Molecular-level insights of early-stage prion protein aggregation on mica and gold surface determined by AFM imaging and molecular simulation

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By in situ time-lapse AFM, we investigated early-stage aggregates of PrP formed at low concentration (100 ng/mL) on mica and Au(1 1 1) surfaces in acetate buffer (pH 4.5). Remarkably different PrP assemblies were observed. Oligomeric structures of PrP aggregates were observed on mica surface, which was in sharp contrast to the multi-layer PrP aggregates yielding parallel linear patterns observed Au(1 1 1) surface. Combining molecular dynamics and docking simulations, PrP monomers, dimers and trimers were revealed as the basic units of the observed aggregates. Besides, the mechanisms of the observed PrP aggregations and the corresponding molecular-substrate and intermolecular interactions were suggested. These interactions involved gold–sulfur interaction, electrostatic interaction, hydrophobic interaction, and hydrogen binding interaction. In contrast, the PrP aggregates observed in pH 7.2 PBS buffer demonstrated similar large ball-like structures on both mica and Au(1 1 1) surfaces. The results indicate that the pH of a solution and the surface of the system can have strong effects on supramolecular assemblies of prion proteins. This study provides in-depth understanding on the structural and mechanistic nature of PrP aggregation, and can be used to study the aggregation mechanisms of other proteins with similar misfolding properties.

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1. Introduction

Prion protein (PrP) has drawn great attention due to its pathological potential to prion diseases, such as Creutzfeld–Jakob disease and “mad cow disease” [1]. Studies have shown that prion diseases are induced by the formation of extended β-sheet rich fibrillar PrP aggregations [2–4], as well as various on-pathway intermediates prior to the PrP fibrils [5–8]. The detailed understanding of these aggregates may help in the search for anti-prion disease treatments. Different types of highly ordered PrP aggregations have been investigated by conventional techniques, such as X-ray diffraction, nuclear magnetic resonance and electron diffraction [9–11]. And compared with these traditional technologies, the atomic force microscopy (AFM) provides additional information on 3D morphology structures and conformation evolution of PrP aggregates with time [12–14]. But most AFM-based studies focused on imaging the microfibers or large aggregations in extreme conditions which are far from the physiological environment of human bodies, such as in high concentrations [15], after long-time incubation [16], and at high temperature [17]. As a result, detailed information on the early-stage PrP aggregation involving the on-pathway intermediates is limited.

For in vitro studies, pH is an important factor among many experimental conditions. It may determine whether PrP fibrils and on-pathway intermediates are formed [18]. In 1999, Jackson et al. reported that when GdnHCl-denatured, DTT-reduced recombinant human PrP (91–231) was dialyzed against a pH 4.0 buffer, it can refold to a monomeric β-sheet rich conformer, which shows partial protease resistance and is prone to associating into soluble oligomers or fibrils [19]. In addition, the misfolded PrP molecules are reported to accumulate in the endosomal organelles, where the typical pH is around 5.0 but can be as low as 4.3 [20–22]. However, most studies have focused on characterizing the acid-induced PrP aggregations [2,19,23] and the PrP molecules in these studies were mainly truncated forms of PrP, rather than the entire protein. These truncated forms of PrP lack the flexible portion (N-terminal domain) of the protein while evidence has suggested that N-terminus can chelate copper and thus acquire some structure...
[24,25], and may also play a role in prion pathogenesis [26]. In addition, different segments of protein are of different biochemical properties, for example, the full-length PrP (23–231) is highly basic (isoelectric point of 9.3), whereas the isoelectric point of PrP 90–231 is about 7.2 [27]. Although some recent aggregation studies utilized the full-length PrP [18,28], its aggregation properties have not been studied as extensively as those of the truncated forms and the mechanistic details are missing.

AFM technology is inherently based on the surface. Solid surfaces, such as mica and gold, have been reported to influence aggregation kinetics and the resulting morphology of PrP aggregates [29]. Gold surface has been widely used in biosensors, and specific proteins can self-assemble on gold surface via the gold–sulfur bonds formed by their cysteine residues and gold atoms [30,31]. Our previous investigation has suggested that PrP molecules can form aggregates aligning with the herringbone structures of the reconstructed Au(1 1 1) surface via Au–S bonds [32]. Meanwhile, mica surface is commonly used as substrate for lipid bilayers, modeling the native cell membrane with its unique properties of being atomically flat, and negatively charged [33]. Several investigations using in situ AFM reported aggregates of amyloid proteins with varied morphologies on mica surface [34,35]. Therefore, application of both solid surfaces as model systems provides the opportunity to elucidate how specific surface environments influence the architectural features of early-stage PrP aggregates.

Herein, we investigate the in situ early-stage architectural features of PrP aggregates formed at low concentration (100 ng/mL) on mica and Au(1 1 1) surfaces using time-lapse liquid phase AFM imaging at pH 4.5. On mica surface, individual oligomeric PrP aggregates, some of which have ring-“head” structures were observed; and in sharp contrast, parallel linear PrP aggregates were formed on Au(1 1 1) surface. By the aid of molecular dynamics and docking simulations, the formation mechanisms of the observed PrP aggregates, as well as the molecular-substrate and intermolecular interactions happening at the interfaces were determined. Meanwhile, ball-like structures of PrP aggregates were observed on both surfaces when the AFM imaging was conducted at pH = 7.2.

2. Material and methods

2.1. Materials

Frozen full-length human recombinant prion protein (sequence: 23–231, theoretical PI/Mw: 9.39/23571.92) was purchased from Calbiochem® in Germany with a concentration of 2 mg/mL, which was from Escherichia coli expression and purified as previously described [36]. The purity of >95% was determined by SDS-PAGE. The protein was stored at −20 °C in 10 mM sodium acetate buffer at pH 4.0 before usage. Before each experiment, purchased prion protein was centrifuged (20,000 × g) for 30 min at 4 °C to remove pre-existing aggregates. Phosphate buffered saline (PBS: 100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2, added with 0.05% sodium azide) was purchased from Pierce (Thermo Scientific, Waltham, MA, USA). Triple deionized water was provided by a Barnstead Nanopure Diamond Laboratory Water System. Mica was purchased from Ted Pella, Inc. (Product No: 56), and the Au(1 1 1) structure was attained by annealing a fresh thermal coated gold chip with 2 min hydrogen flame [31].

2.2. Single-molecule AFM experimental settings

An Agilent 5500 Controller combined with a 50 μm by 50 μm Agilent multipurpose AFM scanner (S/N: 325-00388, with 0.5 Å RMS of z noise specification) was used to obtain images in an area as large as 1000 nm by 1000 nm. The whole system was shielded from environmental interference by a PicoPlusSolution Chamber. Silicon cantilevers tip with spring constant of around 0.1 N/m were used for experiments. The detailed parameters for the image were as follows: drive is approximately 20%, resonance gain is 2, resonance frequency is around 6.725 kHz, resonance amplitude is 4.837 V, “Stop At” is set as 0.8, and scan rate is about 1.02 line/s.

2.3. The liquid phase AFM imaging of the PrP aggregates on mica and Au(1 1 1) surfaces

Centrifugation treated PrPs were re-dissolved in 10 mM pH = 4.5 sodium acetate acid buffer or pH = 7.2 PBS buffer to a final concentration of 100 ng/mL. And 400 μL of each sample was deposited onto newly caved mica surface or freshly reconstructed Au(1 1 1) surface in a liquid cell. Then, samples were imaged immediately at room temperature at 20 min intervals, with the AFM tip immersed in the solution. All the images were taken under TopMAC mode with a PicoTREC controller (Agilent Technologies, Santa Clara, CA), which is a low-force touch method [37]. The detailed description of TopMAC mode AFM used here is shown in the Supplementary material (in Fig. S1). The AFM images were obtained with 1024 × 1024 points and processed using WSxM software [38]. If necessary, only first-order plane-fittings were performed with the analytical software. The height values of the PrP aggregates were determined by individual cross-section profiles generated by the software.

3. Results

3.1. Dynamics simulation of PrP monomer at pH 4.5 and pH 7.2 environment

The human PrP sequence comprising amino acid residues (aa) 23–231 is shown in Fig. 1A. The three-dimensional structures of PrP at pH 4.5 and pH 7.2 are simulated by Amber 11, with 60 ns equilibration and the results are shown in Fig. 1B. The experimental details of the simulations are shown in Supplementary material. Dynamics simulations show that, at pH = 4.5, α-helical-dominant prion protein (PrPα) conformer converted into the β-sheet rich conformer, which is normally called PrPSc. This result is consistent with previous studies [39]. The PrPSc monomer has three hydrophilic α-helices and two hydrophobic β-sheets, implying its

![Fig. 1. (A) The amino acid sequence of the full-length PrP molecule used in the experiment. The residues involved in the β-sheet structures are labeled. (B) The three-dimensional structures of the PrP molecule at pH = 7.2 and 4.5, where three α-helices are in blue and β-sheets in purple. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)](Image 350x608 to 588x784)
amphiprotic property in aqueous solution. These monomers can assemble into dimers or trimers by forming a hydrophobic core with their β-sheets, and leave the more hydrophilic α-helix regions facing the aqueous phase (see Supplementary material, Fig. S2). Previous studies have shown that these dimers and trimers act as the nuclei or growth units to form the fibrils [40].

3.2. Oligomeric PrP aggregations on mica surface at pH 4.5

Fig. 2A shows the real-time evolution of PrP aggregation on mica at pH 4.5, monitored by time-lapse in situ AFM imaging.

3.2.1. Determination of the basic units in observed simple PrP oligomers

Fig. 3A shows the zoom-in profile of the marked area in Fig. 2A.1. Each aggregation unit has different sizes under the same off-set in z direction of the AFM image (trimer > dimer > monomer, Fig. 5A in the Supplementary material), so we can recognize and distinguish PrP monomer (M), dimer (D), and trimer (T) components in these oligomeric PrP aggregates by reading the height of each component from the cross-section profiles (Fig. 3B). To ensure the reliable height analysis, we generated the distribution of the geometrical height values for these basic oligomeric units using more than 350 of these oligomers from 10 high-resolution images (Fig. 3C). The detailed method on how we created the histogram in Fig. 3C is described in Supplementary material. Monomer, dimer and trimer units were recognized from Gauss fittings of the height histograms (Fig. 3C), which clearly show the most probable heights of 0.80 ± 0.01 nm, 1.01 ± 0.01 nm and 1.19 ± 0.02 nm, respectively. By examining the height values of these oligomers in Fig. 3B, we found that the structure labeled as α in Fig. 3A represents a D (1.07 nm), γ is a 3-unit aggregate including one M (0.78 nm) and two Ds (1.09 nm and 1.05 nm) laying on the surface side by side, and β is a 2-unit aggregate containing one D (1.05 nm) and one T (1.2 nm). These observed conformations of oligomers α, β, and γ are further confirmed by docking simulations in Fig. 3D. The intermolecular interactions, which hold together aggregation units (M, D, T) to form the oligomers, are supposed to be hydrogen bonding at the interfaces of the aggregation units. This will be further discussed in Section 4. A list of the predicted residue forming the intermolecular hydrogen bonds at the interfaces is listed in Table S1 (see Supplementary material). Therefore, combining AFM high-resolution images and molecular simulations, we clearly distinguished PrP monomer, dimer and trimer units in each individual oligomer. More representative individual oligomers were selected from the AFM image in Supplementary material Fig. S5. Corresponding simulated structures are provided and compared with the AFM height results in Supplementary material, Fig. S6.

3.2.2. AFM-observed elongation process and “ring-head” oligomers

The oligomers of PrP were observed to continue aggregating with time (Fig. 2A (2)–(4)). With the magnified topographic images in Fig. 4A, two oligomeric PrP aggregates (highlighted by yellow arrows) are found to gradually combine with each other at 40 min and finally turned into an elongated one at 160 min. The zoom-in view (white square) shows that a trimer (T) unit of one oligomeric PrP aggregate combined with a dimer (D) unit of another oligomeric PrP aggregate (see Supplementary material, Fig. S7). This implies that the growth of PrP aggregates is through the assemblies of two or more oligomers. Fig. 4B shows the magnified images of oligomers selected with red squares in Fig. 4A. Interestingly, these oligomers appear to have ring-“head” structures formed by strings of spherical particles and the ring structures have been suggested as soluble oligomeric intermediates which is associated with the toxicity of PrP [41]. High-resolution AFM images show these ring-“head” structures are composed with four to six subunits (spherical particles) (Fig. 4B and Supplementary material, Fig. S8B). The height values of each subunit of these ring structures shown in Fig. 4B are consistent with the ones in the height distribution histogram in Fig. 3C, indicating that the basic units of these ring conformations are also monomer, dimer and trimer. It is also worth noticing that slight heighter values of corresponding subunits could be due to the probability that some subunits overlaid on top the others in ring conformations, resulting in higher “altitude” than their heights obtained from the simple PrP oligomers mentioned in Section 3.2.1 (marked with red arrows in Fig. 4B). Corresponding molecular docking simulations revealed the proper combining conformations for these ring-like structures in Fig. 4C. More representative “ring-head” oligomers are provided in Supplementary material, Fig. S8.

3.3. Architectural features of PrP aggregations with multiple layers on Au(1 1 1) surface at pH 4.5

On Au(1 1 1) surface, at pH = 4.5, regular PrP aggregates in the morphology of highly ordered parallel patterns with hundreds of nanometers in length were observed (Fig. 2B), which is in sharp contrast to the case on mica surface. Zoom-in views of these aggregates and the evolution process are shown in Fig. 5. PrPs were observed to form an orderly patterned first layer fairly quickly after PrP solution was dropped on the surface (Fig. 5A). According to the cross-section profiles of the surface collected at different time points, we see that the height of the architecture increased from around 1.0 nm at 40 min (Fig. 5A) to around 2.2 nm at 80 min (Fig. 5B) and further increased up to around 4.3 nm at 160 min (Fig. 5C). This trend implies that more PrP molecules deposited on top of the first layer from the solution, and then the second layer and multiple layers were formed as time lapsed. For the first layer, the surface height determined by the cross-section profile (Fig. 5A) ranges from 0.8 nm to 1.2 nm, consistent with those of monomer,
Fig. 3. (A) Zoom-in image of the yellow squared area in Fig. 2A (1); (B) Corresponding cross-section profiles for the oligomers in (A); (C) height distribution histogram for each components of the small oligomers which was fitted to three Gaussian yielding heights of 0.80 ± 0.01 nm, 1.01 ± 0.01 nm and 1.19 ± 0.02 nm (R-squared: 0.98); (D) molecular docking simulations for the observed oligomers in (A), monomers (M), dimers (D) and trimers (T) are presented in different colors. The zoom-in images of the interfaces squared in the yellow dashed areas along with hydrogen bonds are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

dimer and trimer units determined on mica surface (Fig. 3C), implying no significant difference in the height values of basic combining units observed between mica and gold surface system. Meanwhile, the linear patterns are generally oriented with a cross-angle of 60° or 120° (marked in Fig. 2B), which coincides with the angle of the reconstructed Au(1 1 1) surface [42]. Combining with the fact that human prion protein monomer has one disulfide bond connecting Cys 179 and Cys 214 [43], the architectural patterns of PrP formed in the first layer were probably induced by the Au–S bonds at the interface between PrP units (monomer, dimer and trimer) and the reconstructed Au(1 1 1) surface, though no chemical evidences are available for the studied system in this work [32]. For the second layer, our previous work showed that the fresh PrP units along with their combinations interacted with the first layer not via Au–S bonding, but via the hydrogen bonding, of which the mechanism is similar to the interactions between PrP oligomers on mica surface as mentioned in Section 3.2. This mechanism explains the phenomenon that the second and the multiple layers have shown similar architectural features to those of the first layer.

3.4. Aggregations on mica and Au(1 1 1) surfaces at pH 7.2

Increasing pH from 4.5 to 7.2 leads to dramatic change in the morphologies of PrP aggregates on both mica and Au(1 1 1) surfaces.
On mica surface, ball-like aggregates of PrP molecules in different sizes were observed instead of any individual oligomers (Fig. 2C). The molecular dynamics simulation shows that monomeric PrP is not able to transform to a stable β-sheet isoform within 60 ns at pH 7.2 (Fig. 1B). The three α-helices of PrP are more hydrophilic, suggesting that monomeric PrP molecule is highly soluble at neutral pH [44]. As a result, these α-helix rich PrP monomers can combine with each other freely to form the ball-like aggregates via intermolecular hydrogen bonding in the solution, and deposit onto the mica surface prior to AFM imaging. Similar ball-like aggregates are observed on Au[111] surface at pH 7.2, in contrast to the parallel patterns observed at pH = 4.5. It implies that no gold–sulfur bonding happened in neutral environment. Fig. S9 in the Supplementary material provides the zoom-in top view of the predicted 3D structure of PrP monomer in the orientation, following which the PrP monomers are predicted to combine with the Au[111] surface via gold–sulfur bonds at pH 4.5. Fig. S9B shows the same region including the disulfide bond at pH 7.2. It shows clearly that at pH 4.5, the
two residues (Cys179 and Cys214) are exposed and stabilized by disulfide bond and β-sheet structures, which make them accessible to Au(1 1 1) surface and bind via gold–sulfur bond as discussed in Section 3.3. In contrast, these two residues are partly enveloped by the α-helices at pH 7.2, and this more flexible structure without β-sheets additionally prevent Cys residues from contact to the Au(1 1 1) surface. Therefore, the ball-like aggregates in Au(1 1 1) surface system are formed in a similar way as in the mica surface system.

4. Discussion

In this study, we investigated the early-stage architectural features of PrP aggregations at molecular level on both mica and Au(1 1 1) surfaces using in situ liquid phase AFM imaging. AFM provides a capability to image PrP aggregates in a hydrated state, without the need for complex pretreatments involved in the traditional technologies mentioned in Section 1 [9–11]. Only low force contacts with the samples are made, minimizing disruption due to impact of the AFM tip (see the detailed description of TopMAC mode AFM in the Supplementary material). Though measured dimensions of samples in AFM images display larger values due to the unavoidable tip broadening effect, their dimensions can still be estimated based on spherical [45] and half-spherical [46] molecular shapes models. But in our work, most of PrP basic units attach to each other, forming oligomeric aggregates. As a result, the rest individual basic units are not enough for us to do the statistics of their dimensions. Here, the high-resolution AFM data provides the height distribution histogram for each component of the small oligomers in liquid that are used to distinguish and recognize the PrP basic units monomer, dimer, and trimer. The obtained mean height of monomer (0.80 ± 0.01 nm, Fig. 3C) is very close to previous work [47].

Previous structural and biochemical analysis of the prion proteins indicate that the assembly of misfolded protein conformers into fibrils is a complex and multistep process involving the population of transient or metastable intermediates prior to the formation of fibrils [48]. Recent studies have suggested that these intermediates exhibit greater cytotoxicity than either the monomeric molecules or fibrils [5–8]. One important advantage of our study is that, a lower concentration (100 ng/mL) was induced to slow down the aggregation process so as to directly follow these intermediates. This low concentration prevents the very fast aggregations by too many PrP molecules, providing opportunities to follow more individual early-stage PrP aggregates (Figs. 3A and 4B and Supplementary material, Figs. S5, S6 and S8) and follow the aggregation processes in both mica and gold surface systems with time-lapse in situ AFM imaging (Figs. 4A and 5). Especially due to the hydrophilicity of mica, the formed PrP aggregates can move freely and further combine with each other into more complex oligomeric aggregates on mica surface (see Section 3.2.2).

Both hydrophilic α-helix structures and hydrophobic β-sheet structures of the three basic units (M, D, and T) at pH 4.5 are essential for the intermolecular interactions to occur at their interfaces. The intermolecular interactions can be classified into two non-covalent types: (1) α-helix induced hydrogen bonding interactions and (2) β-sheet interactions. Based on the results of the docking simulations for PrP dimers and trimers (see Supplementary material, Fig. S2), we determined the number of the solvent-exposed residues in α-helix structures and β-sheet structures of each basic unit in Supplementary material, Table S3. As shown in Table S3, the residues from the α-helix structures dominate the surfaces of these units (91.2% in dimers and 88.9% in trimers), suggesting that the α-helix induced intermolecular hydrogen bonding happens more frequently than the β-sheet interactions. This mechanism leads to the formation of the oligomers observed on mica surface (Figs. 3A and 4B, and Supplementary material, Figs. S6E–G and S8). Similarly, the interaction between the first and second PrP layers in Au(1 1 1) surface system is also caused by hydrogen bonding [32], which is much weaker than the Au–S bonding at the interfaces between first PrP layer and the Au(1 1 1) surface. Thus, the oligomeric PrP units in the higher layers could change their orientations freely after they deposited on the lower layer, resulting in the phenomenon that the observed number of the linear patterns crossed the red dashed line decreases from 8 (Fig. 5B) to 7 (Fig. 5C).

 Gathering all the information of high-resolution single-molecule AFM images and the molecular simulations discussed above, we can construct a schematic illustration for PrP aggregations on mica and Au(1 1 1) surface at pH 4.5 as shown in Fig. 6. Firstly, acid-induced PrP basic units are formed rapidly in solution in both mica and Au(1 1 1) surfaces systems (Fig. 6A). Then individual ring-like, rod-like oligomers, and their combinations (“ring-head” oligomers) are formed in the solution, by these basic units via intermolecular hydrogen bonding at their interfaces.

Fig. 5. Time-lapse in situ PrP aggregation on Au surface. Upper panel: magnifications of the squared areas in Fig. 2B (2–4); bottom panel: corresponding cross-section profile. 50-nm scale bars are indicated. (For interpretation of the references to color in the text, the reader is referred to the web version of the article.)
(Fig 6A). The formation of such “ring-head” structures was supposed to involve the spatial rearrangement of the units to provide a more compact conformation minimizing the surface exposure of hydrophobic areas to water [13]. The isoelectric point of human full-length PrP is 9.39, implying the obtained PrP aggregates are positively charged in pH = 4.5 buffer. Meanwhile, fresh mica surface is negatively charged [49], making the oligomers easily deposit onto the surface via weak electrostatic interactions and can be observed immediately after PrP samples were dropped on the substrate (Fig 6B). In contrast, the reconstructed Au surface is rich in Au(1 1 1) lattice planes [50], which are able to align PrP aggregates via a surface assisted coupling reaction between the disulfide bond and gold atoms (Fig 6C). Thus, the length of each linear pattern is only restricted by the area of the Au terrace. As the green solid line in Fig 5A exemplifies one of linear aggregates with the length of 130 nm.

5. Conclusions

The early-stage architectural features of full-length PrP aggregates were investigated at the molecular level using in situ liquid phase AFM imaging in both mica and Au(1 1 1) surface systems. Oligomeric PrP aggregates, some of which have “ring-head” structures with strings of spherical particles, were observed on mica surface at pH 4.5. Two individual oligomers were observed to combine with each other into an elongated one via time-lapse AFM imaging. In contrast, highly-ordered parallel linear patterns following the underlying Au(1 1 1) herringbone geometry are formed on Au(1 1 1) surface at pH = 4.5. And multiple layers yielding similar patterns were formed with time going on. The results indicate a strong surface effect on the supramolecular assemblies of prion proteins. Based on the results from molecular dynamics, docking simulations, and high-resolution AFM images, we propose that PrP monomers, dimers and trimers perform as basic units for the formation of the observed aggregates. Intramolecular hydrogen bonding interactions happen in both surface systems, but play different roles. For mica surface, the basic units assembled into individual oligomers with unique structures (“ring-head” oligomers) via hydrogen bonding in the solution, then these oligomers deposited onto mica surface via weak electrostatic interaction. However, for Au(1 1 1) surface, the hydrogen bonding interactions happen at the interfaces between the two PrP layers upon the surface. The first PrP layer yielding linear patterns was formed via a surface assisted coupling reaction between the disulfide bond and gold atoms. Meanwhile, ball-like structures of PrP aggregates were observed on both surfaces when the AFM imaging was conducted at pH = 7.2, suggesting the strong influence of environmental pH. These results could help us gain deep insight into structural and mechanistic understanding on PrP aggregation related to prion diseases, and provide a way to identify specific inhibitors against the aggregation interaction of PrPs. We also suggest that this TopMAC mode, liquid phase AFM methodology is applicable to study the effects of the substrate and pH on architectural features of aggregations of other proteins with similar misfolding properties.

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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.colsurfb.2015.07.053

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