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Natural melanin: A potential pH-responsive drug release device

Marco Araújo [a], Raquel Viveiros [a], Tiago R. Correia [b], Ilídio J. Correia [b], Vasco D.B. Bonifácio [a], Teresa Casimiro [a], Ana Aguiar-Ricardo [a,*]

[a] REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, FCT, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[b] CICS-UBI - Centro de Investigação em Ciências da Saúde, Universidade da Beira Interior, Avenida Infante Dom Henrique, 6200-506 Covilhã, Portugal

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ABSTRACT

This work proposes melanin as a new nanocarrier for pH-responsive drug release. Melanin is an abundant natural polymer that can be easily extracted from cuttlefish as nanoparticles with a suitable size range for drug delivery. However, despite its high potentiality, the application of this biopolymer in the pharmaceutical and biomedical fields is yet to be explored. Herein, melanin nanoparticles were impregnated with metronidazole, chosen as model antibiotic drug, using supercritical carbon dioxide. The drug release profile was investigated at acidic and physiologic pH, and the dominant mechanism was found to follow a non-Fickian transport. Drug release from melanin shows a strong pH dependency, which allied to its biocompatibility and lack of cytotoxicity envisages its potential application as nanocarrier in formulations for colon and intestine targeted drug delivery.

1. Introduction

Melanins are natural biopolymers that can be found in a large number of organisms being one of the most abundant pigments in nature (Araújo et al., 2012) The most common type is eumelanin, also called black melanin, that results from the oxidative polymerization of 5,6-dihydroxyindole and 5,6-dihydroxyindole carboxylic acid (Shannon and Semiat, 2008; Meredith et al., 2006). In nature, melanin pigments are usually associated with photo-protection, but they also play a major role in the regulation of a wide range of metabolic processes and molecular interactions (Barr, 1983). Other interesting melanin properties have been reported such as free radical scavenging, antioxidant capacity and amorphous semiconductivity (Tran et al., 2006; Blois et al., 1964) which are attributed to its disordered and heterogeneous polymeric structure. Despite being abundant and easily accessible, the use of melanin in pharmaceutical and biomedical applications reported in literature is scarce. The few studies on melanin drug conjugates that have been performed are mainly related with binding affinity through adsorption (Ings, 1984), as they are useful to study drug accumulation into tissues where this pigment is particularly dominant, mainly in eyes (Pescina et al., 2012), hair (Joseph et al., 1996) or skin (Banning and Heard, 2002).

Herein, we evaluated for the first time melanin nanoparticles as biocompatible drug nanocarriers, using metronidazole (MZ) as a model drug. MZ is a nitroimidazole antibiotic for antibacterial and antiprotozoal therapeutics (Tripathi et al., 2012), used in the treatment of amoebiasis (Krishnaiah et al., 2002), giardiasis and Crohn's disease (Freeman et al., 1997). It was also reported as a radiosensitizer due to its capacity to enhance radiation damage of tumoral cells, being applied in preoperative radiotherapy for gastric cancer (Skoropad et al., 2003). In this study, MZ was loaded into nano-sized melanin particles using supercritical CO_2 ($scCO_2$) technology. The high diffusivity and low viscosity of $scCO_2$ decrease the mass transfer limitations found in conventional impregnation (Soares da Silva et al., 2011).

The interest in using melanin as a drug release agent is related to its nature, as it is an abundant biopolymer derived from natural sources. In recent years, several biopolymers, such as starch and chitosan, have been extensively investigated as drug delivery systems. However, most of them require further structural and/or morphologic modifications that are difficult to perform and to obtain in good yield (Malafaya et al., 2007). Melanin has the advantage of being easily obtained from the cuttlefish or octopus ink, through a simple extraction method that involves successive dilutions and washings of the ink with distilled water (Araújo et al., 2012). This is an economical advantage when compared to other

* Corresponding author at: Universidade Nova de Lisboa, Faculdade de Ciências e Tecnologia, Departamento de Química, Campus da Caparica, 2825-516 Caparica, Portugal. Tel.: +351 21 2949648.
E-mail addresses: teresa.casimiro@fct.unl.pt (T. Casimiro), air@fct.unl.pt (A. Aguiar-Ricardo).

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natural polymers as melanin is obtained ready-to-use, without the need of further structure modification. In addition by using $scCO_2$, a clean technology, it is possible to prepare drug-melanin formulations without making use of any solvent and thus, with no further drying or purification steps.

2. Materials and methods

2.1. Materials

Carbon dioxide was obtained from Air Liquide with purity better than 99.998%. Amphotericin B, Eagles minimum essential medium with Earle's balanced salt solution (EMEM(EBSS)), human colorectal carcinoma-derived Caco-2 cells, Luria Bertani (LB) broth, L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphofenyl)-2H-tetrazolium (MTS), metronidazole (MZ, 98% purity), non-essential amino acids (NEAA), penicillin G, resazurin sodium salt, streptomycin, and trypsin were purchased from Sigma–Aldrich and used without further purification. Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany). Bacterial strain *Escherichia coli* (*E. coli*) DH5α was purchased from ATCC. Finally, LB agar was purchased from Pronadise. Sepia melanin was obtained from *Sepia officinalis* ink sacs. Its extraction was achieved through several washing steps of the ink with Milli-Q water, followed by centrifugation (8000 *g*, 20 min) to obtain a black precipitate, which was then lyophilized.

2.2. Supercritical CO_2-assisted drug impregnation

Sepia melanin was impregnated with metronidazole using $scCO_2$ in batch mode. 200 mg of melanin were packed in a cellulose membrane (3500 MWCO) and 450 mg of MZ were loaded into a stainless steel high-pressure cell equipped with two aligned sapphire windows, as previously described (Temtem et al., 2009, 2012). The cell is divided into two compartments by a macroporous support in order to prevent physical contact between the drug and the biopolymer, the drug is placed in the lower part and melanin in the upper compartment. Excess MZ was always present at the bottom of the cell during the experiment to ensure saturation of the medium at the *p*, *T* impregnation conditions. After loading the reactants, the cell was immersed in a thermostated water bath set to 40.0 ± 0.1 °C and filled with carbon dioxide up to 20 MPa. After 24 h, the cell was quickly depressurized and allowed to cool to room temperature. The total amount of drug impregnated in melanin was quantified by UV–vis, after crushing the polymer at the end of the drug release experiments.

2.3. Drug-release profile

The drug release experiments were carried out at physiologic and acidic pHs. The impregnated melanin (20 mg) and the control neat melanin were immersed in 200 mL of phosphate buffer saline solutions (pH 7.4) and glycine–hydrochloric acid buffer solutions (pH 2.2) at 37 °C. 1 mL sample aliquots were withdrawn at different time intervals and quantitatively replaced by a fresh medium. The drug release quantification was evaluated by building calibration curves considering λ = 319 nm, which corresponds to the maximum absorbance of MZ in phosphate and glycine–HCl buffers. All measurements were carried out in triplicate and values plotted with standard deviation errors. The diffusion of metronidazole through the polymeric network in both PBS and glycine–hydrochloric acid buffers was modeled using the empirical Korsmeyer–Peppas equation (Eq. (1)),

$$\frac{M_t}{M_\infty} = kt^n \quad (1)$$

where M_t is the absolute cumulative amount of drug released at time t, M_∞ is the total amount of drug impregnated in the polymer samples, k is the diffusion coefficient that reflects the structural and geometric characteristics of the device, and n is the release exponent, which gives an insight on the specific transport mechanism (Ritger and Peppas, 1987; Restani et al., 2010).

2.4. Cytotoxicity assays

The human colorectal carcinoma-derived Caco-2 cells were seeded with 6 mL of EMEM (EBSS), supplemented with 2 mM L-glutamine, heat-inactivated FBS (10% v/v), NEAA (1% v/v) and 1% antibiotic/antimycotic solution in T-flasks of 25 cm^2, incubated at 37 °C, using a 5% CO_2 humidified atmosphere. After cells attained confluence, they were subcultivated by 3–5 min incubation in 0.18% trypsin (1:250) and 5 mM ethylenediaminetetraacetic acid (EDTA). Then they were centrifuged, resuspended in culture medium and then seeded in T-flasks of 75 cm^2.

Subsequently, cell behavior in the presence of neat and impregnated melanin was characterized by monitoring their growth using an Olympus CX41 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera. To further characterize the cytotoxic profile of materials, an MTS assay was also performed accordingly to a procedure previously described in the literature (Maia et al., 2009; Ribeiro et al., 2009). Cell adhesion and proliferation in the presence of melanin were also characterized by scanning electron microscopy (SEM) using a Hitachi S-2700 (Tokyo, Japan) scanning electron microscope operated at an accelerating voltage of 20 kV at various amplifications.

2.5. Determination of antibacterial activity

In order to characterize the antibacterial properties of neat and impregnated melanin, a resazurin assay was performed. Briefly, bacterial cultures (*E. coli*) were seeded in 96 well plates at a density of 5×10^6 colony-forming unit (CFU)/mL under aseptic conditions. After 24 h of incubation, 100 μL of medium of each well were transferred to another 96 well plates, and 20 μL of 0.1% resazurin solution were added to each sample, followed by the plates being incubated at 37 °C during 1–4 h. Finally, bacterial growth was followed using a Nikon digital camera (Nikon D50, Ayuthaya, Thailand) (Alves et al., 2014).

2.6. Melanin characterization

The UV–vis spectra for the drug release quantification were carried in a Lambda 25 PerkinElmer spectrophotometer. ATR–FTIR analyses were performed on a Nicolet spectrophotometer model IS-10. The deepness of analysis is about 2 μm. X-ray powder diffraction was performed using a RIGAKU X-ray diffractometer MiniFlex II with automatic data acquisition (peak search for Windows v. 6.0 Rigaku) using Cu Kα radiation (λ = 0,15406 nm) and working at 30 kV/15 mA. The diffraction patterns were collected in the range $2\theta = 5–50°$ with a 0.02° step size and an acquisition time of 1 min/step. For SEM analysis, samples were coated with a carbon film, and the images were obtained using a Cross Beam Workstation (SEM)-Zeiss Auriga equipment, with a Schottky field emitter, resolution of 1.0 nm @ 15 kV, 1.9 nm @ 1 kV, acceleration voltage between 0.1 and 30 kV.

The water uptake performance of the polymer was determined both at pH 7.4 and pH 2.2 in similar conditions to those used for the drug release experiments. Briefly, 20 mg of melanin were put on a sleeve filter of 0.1 μm mesh and immersed in buffer solution at 37 °C for 8 h. The water content was estimated by the difference between the weight of the swollen polymer samples (W) after

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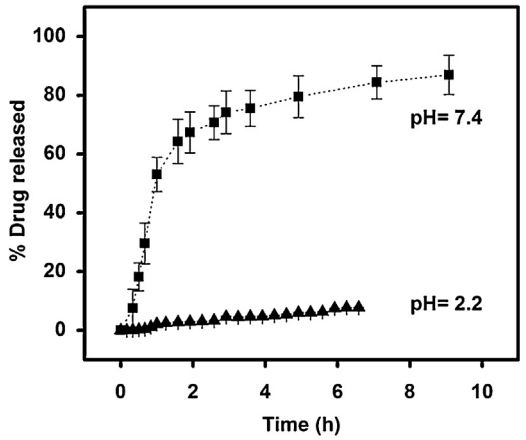

Fig. 1. MZ release profile from melanin nanoparticles at pH 7.4 (◆) and pH 2.2 (■).

careful wiping the filter with a soft tissue, and the weight of the dry polymer samples (W_0) (Eq. (2)).

$$\text{Swelling} = \frac{W - W_0}{W_0} \quad (2)$$

3. Results and discussion

Melanin was loaded with 2.3 wt% of MZ by $scCO_2$-assisted impregnation. Fig. 1 shows the obtained MZ release profiles from melanin samples at pH 7.4 and 2.2 for 10 h of release.

The drug release was continued for 60 h showing a well-defined plateau (see Graphical abstract). It is a remarkable pH-trigged response of melanin in MZ release. As MZ solubility in different buffers is nearly pH-independent (Wu and Fassihi, 2005), this remarked difference in the drug release kinetics must be related with the behaviour of the biopolymer when exposed to physiological and acidic media.

The release plateau at physiologic pH 7.4 was obtained in about 9 h, corresponding to 87% of MZ release. In the first 1.5 h, there was a fast but controlled release of ca 65% of MZ, presenting after that was a slower release till it reached the plateau. At pH 2.2, the amount of drug released does not exceed 10% for the same period of time. This release profile is comparable to other natural derived drug carriers (Elzatahry and Eldin, 2008; Krishnaiah et al., 2001; Perera et al., 2010), suggesting that formulations of melanin could be potentially used as pH-targeted drug release devices to colon and intestines, either by rectal administration or orally, sustaining drug release through stomach (pH 2) and releasing the drug at those targets where pH is around 7.4.

This pH-dependent release may be explained by conformational rearrangements in melanin structure. This biopolymer is composed by DHI and DHICA monomers (Fig. 2) and thus, carboxylic acid ($pK_a \approx 4.5$) and phenolic ($pK_a \approx 9$) groups are directly responsible for the surface physical and chemical properties.

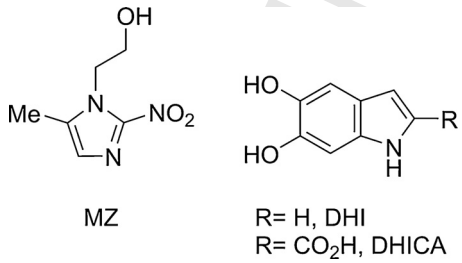

Fig. 2. Chemical structures of metronidazole (MZ) and structural units of melanin: 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA).

Table 1
Korsmeyer–Peppas equation parameters and drug release at pH 7.4 and 2.2.

pH	Korsmeyer–Peppas parameters			Drug release (mg/g)[d]
	r^2[a]	k (h^{-1})[b]	n[c]	
7.4	0.954	0.4420	0.89	19.9
2.2	0.942	0.0163	0.89	3.4

[a] Correlation coefficient.
[b] Constant incorporating structural and geometric characteristics of the system.
[c] Diffusion exponent.
[d] Maximum mass of MZ released per mass of impregnated melanin.

The presence of such units, specially the carboxyl groups (which pK_a is in the pH range of the tested buffered solutions), may force the biopolymer to acquire different conformations upon variation in the pH of the medium. In PBS buffer (pH 7.4), the carboxyl groups are negatively charged due to the deprotonation and consequently, the repulsion between these groups at the polymer's surface are higher, providing changes in the polymer chain conformation that favour the release of the drug from its internal structure. On the other hand, in the acidic medium, the minimization of electrostatic effects, due to the protonation of the carboxyl groups, seems to promote novel biopolymer conformations with increased ability to retain the drug.

No significant swelling was obtained at both pHs (in 10 h, data not shown). The absence of swelling in melanin was predictable due to its high rigidity and cross-linked structure. It seems that the drug diffusion from the structure is enhanced by the higher surface hydrophilicity at physiologic pH. The in vitro release data was correlated using the Korsmeyer–Peppas model (Fig.S1, Supporting Information), commonly used to describe the drug release kinetics from non-swellable polymeric systems (Eq. (2)), and the calculated Korsmeyer–Peppas constants are included in Table 1.

For a non-swellable spherical shaped system, as is the case of melanin, the results obtained for the diffusion coefficient and release exponent suggest that the dominant mechanism for drug release corresponds to a non-Fickian or anomalous transport (Slepmann and Peppas, 2001), where Fickian diffusion through the hydrated layers of the matrix and polymer relaxation are both involved. The data was computed using Excel Solver add-in module. These results are in accordance with the hypothesis proposed above, where a change in the conformation of the biopolymer, inducing its relaxation, can be the basis of its different behavior in the acidic and the neutral media.

Fig. 3 shows the X-ray diffraction spectra of melanin before and after impregnation, MZ processed in $scCO_2$ and the physical mixture. The XRD spectrum of melanin shows the characteristic diffraction peaks ($2\theta = 27$, 32 and 45°) of crystalline NaCl (halite, JCPDS 5-628).

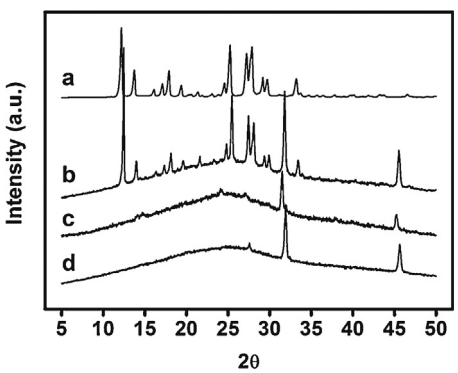

Fig. 3. XRD of metronidazole (A) and melanin after (B) and before (C) the drug impregnation compared to the physical mixture (D).

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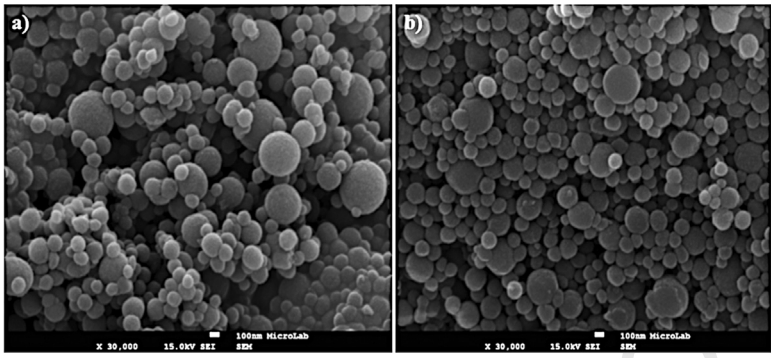

Fig. 4. SEM images of melanin extracted from cuttlefish: a) neat, b) impregnated with MZ.

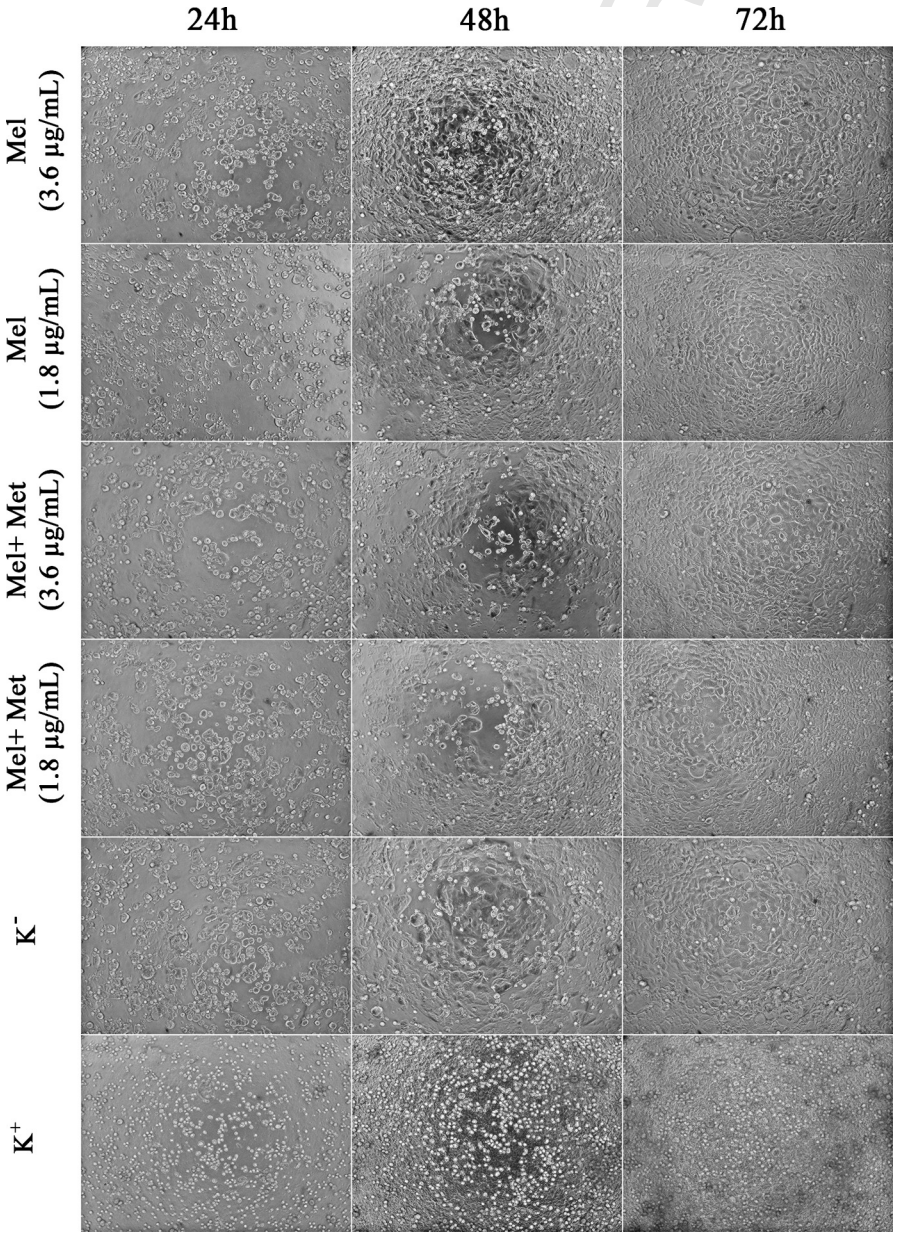

Fig. 5. Microscopic photographs of Caco-2 cells after being seeded in the presence of the culture medium that was previously in contact with MZ and MZ-impregnated melanin during 24, 48 and 72 h. (K^-) the negative control (live cells) and (K^+) the positive control (dead cells). Original magnification ×100.

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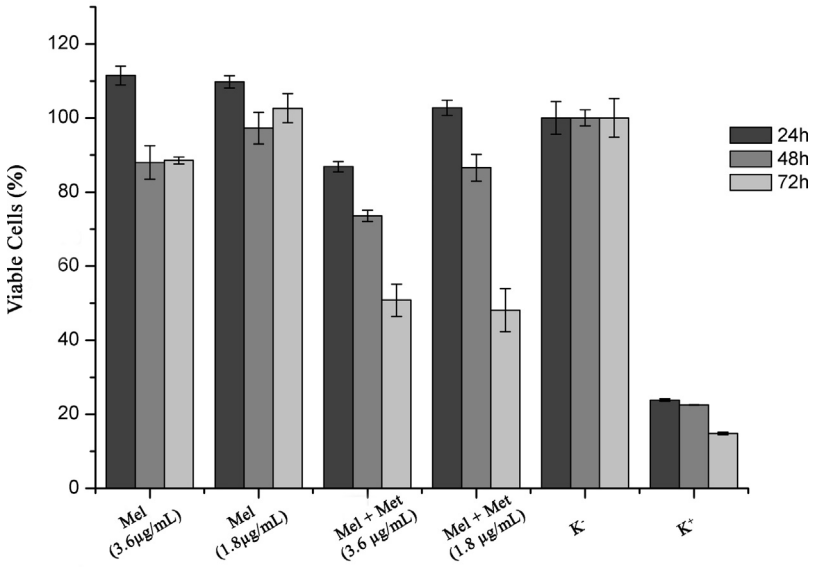

Fig. 6. Cellular activities measured by the MTS assay after 24, 48 and 72 h in contact with neat and MZ-impregnated melanin. K^- – negative control (live cells), and K^+ – positive control (dead cells), are also represented. Each result is the mean ± standard error of the mean of at least three independent experiments.

The amorphous nature of melanin (wide band from $2\theta = 15–30^\circ$) was observed for all diffractograms, which is indicative of its characteristic heterogeneous and disordered structure. By comparing the physical mixture of MZ and melanin with MZ-impregnated melanin, it is possible to observe a notorious shift of the diffraction peaks toward lower 2θ values. The distribution of MZ within the polyaromatic sheets may induce an increase of the interplanar spacing, which in turn, according to Bragg's law, leads to a decrease of the scattering angle. Although the spacial arrangement of the drug inside the melanin structure is not clear, it is believed that π–π interactions (imidazole scaffold) and hydrogen bonding (hydroxyl and/or nitro groups) may be involved in the stabilization of MZ in the cavity that lies between the indole-quinone sheets.

Morphologic characterization of extracted melanin, before and after impregnation, was accessed by SEM analysis and shown in Fig. 4.

No relevant change at morphological level was observed after drug impregnation. The extracted melanin is obtained as discrete spherical granules with diameters ranging from 100 to 250 nm. Its nanostructure was already elucidated (Clancy et al., 2000). The melanin morphology is ideal for vascular and gastro-intestinal drug delivery systems, which is normally optimized between 100–300 nm (Desai et al., 1996; Champion et al., 2007). In addition, its round shaped geometry is favored when compared to rod shaped particles, as the first are quickly uptaken by the cells through endocytosis (Muro et al., 2008). These parameters can influence several processes that occur during the drug release, from the transport and biodistribution of the particles at vascular level, to the strength of adhesion and internalization at the cellular level (Decuzzi et al., 2009).

As MZ is commonly classified as an antibacterial and antiprotozoal agent, the behavior of neat and impregnated melanin was characterized by using a bacterial strain *E. coli* that is usually found in the colon. The antimicrobial activity of melanin was assessed through a Resazurin assay using different concentrations of the material (3.75 mg/mL, 5.00 mg/mL, 6.25 mg/mL, 7.50 mg/mL, 8.75 mg/mL and 10.00 mg/mL). The results obtained (see Fig. S3 from Supporting Information) demonstrated that only melanin impregnated with MZ has antibacterial activity.

Fig. 5 shows that Caco-2 cells adhered and proliferated in the presence of melanin for 72 h, demonstrating its biocompatibility. However, when melanin was impregnated with 3.6 μg/mL of metronidazole, few cells adhered and proliferated. In the presence of melanin loaded with 1.8 μg/mL of the drug, the number of viable cells was comparable to that of the negative control (100% live cells).

The MTS results (Fig. 6) also demonstrated that the medium that was in contact with melanin did not have an acute cytotoxic effect for cells during 72 h, showing cellular viabilities comparable to that of the negative control. When cells were incubated with the medium that was previously in contact with the impregnated melanin (containing 1.8 and 3.6 μg/mL of MZ), cellular viability started to decrease after 24 h, which may be caused by MZ release from melanin. Such results demonstrate the potential of melanin-based drug delivery systems for colorectal cancer therapy.

To further characterize the cytotoxic profile of melanin devices, SEM images were also acquired (Fig. 7), where it is visible when the adhesion and proliferation of Caco-2 cells were seeded in contact with melanin.

Our findings corroborate the excellent biocompatibility of melanin, fully supporting its application in the drug delivery systems. Furthermore, melanin is biodegradable, which can be of an advantage as it is eliminated from the body as small, nontoxic fragments after having served their intended purpose (Bettinger et al., 2009).

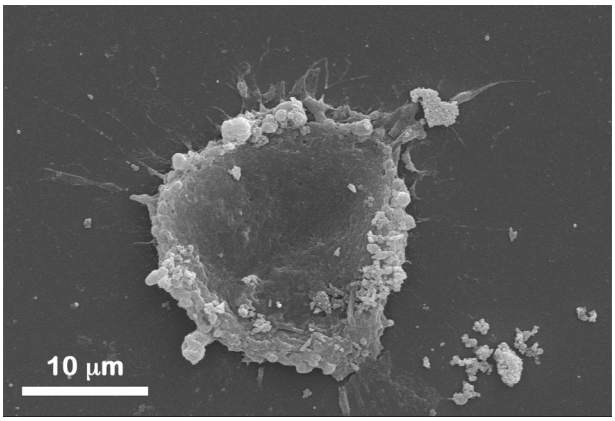

Fig. 7. SEM image of Caco-2 cell growing in contact with melanin (30,000×, 20 kV).

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The development of novel stimuli-responsive drug delivery systems has received significant attention from the pharmaceutical industry, mainly due to their potential for precise targeting with less severe side effects (Lee et al., 2011). In this case, melanin has proven its ability to deliver the drug upon pH stimulus, as less than 10% of MZ is released in acidic conditions (pH 2.2) while 87% of the drug is released at physiologic pH (pH 7.4), pH conditions similar to stomach and intestine environments.

4. Conclusions

In this work we showed that melanin, which can be easily extracted from cuttlefish directly as spherical nanoparticles, could be a very interesting nanocarrier drug release device. Melanin could be straightforwardly impregnated with a drug using supercritical fluid technology, a clean route for impregnation, taking advantage of the high diffusivity, low viscosity and high density of $scCO_2$, leaving no residues in the final product. In addition, we showed that melanin strongly responds to pH thus, having a significant control on drug release, which is a very interesting feature for the treatment of intestine and colon diseases, which would greatly benefit with pH-targeting. These interesting results allied to its high biocompatibility can prompt the use of melanin as a novel biomaterial for the potential use in the pharmaceutical and biomedical fields.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2014.04.051.

References

Alves, P., Cardoso, R., Correia, T.R., Antunes, B.P., Correia, I.J., Ferreira, P., 2014. Surface modification of polyurethane films by plasma and ultraviolet light to improve haemocompatibility for artificial heart valves. Colloids and Surfaces B 113, 25–32.
Q10 Araújo, M., Xavier, J., Nunes, C., Vaz, P., Humanes, M., 2012. Marine sponge melanin: a new source of an old biopolymer. Journal of Structural Chemistry 23, 115–122.
Banning, T.P., Heard, C.M., 2002. Binding of doxycycline to keratin, melanin and human epidermal tissue. International Journal of Pharmaceutics 235, 219–227.
Barr, F.E., 1983. Melanin: the organizing molecule. Medical Hypotheses 11, 1–139.
Bettinger, C.J., Bruggeman, J.P., Misra, A., Borenstein, J.T., Langer, R., 2009. Biocompatibility of biodegradable semiconducting melanin films for nerve tissue engineering. Biomaterials 30, 3050–3057.
Blois, M.S., Zahlan, A.B., Maling, J.E., 1964. Electron spin resonance studies on melanins. Biophysical Journal 4, 471–490.
Champion, J.A., Katare, Y.K., Mitragotri, S., 2007. Particle shape: a new design parameter for micro- and nanoscale drug delivery carriers. Journal of Controlled Release 121, 3–9.
Clancy, C.M.R., Nofsinger, J.B., Hanks, R.K., Simon, J.D., 2000. A hierarchical self-assembly of eumelanin. The Journal of Physical Chemistry B 104, 7871–7873.
Decuzzi, P., Pasqualini, R., Arap, W., Ferrari, M., 2009. Intravascular delivery of particulate systems: does geometry really matter? Pharmaceutical Research 26, 235–243.
Desai, M.P., Labhasetwar, V., Amidon, G.L., Levy, R.J., 1996. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharmaceutical Research 13, 1838–1845.
Elzatahry, A.A., Eldin, M.S.M., 2008. Preparation and characterization of metronidazole-loaded chitosan nanoparticles for drug delivery application. Polymers for Advanced Technologies 19, 1787–1791.
Freeman, C.D., Klutman, N.E., Lamp, K.C., 1997. Metronidazole. A therapeutic review and update. Drugs 54, 679–708.
Ings, R.M.J., 1984. The melanin binding of drugs and its implications. Drug Metabolism Reviews 15, 1183–1212.
Joseph Jr., R.E., Su, T.P., Cone, E.J., 1996. In vitro binding studies of drugs to hair: influence of melanin and lipids on cocaine binding to Caucasoid and Africoid hair. Journal of Analytical Toxicology 20, 338–344.
Krishnaiah, Y.S.R., Seetha, A.D., Nageshwara, R.L., 2001. Guar gum as a carrier for colon specific delivery; influence of metronidazole and tinidazole on in vitro release of albendazole from guar gum matrix tablets. Journal of Pharmacy and Pharmaceutical Sciences 4, 235–243.
Krishnaiah, Y.S.R., Reddy, P.R.B., Satyanarayana, V., Karthikeyan, R.S., 2002. Studies on the development of oral colon targeted drug delivery systems for metronidazole in the treatment of amoebiasis. International Journal of Pharmaceutics 236, 43–55.
Lee, B.S., Yoon, C.W., Osipov, A., Moghavem, N., Nwachokor, D., Amatya, R., Na, R., Pantoja, J.L., Pham, M.D., Black, K.L., Yu, J.S., 2011. Nanoprodrugs of NSAIDs preparation and characterization of flufenamic acid nanoprodrugs. Journal of Drug Delivery Article ID 980720. Q11
Maia, J., Ribeiro, M.P., Ventura, C., Carvalho, R.A., Correia, I.J., Gil, M.H., 2009. Ocular injectable formulation assessment for oxidized dextran-based hydrogels. Acta Biomaterialia 5, 1948–1955.
Malafaya, P.B., Silva, G., Reis, R.L., 2007. Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Advanced Drug Delivery Reviews 59, 207–233.
Meredith, P., Powell, B.J., Riesz, J., Nighswander-Rempel, S.P., Pederson, M., Moore, E. G., 2006. Towards structure-property-function relationships for eumelanin. Soft Matter 2, 37–44.
Muro, S., Garnacho, C., Champion, J.A., Leferovich, J., Gajewski, C., Schuchman, E.H., Mitragotri, S., Muzykantov, V.R., 2008. Control of endothelial targeting and intracellular delivery of therapeutic enzymes by modulating the size and shape of ICAM-1-targeted carriers. Molecular Therapy 16, 1450–1458.
Perera, G., Barthelmes, J., Bernkop-Schnürch, A., 2010. Novel pectin-4-aminothiophenole conjugate microparticles for colon-specific drug delivery. Journal of Controlled Release 145, 240–246.
Pescina, S., Santi, P., Ferrari, G., Padula, C., Cavallini, P., Govoni, P., Nicoli, S., 2012. Ex vivo models to evaluate the role of ocular melanin in trans-scleral drug delivery. European Journal of Pharmaceutical Sciences 46, 475–483.
Restani, R.B., Correia, V.G., Bonifácio, V.D.B., Aguiar-Ricardo, A., 2010. Development of functional mesoporous microparticles for controlled drug delivery. Journal of Supercritical Fluids 55, 333–339.
Ribeiro, M.P., Espiga, A., Silva, D., Baptista, P., Henriques, J., Ferreira, C., Silva, J.C., Borges, J.P., Pires, E., Chaves, P., Correia, I.J., 2009. Development of a new chitosan hydrogel for wound dressing. Wound Repair and Regeneration 17, 817–824.
Ritger, P.L., Peppas, N.A., 1987. A simple equation for description of solute release. I. Fickian and non-Fickian release from non-swellable devices in the form of slabs, spheres cylinders or disks. Journal of Controlled Release 5, 23–26.
Shannon, M.A., Semiat, R., 2008. Advancing materials and technologies for water purification. Materials Research Bulletin 33, 9–12.
Skoropad, V.Y., Berdov, B.A., Zagrebin, V.M., 2003. Preoperative radiotherapy in combination with metronidazole for resectable gastric cancer. European Journal of Surgical Oncology 29, 166–170.
Slepmann, J., Peppas, N.A., 2001. Modelling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). Advanced Drug Delivery Reviews 48, 139–157.
Soares da Silva, M., Viveiros, R., Morgado, P.I., Aguiar-Ricardo, A., Correia, I.J., Casimiro, T., 2011. Development of 2-(dimethylamino)ethyl methacrylate-based molecular recognition devices for controlled drug delivery using supercritical fluid technology. International Journal of Pharmaceutics 416, 61–68.
Temtem, M., Pompeu, D., Jaraquemada, G., Cabrita, E.J., Casimiro, T., Aguiar-Ricardo, A., 2009. Development of PMMA membranes functionalized with hydroxypropyl-beta-cyclodextrins for controlled drug delivery using a supercritical CO_2-assisted technology. International Journal of Pharmaceutics 376, 110–115.
Temtem, M., Barroso, T., Casimiro, T., Mano, J.F., Aguiar-Ricardo, A., 2012. Dual stimuli responsive poly(*N*-isopropylacrylamide) coated chitosan scaffolds for controlled release prepared from a non-residue technology. Journal of Supercritical Fluids 66, 398–404.
Tran, M.L., Powell, P.J., Meredith, P., 2006. Chemical and structural disorder in Eumelanins: a possible explanation for broadband absorbance. Biophysical Journal 90, 743–752.
Tripathi, G.K., Singh, S., Saroh, S., Singh, A., Dubey, R.K., 2012. Synthesis and design of chitosan derivative pH stimuli sensitive microparticles for colon targetemetronidazole delivery. Journal of Chemical and Pharmaceutical Research 4, 2656–2665.
Wu, Y., Fassihi, R., 2005. Stability of metronidazole, tetracycline HCl and famotidine alone and in combination. International Journal of Pharmaceutics 290, 1–13.