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Human serum albumin and other proteins as templating agents for the synthesis of nanosized dopamine-eumelanin

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ABSTRACT

Eumelanin like materials are known to be heterogeneous and highly insoluble materials and hence it was difficult to use them for applications even if they display fascinating properties as photoprotection and photoconductivity. Owing to the known reactivity of quinones available on the surface of dopamine-eumelanin particles with nucleophiles, we propose and demonstrate that proteins (among them human serum albumin, hen egg white lysozyme and α-lactalbumine from bovine milk) are able to control the size of dopamine-eumelanin aggregates formed in dopamine solutions upon oxidation. The particles obtained in the presence of human serum albumin can be as small as 30 nm in diameter and the viability of human gingival fibroblasts is not significantly affected (with respect to pure dopamine-eumelanin) in the presence of such particles provided they are diluted enough.

1. Introduction

Eumelanin and pheomelanin are the brown–black and the yellow–reddish pigments of the skin, eyes and hairs [1]. They play the role of photoprotectants [2] and eumelanin is known for its antioxidant [3] properties whereas pheomelanin is pro-oxidant [4]. Natural eumelanins and pheomelanins are synthesized from the amino acids tyrosine and cysteine respectively in complex pathways called melanogenesis [5]. The basic building blocks of both pigments are 5,6-dihydroxyindole [6] and 5-*S*-cysteinyldopa [7] for eumelanin and pheomelanin respectively. The formation mechanism of these building blocks is well known from a kinetic and hence from a mechanistic point of view but their oligomerization to yield eumelanin and pheomelanin is the subject of intensive research both from an experimental [8,9] and from a numerical simulation point of view [10,11]. Nevertheless the material obtained from catechol amine solutions (among which dopamine or norepinephrine, L-DOPA...), the usual synthetic precursors of eumelanins, results from a heterogeneous population of aggregates a part of which (i.e., particles a few micrometer in diameter) precipitate out of the solution. It has been found recently that polyvinyl alcohol (PVA) is able to modify the self-assembly of dopamine-eumelanin particles notably reducing their hydrodynamic diameter [12]. Simultaneously, when dopamine is oxidized in basic solutions (0_2 being used as an oxidant) there is not only the formation of dopamine-eumelanin aggregates in solution but also deposition of a brown–black coating on the walls of the reaction vessel [13]. The formation of the so-called polydopamine film is also inhibited when the synthesis is performed in the presence of poly(N-vinylpyrrolidone) but not inhibited in the presence of PVA [14]. There seems hence possible to control the degree of self-association of small eumelanin clusters in the presence of well suited additives. This is of the highest practical interest because it will allow to handle the almost insoluble eumelanin like materials in a controlled and reproducible manner, for instance in the form of stable colloids. Among possible additives to control the size of eumelanin like materials, polyamines seem natural candidates owing to the reactivity of quinone groups, present on the surface of dopamine-eumelanin, for nucleophiles like amines and thiols [15,16]. Hence proteins seem also good candidates to modify the formation process of dopamine-eumelanin aggregates, owing to the presence of free amino groups (due to the presence of lysine and histidine residues and the $-NH_2$ terminal group) on the surface of most of them. In addition natural eumelanins are always found and isolated with bound proteins on their surface [17]. It is the aim of this preliminary investigation to show that the reactivity of dopamine-eumelanin for proteins is an efficient way to control the size of the obtained dopamine-eumelanins. Above a critical concentration of Human serum albumin (HSA) dopamine-eumelanin nanoparticles are obtained. Those particles display an excellent biocompatibility and may hence be used for biomedical applications. The kinetics of dopamine-eumelanin formation in solution is also accelerated in the presence of HSA as well as in the presence of other proteins like

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hen-egg white lysozyme and α-lactalbumin from bovine milk. However, the presence of proteins in the solution during the oxidation of dopamine slows down the deposition kinetics of dopamine-eumelanin on solid surfaces in contact with these solutions.

2. Materials and methods

2.1. Synthesis of dopamine-eumelanin and characterizations

Dopamine hydrochloride (ref. H 8502) was purchased from Sigma Aldrich and used without further purification. Poly(allylamine hydrochloride) (PAH, Aldrich, ref. 283215, weight average molecular mass: 15,000 g mol^{-1}) was dissolved at a concentration of 1 mg mL^{-1} in distilled water. All solutions were prepared from doubly distilled and deionized water (Milli Q plus, ρ = 18.2 MΩ cm). Dopamine was dissolved at a concentration of 2 mg mL^{-1} (10.6 mM) in 50 mM Tris buffer (Prolabo France) at pH = 8.5. The dissolution process of dopamine was kept as time t = 0 for the formation kinetics of dopamine-eumelanin. This solution was shaken with a magnetic stirrer (300 rpm) and some solution was removed at regular time intervals and diluted by a factor of 20 in Tris buffer. The UV–visible spectra of these solutions were immediately measured after the dilution process (required to obtain an absorbance value lower than 2), with respect to Tris buffer put in the reference cuvette of the double beam spectrophotometer (UV mc^2, Safas, Monaco). The absorbance at λ = 350 nm, at which dopamine does not absorb any more (it displays an intense absorption peak with a maximum at 280 nm) was then plotted as a function of the reaction time.

The same experiments were performed with dopamine solutions containing either human serum albumin (HSA, Sigma–Aldrich, ref. A8763), hen egg white lysozyme (Sigma–Aldrich, ref. L6876) or α-lactalbumine from bovine milk (Sigma–Aldrich, ref. L5385). The proteins were used as received and dissolved in Tris buffer at concentrations between 0.1 and 2 mg mL^{-1}. The protein solutions were left to equilibrate at ambient temperature for at least half an hour then they were shaken at 300 rpm on a magnetic stirrer. The dopamine hydrochloride powder was then added to reach a constant concentration of 2 mg mL^{-1} (corresponding to 10.6 mM). During the synthesis, the solution was shaken at 300 rpm as for the synthesis of dopamine-eumelanin. We are aware that shaking the protein solution may induce some important conformational changes in the proteins secondary and tertiary structures, but our aim is to investigate the influence of polypeptide chains on the kinetics of dopamine-eumelanin formation as well as on the size and surface potential of the obtained particles.

The solutions containing dopamine and HSA were characterized by means of dynamic light scattering (DLS) and zeta potential measurement using a Nano-ZS device (Malvern Instruments, UK). The size distribution was calculated from the intensity autocorrelation function using the CONTIN algorithm. Note that the DLS and zeta potential measurements were performed on the solutions containing the dopamine-eumelanin and the added proteins, i.e. no trial was made to separate the colloidal dopamine-eumelanin particles from the unbound proteins. The intensity autocorrelation function was analyzed by taking into account that dopamine-eumelanin has a real refractive index of 1.73–0.02i [20] at a wavelength of 589 nm, i.e. close to the 632.8 nm of the He–Ne laser used in Nano-ZS device. The imaginary part of the refractive index takes into account for the substantial light absorption by the dopamine-eumelanin suspension.

In some experiments, cleaned quartz slides were immersed in the dopamine and dopamine + HSA containing solutions and the absorption spectra of these quartz slides were then measured after a given reaction time taking an uncoated quartz slide as the reference. The aim of these experiments was to investigate whether the presence of the protein in solution affects the deposition of dopamine-eumelanin on solid surfaces.

2.2. Atomic force microscopy

To further characterize the dopamine-eumelanin colloids obtained in the absence or in the presence of proteins, they were synthesized during 72 h in the absence of proteins or in the presence of HSA at 1 mg mL^{-1}. The reaction time of 72 h was shown to be sufficient to reach a steady state in the spectra evolution of the system as shown in Fig. 1. 12 mm glass slides (Microscopoe cover glasses, VWR, Germany) precoated with a layer of positively charged poly(allylamine hydrochloride) were then immersed in the obtained dopamine-eumelanin solutions to allow for the adsorption of the colloids. The adsorption process was allowed to occur for 1 min. Longer adsorption times did not change markedly the surface coverage of dopamine-eumelanin on the substrate (data not shown). The coated glass slides were then intensively rinsed with distilled water and gently dried under a stream of nitrogen before imaging with an atomic force microscope (Bioscope Catalist, Bruker) in ambient air and in the contact mode. The cantilevers were of the Scansist type with a nominal spring constant of 0.4 N m^{-1} and fitted with a silicon tip. The scan frequency was of 0.5 Hz with a resolution of 256 × 256 pixels over an image size of 1 μm × 1 μm.

The AFM data allow to estimate the size distribution of the particles that have been captured on the modified glass substrate by means of adsorption. This may however induce some bias in the estimated size because the small and large colloids may have a different adsorption behavior on the PAH modified substrate. These experiments were hence performed to complement the DLS experiments in order to show that the presence of HSA in the reaction mixture modifies significantly the size distribution of the dopamine-eumelanin colloids.

2.3. Cell culture

Primary human gingival fibroblasts (HGF) were isolated from human gingival connective tissue of healthy donors. Gingival

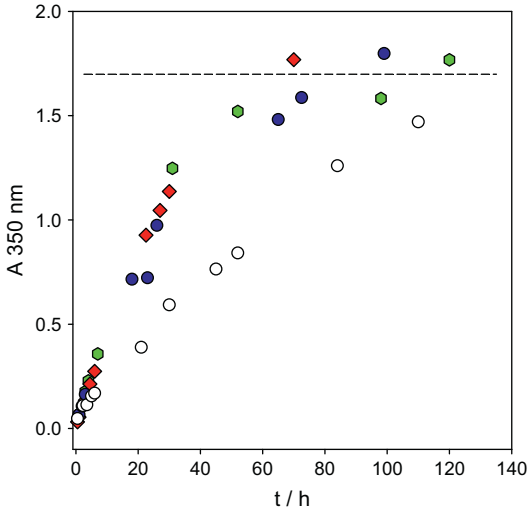

Fig. 1. Absorbance at λ = 350 nm versus time for dopamine solutions put in oxidizing conditions (in the presence of air and Tris buffer at pH = 8.5) in the absence of HSA (○) and in the presence of HSA at 0.2 mg mL^{-1} (●), 1 mg mL^{-1} (◆) and 2 mg mL^{-1} (⬢). The horizontal dashed line shows the plateau value reached in the absorption versus time curves. The given data are the average of two independent measurements, the relative variation between the absorbance values is lower than 15%.