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Artificial pheomelanin nanoparticles and their photo-sensitization properties

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ABSTRACT

Pheomelanin-type nanoparticles (PMNPs) were synthesized through a simple oxidative polymerization of 3,4-dihydroxyphenylalanine (DOPA) in the presence of cysteine by $KMnO_4$. The synthesized PMNPs had a diameter of approximately 100 nm, exhibited high dispersion stability in neutral water and various culture media and possessed similar morphology to naturally occurring pheomelanins. The efficiency of photoinduced generation of hydroxyl radicals from PMNPs was determined and related in vitro cell experiments that were carried out, with data being compared to those from eumelanin-type nanoparticles (EMNPs) and natural sepia melanin nanoparticles. Endocytosed PMNPs showed the highest phototoxicity (~50% viability) to UV-irradiated HeLa cells, confirming the direct relationship between phototoxic efficiency and the generation of hydroxyl radicals through the complex processes of the O_2 sensitization.

1. Introduction

Melanins are well-known biopolymers that are present in most organisms and play diverse and important roles, including in skin pigmentation [1]. They are used by organisms for, among other things, photosensitization, thermoregulation, radical scavenging, and photoprotection, due to their ability to absorb light throughout the UV and visible regions [2,3].

Melanins are generally classified either black-brown eumelanins or yellow-red pheomelanins. Eumelanin is believed to result from the oxidation of tyrosine by tyrosinase forming dopaquinone followed by the cyclization to generate 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and the successive polymerization.4 Meanwhile, pheomelanin derives from benzothiazine units, the compound that results from the incorporation of the sulfur-containing amino acid cysteine into quinones [4,5].

The difference in the structure and physical properties of these two kinds of melanin suggests that they may react differently, both chemically and biologically, when they are exposed to light [6]. For example, while eumelanin acts as a sunscreen against UV radiation, pheomelanin is seemingly more sensitive to radiation and may in fact add to the harmful effects of radiation exposure, including in the formation of carcinoma [7–10]. Differences in chemical composition of various types of melanin can result in different responses to UV excitation. Such excitation is often thermally dispersed, but may also trigger the generation of reactive oxygen species (ROS), including superoxide and hydroxyl radicals, through catalyzed oxygen reduction. This can induce single strand breaks in DNA [7,11]. Pheomelanin is more efficient at producing oxyradicals than eumelanin [12,13]. Therefore, it is very important to precisely understand the relationship of the structures of different melanins with their corresponding biological function.

Unfortunately, melanins are difficult to study given their strong binding to proteins in biological environments [14]. Melanin has traditionally either been extracted from organisms in which it occurs naturally or synthesized artificially using a method that mimics biosynthetic pathways. Unfortunately, biological extraction generally requires the repetitive use of strong acids and bases, with no standardized method having been established that leaves the structure intact [14,15]. Meanwhile, synthetic techniques, which generally involve the chemical or enzymatic oxidation of the necessary precursors, tend to result in structures that lack the necessary particle characteristics of naturally produced melanin [16,17]. The study is further complicated by the dispersibility of melanins in most solvents, including neutral water, a problem that has been imperfectly addressed through the use of non-biological aqueous alkaline environments [18,19].

Herein, we report a facile synthetic method by which to prepare pheomelanin-type nanoparticles (PMNPs) with high dispersion stability in both water and biological media. Various properties of the prepared PMNPs were investigated by comparing them with eumelanin-type nanoparticles (EMNPs), which had already been successfully synthesized by our group and which had also exhibited

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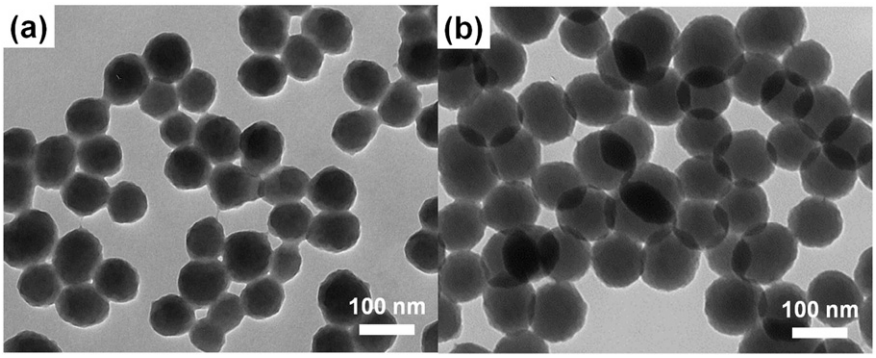


Fig. 1. TEM images of (a) EMNPs and (b) PMNPs.

excellent dispersion stability [20], as well as with natural sepia melanin, so as to determine the differences in photophysical properties that derive from the altered structural composition.

2. Results and Discussion

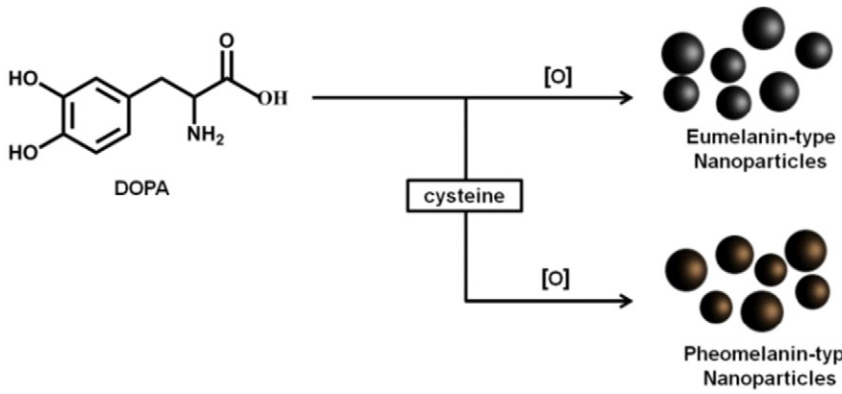
TEM images in Fig. 1 show the morphology of prepared PMNPs and EMNPs, demonstrating that both have a similar spherical shape and size of approximately 100 nm. This suggests their suitability as models for naturally occurring melanins, as most natural melanins with living organisms that demonstrate a comparable morphology [16,17]. The size of PMNPs is slightly larger than the EMNPs probably due to the different kinetics and reaction mechanisms during the synthesis (Scheme 1). However, the size increase is not proportional to the amount of the incorporated cysteine (see Fig. S1 in Supporting Data).

Elemental analysis (EA) data (Table 1) show additional differences, in this case with regard to chemical composition. As expected, sulfur was not detected in the EMNPs, while the relative ratio of sulfur in the PMNPs directly related to the amount of cysteine added, reaching a maximum in samples with an equivalent or great amount of cysteine added with regard to DOPA during synthesis. To ensure total conversion to PMNPs, all following samples were prepared using a cysteine/DOPA molar ratio of 2:1. PMNPs show a similar absorption to EMNPs in the UV–Vis region (Fig. 2a). This broad and featureless absorption band is uncommon among organic chromophores and suggests that PMNPs contain a large number of energy states due to the heterogeneity of their chemical compositions [21]. Meanwhile, ESR data indicate the presence of stable free radical centers within the melanin nanoparticle structures, with the PMNP data being distinct from that for the EMNPs (Fig. 2b). This distinctive ESR signal with hyperfine splitting is associated with delocalization of an unpaired electron on the nitrogen of a semiquinoneimine moiety [22].

Synthetic pheomelanin synthesized by tyrosinase (Enzyme-PM) [23], which has been already proved to be very similar to natural pheomelanin [24], was used for comparison with the synthesized PMNPs. Although Enzyme-PM formed a cloudy mass that looked very different when compared to PMNPs (see SEM and TEM images of Fig. S2 in Supporting Data), the spectroscopic features and chemical analysis data proved to be very similar confirming that synthesized PMNPs have pheomelanin characteristics (see UV–Vis absorption and ESR spectra of Fig. S3, and HPLC analysis result after HI reduction hydrolysis of Fig. S4 in Supporting Data). Electrophoretic Light Scattering (ELS) method was used to determine the long-term dispersion stability of PMNPs in various media, with results showing size distribution after storage for one day at room temperature in water, PBS buffer, and RPMI 1640 cell culture media (Fig. 3). PMNPs dispersed in cell culture media seem slightly bigger than those in other solutions; the possible interaction with proteins in the culture media might produce this result which is very similar to the surface adsorption of various materials on the surfaces of nanoparticles [25].

Photophysical properties of the prepared water-dispersible PMNPs and EMNPs, as well as the purified sepia melanin, were investigated by monitoring the generation of hydroxyl radicals. Terephthalate (TA) was used as a probe molecule as it is known to generate fluorescent hydroxylterephthalate (HTA) through the interaction with hydroxyl radicals [26], which is considered as one of the major ROS through the complex processes of the O_2 sensitization by melanins [7–10].

HTA emits at 425 nm; the change in fluorescence intensity at this wavelength for PMNPs, EMNPs, and sepia melanin is shown in Fig. 4a. PMNPs produced more hydroxyl radicals than the others; the amount produced by the natural sepia melanin is consistent with previously reported data [12,13], which is equivalent to the amount of H_2O_2 (~0.5 mM solution) by the generation of HTA (see Fig. S5 in Supporting Data).



Scheme 1. Synthetic procedure for the preparation of eumelanin-type nanoparticles (EMNPs) and pheomelanin-type nanoparticles (PMNPs).