Surface-facilitated formation of polydopamine and its implications in melanogenesis

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ABSTRACT

This manuscript examines influences of differently functionalized surfaces on the formation of solution-dispersed polydopamine (pDA). Glass vials functionalized with different functional groups provided a set of conditions with which the relationship between the area of active surface and the rate of pDA formation could be systematically studied. The results suggest that charged and polar surfaces accelerate pDA formation in solution, with the effect of -NH2 surfaces being exceptionally strong. In the vials, pDA formed as both forms of dispersions in solution and films at solid-liquid interface. Further analyses confirmed that both forms of pDA formed with -NH2 surfaces were chemically similar to conventional pDA synthesized without help of functional surfaces. Among short peptide-based amyloid fibers with defined surface functional groups, and those displaying lysines (-NH2) greatly accelerated the formation of pDA, consistent with the results of -NH2-functionalized vials. The results suggest that pDA formation may be facilitated by surface functional groups of solid-liquid interfaces, and have implications for the overlooked roles of amyloid fibers in biological melanogenesis.

1. Introduction

Melanins—a family of natural pigments synthesized from L-dopa—have consistently attracted research interest since their discovery, because of their functional versatility and wide occurrence in many organisms [1]. Depending on the molecular composition, melanins in humans are categorized as eumelanin (synthesized primarily from L-dopa), phaeomelanin (including L-cysteine as an additive), and neuromelanin (molecularly similar to eumelanin, but contain more proteins and lipids) [2]. Melanins also exist in other organisms carrying out diverse functions, such as coloration of bird feathers [3], functions of cephalopod ink [4], cuticle sclerotization [5], pathogenic activities of fungi [6,7], and self-protection of bacteria [8]. Inspired by various superior materials properties of melanins, efforts to chemically synthesize artificial versions of melanins—mostly eumelanin—have been increasingly attempted. Among others, polydopamine (pDA), which is synthesized by oxidation of dopamine in a basic buffer solution, is being utilized in various forms (e.g., films, particles, and fibers) for an enormously broad range of applications, including functional coatings [9–11], photothermal therapy [12–15], energy materials [16–20], and other functional nanostructures [21–25]. Despite these numerous efforts, it is still difficult to fully recapitulate diverse functions of natural melanins, because of the complexity that resides in their molecular composition and structure.

Natural eumelanin and its chemically synthesized equivalents are roughly similar but are different in a few aspects, such as detailed morphological features, light absorption, radical scavenging efficiency, and water sorption [26,27]. Biological melanogenesis relies on a cascade of enzymatic reactions, whereas chemical synthesis of melanins is conducted by simple oxidation of a dopamine derivative. This causes differences in chemical composition between them, among which the most remarkable is the relative composition of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole carboxylic acid (DHICA) [28–30]. A relatively underappreciated—but critical—aspect of biological melanogenesis is the involvement of a proteinaceous scaffold that exhibits an amyloid structure. In a melanosome, a melanosomal protein, Pmel17, is
expressed intraluminally and spontaneously assembled to form amyloid fibers [31–33]. The role of Pmel17 fibers in melanogenesis has long been considered somewhat less significant; previous related works suggested that Pmel17 fibers primarily act as a matrix for sequestering potentially harmful intermediates that appear during melanogenesis [34–36]. The functions of Pmel17 fibers, however, may be more diverse and crucial than expected; they survived the evolutionary process from bacteria to humans. Throughout a series of previous works, we have recently shown that amyloid fibers (of Pmel17 and other proteins) have diverse functions regarding the formation process and properties of pDA, including catalytic acceleration of its formation and regulation of its morphology, water-dispersibility, and even spin concentration [37–39]. The molecular principles of such multifaceted functions are, however, far from understood, albeit its potential relevance to other supramolecular functionalizations prevalent in nature.

To this end, we started with an assumption that the spatial orientation of functional groups is the key in the observed functions of amyloid fibers. Amyloid fibers tend to present polar (charged, in particular) residues on their surface, while burying nonpolar residues inside [40]. Periodic and proximal molecular configuration of polar residues often leads to unexpected catalytic activities, as in some cases of enzymes or supramolecular materials. We used glass vials as a simplified model solid-liquid interface (i.e., surface) whose molecular structure is carefully controlled. Facilitating influences of molecularly derivatized surfaces for pDA formation were assessed, and their chemical properties on surface and in solution were comparatively investigated. To verify our initial assumption, we also synthesized short peptide-based amyloid fibers designed to display certain polar side chains on their surface. This study reveals that solution-phase pDA formation can be accelerated by surface-oriented functional groups, which has implications in the role of Pmel17 fibers in biological melanogenesis. It also emphasizes the importance of local molecular orientation in supramolecular catalysis.

2. Materials and methods

2.1. Materials

The following chemicals, phosphate-buffered saline (PBS; pH 7.4) and dopamine hydrochloride were purchased from Thermo Fisher Scientific. Rink Amide MBHA resin HL and N,N,N,N′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU, 98%) were purchased from Alfa Aesar (USA). 1 M Tris-HCl, pH 8.5 was purchased from Biosesang (South Korea). Fmoc-protected amino acids and N,N-diisopropylethylamine (DIPEA, 99%) were purchased from TCI (Japan). Acetic anhydride (99.5%) was purchased from Sigma-Aldrich (USA). N,N-dimethylformamide (DMF, 99.0%) for peptide synthesis (99.8%) was purchased from Acros Organics (USA). Trifluoroacetic acid (99.0%), methyl alcohol (HPLC grade), acetonitrile (HPLC grade), water (HPLC grade), dichloromethane (99.5%) and ethyl ether (99.5%) were purchased from Samchun (South Korea). All reagents were used without further purification. Microscope slides (DU. 2355031) were purchased from Daihan Scientific (South Korea). 30 mL-vials (SCV-30) were purchased from Dawonscience (South Korea). A C18 column was purchased from Thermo Fisher Scientific (USA).

2.2. Synthesis and purification of peptides

[Ac-IHIHIYI-NH₂] and [Ac-IKIYI-NH₂] sequences were synthesized by Fmoc-based solid-phase synthesis, using Rink-amide MBHA resin at room temperature. Fmoc groups were removed by repeated reactions (10 min) with 20% piperidine in N-methyl-2-pyrrolidone. Amide coupling was conducted by mixing a protected amino acid, HBTU, and DIPEA in DMF. The coupling reaction took 2 h at room temperature. After all the seven amino acids were coupled, the N-terminal of the peptide was acetylated with acetic anhydride and pyridine in DMF. Cleavage and side chain deprotection were accomplished by treating the cleavage cocktail for 2 h at room temperature after acetylation. The cleavage cocktail was prepared by mixing trifluoroacetic acid, triisopropylsilane, and DCM (95:2.5:2.5, v/v). After the synthesis was complete, crude products were purified via reverse-phase high-performance liquid chromatography with a C18 column. Peptides were lyophilized after purification.

2.3. Synthesis of amyloid fibers and subsequent pDA formation

Amyloid fibers were prepared by dissolving purified peptides in 25 mM Tris-HCl buffer (pH 8.5) and incubated at 37 °C, 200 rpm for 1 h to make peptides self-assemble. After incubation, the solution was centrifuged (15,000 rpm, 15 min) and the supernatant was discarded. The concentration of amyloid fibers was indirectly calculated by measuring the absorbance at 280 nm of the supernatant (i.e., the concentration of the remaining peptide monomers) after incubation. The precipitated amyloid fibers were redispersed in a PBS solution (pH 7.4) and mixed with dopamine in a cuvette. The final concentrations of amyloid fibers and dopamine were 150 μM. Cuvettes were placed in an orbital shaker (250 rpm) at room temperature.

2.4. UV-visible (UV-VIS) spectroscopy

The UV-VIS absorbance spectra of solutions containing pDA were taken for a wavelength range of 300–800 nm by using Lambda 465 (PerkinElmer, Inc., USA). The absorbance spectra of pDA synthesized with amyloid fibers were taken for a wavelength range of 250–800 nm. Before measuring each absorbance, cuvettes were vortexed and sonicated for 10 s.

2.5. Atomic force microscopy (AFM) analysis

AFM imaging of substrates was performed on an atomic force microscope (XE-150, Park systems, South Korea). An NCHR-50 (NanoWorld, Switzerland) AFM cantilever (typical resonant frequency of 320 kHz and force constant of 42 N m⁻¹) was employed in the non-contact mode for the determination of topographic profiles. The root-mean-square roughness (Rq) was determined over a 5 × 5 μm² area.

2.6. Field-emission scanning electron microscopy (FE-SEM)

A solution containing pDA was centrifuged at 13,000 rpm for 10 min, and the pellet was rinsed with DI water. The resultant pDA aggregates were re-dispersed in 2 mL of DI water and deposited on a Si wafer for drying in an oven (80 °C). The morphology of pDA was analyzed with FE-SEM S-4800 (Hitachi, Japan).

2.7. Laser desorption/ionization time-of-flight mass spectrometry (LDI-TOF-MS)

LDI-TOF-MS analysis was performed using IDSYSs (ASTA, Korea) with a Nb:YAG laser having a wavelength of 343 nm, a pulse rate of 1 kHz, and 50 μm in spot diameter at a target plate. The accelerating voltage was 18 kV in positive ionization mode. All mass spectra were obtained by averaging 100 laser shots. For LDI-TOF-MS analysis, 1 μL of reaction mixtures of pDA was spotted on a stainless-steel target plate, dried under ambient condition, and subjected to LDI-TOF-MS analysis, respectively.

2.8. Ellipsometry

The thickness and refractive index of the coatings were determined with an alpha-SE spectroscopic ellipsometer (J. A. Woollam) in the wavelength range of 390–900 nm at an incident angle of 70°. The data were fitted to a Cauchy model using the CompleteEASE software. Refractive index n(λ) = 1.45 + 0.01/λ². 
2.9. Water contact angle measurement

Water contact angles were measured using a phoenix 300 (SEO co., Ltd, Korea). 10 μL of deionization water was dropped on each sample and the static contact angle was measured with ImageXP software.

3. Results

To quantitatively assess the influences of a polar surface on pDA formation, we first designed a minimalistic experiment using a glass vial. By injecting different volumes of an aqueous solution of dopamine (2 mg/mL in DI water) into O₂ plasma-treated glass vials, solutions having different ratios of solid-liquid interface area (i.e., active surface area) and volume (A/V) could be conveniently generated (Fig. 1a), which provide a set of reaction conditions with which influences of the area of the surface can be systematically assessed. Prolonged incubation of dopamine solutions exhibited a linearly decreasing trend in the darkness of solution (which reflects the amount of pDA dispersed in the solution) in respect to the volume of the injected solution (Fig. 1b), indicating a proportional relationship between the area of polar surface and the amount of pDA dispersed in solution. UV–VIS spectra collected from each solution, which commonly exhibited a gradual increase of broad and monotonic light absorption (a representative feature of pDA derivatives), showed such a trend more clearly (Figs. 1c and S1). Plotting light absorption at 450 nm (A₄₅₀; as a quantitative metric for the amount of solution-phase pDA) against the calculated A/V ratios of different solutions revealed their linear correlation (Fig. 1d). To rule out the potential influence of the accessible amount of O₂ in each solution, we conducted the same experiment with vials capped with a punched top and found that punched tops did not cause significant changes in the amount of pDA (Fig. S2). The results suggest that the pDA formation in deionized water (DI water) is quantitatively correlated to the area of polar (in this particular case, negatively charged) surface.

As the O₂ plasma-treated glass surface is competent with the well-known silane-based covalent modifications, we next sought to compare a set of surfaces with different terminal functional groups for their influences on solution-dispersed pDA formation. The set of functional groups was chosen to include non-polar, polar, charged, and nucleophilic moieties (Fig. S3 and S4). Aqueous solutions of dopamine (4 mL, 2 mg/mL) were incubated in vials functionalized with different functional groups. As shown in Fig. 2a and b, the set of functional groups could be grouped into three categories in respect to their influence on pDA formation: (i) -CH₃, -OH, -SH, and -COOH surfaces promoted pDA formation to the extent similar to the bare (O₂ plasma-treated) vial. (ii) Only -NH₂ surface promoted pDA formation better in respect to the bare vial, while (iii) the most hydrophobic -CF₃ surface did adversely so (Fig. S5). The -NH₂ surfaces showed the same trend between A/V ratio and the rate of pDA formation, as the case of the bare vial (Fig. 2c). The results suggest that surfaces with a broad range of terminal functional groups are capable of moderately promoting pDA formation, with an exception of the very hydrophobic -CF₃ surface. The molecular origins of such global surface activity of many functional groups are unclear, but are presumably related to the multimodal repertoires of a catechol group for interacting with various other functional surfaces, which form a basis for material-independent formation of pDA coating [41]. The highest activity of the -NH₂ surface was expectable from previous reports [39], and strongly suggests that amine groups play roles as a nucleophile or a positive charge, which covalently (via imine formation) or non-covalently (via cation-π interactions) recruits dopamine and its oxidative intermediates near the surfaces, respectively. Such an influence of the -NH₂ surface remained consistent over a broad range of pH (7–11).
while being most remarkable at pH 7 (Fig. S6).

Having observed that the formation of solution-phase pDA may be regulated by functional groups at a solid-liquid interface, we next asked if it is chemically equivalent to conventionally synthesized pDA. We chose the -NH$_2$ surface for further analyses, as it was shown the most efficient in accelerating pDA formation. Dispersions of synthesized pDA were dried and the residual powders were analyzed using FE-SEM and LDI-TOF-MS. Fig. 3 a shows the morphology of the solution-dispersed phase of pDA synthesized with an -NH$_2$ surface, showing spherical structures (with diameters of 60–70 nm) adhered to each other. Such morphological features of the pDA were comparable with previously reported pDA synthesized by using an oxidant in an aqueous or a mixed solution. LDI-TOF-MS spectra of pDA synthesized with the -NH$_2$ surfaces presented a typical mass peak of pDA subunit at m/z 402 corresponding to a trimer of two DHIs and a pyrrolecarboxylic acid (Fig. 3 b) [42]. In addition, there were two mass peaks at m/z 142 [dopamine - 2OH + Na]$^+$ and 177 [dopamine - Na]$^+$ which originated from fragmentation and sodiation of dopamine, respectively [43]. The results imply that the pDA synthesized with the –NH$_2$ surface has the characteristic substructures of conventional pDA along with physically intercalated dopamine monomers. By contrast, the typical mass peak of pDA subunit was not detected from pDA synthesized with the bare surface, while the mass peak at m/z 142 [dopamine - 2OH + Na]$^+$ was intensified with appearance of a new peak at m/z 159 [dopamine - OH + Na]$^+$. This indicates the pDA synthesized with the bare surface was mainly composed of monomeric dopamine with a much lower degree of polymerization than the pDA synthesized with the –NH$_2$.
surface. LDI-TOF-MS analyses further confirm that the -NH$_2$ functional groups on the surface of a glass vial promote polymerization of dopamine. Collectively, the data in Fig. 3 suggests that the surface modified with -NH$_2$ groups accelerates pDA formation, not much altering its morphological features and molecular components.

For decades, pDA has attracted interest from various fields for its ability to form a thin film compatible with further covalent functionalization in a substrate type-independent manner [9]. An interesting aspect of pDA film is that it generally requires a moderately basic pH and amine-containing molecules in solution, except occasions using highly oxidizing and extreme hydrothermal conditions [44,45]. Lee et al. have suggested that the amine molecules may work as a crosslinker connecting oxidized dopamine derivatives via bivalent cation-π interactions [46]. The mechanism of film deposition depends on a sedimentation-like process of oligomeric molecular complexes, which makes amine moieties (or other molecules that enable the crosslinking) crucial in the formation of pDA films. Amines that are immobilized on surface, on the other hand, are expected to be less effective in crosslinking aromatic intermediates, and thus less so in promoting the deposition of pDA films. To characterize the film-phase of pDA, we prepared -NH$_2$-modified Si wafers and analyzed them after incubating in an aqueous solution (pH 7) of dopamine. Albeit low pH and the absence of amine-containing molecules in solution, a very thin film was deposited onto the surfaces, which we could identify by observing a slight color change (Fig. 4a) and conducting ellipsometric measurements. The films became gradually rougher, thicker, and hydrophilic (Fig. 4b). However, the changes were minute, indicating that the deposition occurred at the very initial period and did not proceed significantly afterwards. This was opposed to the case of solution-phase amines, which led to the deposition of much thicker multi-layered films. To examine detailed morphological features of the surfaces, we conducted AFM analysis. As shown in Fig. 4c, spherical pDA species with diameters of 10–20 nm were observed on both surfaces, with their numbers increasing for longer incubation. The pDA species adhered to the surfaces, on the other hand, are likely unable to do so. They would rather coalesce into larger aggregates that dispersed much better in an aqueous solution than in the case without (Fig. 5c). When Lys was replaced with His (Ac-IHIHIYI-NH$_2$; IHY), the influence became weaker, indicating surface-enriched amine moieties are superior to simple positive charges in accelerating pDA formation (Figs. 5c and 58). In addition, IKY or HYF fibers led to changes in morphology and water-dispersibility of pDA. Inclusion of fibers in pDA resulted in larger aggregates that dispersed much better in an aqueous solution than in the case without (Fig. 5d), which is consistent with our previous results with amyloid fibers of various proteins [37]. Overall, the data with IKY fibers support that amine moieties located on surfaces of amyloid fibers play a decisive role in regulating pDA formation, as we observed in amine-modified vial surfaces.

4. Discussions

The analytic results of the solution-dispersed and surface-coated phases of pDA show that the roles of amine moieties may depend drastically on where they are located (i.e., those in solution and at surface). Amines in solution can participate into pDA and crosslink its oligomeric structures via cation-π interactions, altering their morphological and paramagnetic properties [48]. Amines at solid-liquid interfaces, on the other hand, are likely unable to do so. They would rather covalently capture oxidized products of dopamine, increasing their local effective concentrations. Reflecting this difference, amines immobilized on solid-liquid interfaces led to the formation of extremely thin pDA films, leaving the majority of pDA as dispersions in the solution. This is opposed to the deposition of thick films in previously reported cases using an amine-containing buffer at basic pHs [49–51]. Interestingly, the immobilized amines were rather functional for accelerating the formation of solution-dispersed pDA formation. Influences of the chemical structure of the substrate on the formation of pDA films

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**Fig. 4.** (a) Photos of -NH$_2$-derivatized coverslips incubated in a dopamine solution (4 mL, 2 mg/mL) for 0, 24, 96, and 120 h (from the left to the right). (b) Measured values of roughness, thickness, and water contact angles (WCA) of the -NH$_2$ modified surfaces. (c) AFM images of -NH$_2$ surfaces incubated in the dopamine solution.
ural melanins, but have characteristics not compatible with intracellular
thereon have recently started to be examined by spectroscopic methods
The mussel-inspired pDA films are chemically similar to natural melamins, but have characteristics not compatible with intracellular
environment, such as nonspecific adhesiveness toward solid surfaces and uncontrollable formation kinetics. Amyloid scaffolds of Pmel17 provide a plenty of lysines—along with other polar residues—displayed on their surface, which are expected to have critical influences on the kinetic and materials properties of biosynthesized melamins.

5. Conclusions
In summary, we showed that the formation of solution-dispersed pDA was facilitated by functional groups at solid-liquid interfaces, and the identity of the functional group determined the strength of such an influence. Amine-functionalized surfaces were most effective in the facilitating function, whereas non-polar moieties were measured to be less so. Further analyses of pDA formed with the NH
short-peptide-based amyloids. Background absorbance of amyloid fiber is corrected. (d) Photos of solutions with and without each amyloid fiber incubated with dopamine (2 mg/mL) for desired times.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability
Data will be made available on request.

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Appendix A. Supplementary material
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.colsurfb.2022.113068.

References

CRediT authorship contribution statement
Chan Yeon Kim: Methodology, Data curation, Formal analysis, Investigation, Writing – original draft. Yoonyoung Kim: Methodology, Data curation, Formal analysis. Min Young Lee: Methodology, Validation, Investigation, Resources. Seong Ah Jo: Methodology, Validation, Investigation, Resources. Seung-Woo Kim: Formal analysis, Writing - review & editing. Young-Kwan Kim: Supervision, Writing – review & editing. Kyungtae Kang: Conceptualization, Project administration, Supervision, Funding acquisition, Writing – review & editing.