaces with oligopeptides [27]. However, at the moment there is any report of the use of LM2 cells and HaCaT cells attached on cysteine surface modified. The result of this study demonstrates that it is not necessary to use the cysteine as a linker to improve the viability on this kind of cell. When the surface is modified with cysteine the viability increases up to 100%. Taking into account, the results demonstrate and ensure that with only modification of the surface polymer with cysteine it is possible to reach 100% of viability and no further modification with peptides or other molecules to improve viability is necessary. The hypothesis was that surface modification would have a positive impact on cell–substrate interactions. The present study shows the synthesis and characterization of a new biocompatible material composed of a PANI backbone modified with cysteine. Moreover, the biocompatibility of PANI-Cys films, using two cell lines LM2 and HaCaT is probed by assessing the adhesion to substrate. The cellular morphology of both cell lines is also studied in situ by fluorescence and AFM microscopies. All these tests show that this new material is suitable to attach and grow cells, indicating their potential use as biocompatible surfaces and tissue engineering.

2. Materials and methods

All the reagents were of analytical grade.

2.1. Synthesis and modification of polyaniline films

Polyaniline (PANI) films were obtained by in-situ polymerization of aniline onto polyethylene terephthalate (PET) and polyethylene (PE). Commercial films of PET and PE (Goodfellow) were submerged in an aqueous solution containing 50 mM aniline. To initiate the polymerization, ammonium persulfate (APS) as oxidant (50 mM) was added. Different pieces of film were left to react for 30 min to 4 h at room temperature. The optimal polymerization time to obtain uniform films was found to be 2 h. The films polymerized during 2 h were rinsed thoroughly with a solution 1 M of hydrochloric acid. Then PANI films were functionalized by nucleophilic addition [28,29]. The films were immersed into a stirred aqueous solution of L-cysteine 1 M for 24 h at room temperature. Then, they were washed with deionized water (DI), dried in air and stored at room temperature. Finally, these films were sterilized overnight with UV light in the laminar flow.

2.2. Characterization of chemical structure

The chemical structures of all samples were determined by Fourier-transform infrared spectroscopy (FTIR) and the spectra were collected on a Nicolet Impact 410 spectrometer in transmission mode in air of the polymer and modified polymer supported onto PE. A total of 200 scans were averaged at a resolution of 4 cm^{-1}.

UV–visible spectra were recorded on a Shimadzu UV-2401PC spectrometer in the wavelength range from 250 nm to 1000 nm. Three films for each formulation were tested using air as a background, by transmission of the polymer deposited onto PET.

2.3. Contact angle measurement

The measurements were carried out at 25 °C, using 5 μl of DI water deposited onto the surface. A microscope Intel Play QX3 with 60× objective was used for photographing the drop image. The images (tiff format) were analyzed using "Drop Analysis", an add-in of ImageJ® image processing software. At least five readings ($n = 5$) were made on different parts of the films and the results informed were averaged of these 5 measurements [30].

2.4. Cell lines

HaCaT or LM2 cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% of an antibiotic–antimycotic solution (Gibco) at 37 °C in a humidified 5% CO_2 containing atmosphere. Cells were seeded onto unmodified TCP and PANI-Cys films. The modified films were sterilized on UV light overnight, and then placed in a dish culture.

2.5. Cell viability

Tetrazolium dye (MTT; 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay was generally used for the determination of cytotoxic effects on the growth and cell viability [31]. The HaCaT or LM2 cell line was cultured on PANI and PANI-Cys in 96-well plates at a density of 2.5×10^5 cells/well in DMEM. The TCP was used as the control. The MTT was added to each culture medium to obtain quantitative values of both the HaCaT cells and the LM2 cells on the 96 wells, PANI-Cys films after 24 and 36 h of culture and PANI films after 36 h of culture. To assay the attached cells, the cultured medium was discarded. After culturing the cells were rinsed three times with sterile phosphate-buffered saline (PBS). Then, a medium in MTT was added and the cells were continued to culture for 3 h. After removing the medium, dimethylsulfoxide was added, and the absorbance was read at 550 nm using a microplate reader (Thermo Lab systems, Finland). Ten parallel replicates were read for each sample.

2.6. Adhesion of HaCaT and LM2 cell lines to PANI-Cys surface

Cell count was based on incorporation of 5 μg/mL Hoechst 33342 (Sigma-Aldrich Ltd.) that labels the DNA and serves for visualization of nuclei. The samples were fixed in cold methanol for 15 min then this fluorescent dye was added for 1 min to the cells that were finally washed. Fluorescence was recorded using a Nikon microscope, using an excitation filter BP340-380, with a color CCD camera. Cell counting was done using ImageJ® 1.34 s image analysis software. The cell number was counted on average in ten fields.

2.7. Atomic force microscopy, cell morphology studies

After the HaCaT and LM2 cells were grown in PANI-Cys films, 1 mL fixation solution (3.7% formaldehyde) was added to the sample for 15 min. The samples were then rinsed with PBS. Finally the fixed cells were studied by atomic force microscopy (AFM). The AFM measurements were made with an Agilent 5420 AFM/STM microscope in ambient conditions. The analysis of fixed cells was carried out at room temperature (20–25 °C) using a commercial Point Probe® Plus Non-Contact/Tapping Mode-Long Cantilever (PPP-NCL) with a force constant of 6 N m^{-1} and resonance frequency of 156 Hz in the Acoustic AC (AAC) mode.

3. Result and discussion

3.1. Characterization PS-PANI-Cys films

The UV–visible absorption spectrum of PANI and PANI-Cys film is shown in Fig. 1A. This figure shows the typical electronic transitions of the PANI. A typical UV–vis spectrum of the PANI in the doped state (dot gray line), undoped state (solid black line) and PANI-Cys (dot black line) is shown. For the doped PANI, the shoulder at ca. 349 nm is attributed to the $\pi \rightarrow \pi^*$ transition of the aniline ring and the band at ca. 413 nm is assigned to the $n \rightarrow \pi^*$ transition of the localized cation radical. On the other side, the band at ca. 850 nm is assigned to the excitation from the highest occupied molecular orbital (HOMO) of the benzenoid rings (pb) to the lowest unoccupied molecular orbital (LUMO) of the quinoid rings (p q) in the quinoneimine units [32]. It is

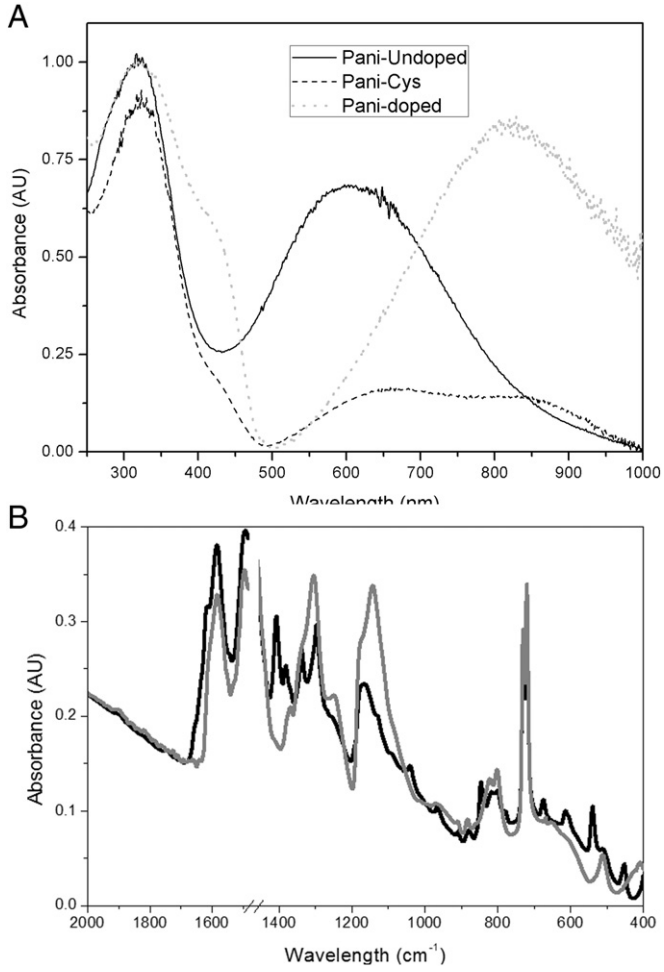

Fig. 1. A) Uv–visible spectroscopy of dopped PANI (solid black line), undopped PANI (dot gray line) and PANI-Cys (dot black line) B) Fourier transforms infrared (FT-IR) spectra of PANI (solid gray line) and PANI-Cys (solid black line).

possible to observe a bathochromic shift of 850 nm band to 630 nm when PANI is in the undoped state this behavior is due to the lack of the conjugation of the backbone. In the PANI-Cys spectrum, the band occurs at 850 nm, suggesting that the carboxylic acid group in cysteine self-dopped the polymer. Moreover, an additional band at 630 nm suggests that some domains of the polymer are not doped. On the other hand, these results indicate that the electronic properties of the polymers do not change significantly after the modification of the backbone.

In Fig. 1B is shown the FTIR spectra of: PANI (solid gray line) and PANI-Cys (solid black line). The spectrum of PANI shows all characteristic band of polyaniline: at 1588 cm^{-1} (assigned as C═C stretching of the quinoid rings), 1496 cm^{-1} (C═C stretching of benzenoid rings), 1306 cm^{-1} (C–N stretching mode) and 1164 cm^{-1} (ring stretching, N–Q–N, Q representing the quinoid ring) [33]. The spectrum of modified PANI-Cys shows additional bands at ca 674 cm^{-1} that could be assigned to the C–S linkage of the Cysteine group to the polymer. A shoulder at 1230 cm^{-1} and 1663 cm^{-1} could be assigned to the stretching of the C–O and C═O groups present in the cysteine. The infrared spectrum reveals that PANI-Cys presents new functional groups in comparison with unmodified PANI. The weak absorption of the C═O group suggests that the degree of modification is small, likely due to the modification confined to the surface of the film.

3.2. Contact angle measurements

Contact angle was measured to evaluate the surface hydrophobicity/hydrophilicity of the base material polystyrene tissue culture plastic (TCP) and those modified with PANI-Cys films. For TCP the contact angle measured is 68 ± 1° (not shown). These results are consistent with previous studies showing that TCP has drop water contact angle of 65.8° [34]. The contact angles measured for PANI have a value of 86 ± 1° and for the PANI-Cys film is 90 ± 1° (Fig. 2).

These results indicate that PANI film is less hydrophobic than PANI-Cys films. It is well accepted that both very hydrophobic and very hydrophilic surfaces are suitable for cell attachment [35–37]. Surfaces with moderate wettability are able to adsorb proteins, and at the same time preserve their natural conformation, resulting in positive cell attachment. Zuwei et al. [38] have shown that the best water contact angle for endothelial cell attachment and proliferation is 70°, on the polycaprolactone graft poly(methacrylic acid) (PCL-g-PMAA) [39], while for chondrocytes it is c.a. 76°, used as a support for poly-L-lactic acid graft poly(methacrylic acid) (PLLA-g-PMAA) prepared by the same grafting method. The authors shown that by changing the quantities of the graft polymer the water contact angle decreases to 65°, and this surface exhibits very poor chondrocyte spreading and attachment on all cell types. All these results support the fact that different cells prefers to attach at surfaces with contact angle between 76 to 70°. It has to note that the cell material interactions are not only governed by the hydrophilic character of material surfaces, even though moderately hydrophilic surfaces have been found to promote cell adhesion best. Other surface properties such as roughness [40] and surface charge [41] also have influences on cell behaviors.

3.3. Cell viability

The cell viability of both cell lines (HaCaT and LM2) on PANI, PANI-Cys surfaces and plastic control were studied by MTT assay. The MTT analysis results for both the HaCaT and LM2 cell lines are displayed in Fig. 3. The HaCaT and LM2 cell lines show a viability level of approximately 81.31% and 79.7%, respectively on the PANI surfaces after 36 h compared to the TCP control (Fig. 3A). While the assay for both cell lines on the PANI-Cys shows a viability level of 100% compared to the TCP control (Fig. 3B). MTT assay shows that the percentage cell viability increases with culturing time in both surfaces on TCP, and PANI-Cys surfaces. No remarkable cytotoxicity is noticed after 24 and 36 h of incubation for both cell types. These results clearly indicate that PANI-Cys surface is biocompatible and this surface increases the viability cellular in both cell lines by approximately a 20% in comparison with PANI surface. These results suggest that PANI-Cys surface offers a good biological environment and improves the viability of cells without cytotoxicity compared with PANI surfaces.

3.4. Adhesion of HaCaT and LM2 cell lines to PANI-Cys surface

Both cells lines were incubated on TCP or PANI-Cys films for periods of 24 h and 36 h, stained with Hoechst 33342 and counted after both time intervals. The relative number of cells adhering to the substrata at different times is shown in Fig. 4.

In the current study, almost the same relative cell density is found for keratinocytes on TCP with 100 ± 0.97 compared to PANI-Cys films with 100 ± 1.38%. The relative cell density of LM2 cell line is comparable on both materials (TCP: 100 ± 1.15, PANI-Cys 100 ± 1.39). Considering this result, it is possible to conclude that both cell lines show the same cell adhesion on PANI-Cys films when compared to TCP.

Our studies show that in serum-containing medium, the PANI-Cys surface presents the same adhesion than those growing on TCP having different hydrophobic natures. Zhou et al. reported that surface modified with L-Cys presents better cell adhesion due to protein adsorption differences that could be beneficial by adhesion cell [42]. It has been

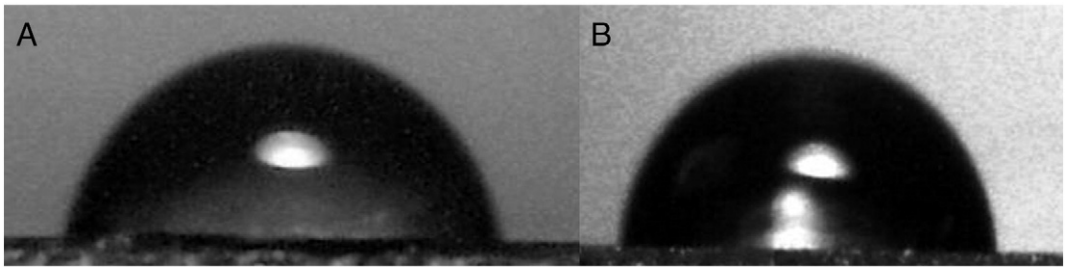

Fig. 2. Contact angle measurement, A) PANI and B) PANI-Cys.

confirmed that material surface properties including surface chemistry, hydrophobicity and topography are important parameters affecting the adhesion proteins, thus affecting the cell behaviors [43,44].

3.5. HaCaT and LM2 cell attachment and proliferation

In vitro biocompatibility was assessed by examining the potential for PANI-Cys films to support the growth and attachment of two cell types (HaCaT and LM2 cells). The attachment behavior of the cells was further characterized by immune fluorescence. Cell attachment, is best viewed via fluorescence microscopy after staining with the nucleic acid dye, Hoechst 33342, as shown in Fig. 5. The micrographs in Fig. 5 show comparable nuclear morphologies for cells grown on both TCP (Fig. 5A and C) and PANI-Cys (Fig. 5B and D). The cells cultured within 36 h, have assumed the typical nuclear morphology of cultured HaCaT (Fig. 5A and B) and LM2 (Fig. 5C and D) cell lines. The morphology of cells growing on TCP is indistinguishable from that of cells growing on the modified film. Moreover, the morphology, density and proliferation of both cell lines adhere to the PANI-Cys films are similar on TCP and they reached similar levels of confluence to that on the polymer films.

The investigation shows that a number of cells are well attached to PANI-Cys surfaces and the cellular density on the surfaces is significant within 36 h of culture, implying that the surfaces have favorable biological properties by both cell lines. This suggests that the prepared specimens provide a biocompatible environment, which favors cell attachment. Our results show that both cell lines are attached in high numbers, showing cell morphology that is comparable to controls.

Results obtained by fluorescence microscopy have been validated by AFM thus demonstrating the high potential of the AFM technique. The effect of PANI-Cys films on the adhesion of LM2 and HaCaT cells was investigated through morphological observations with an AFM (Fig. 6B and D, respectively). Fig. 6 shows the morphology cells by both cell lines on PANI-Cys films have the same cell morphology like the cells on TCP. The cell shape, such as cell elongation, cell widening and sinking, lamellipodium or filopodium generation, and others, are essential for cell migration and invasion, indicating the same effects of PANI-Cys surface and TPC on migration of both cell lines.

It is noteworthy that PANI and PANI-Cys films can be made onto more hydrophobic surfaces (e.g. PE or PP) [45] where cell attachment is poor. In that way, the surface of polymer samples can be modified to improve cell growth and proliferation.

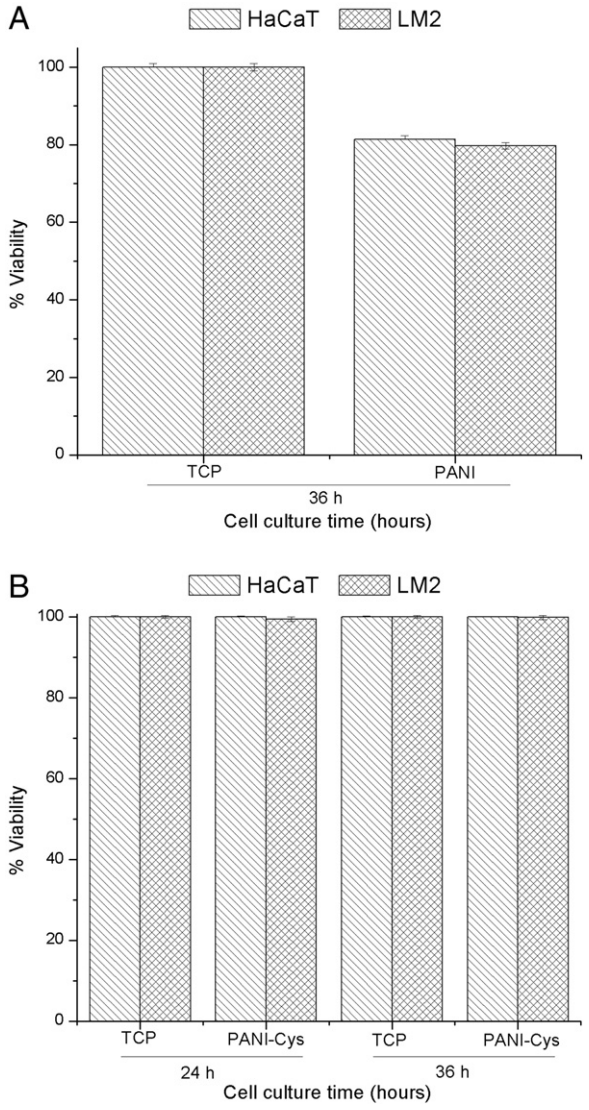

Fig. 3. Cell viability measured with MTT assay of A) cells cultured on PANI after 36 h with a control cultured on plastic (TCP) and B-cells cultured on PANI-Cys after 24 h and 36 h with a control cultured on plastic (TCP). Data represent the mean ± SD for ten samples.

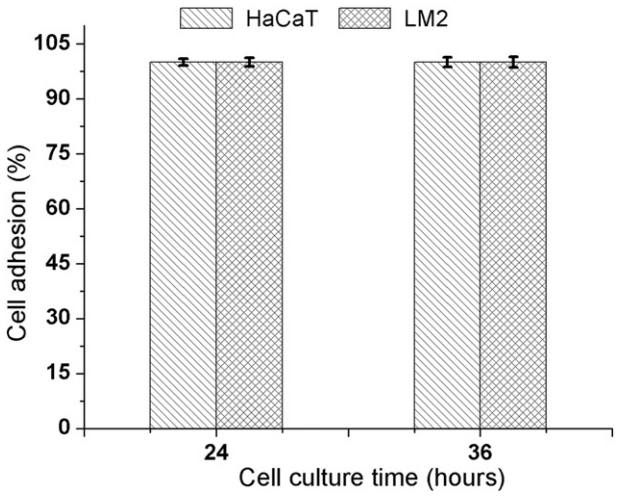

Fig. 4. HaCaT and LM2 cells adhesion at 24, and 36 h of culture on PANI-Cys and TCP as control surface. Data represent the mean ± SD for six samples.

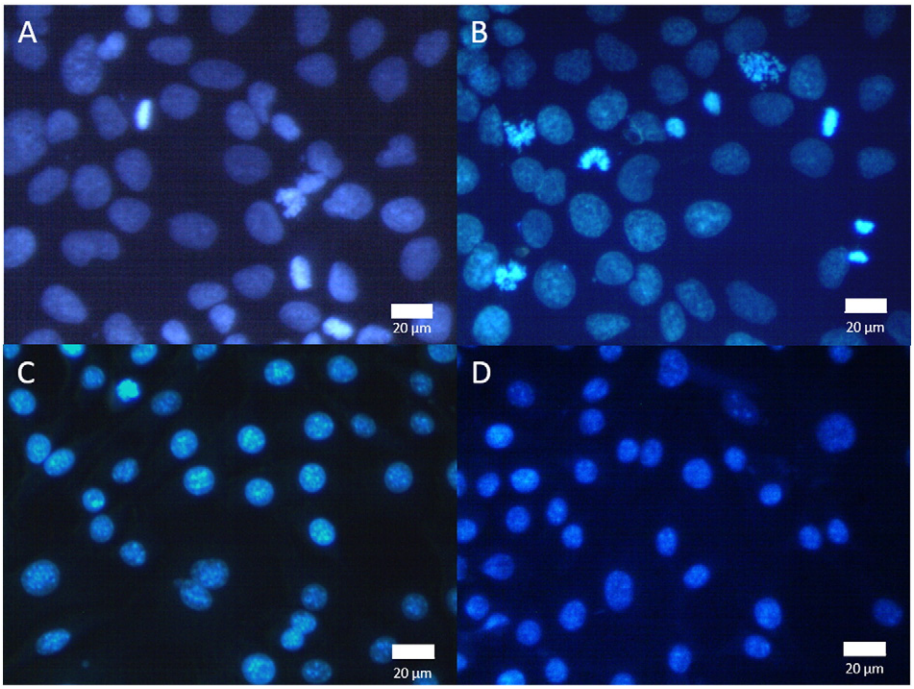

Fig. 5. Fluorescence images of the morphology of A) HaCaT cells on TCP, B) HaCaT cells on PANI-Cys film, C) LM2 cells on TCP and D) LM2 cells on PANI-Cys film.

4. Conclusion

Polymers have gained a remarkable place in the biomedical field as materials for the fabrication of various devices and for tissue engineering applications. We have successfully polymerized the PANI and functionalized with Cys into films. Therefore, in this work the new material PANI-Cys has been characterized, the physical and chemical properties of PANI-Cys films including biocompatibility in vitro. We conclude that PANI-Cys is a suitable material for future in vitro study on cell growth. Although, the in vivo biocompatibility of undoped PANI has previously been reported, we believe that this work is the first study of biocompatibility of LM2 and HaCaT cell lines culturing onto PANI-Cys films and that it demonstrates in-vitro cytocompatibility.

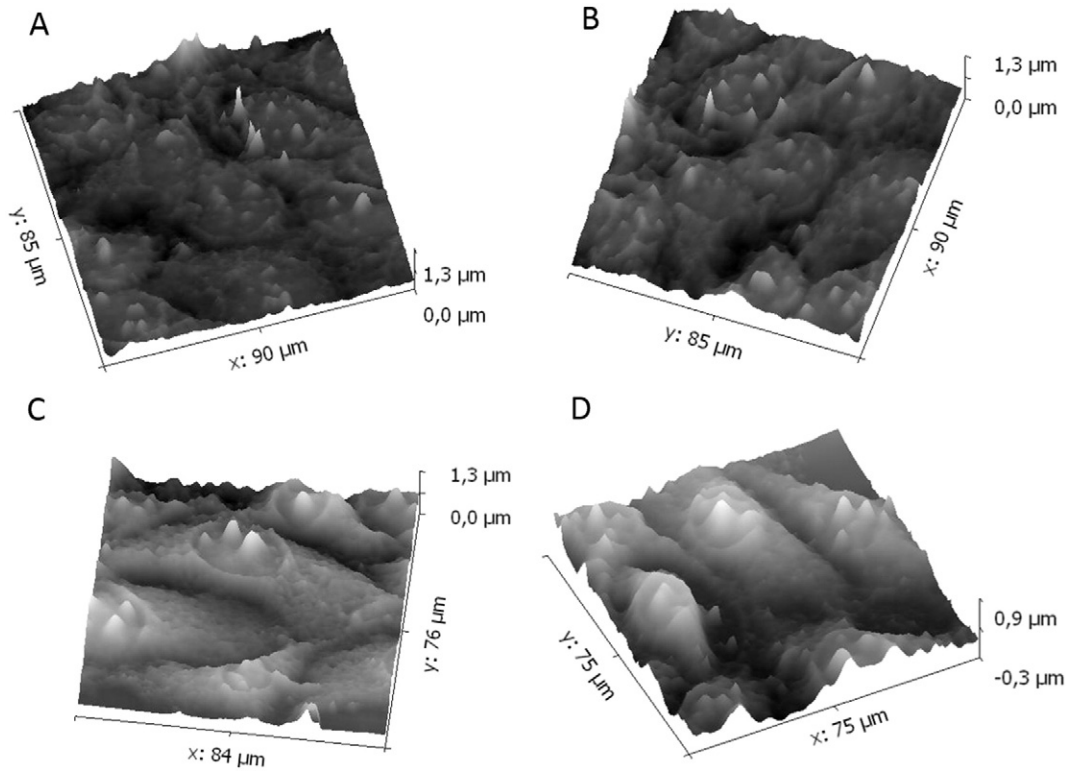

Fig. 6. Visualization AFM images of the morphology of A) HaCaT cells on TCP, B) HaCaT cells on PANI-Cys film, C) LM2 cells on TCP and D) LM2 cells on PANI-Cys film.

PANI-Cys substrate is found to be biocompatible for both healthy and cancer cells according to results of the morphology. Cells grow equally well on PANI-Cys compared with cells grown on TCP and in minor grade when they are cultivated on PANI surface. However, this conclusion may not be generalized to all cell types. This surface exhibited an excellent applicability for cell adhesion, growth, and proliferation by both cell lines. The results of this study indicate that modifying the surface with PANI-Cys enhances cell attachment and this substrate can be used in tissue engineering applications.

Conflict of interest

Authors declare that they have no conflicts of interest.

Acknowledgment

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