Research Article

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Biocompatibility investigations of synthetic melanin and melanin analogue for application in bioelectronics

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Abstract

Biocompatibility tests were performed for melanin synthesized in water (W-Mel) and dimethylsulfoxide (D-Mel) aiming to evaluate its potential for bioelectronics application. *In vitro* biocompatibility of melanin was assessed using crystal violet assays with fibroblast cells (NIH3T3) cultured with W-Mel and D-Mel extracts in various dilutions. The results show that a high concentration of melanin kills the cells. However, after periods of 48 h incubation, cell viability is significantly favored after treatment with D-Mel at low concentrations (1:16, 1:32 and 1:64), as opposed to treatment with W-Mel extracts. Cellular adhesion tests show that fibroblast cells adhere to melanin thin films. These results show that D-Mel may be an interesting material for application in bioelectronic devices targeted for implants.

Keywords: biocompatibility; melanin; polymer; bioelectronics

INTRODUCTION

In recent decades, conductive polymers have gained attention because of their good optical and electrical properties, low cost, easy synthesis and processing, as well as for being flexible, porous, biodegradable and biocompatible, serving as an alternative in electronic devices for implants.[1–3] Currently, there is a great demand for new materials in the production of biosensors, since the combination of electronic devices and organic materials can be more advantageous when compared to the conventional biodetection methods, as it simplifies the detection of biological species.[3]

An increasingly prominent material is melanin. Melanin is a natural pigment present in the human body, which has several functions, such as photoprotection, photosensitivity, metal chelation and thermoregulation, making it a potential polymer for applications in implanted devices.[4–6] Nowadays, this biomolecule is used in several applications, such as batteries,[7] sensors,[8] drug release,[9] tissue engineering[10] and biomimetic interfaces.[11,12] Therefore, melanin is an attractive candidate for organic implantable devices due to its interesting electrical, optical and physicochemical properties, such as photoconductivity in the solid state, hybrid ionic–electronic conduction and biocompatibility.[3,13,14] Studies concerning melanin biocompatibility indicate good cellular responses in the presence of the pigment, which makes it suitable for implants.[10,15,16]

In natura, after a series of oxidative processes, melanin is formed from tyrosine.[4,17] However, given the difficulty of extraction of natural melanin, much research uses the material synthesized from the oxidation of L-3-(3,4-dihydroxyphenyl)alanine (L-dopa) in aqueous medium.[17] Melanin obtained during this process is insoluble in water and other solvents, which limits the production of devices based on melanin thin films.

A simple way to use melanin in technological applications is by obtaining soluble melanin analogues, which facilitates its deposition as thin films.[18–21] In 2004, a soluble melanin analogue was obtained through organic synthesis using dimethylsulfoxide (DMSO) and benzoyl peroxide.[18] The melanin-like derivative from this procedure (D-Mel) is a product similar to melanin, with interesting electronic properties, greater solubility in DMSO and with good thin-film properties.[18,22–24] D-Mel is comparable to traditional melanin, differing only in the functionalization of the 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) structures with sulfonated groups ($=SO_2CH_3$).[24] Figure 1 illustrates the structure of melanin monomers.

Thus, since there is limited work on melanin biocompatibility and no reports of the study of melanin-like polymers corresponding to D-Mel, the work reported here aimed to evaluate its

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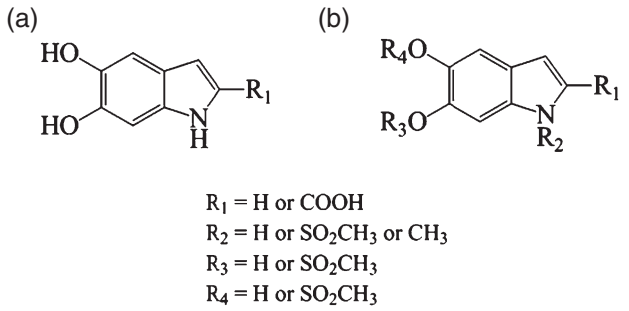

Figure 1. Monomers that constitute melanin. Monomers obtained by synthesis in (a) water and (b) DMSO.

biocompatibility and thin-film adhesion, comparing with traditionally synthesized melanin, aiming at the implementation of melanin analogues in implantable devices.

METHODS

Melanin synthesis

The synthesis of traditional melanin in aqueous medium (W-Mel) was carried out using a known procedure,[25] described briefly as follows. Initially, 1.00 g of L-dopa was dissolved in 200 mL of deionized water (Milli-Q), at pH 8 to 10. This mixture was stirred for 3 days at room temperature under oxygenation, utilizing an air pump. After the synthesis was completed, the solution was placed in a 5000 Da dialysis membrane and immersed in Milli-Q water, in order to eliminate synthesis residues. The product was dried in an oven at 80 °C.

In order to realize the synthesis of melanin in organic phase, the reaction of 1.500 g of L-dopa with 0.925 g of benzoyl peroxide and 200 mL of DMSO was performed. The reaction was maintained under agitation at elevated temperature (100 °C) for 8 days.[26] Subsequently, the solution was heated until 25% of the initial amount was left. After reaching room temperature again, 150 mL of acetonitrile was added and left for 12 h in order to precipitate melanin. Finally, the sequence of centrifugation/extraction of the precipitate resulted in the purified powder of synthetic D-Mel.

Experimental groups

Cellular tests with melanin were carried out with powdered samples (extracts). Both W-Mel and D-Mel were dispersed in Dulbecco's modified Eagle medium (DMEM; Nutricell) with 10% fetal bovine serum (FBS; Nutricell) at 50 mg mL^{-1} (initial dilution). After vigorous stirring using a vortex, the solutions were left for 24 h in a CO_2 incubator at 37 °C. From the initial dilution, serial dilutions of 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 were made. These dilutions, as well as positive and negative controls, were used as experimental groups.

Cell culture

Cellular viability tests were performed according to ISO 10993 – 5,[27] using mouse fibroblast cells (NIH3T3) from an immortalized culture (ATCC). Fibroblasts were grown in DMEM supplemented with 10% FBS. Cells were maintained and incubated in a culture flask at 37 °C in a 5% CO_2 humidified atmosphere. After subconfluence, the cells were removed from the culture flask using a trypsin enzyme solution (0.25% trypsin, 1 mmol L^{-1} EDTA; Sigma-Aldrich) diluted in phosphate-buffered saline (PBS; 2:8). Subsequently, these cells were maintained for 5 min in a CO_2 incubator at 37 °C, and then the enzyme was inactivated with DMEM culture medium.[28] The cells detached from the bottle were centrifuged at 1200 rpm for 5 min at 4 °C. The centrifuging provided a cell pellet which was suspended in DMEM with 10% FBS.

Crystal violet tests

The effects of W-Mel and D-Mel extracts on the viability of cells were assessed using crystal violet tests.[29] Fibroblast cells were plated at a density of 2×10^3 cells per well in eight 96-well plates each. After 24 h of incubation in CO_2 at 37 °C, the medium was replaced by DMEM with 10% FBS and the various dilutions of melanin extracts were also added. Positive (DMEM with 10% FBS) and negative (DMEM with 1% FBS) control groups were also present. After addition of media containing melanin extracts, plates were incubated for various experimental periods: 6, 12, 24 and 48 h. After each period, the culture media were removed, and cells were rinsed twice with PBS and fixed with 100% methanol for 10 min. Subsequent to methanol removal, a 0.2% crystal violet solution in 20% ethanol was added in each well for 3 min. The excess of this dye was removed by washing with PBS. Finally, 0.05 mol L^{-1} sodium citrate in 50% ethanol was added over 10 min. All assays were done in triplicate. The absorbance of each well was assessed using a spectrophotometer (Synergy MX Monochromator-based Biotek), at 540 nm.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. Results of cellular viability tests showed a normal distribution, then an ANOVA test (one-way and two-way ANOVA), with Bonferroni and Tukey post-test, with $p < 0.05$, was used.[30] The hypothesis was that the means of several groups were equal, and the hypothesis was rejected when the difference was statistically significant.

Melanin thin films for cellular adhesion tests

Cell adhesion tests were performed on melanin thin films, prepared from four precursor solutions: W-Mel solutions (concentrations of 5 and 30 mg mL^{-1} in aqueous solutions of NH_4OH), representing groups 1 and 2, and D-Mel solutions (concentrations of 5 and 30 mg mL^{-1} in DMSO), representing groups 3 and 4, respectively. Aqueous solutions of W-Mel were obtained following a procedure adapted from Bothma *et al.*[31] Briefly, group 1 solution was prepared from 75 mg of W-Mel diluted in a solution of 5 mL of water and 10 mL of NH_4OH. The mixture was stirred at room temperature for 1 h and a further 1 h in an ultrasonic bath. The group 2 solution was prepared similarly, and solutions 3 and 4 (D-Mel solutions) were obtained according to the same procedure, however using only DMSO as solvent.

The solutions were deposited onto glass substrates using the spin-coating technique with a rotation speed of 2000 rpm for 60 s. The surface morphology of the melanin films was investigated using an AFM instrument (AFM xe7, Park Systems) working in non-contact mode.

Cell culture

Primary human gingival fibroblasts (hGF) were obtained from samples of healthy tissue from patients. The experimental protocol used in this study was approved by the Committee on Ethics in Human Experimentation, Bauru School of Dentistry, University of São Paulo (Protocol Number 6706412.2.0000.5417, 8 November 2012), and written informed consent was obtained from all

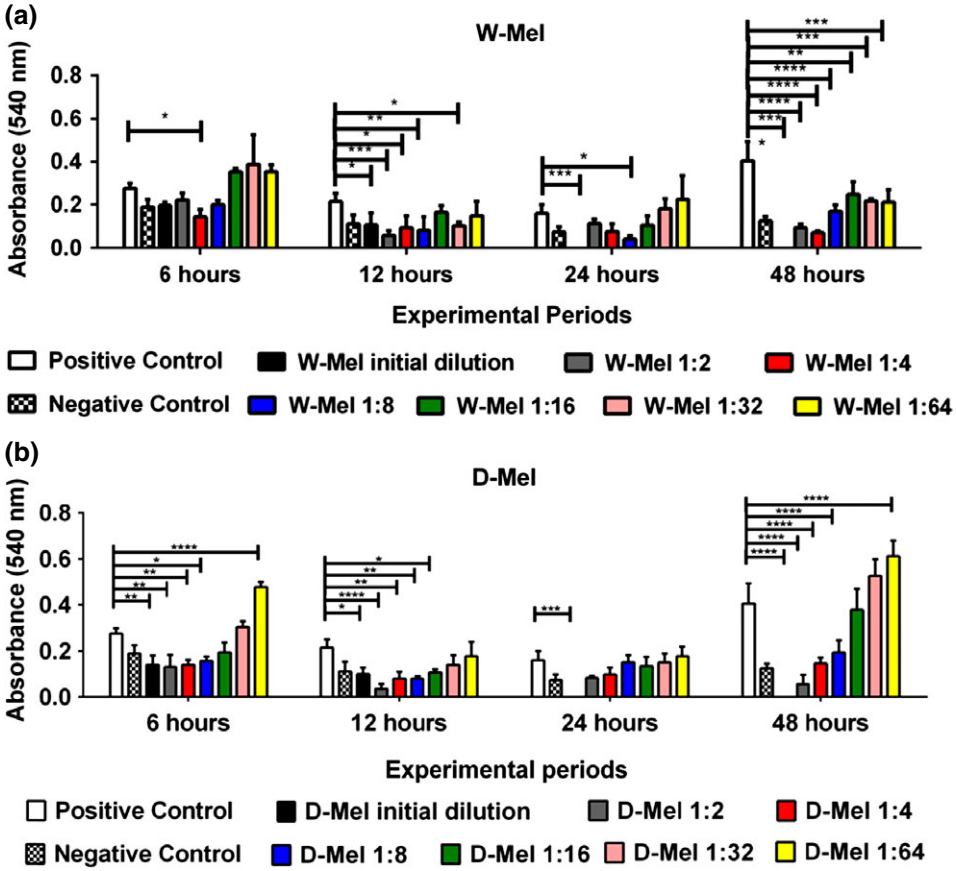

Figure 2. Cell viability using crystal violet tests. Effects of (a) W-Mel and (b) D-Mel on fibroblasts. All graphs are on the same scale to facilitate comparisons. Initial dilution refers to W-Mel (or D-Mel) extracts dispersed in DMEM with 10% FBS at 50 mg mL^{-1}. Asterisks represent significance levels of the tests: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

patients. This study was performed in accordance with the Declaration of Helsinki.

The harvested tissue was rinsed several times in sterile saline solution (0.9% sodium chloride) and antibiotics (100 U mL^{-1} penicillin, 125 µg mL^{-1} streptomycin and 5 µg mL^{-1} amphotericin). The tissue was cut into small pieces and cultured with DMEM containing 10% FBS. The cells grew from explants until reaching confluence; then they were detached with 0.025% trypsin (Sigma Chemical Co.) for 10 min and subcultured in T-75 cell culture flasks. The hGF were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cell cultures used in experiments were in passage 5.

Cells (1×10^4 hGF per well) were transferred to 24-well plates, containing sterile spherical glass coverslips (13 mm in diameter), and coated with films, in duplicate ($n = 8$). Spherical glass coverslips without films were used as control (negative control). The cells remained in seeding media from 24 to 48 h.

SEM analysis

A morphological study of the cells cultured on melanin films was carried out after 24 and 48 h of cell culture using SEM (T 220-A, Jeol, Tokyo, Japan).[32,33]

After the 24 and 48 h test periods, the coverslips were carefully removed and 20 specimens (eight for each group and four negative controls) were used for SEM. Each specimen was fixed over osmium tetroxide vapor (OsO_4) for 24 h and then dried for 72 h in a glass desiccator with silica gel, gold-coated in a sputter coater (Balzers, Hammer VII, Alexandria) for 120 s and observed.

RESULTS

Figure 2 shows the results of cell viability using the crystal violet tests. Crystal violet is a dye that electrostatically binds to nuclear DNA and cellular proteins. Therefore, in absorbance studies, lower absorbance values indicate a decrease in the number of viable cells after treatment with melanin extract. Asterisks indicate the groups that showed significant differences in the variance analysis test (ANOVA).

In all tests, cells from positive and negative control groups are viable, the difference is the number of viable cells between these groups. Results show that the negative control groups have lower absorbance, always justified by the small amounts of available growth factors (1% FBS). Despite the viability in both control groups, cells experienced less stimulation in the negative control group, thus causing them to proliferate less, which is expected and validates the methodology. During periods of 24 and 48 h, groups treated with the initial dilution of W-Mel and D-Mel show negative results, i.e. a high concentration of melanin kills the cells.

Figure 2(a) indicates that after treatment with W-Mel extract, most cells show lower viability than the positive control group, with some exceptions. For 6 h of incubation, groups treated with 1:16, 1:32 and 1:64 concentrations of W-Mel have a higher viability than the control group, although these differences are not significant. Even so, a higher viability is also observed in the group treated with 1:64 concentration after 24 h. It is interesting to note that after 48 h of treatment, all W-Mel extract concentrations promote the reduction of cell viability, with significant differences

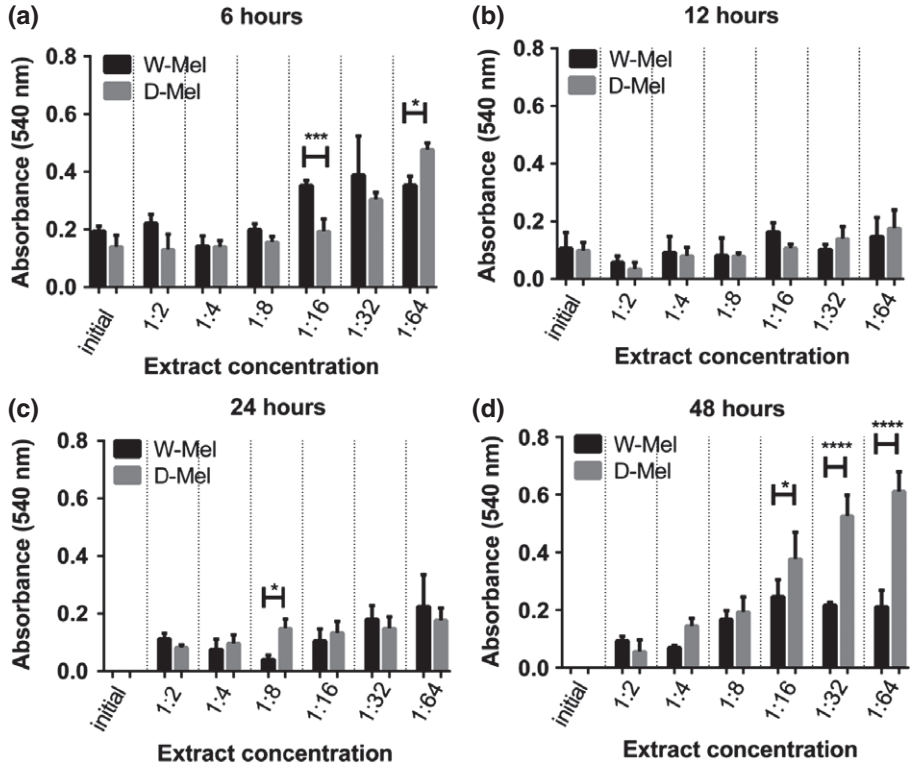

Figure 3. Cell viability using crystal violet tests. Effects of W-Mel and D-Mel on fibroblasts after incubation periods of (a) 6 h, (b) 12 h, (c) 24 h and (d) 48 h. All graphs are on the same scale to facilitate comparisons. Asterisks represent significance levels of the tests: *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

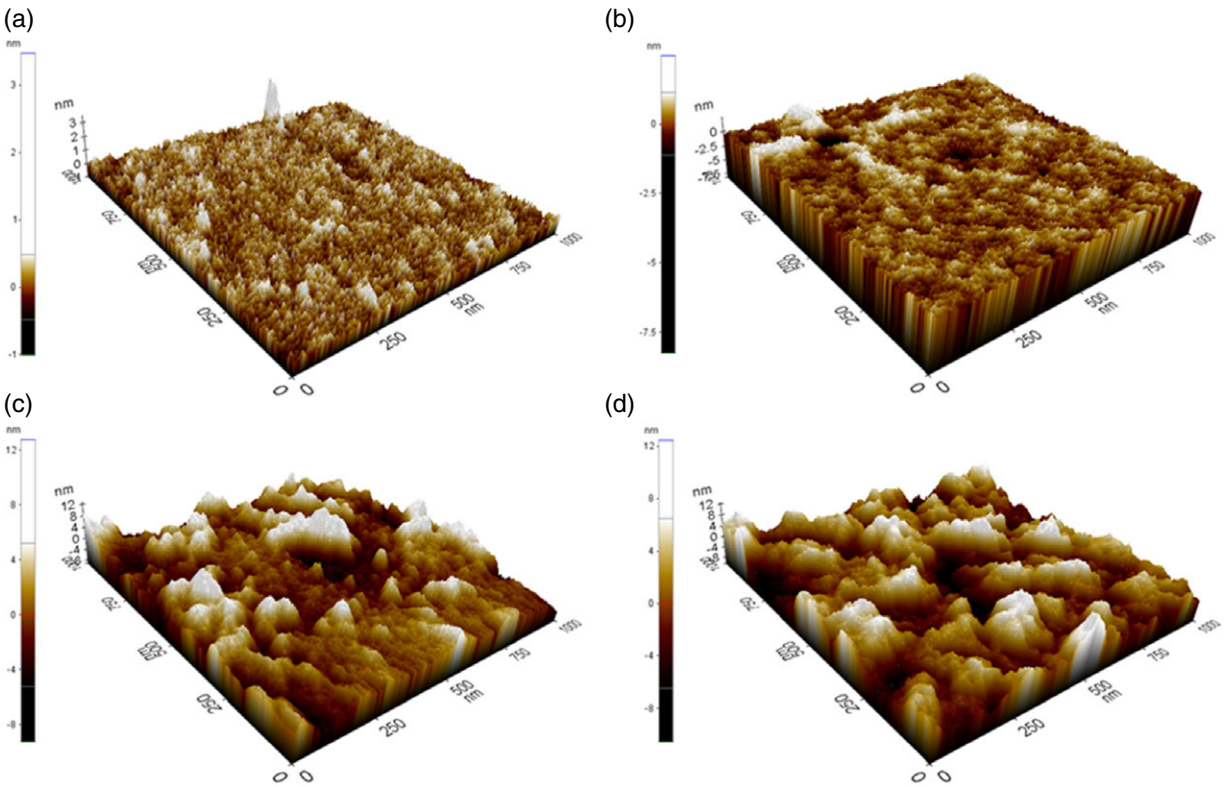

Figure 4. AFM images (1.0 μm × 1.0 μm) of melanin thin films: (a) W-Mel 5 mg mL^{-1} (group 1); (b) W-Mel 30 mg mL^{-1} (group 2); (c) D-Mel 5 mg mL^{-1} (group 3); (d) D-Mel 30 mg mL^{-1} (group 4).

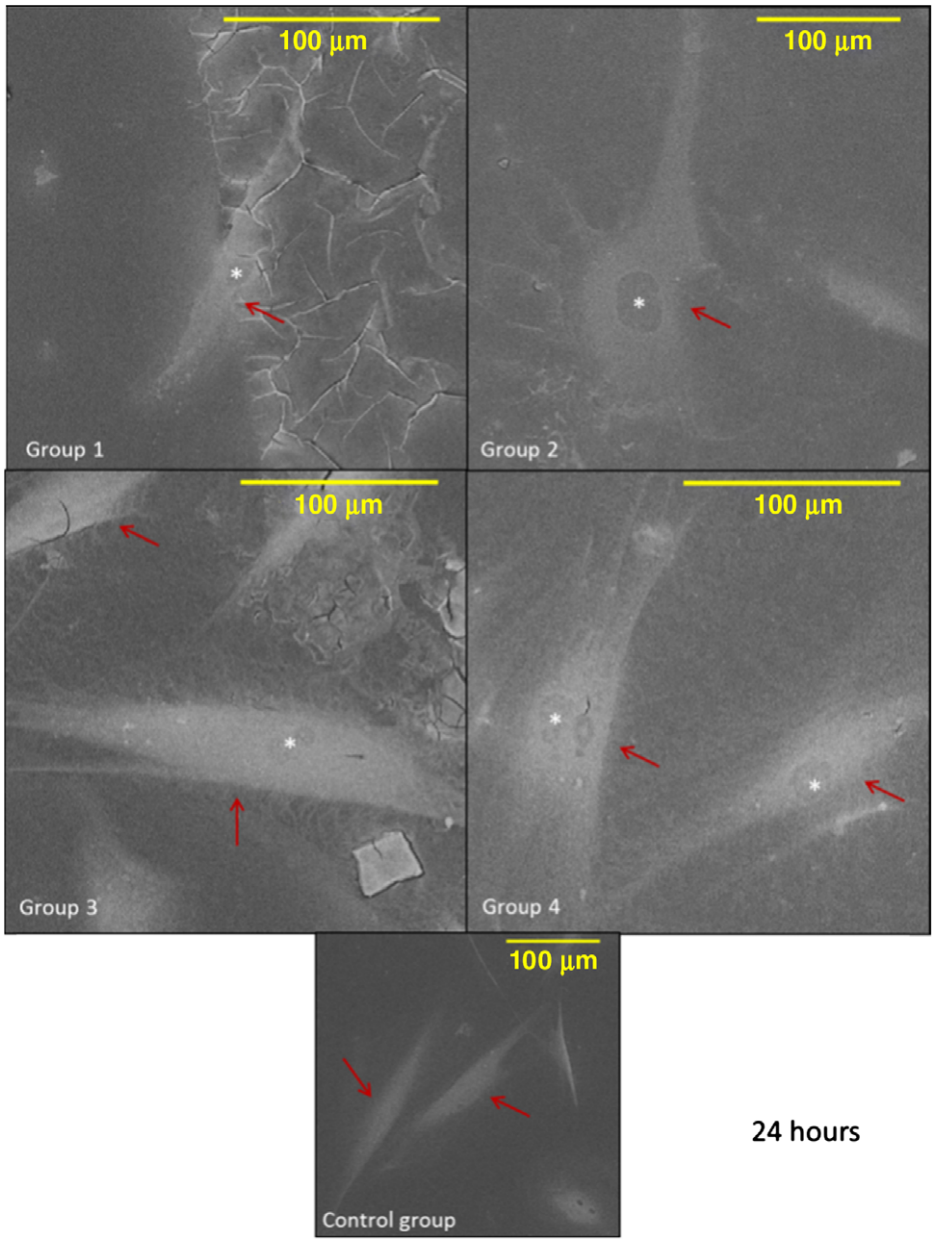

Figure 5. Within 24 h, fibroblasts are observed (red arrows) in all groups (experimental and control). Groups 1 to 4 represent different concentrations from precursor solutions of melanin thin films. Fibroblasts show characteristic morphology with cytoplasmic extension and suggestive region of the cell nucleus (white asterisk).

when compared to the positive control group. Other treatments with W-Mel show less cell viability than the positive control group.

From Fig. 2(b) it can be observed that treatment with D-Mel extracts leads to different reactions depending on extract concentration and incubation time. For incubation times of 12 and 24 h, the presence of melanin in any concentration decreases cell viability when compared to the positive control group. During incubation of 6 and 48 h, the extracts with initial, 1:2, 1:4, 1:8 and 1:16 dilutions show a lower viability than the control group. On the other hand, for periods of 6 and 48 h, the cells incubated with D-Mel extracts in 1:32 and 1:64 concentrations present a higher viability. It is noteworthy that the difference between the positive control and the 1:64 groups is significant.

Figure 3 shows the results of crystal violet tests, directly comparing the treatments with W-Mel and D-Mel, for various incubation periods. Although differences are not significant, it is important to emphasize that, generally in high concentrations, D-Mel extracts provide a worse cell viability than W-Mel extracts for incubation periods of 6, 12 and 24 h. After 48 h incubation, cell viability is significantly favored after treatment with D-Mel at low concentrations (1:16, 1:32 and 1:64).

Morphological characterization of melanin thin films before cell culturing performed using AFM is shown in Fig. 4. Thin-film roughness from groups 1 to 4 are respectively 0.239, 0.377, 3.328 and 4.466 nm. Note that the higher surface roughness obtained in D-Mel films, comparing with W-Mel films, may be a result of the higher aggregation of melanin synthesized in DMSO at high temperature (100 °C). This synthesis promotes an increase of DHI

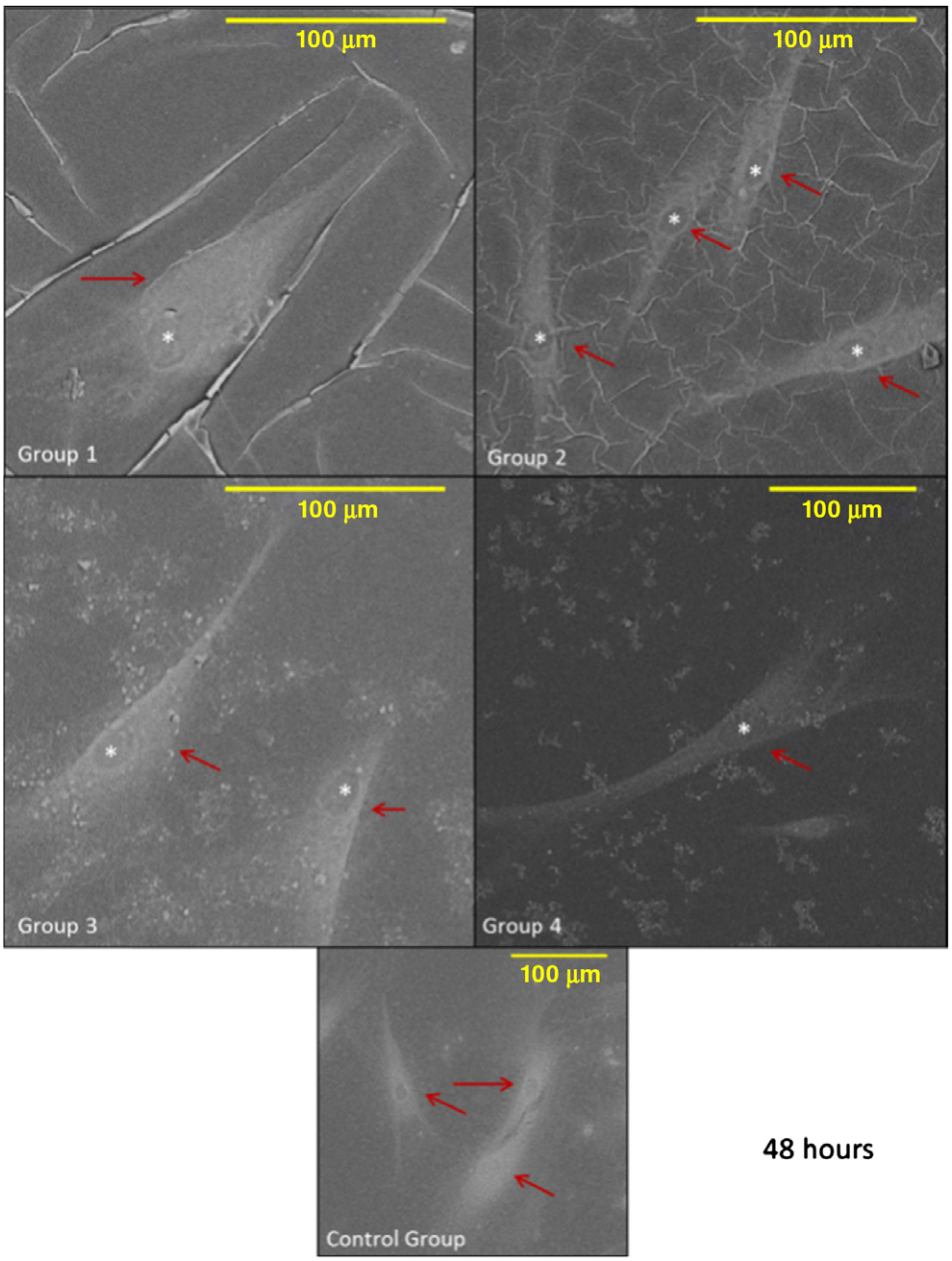

Figure 6. In 48 h, cells are observed (red arrows) in all groups, including control. Groups 1 to 4 represent different concentrations from precursor solutions of melanin thin films. The cells, in all groups, show cytoplasmic extension and suggestive region of the cell nucleus (white asterisk).

structures in the polymer,[26] which may lead to molecules with a higher degree of polymerization.

Figures 5 and 6 show images obtained from tests of cell adhesion on melanin thin films. For all groups in the two periods (24 and 48 h), the fibroblasts are found to be adhered and present apparently normal morphology. The cytoplasmic extensions, as well as suggestive cell nucleus regions, are general findings for all samples. In comparison, the 48 h samples reveal a more uniform distribution and also more cells on the studied surfaces.

DISCUSSION

Melanin and melanin analogue are compounds that have potential for a wide range of biomedical applications. However, the biocompatibility of these materials has not been adequately characterized and studies addressing melanin biocompatibility are scarce. The goal of our study was to explore the cellular viability and cell adhesion of fibroblast cells in melanin extracts and thin films.

In vitro tests of melanin synthesized in water and in DMSO assessed using crystal violet assays with fibroblast cells show that after 48 h in incubation, cell viability is significantly favored after treatment with D-Mel at low concentrations (1:16, 1:32 and 1:64), as opposed to treatment with W-Mel extracts.

The observed effect of reduction of cell viability after 48 h of treatment with W-Mel extract can be explained by the inherent cytotoxicity of primary constituents of melanin, observed for various cell lines.[34–37] Studies concerning the inherent cytotoxicity of the monomers DHI and DHICA against various cell lines show that, under usual assay conditions, the apparent cytotoxicity of these two indole moieties reflects their instability in the culture

medium. The increase of the cytotoxic effects observed with incubation time is justified by the generation of reactive oxygen species (especially H_2O_2) during melanin precursor auto-oxidation outside the cells.[34]

Regarding the differences between the W-Mel and D-Mel extracts, the higher viability of cells incubated with D-Mel can be explained by the effect of sulfate groups attached to monomers that constitute D-Mel. Structurally, the main difference when melanin is synthesized in DMSO is the incorporation of sulfonated groups coming from DMSO oxidation, at the phenolic hydroxyl group of DHI and DHICA.[18,24,26] This functionalization of D-Mel can enhance cell viability, since chemical structure and functional groups play a very important role in biocompatibility.[38] For instance, melanin colloidal nanospheres, shown to be biodegradable, presented a median lethal dose and induced no toxicity during long-term retention in rats.[39] Extracts of poly(2-hydroxyethyl methacrylate) containing adrenochrome-melanin showed no cytotoxicity to human choroidal fibroblasts, only for the extracts of polymers with a high melanin content.[40]

In the present study, the sulfonation of DHI and DHICA monomers may also increase the cellular viability due to the fungicidal effect presented by organosulfur compounds.[41] Other studies supporting this hypothesis show that surface-modified sulfur nanoparticles also expressed promising inhibitory effects on fungal growth.[42]

Cellular adhesion tests show the ability of fibroblast cells to adhere to melanin thin films. Apparently, there is no difference between cell adhesion on films of W-Mel and D-Mel. The panorama, cell number and morphology are very similar. Thus, the results indicate that different films allow adhesion of fibroblasts, since normal morphology is observed for this cell type.

These results are similar to other studies that indicate cell viability and cell adhesion in melanin thin films. Bettinger and colleagues evaluated the potential use of melanin films as a material for tissue engineering applications by using *in vitro* biocompatibility in terms of cell attachment and growth.[10] The melanin for thin-film production was also dissolved in DMSO and their results suggest that solution-processed melanin thin films have the potential to be used as a biodegradable semiconducting biomaterial in tissue engineering applications. *In vitro* biocompatibility assessed by examining the potential for melanin films to support the growth and attachment of cells suggests that, cultured on melanin thin films, cells exhibit a more activated phenotype than on collagen-coated glass and uncoated glass.[10] Other results show that melanin films support adhesion, regular growth, survival and, mainly, the formation of neuronal precursors and neurons starting from undifferentiated cells.[16]

CONCLUSIONS

We report the biocompatibility of melanin synthesized in water (W-Mel) and in DMSO (D-Mel) through crystal violet assays and cellular adhesion tests. *In vitro* tests allowed us to verify that melanin extract at high concentrations did not allow cell viability of fibroblast cells. However, cell viability is significantly favored after treatment with D-Mel at low concentrations (1:16, 1:32 and 1:64). Comparing treatments with W-Mel and D-Mel, the cells incubated with melanin extract synthesized using an organic route (D-Mel), which leads to functionalization with sulfate groups, provided better results when compared to the treatment with melanin synthesized in water (W-Mel).

The statistical tests performed indicated that cellular viability is affected by incubation time, extract concentration and melanin structural characteristics. The results indicate that when in contact with the melanin extract at low concentrations, fibroblasts may exhibit a more active phenotype, increasing their proliferation as compared to control group cells.

To our knowledge, this is the first study of the cytotoxicity of melanin functionalized with sulfate groups. Since the organic route promotes structural differences in D-Mel, the effects of these changes in biocompatibility properties are promising, with better cell viability and adhesion than observed for traditional melanin. These results show that D-Mel may be an interesting material for application in implantable devices.

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REFERENCES

1 Balint R, Cassidy NJ and Cartmell SH, *Acta Biomater* **10**:2341–2353 (2014).
2 Qin YH, Howlader MMR, Deen MJ, Haddara YM and Selvaganapathy PR, *Sens Actuators B* **202**:758–778 (2014).
3 Bettinger CJ, *Trends Biotechnol* **33**:575–585 (2015).
4 d'Ischia M, Wakamatsu K, Cicoira F, Di Mauro E, Garcia-Borron JC, Commo S *et al.*, *Pigm Cell Res* **28**:520–544 (2015).
5 Tarabella G, Pezzella A, Romeo A, D'Angelo P, Coppede N, Calicchio M *et al.*, *J Mater Chem B* **1**:3843–3849 (2013).
6 Rivnay J, Owens RM and Malliaras GG, *Chem Mater* **26**:679–685 (2014).
7 Kim YJ, Wu W, Chun S-E, Whitacre JF and Bettinger CJ, *Proc Natl Acad Sci USA* **110**:20912–20917 (2013).
8 da Silva MP, Fernandes JC, de Figueiredo NB, Congiu M, Mulato M and de Oliveira Graeff CF, *AIP Adv* **4**:037120 (2014).
9 Araujo M, Viveiros R, Correia TR, Correia IJ, Bonifacio VDB, Casimiro T *et al.*, *Int J Pharm* **469**:140–145 (2014).
10 Bettinger CJ, Bruggeman PP, Misra A, Borenstein JT and Langer R, *Biomaterials* **30**:3050–3057 (2009).
11 Wuensche J, Cardenas L, Rosei F, Cicoira F, Gauvin R, Graeff CFO *et al.*, *Adv Funct Mater* **23**:5591–5598 (2013).
12 Ambrico M, Ambrico PF, Cardone A, Cicco SR, Palumbo F, Ligonzo T *et al.*, *J Mater Chem C* **2**:573–582 (2014).
13 Nighswander-Rempel SP, Riesz J, Gilmore J, Bothma JP and Meredith P, *J Phys Chem B* **109**:20629–20635 (2005).
14 Nofsinger JB and Simon JD, *Photochem Photobiol* **74**:31–37 (2001).
15 Kai D, Prabhakaran MP, Jin G and Ramakrishna S, *J Mater Chem B* **1**:2305–2314 (2013).
16 Pezzella A, Barra M, Musto A, Navarra A, Alfe M, Manini P *et al.*, *Mater Horiz* **2**:212–220 (2015).
17 d'Ischia M, Wakamatsu K, Napolitano A, Briganti S, Garcia-Borron J-C, Kovacs D *et al.*, *Pigm Cell Melanoma Res* **26**:616–633 (2013).
18 Deziderio SN, Brunello CA, da Silva MIN, Cotta MA and Graeff CFO, *J Non-Cryst Solids* **338**:634–638 (2004).
19 Pezzella A, Ambrogi V, Arzillo M, Napolitano A, Carfagna C and d'Ischia M, *Photochem Photobiol* **86**:533–537 (2010).
20 della Vecchia NF, Cerruti P, Gentile G, Errico ME, Ambrogi V, D'Errico G *et al.*, *Biomacromolecules* **15**:3811–3816 (2014).
21 Cicco SR, Ambrico M, Ambrico PF, Talamo MM, Cardone A, Ligonzo T *et al.*, *J Mater Chem C* **3**:2810–2816 (2015).
22 da Silva MIN, Deziderio SN, Gonzalez JC, Graeff CFO and Cotta MA, *J Appl Phys* **96**:5803–5807 (2004).
23 Wuensche J, Cicoira F, Graeff CFO and Santato C, *J Mater Chem B* **1**:3836–3842 (2013).
24 Bronze-Uhle ES, Batagin-Neto A, Xavier PHP, Fernandes NI, de Azevedo ER and Graeff CFO, *J Mol Struct* **1047**:102–108 (2013).
25 Felix CC, Hyde JS, Sarna T and Sealy RC, *J Am Chem Soc* **100**:3922–3926 (1978).

26 Piacenti-Silva M, Bronze-Uhle ES, Paulin JV and Graeff CFO, *J Mol Struct* **1056**:135–140 (2014).
27 ISO 10993–5: Biological evaluation of medical devices – Part 5: Tests for cytotoxicity: in vitro methods. International Organization for Standardization, Geneva (1992).
28 Ricci Volpato LE, de Oliveira RC, Espinosa MM, Bagnato VS and Machado MAAM, *J Biomed Opt* **16**:075004 (2011).
29 Kueng W, Silber E and Eppenberger U, *Anal Biochem* **182**:16–19 (1989).
30 Glantz SA and Slinker BK, *Primer of Applied Regression and Analysis of Variance*, 2nd edition. McGraw-Hill, New York (2000).
31 Bothma JP, de Boor J, Divakar U, Schwenn PE and Meredith P, *Adv Mater* **20**:3539–3542 (2008).
32 Quattlebaum EC and Carner GR, *Can. J. Botany Rev. Canad. Botan.* **58**:1700–1703 (1980).
33 Alves SB, Rossi LS, Lopes RB, Tamai MA and Pereira RM, *J Invertebr Pathol* **81**:70–77 (2002).
34 Urabe K, Aroca P, Tsukamoto K, Mascagna D, Palumbo A, Prota G *et al.*, *Biochim Biophys Acta Mol Cell Res* **1221**:272–278 (1994).
35 Hochstein P and Cohen G, *Ann NY Acad Sci* **100**:876–886 (1963).
36 Pawelek JM and Lerner AB, *Nature* **276**:627–628 (1978).
37 Heiduschka P, Blitgen-Heinecke P, Tura A, Kokkinou D, Julien S, Hofmeister S *et al.*, *Toxicol Pathol* **35**:1030–1038 (2007).
38 Wang Y-X, Robertson JL, Spillman WB and Claus RO, *Pharm Res* **21**:1362–1373 (2004).
39 Liu Y, Ai K, Liu J, Deng M, He Y and Lu L, *Adv Mater* **25**:1353–1359 (2013).
40 Chirila TV, Thompson DE and Constable IJ, *J Biomater Sci Polym Ed* **3**:481–498 (1992).
41 Ayodele ET, Hudson HR, Ojo IAO and Pianka M, *Phosphorus Sulfur Silicon Relat Elem* **159**:123–142 (2000).
42 Roy Choudhury S, Ghosh M, Mandal A, Chakravorty D, Pal M, Pradhan S *et al.*, *Appl Microbiol Biotechnol* **90**:733–743 (2011).

