http://dx.doi.org/10.5012/bkcs.2012.33.11.3788

The Synthetic Melanin Nanoparticles Having An Excellent Binding Capacity of Heavy Metal Ions

Da Jeong Kim,† Kuk-Youn Ju, and Jin-Kyu Lee*

*Department of Chemistry, Seoul National University, Seoul 151-742, Korea. *E-mail: jinklee@snu.ac.kr*
†Current address: LG Chem Research Park, #104-1 Munji-dong, Yuseong-gu, Daejeon 305-380, Korea
Received July 16, 2012, Accepted August 20, 2012

Spherical-shape melanin nanoparticles with good water-dispersibility were successfully synthesized by a simple oxidation polymerization of 3,4-dihydroxy-phenylalanin (DOPA) with $KMnO_4$. Similar features to those known from natural and synthetic melanin polymers were observed from prepared melanin nanoparticles by FT-IR, UV-Vis., and ESR spectroscopic methods. Their binding ability with several heavy metal ions from aqueous solution was quantitatively investigated, and the maximum binding capacities with melanin nanoparticles to lead, copper, and cadmium ions were obtained as 2.45, 2.17 and 1.88 mmol/g, respectively, which are much larger values than those reported from natural and synthetic melanin polymers. The large binding capacity and fast binding rate of melanin nanoparticles to metal ions can make them an excellent candidate for the remediation of contaminated water.

Key Words : Melanin nanoparticles, Artificial melanin, Oxidation-polymerization, Heavy metal removal

Introduction

Melanins are natural biopolymers, having black or dark brown in colour, which are widely distributed throughout living organisms. They have attracted a great deal of attention due to their various biological features, including photosensitization, metal ions chelation, anti-oxidizing and free radical scavenging behaviour, photo-protection, and strong non-radiative relaxation of photo-exited electronic states.[1] It has been suggested that the biogenesis of melanins seem to occur through the enzymatic oxidation of DOPA or tyrosine to dopachrome followed by an intramolecular conversion to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxy-indole-2-carboxylic acid (DHICA), which reacts further through oxidative polymerization to melanin.[2] For the detailed investigation of melanin bio-functions, natural melanins isolated from various sources such as sepia,[3] retinal pigment epithelium of the eyes, organelles from the substantia nigra of the brain,[4] fungi,[5] and leaves of green tea[6] have been utilized. Interestingly they show a distinctive nanometer-sized granular shape, which is expected to play an important role in the bio-functions of natural melanins.[4] However, there is a limitation to the interpretation of the intrinsic properties of natural melanins due to the possible alternation of their inherent properties during the isolation processes and the lack of standardized isolation protocols to compare results from different sources.[7] On the other hand, synthetic melanins, which have been prepared by chemical or enzymatic oxidation of tyrosine or 3,4-dihydroxy-L-phenylalanin (L-DOPA),[8] have provided much information about the intrinsic properties of melanins. Unfortunately, the precise reaction mechanism was not fully understood due to the paramagnetic characteristics of melanins and most of the synthetic melanins prepared by these processes do not have the characteristic particle-shape observed in natural ones. Therefore they cannot offer much information about melanin in terms of morphology, shape and size. Furthermore, most synthetic melanins as well as isolated natural ones are not stably dispersed in water,[9] restricting the analysis and related experiments to homogeneously dispersed systems. The ability of melanins to bind metal ions has been considered to be one of their unique biological functions, allowing the reservation of some metal ions and the quenching of redox reactive metals, thereby releasing the oxidative stress on biological systems.[10] The binding capacity of melanin toward the various metal ions, including heavy metals, has been characterized through direct or indirect experimentation from a lot of the melanin models mentioned above even though they have some limitations of reproducibility and comparability.[10,11] Therefore, it is important and very interesting to develop a new synthetic method for artificial melanins with nanoparticle shape and excellent dispersibility in aqueous solution and to check their binding ability to metal ions.

From this motivation, we have developed a novel synthetic method to prepare artificial melanin nanoparticles having a good dispersibility in water by a deliberately selected oxidation-polymerization reaction of DL-DOPA with $KMnO_4$. We have also achieved excellent binding capacity to heavy metal ions such as lead, copper, and cadmium.

Experimental

Materials. $Cd(NO_3)_2{\cdot}4H_2O$, $Cu(NO_3)_2{\cdot}5/2\ H_2O$, $Pb(NO_3)_2$, and 3,4-Dihydroxy-DL-phenylalanine (DL-DOPA) were purchased from the Sigma Aldrich. $KMnO_4$ was purchased from Dae-Jung. All chemicals were analytical grade and used without further purification.

Synthesis of Melanin Nanoparticles. 2 mmol of DL-DOPA was dissolved in 200 mL of deionized water by heating at 50 °C. Under vigorous magnetic stirring, 6 mL of 0.1 N $KMnO_4$ was added to this solution. The solution quickly turned dark purple, and after few minute became black. After 6 h, the black product was centrifuged (20000 rpm, 10 min) and washed several times with deionized water. Finally, the nanoparticles were stored in deionized water.

Binding of Heavy Metal Ions. For metal binding reactions in a batch reactor, 4 mL of aqueous Pb^{2+} solutions of various concentrations in the range of 2.5-40 mM were added to 10 mL of melanin nanoparticle solution (2 mg/mL) in deionized water. After 1 min of shaking, the melanin nanoparticles binding to Pb^{2+} were separated from the media using centrifugation (20000 rpm, 10 min), and the amount of unbound Pb^{2+} in the supernatants was analyzed by inductively coupled plasma atomic emission spectrometer (ICP-AES). The amounts of metal ions bound to the synthetic melanin nanoparticles were calculated by conducting a mass balance on the metal ions before and after the binding. The binding capacities of synthetic melanin to Cu^{2+} and Cd^{2+} were also determined by similar processes.

Characterization. The morphology of melanin nanoparticles was characterized by field emission scanning electron microscopy (FE-SEM; Hitachi SU-70) and transmission electron microscopy (TEM; Hitachi-7600). The sizes of the melanin nanoparticles were also measured by electrophoretic light scattering spectrophotometer (ELS; ELS-8000). Infrared spectra were recorded by a fourier transform infrared (FT-IR) spectrometer (JASCO FT-IR-600), and UV-vis spectra were recorded by a SINCO S-3100. X-ray photoelectron spectra were recorded by an X-ray photoelectron spectrometer (XPS; AXIS-HSi), and electron spin resonance (ESR) spectra were recorded by a JEOL JES-TE200. The concentration of metal ions was measured by ICP-ES (ICPS-7510).

Results and Discussion

Artificial melanins were successfully synthesized as a spherical particles in the size range of 100-150 nm through the oxidation of DOPA with $KMnO_4$ followed by polymerization in water. As soon as $KMnO_4$ solution (6 mL of 0.1 M solution) was added into the DOPA solution (200 mL of 10 mM solution in deionized water) with vigorous stirring, the colourless solution of DOPA rapidly turned to purple due to the colour of $KMnO_4$ and gradually changed to a black solution as the reaction progressed. The amount of $KMnO_4$ was optimized to generate the largest amount of artificial melanin in the nanoparticle shape. The reaction of DOPA with $KMnO_4$ was carried out for 6 h, which was confirmed by checking the change of the absorption peak from the precursor molecule (DOPA) at 285 nm with UV-vis spectroscopy (Figure S1). After 6 h, the intensity of the peak at 285 nm did not change significantly, probably due to embedded DOPA or small oligomers inside the melanin nanoparticles, and ~160 mg of melanin nanoparticles could be obtained after several centrifugation and re-dispersion processes for purification and size-selection. As shown in Figure 1, transmission electron microscope (TEM) and scanning electron microscope (SEM) images show the size of the synthesized melanin nanoparticles was in the range 100-150 nm. In order to measure their actual dispersed sizes in water solution, the hydrodynamic sizes of synthesized melanin nanoparticles were also measured by electrophoretic light scattering (ELS) spectrophoto-meter, and shown to be 135 nm on average. Even though the size of nanoparticles measured by ELS is usually reported to be bigger than that from TEM due to the surrounding water molecules and swollen effect especially in polymeric materials, the hydrodynamic size of the synthesized melanin nanoparticles from ELS measurement is not significantly different from TEM; it might indicate that the synthesized melanin nanoparticles are 3-dimensionally cross-linked polymers with a high cross-linking density or tightly stacked by the interaction of oligomeric conjugated structures as suggested in the literature.[12]

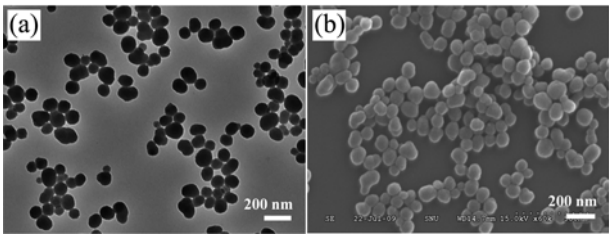

Figure 1. Images of melanin nanoparticles observed by (a) TEM and (b) SEM.

The chemical functionalities of the synthesized melanin nanoparticles were characterized by FT-IR spectra (Figure 2(a)). Characteristic IR peaks for the functional groups of the synthetic melanin nanoparticles are almost identical with previously reported values for melanins:[13] 3300-3500 cm^{-1} for NH (~3300 cm^{-1}) and OH stretching (~3400 cm^{-1}) in indole or pyrrole; 1710 cm^{-1} for C=O stretching in COOH; 1610-1690 cm^{-1} for aromatic C=C and C=N bending and C=O stretching and 1580 cm^{-1} for the asymmetrical bending of COO^- (salts). Due to a conjugated π-electron system to stabilize free radicals, melanins have been demonstrated to be a unique biopolymer exhibiting a relatively large amount of free radical sites. A single-line electron spin resonance (ESR) signal, which originates from stable free radical centers, has been suggested as one of unusual features indentifying melanins from other biomaterials.[14] As shown in Figure 2(b), melanin nanoparticles synthesized by oxidation-polymerization of DOPA in our experiment showed a broad single-line ESR spectrum similar to the reported data from natural and synthetic melanins. Characteristic broad absorption bands in the UV-vis spectrum, known for all the reported melanins, presumably due to the conjugated systems by the oxidative-polymerization of DHI and DHICA,[1] were also observed from the synthesized melanin nanoparticles (Figure 2(c)). Based on the results from all these analyses, artificial melanin nanoparticles synthesized from the reaction of DOPA with $KMnO_4$ seem to be almost identical to reported

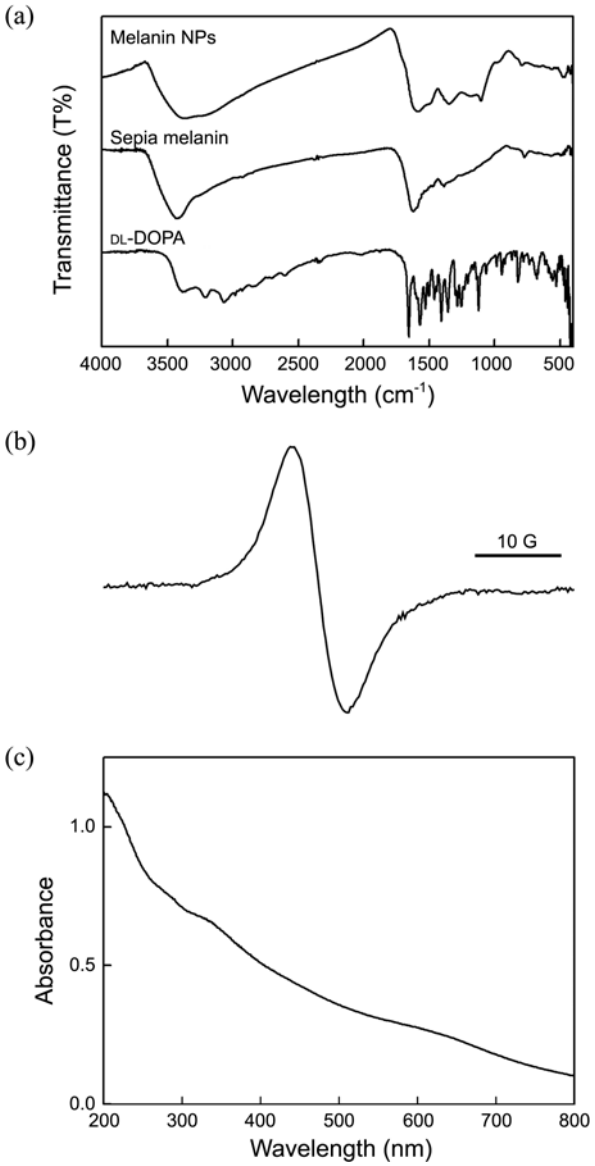

Figure 2. The characterization results of synthetic melanin nanoparticles. (a) FT-IR spectra of synthesized melanin nanoparticles, sepia melanin, and DL-DOPA, (b) ESR spectrum and (c) UV/Vis spectrum of synthetized melanin nanoparticles.

melanin biopolymers.

Although the definite chemical mechanism for synthesizing melanins is not clear yet, it has been suggested that it involves an oxidation of catechol to benzoquinone followed by an intramolecular cyclization through a Michael addition to give indole units and further polymerization to melanin.[15] Interestingly, it has been an important research topic to synthesize mussel-inspired adhesive materials by the oxidation of the catechol units in dopamine followed by cross-linking in the presence of various oxidants;[16] the basic chemistry of both reactions are expected to be very similar. When other oxidants having a strong oxidation power such as H_2O_2, $NaIO_4$, or $(NH_4)_2S_2O_8$ were employed to prepare artificial melanins from DOPA, unlike the results from mussel-inspired adhesive materials, the particle-shaped melanins were not effectively generated even though the colour of the reaction mixture turned to black. In the particular case of H_2O_2, no colour change was observed. Although more detailed investigations are necessary to understand better the differences between these reaction conditions, it seems to be related to the pH of the reaction conditions needed to accelerate the intramolecular cyclization to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by removing a proton from the amine site as suggested in the literature;[15c] for the oxidization of catechol to benzoquinone followed by intra/intermolecular addition of catechol amines, a basic condition of inducing the deprotonation of the amine group has been suggested to be an important factor. In order to check this effect, pH changes during the reaction of DOPA with $KMnO_4$ were monitored. It showed that a slightly acidic DOPA solution immediately turned basic as soon as $KMnO_4$ was added, and the pH of the solution gradually decreased back to slightly acidic as the reaction progressed (Figure S2). The acidic pH might have originated from the hydrogen atom abstraction as the oxidation of DOPA and the subsequent ring closing reaction progressed, based on the suggested reaction mechanism in the literature.[17]

Since it was reported that natural and synthetic melanins can incorporate a variety of metal ions in a similar manner,[10c,18] many research groups have investigated this properties and shown that the good binding ability of melanins to heavy metal ions have been attributed to various functional groups such as carboxyl, amine, quinone, and semiquinone groups in the melanin polymer.[19] Strong interactions of melanin radicals with paramagnetic[19c,20] or diamagnetic ions[10d,21] have also been studied using deliberately designed ESR experiments, confirming that the binding of metal ions on melanin polymer is based on a chemical interaction through the chelation of metal ions by free radicals rather than by physical adsorption or chemical oxidation.

Once it was confirmed that the synthesized melanin from our method had a nanoparticle shape with an excellent water-dispersibility and a characteristic stable free radical feature, the binding abilities of melanin nanoparticles towards various heavy metal ions including Pb^{2+}, Cd^{2+} and Cu^{2+} were investigated. The binding capacities of melanin nanoparticles to heavy metal ions are given as a function of initially loaded amounts of metal ions added on the specific amount (20 mg) of melanin nanoparticles (Figure 3). The binding capacities which are calculated from the amounts of metal ions bound per unit gram of melanin nanoparticles steadily increased as the loaded amount of metal ions increased from 10 to 80 μmol. When the loaded amounts of metal ions were larger than 100 μmol to 20 mg of melanin nanoparticles, it seemed to reach to the saturation point of binding as excess amounts of metal ions were detected from the supernatant. Before the melanin nanoparticles were saturated with metal ions, they were able to maintain good dispersibility in water. However, after the saturated point, they tended to aggregate, producing precipitates probably due to a change of surface charges (Figure 4). In agreement to the reported results in the literature,[10d,21] the integrated intensity of the free-radical signal

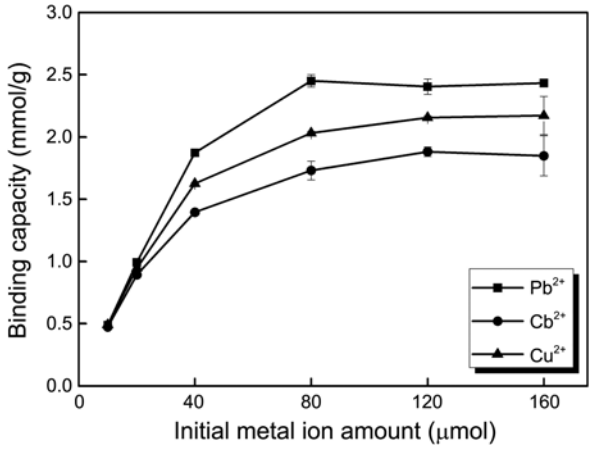

Figure 3. Binding capacities of synthetic melanin nanoparticles. Binding capacities of melanin to heavy metal ions were determined at various amount of metal ions added on 20 mg of melanin nanoparticles.

increased when the amount of diamagnetic Pb^{2+} ion added to melanin nanoparticles increased; this result could confirm that the binding mode of metal ions on melanin nanoparticles synthesized by our method is similar to the known chemical interaction through the chelation of metal ions by free radicals (Figure S3 and Table S1).

The maximum binding capacities of melanin nanoparticles to Pb^{2+}, Cd^{2+} and Cu^{2+} ions are calculated as 2.45, 2.17 and 1.88 mmol/g melanin nanoparticles, respectively. Based on the simplified assumption that melanin nanoparticles are composed of DHI and DHICA, because the exact chemical structure is not clear and the formula weight of the repeating unit in melanin polymer might not be very different from that of DHI or DHICA, the maximum binding capacity values of those metal ions correspond to the binding of one metal ion per every two or three repeating units (DHI or DHICA units) in the melanin nanoparticles. It may imply that the metal ions are bound not only on the surface but also

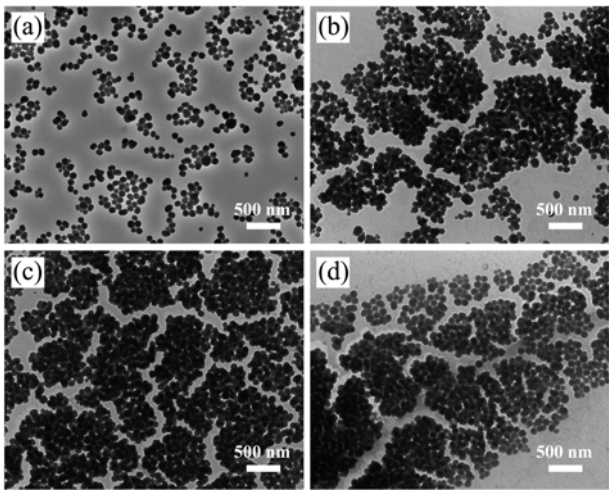

Figure 4. TEM images of melanin nanoparticles: (a) before treating with metal ions, (b) after treating with Pb^{2+} ions, (c) Cd^{2+} ions, and (d) Cu^{2+} ions with far more than saturated amounts.

Table 1. The maximum binding capacities (mmol/g) of melanin nanoparticles and other materials to various metal ions

	Pb(II)	Cu(II)	Cd(II)	Fe(III)
Melanin NPs	2.45	2.17	1.88	
Sepia melanin[22]		1.1		1.2
Choroid granules[10c]	0.93	0.96		
Iris granules[10c]	0.51	0.68		
Ciliary body granules[10c]	0.42	0.6		
Synthetic melanin[10c]	0.70	0.71		
Starch graft copolymer[23]	2.09	2.12		
Hydrated Fe(III) oxides[24]	1.6	1.4	1.4	

inside of the melanin nanoparticles, probably due to the swolling of nanoparticles as metal ions are bound. Compared to the reported binding capacities of melanin polymers, either isolated from natural sources or synthesized by enzymatic method, therefore, the maximum binding capacities of melanin nanoparticles in our study are significantly higher than those values (Table 1). Most of the melanin polymers, either isolated from natural sources or synthesized by an enzymatic method, showed small binding capacities in the range of 0.42-0.96 mmo/lg. Interestingly, melanin polymer isolated from the sepia ink sac showed the largest binding capacity value, 1.1-1.2 mmol/g, probably due to the well-isolated particular shape and simple isolation process accomplished without treatment with harsh chemicals such as strong acids. The large binding capacities of synthesized melanin nanoparticles by our method can also be attributed to the particular shape as well as the excellent dispersibility in water, which can increase the effective surface-to-volume ratio. This large binding capacity is even larger than capacity values of other adsorbents which have been recently synthesized for the special purpose of metal ion removal (Table 1).

The removal of toxic metal ions such as lead, cadmium, and copper from water has received a great deal of attention because they can act as enzyme inhibitors causing a range of health problems.[23,25] Accordingly, there has been much effort to develop removal methods of heavy metal ions such as ion-exchange, reverse osmosis, and electrodialysis techniques, and to synthesize new adsorbing materials including metal oxides, silica and activated carbon.[23,26] Therefore, based on the high binding capacities and rapid binding rate, the melanin nanoparticles synthesized by our method can be used as an excellent material to remove heavy metal ions from contaminant water.

Conclusion

We have successfully synthesized melanin nanoparticles by the chemical oxidation-polymerization of DOPA using $KMnO_4$ as an oxidizing agent. These melanin nanoparticles have an excellent stability in water and show a large binding capacity to heavy metal ions due to the high surface-to-volume ratio of well-dispersed nanoparticles in the solution. We believe that this method of synthesizing artificial

melanin nanoparticles will accelerate research activities investigating many fundamental properties of natural melanin and create great potential for the remediation of contaminated water.

Supplementary Material. Changes of UV-Vis absorption and pH during the oxidation of DOPA with $KMnO_4$, ESR spectra and change of free-radical concentration of melanin nanoparticles after Pb^{2+} binding can be found in the online version of the supplementary materials associated with this article at http://journal.kcsnet.or.kr.

Acknowledgments. This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean Government (MEST, R01-2008-000-20302-0). D. J. Kim and K.-Y. Ju are grateful for the award of a BK21 fellowship.

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