Evaluating Effect of Metallic Ions on Aggregation Behavior of Amyloid A β 42 by AFM Imaging and SERS

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Abstract

In this study, self-assembled monolayer (SAM) method was employed to fabricate monolayer of β -amyloid peptides A β 42 on gold substrate with a bolaamphiphile named thiol (MHA). Firstly, the samples of gold substrate (blank control), the MHA-modified substrate and the A β 42-modified substrate were detected by X-ray photoelectron spectroscopy (XPS) to track the self-assembly process. The spectra of binding energy measured from these three sample surfaces could be well fitted with the corresponding monolayer's composition, which means A β 42 monolayer is well formed. Aggregation behaviors of A β 42 in the absence and presence of metallic ions (Zn^{2+} , Ca^{2+} , Al^{3+}) were then monitored by atomic force microscopy (AFM) and surface-enhanced Raman Scattering (SERS), respectively. The recorded surface morphology of different experimental groups obtained by AFM showed markedly different nanostructures, indicating occurrence of aggregation behaviors of A β 42. In solutions with added metal ions, the increased size of surface structures was observed, which suggest the presence of metal cations promotes aggregation behavior between A β 42 molecules. Furtherly, the interaction between A β 42 and metal cations was investigated by SERS. The results demonstrate that the Raman strength of A β 42 changes after the metal cation treatment. Taken together, the combined AFM imaging and Raman analyses show that the three kinds of metallic ions promote the process of A β 42 aggregation.

1. Backgrounds

In recent years, Alzheimer's disease (AD) is paid more and more attention for its terrible influence on the health of the elderly. Amyloid plaques with high density of β -amyloid peptides (A β) in the brain tissue of patients have been proved to be the major pathological feature of AD [1, 2]. Aggregation of A β is more and more widely recognized as the culprit of AD. It was reported that the neurotoxicity of A β is related to its aggregation behavior back in the last century [3, 4]. The concentration of deposited A β in the cerebral cortex correlates with the degree of dementia and synaptic loss [5]. Main components of the plaques were studied to be A β 40 and A β 42, which are two homologous isomers of A β [6]. While A β 40 is the most abundant homologous isomers ever discovered, A β 42 has been reported to be the most toxic [7, 8]. The article focuses on the latter species A β 42, whose neurotoxicity has essential correlation with the pathology of AD [9]. Furthermore, investigating the aggregation behavior of A β on a molecular level is critical to exploring the therapeutic schemes of AD.

There remains a key question in the pathology of AD: what are the risk factors that affect the aggregation of A β ? In recent years, several neurotoxic metal ions were proposed as affecting factors in the misfolding and aggregation processes of A β [10, 11]. However, the interactional relation between metals ions and A β is still unclear. Exploring the mechanism of metal cations' effect on A β remains challenging. It has been found through the autopsy of AD patients that abnormally high concentrations of Zn^{2+} , Ca^{2+} are present along with A β in the senile plaques of AD [12, 13], where Al^{3+} is also detected [14]. Concentration levels of metal cations in the neuropil of AD patients are significantly risen as well [15, 16].

Despite the fact that the effects of metal cations on A β misfolding and aggregation have been extensively studied, the underlying mechanism regulating A β aggregation remains elusive. Therefore, a study on aggregation events of A β in the presence and absence of metal ions at molecular level would be

significant. Recently, atomic force microscopy (AFM) is widely used for molecular detection [17, 18]. Since the turn of the century, new perspectives have been opened with the advent of AFM in the investigation of biomolecular interactions [19]. In this paper, studies focused on the effects of several metal ions on A β 42 aggregation. The three-dimensional morphology measured by AFM has high spatial resolution, which can be measured under near-physiological conditions without special sample preparation. By applying AFM, it's achievable to concentrate on what we can find by "looking" at the protein molecules and analyze biological phenomena [20].

However, the main challenge with AFM testing is the sample preparation, especially the stabilization of protein molecules onto the gold substrate. In this study, the possibility of investigating molecular events in physiological solution is realized through a sample preparation method called Self-assembly monolayer (SAM), which has been developed over the last two decades and widely applied by biologists and chemists [21, 22]. To achieve SAM chemically, a cleaned gold substrate should be immersed in a solution of thiols such as 16-Mercaptohexadecanoic acid (MHA), which is followed by spontaneously reaction between the thiol molecules and gold. Gradually, MHA monolayer on the gold surface with ordered and stable bonds (Au-S bond) is formed. Afterwards, the carboxyl terminus of MHA is activated by 1-ethyl-3-(dimethylaminopropyl) carbodi-imide hydrochloride (EDC) and N-Hydroxysulfosuccinimide (NHS) and then immersed into protein (A β 42) solution. Eventually, a stable and ordered monolayer of A β 42 on the thiols-modified gold surface is formed. The mechanism of mercaptan self-assembly method is depicted in Figure 1.

In order to track the formation of A β monolayer, X-ray photoelectron spectroscopy (XPS) were employed. The composition characteristics of the gold surface, the A β 42 monolayer, and the MHA film were studied. By employing AFM imaging, we monitored the nanostructure changes of A β in the presence and absence of metallic ions (Zn²⁺□Ca²⁺□Al³⁺). Until now, the lack of study on revealing molecular events of A β raised a controversy about whether metal cations are helpful to the aggregation of A β molecules.

The mechanism of the influence of metallic ions on A β abnormal aggregation is furtherly probed and discussed by Surface-Enhanced Raman Scattering (SERS), which is an emerging sample surface analysis technique [23]. The changes of chemical bonds and groups in molecules lead to different molecular rotation or vibration states, which can be judged by the change of Raman scattering light frequency [24]. For proteins, Raman scattering can obtain not only important information about amino acid composition, but also secondary structure information such as β - sheet and α -helix. It has been reported that the Raman cross sections of Au are enhanced to some extent when they adsorb different molecules [25]. Because this effect occurs in the metal adsorbed molecular system, many important processes such as surface studies are related to it. The purpose of SERS study is to quantitatively characterize the effect of metal ions on the conformational transition of A β 42, and to probe the role of these metal ions in the process of abnormal aggregation of A β 42. Combined with the results of AFM study, the aggregation behavior of A β in the absence and presence of metallic ions was elucidated.

The results obtained by AFM imaging indicates that the surface nanostructure of A β 42 molecular layer shows more pronounced state of aggregation in physiological solutions with added metal ions, which is characterized by the enlargement of nanoparticles on the surface. Further study by SERS showed that the Raman strength of A β 42 changes significantly after the metal cation treatment. A considerable part of the amide bonds interacts with metal cations, leading to a structural change, which is characterized by the weakened β -fold (1669cm⁻¹) Raman peak. The results are in agreement with those observed by AFM.

These results may have relevance for understanding the pathology of AD and may initiate new therapeutic strategies targeting metal - A β interactions.

2. Materials And Methods

2.1 Materials

Metal salts used in this work are chlorides (ZnCl₂, CaCl₂ and AlCl₃·6H₂O), manufactured by Sigma Aldrich Chemical Co.. All chemicals were used as received. Phosphate buffered saline (PBS, pH 7.4) and absolute ethyl alcohol (guaranteed grade) were produced by Merck Co.. Ultra-pure water was made by Millipore purification system. 1-ethyl-3-(dimethylaminopropyl) carbodi-imide hydrochloride (EDC), N-Hydroxysulfosuccinimide (NHS), 16-Mercapto hexadecanoic acid (MHA) are made by Sigma Aldrich Chemical Co.. The amyloid peptides used in this work (A β 42) was obtained from AnaSpec. (USA).

2.2 Formation of protein films

2.2.1 Preperation of gold film

Besides, vapor deposition method was applied to obtain the film of gold particles. Gold particle spray film deposited on the mica plate in high vacuum by applying turbo evaporator (at $\sim 10^{-7}$ Torr), Using a radiator heater, mica plates were heated to 325°C for 2 h prior to deposition. The velocity of evaporation is limited within the range of 0.1-0.3 nm/s. The thickness of gold granular film is about 200 nm. In addition, a chromium film was deposited between the gold and the surface of mica to increases the adherence. Finally, the obtained gold film was annealed in H₂ flame for one minute.

Before use, the prepared gold plaque was immersed in piranha solution (v/v H₂SO₄:H₂O₂=3:1) for 30 min to remove organic pollutants on the surface. Then the gold surface was washed three times by absolute ethyl alcohol and ultrapure water in turn. Next, it was dried in nitrogen to avoid any pollution. The surface of gold substrate was characterized by XPS.

2.2.2 Formation of MHA film

MHA was dissolved in an ethanol solution at a concentration of 1mM. After that, the gold substrate was immersed into MHA solution to form MHA membrane spontaneously. Ultrasonic cleaning was applied to remove unbound MHA molecules. Then the SAM film was irrigated by absolute ethyl alcohol and ultrapure water successively. Finally, it's necessary to dry the MHA film in nitrogen immediately. The sample of MHA monolayer was characterized by XPS.

2.2.3 A β 42 immobilization onto the MHA film

The carboxyl groups at the end of the MHA film reacts with the amino groups of lysine in A β molecule [26, 27]. Taking advantage of this principle, steady A β 42 monolayer can be acquired. The A β protein was

dissolved in a physiological solution (PBS), which was freshly prepared according to standard method. It should be noted that MHA film were activated for 1 h at normal temperature using the method mentioned above. Afterwards, the activated MHA film was rinsed as mentioned above and then immersed in 10 μ M A β 42 solution and stored in refrigerator at 4°C for 12 h. At last, the A β 42 film was prepared. Besides, it should be pointed out that the A β 42-modified substrates should be rinsed three times by ultrapure water and dried in nitrogen before testing. This step is to remove free protein molecules from the surface of the substrate. The surface of A β 42 monolayer was characterized by XPS.

2.3 AFM imaging

The powder of the three metal salts was dissolved in PBS respectively and finally diluted to 10 μ M before use. For the comparison of A β 42 aggregation events in the absence and presence of metal cations, the A β 42 monolayers were incubated in blank solution (PBS) and metal ionic solutions and placed in 37°C incubator for 24h and 48h respectively. Three-dimensional images of the incubated A β 42 monolayers were achieved at a resolution of 512×512 and a rate of 1 Hz. Surface topography of all samples were scanned by atomic force microscopy (JPK Nanowizard®II, Germany). Three-dimensional topography of blank group and experimental groups recorded in different conditions were analyzed by imaging processing software which is offered by the company.

2.4 SERS determination

A β 42 monolayer was prepared by the method mentioned above. 10 μ M concentration of metal salts was dissolved in PBS solution. The prepared A β 42 monolayer modified substrate was placed in a clean liquid pool, 2 mL of blank solution (PBS) and metal ion solution (Zn^{2+} , Ca^{2+} , Al^{3+}) was added respectively, and immersed in an incubator (37°C) for 24 h.

A confocal Raman spectrometer of Horiba company was used to measure the Raman spectrum of A β 42 molecular film. The laser was a He Ne laser with excitation wavelength of 633nm, laser power of 6.0mw, resolution of 1 cm^{-1} , and the measurement range was 800 ~ 2800 cm^{-1} . With PBS solution as blank control, the conformational changes of A β 42 with the addition of metal cations were determined by Raman spectroscopy. For each experimental group, the Raman experiment was repeated at least three times.

In addition, we used Levenbery-Marquardt algorithm for peak split and fitting of the Raman spectral bands (corresponding to the β -folded conformation) (about 1600 ~ 1700 cm^{-1}) and the characteristic peaks corresponding to amide bond at about 1400 cm^{-1} . During the SERS testing, the same sample parameters were set. In order to get accurate results, each sample was repeated at least three times. The integration time of Raman data was initially set as 10s, and then the integration time was gradually increased to 60s.

3. Results And Discussion

3.1. Preparation of chemically immobilized A β 42 monolayers

It's of crucial importance to consider immobilization carefully for the sensitivity and reproducibility of bioassays. Self-assembly monolayer (SAM), which is reliable for protein immobilization, is a topic of current interest in biological studies. SAM method can be used for self-assembly of protein molecules without altering the stability and activity of protein [28]. It has been studied that thiol concentration of 1 mM and immersion time of 24h are befitting for the formation of Mercaptan molecular film [29]. Besides, MHA with a proper length of the chain serves as a spacer to minimize the interference from gold substrate [30]. Our previous research showed that the chemical SAMs method has good reproducibility [31, 32].

We monitored the self-assembled processes by X-ray photoelectron spectroscopy (XPS) to ensure that Aβ42 was successfully modified on the surface of the gold substrate. Electron emission can be observed when the sample is exposed to electromagnetic waves with short enough wavelengths, i.e. high photon energy. This phenomenon is called photoelectric effect or photoionization because of the presence of observable photocurrents. In this process, the binding energy of material can be expressed by the following equation:

$$E_k = h\nu - E_b - \phi_s$$

E_k -the kinetic energy of photoelectrons (in eV); $h\nu$ -the energy of photons in the X-ray source (in eV); E_b -the binding energy in the specific orbit of an atom (in eV); ϕ_s -the work function of the spectrometer (in eV).

The surfaces of different samples (blank substrate, MHA films, Aβ42 monolayer) were investigated by XPS and all measurements should be repeated 3 times for each sample. Full spectrum of elements obtained by XPS showed changed element binding energy of the bare gold-MHA-modified and the Aβ42-modified gold sample. The full XPS spectra of the three surfaces are shown in Figure 2. It shows that the elements on the surface of the three samples are identical to the modified molecules during self-assembly. The peak values of Au4f binding in the XPS spectra of blank substrate are 86.7 eV and 83.9 eV, respectively, which are consistent with the standard spectra. The peak values of Au4f binding shifted to 87.9eV and 90.8eV after the MHA molecule was modified on the surface of gold film. The formation of Au-S bond should be the cause of the binding-energy shift of Au4f. What's more, peak position of the S2p binding energy spectrum (Figure 2(d)) of the MHA film is lower than 164eV, indicating that there is no unbounded MHA molecule on the sample surface [33]. There are two main peak in the binding energy spectrum of S2p. The peak at 161.2 eV may be due to the bond between MHA and Au, which reduces the binding energy of S2p [34]. The peak at 161eV may be attributed to the C-S bond [35]. The results suggest that the immobilization of Aβ42 on the gold substrate is successful.

3.2. Topographic images of A β 42 monolayer imaged by AFM in solution

Firstly, the topography of A β 42 monolayer in the absence of metal cations display Granular structure after incubating for 24h (Figure 3(A1)). However, the topography of A β 42 monolayer in the presence of Zn $^{2+}$, Ca $^{2+}$ and Al $^{3+}$ showed spherical structures without rules after 24h (Figure 3(B1)–(C1)–(D1)). On the basis of the above results, it can be suggested that in the presence of metallic ions, the aggregation behavior of A β 42 was greatly induced; in other words, the presence of metal ions leads to disorder and clumping on the surface of A β 42 monolayer. What's more, stable aggregation state was observed during the experiment with the extending of incubation time. The results indicated that the addition of Zn $^{2+}$, Ca $^{2+}$ and Al $^{3+}$ drastically destabilized A β 42 and stabilize aggregation of A β 42. The result is consistent with the mode proposed in Bruno' paper [36]. Besides, the aggregation state of A β 42 in 10 μ M Ca $^{2+}$ –Zn $^{2+}$ and Al $^{3+}$ solution differed in size and morphology of nanoparticles imaged by AFM. In conclusion, AFM imaging results showed large amounts of heterogeneous and conglobate-shaped aggregates were produced in the presence of metallic ions. The influence of different kinds of metal ions on the process of A β 42 shows some differences, which may be attributed to the following reasons: 1) different charge amount, different molar ratio of A β 42 to metal ions; 2) action modes and action sites on the A β 42 peptide chain are different in the presence of different metal cations.

3.3. SERS analysis

In general, the enhancement of the surface signal is 106 times, which is equivalent to the amplification of the surface monolayer to more than one million layers. Therefore, the advantage of SERS is that it can avoid the signal interference caused by the same substance in the solution, and obtain high-quality surface molecular vibration and rotation signals, which is of great significance for a detailed understanding of the interaction mode between molecules (such as metal ions) and self-assembled monolayers and the structural changes of molecules Righteousness. Since the discovery of SERS, it has been successfully applied in many fields such as chemistry, biology and so on.

Above all, stable SERS signal is of great significance for accurate analysis results. Hence, the SERS properties of the obtained A β 42 modified-substrates were evaluated in different metal ion environments. In order to investigate the stability of Raman measurement results, the changes of SERS spectra of A β 42 molecular layer were recorded under continuous laser irradiation. As shown in Figure 4, when different integral time was set, no obvious change in the peak shape of Raman spectrum curves for different samples was seen, which means the A β 42 monolayer in these three metal ion solutions has good stability under continuous laser irradiation.

In Figure 5 curve A stands for the Raman spectrum of the blank control group and curve B stands for the experimental group. The results indicated that β -folds (peak at 1669cm $^{-1}$) and Amide II (peak at 1375cm $^{-1}$)

¹) is the characteristic structure for the natural conformation of A β 42. With the addition of Zn²⁺, the peak intensity of the β -folded conformation at 1669cm⁻¹ and amide II band at 1375cm⁻¹ was weakened to a certain extent, respectively (Figure 5(1)). Moreover, in blank control group, the Raman peak signal at 1375cm⁻¹ was determined to be stronger than that at 1669cm⁻¹, which means the vibration attributed to N-H is greater than that attributed to β -fold. On the contrary, the signal intensity of Amide II (1375cm⁻¹) was greatly weakened after adding Zn²⁺, which is lower than that of β -fold (1669cm⁻¹). As shown in Figure 5(2), the addition of Ca²⁺ also caused a structural change of A β 42, which is characterized by the reduced Raman intensity of Amide II from 156.13 to 115.36, and the reduced Raman intensity of β -fold decreased from 144.669 to 117.76. It's worth noting that the Raman peak of Amide II at 1375cm⁻¹ was greatly weakened and almost disappear, and the intensity of the β -fold peak at 1669cm⁻¹ was also decreased compared with the blank control group, indicating that a considerable part of amide bonds in A β 42 molecule probably interacted with Al³⁺ which resulted in a conformational change.

Furthermore, the change of molecular conformation was evaluated by the Raman intensity ratio (I_{1375} / I_{1669}) (displayed in Figure 6). In comparison, I_{1375} / I_{1669} of A β 42 monolayer in the presence of is the lowest, which indicates that Al³⁺ make the greatest effect on the conformation change of A β 42. The result is consistent with Banks's study, found that Al³⁺ can stabilize the aggregation structure of A β to a greater extent [37].

Combined with Figure 3, the nanostructures with aggregation state on the surface in the presence of metallic ions is consistent the results obtained by SERS. Based on these results, it can be inferred the presence of metallic ions plays a vital role in the occurrence of the abnormal aggregation events of A β 42. The implications of this phenomenon are as follows: misfolding of A β conformation at the early stage leads to a destabilization and the interaction between metal cations and A β results in a conformational change of A β , which promotes formation of aggregates. If we can block the abnormal aggregation of A β 42 by chelating metal ions, we may interrupt this pathological change in the early stage of AD disease, so as to achieve the purpose of prevention and treatment of AD.

4. Conclusion

A study of aggregation events between A β 42 immobilized on MHA monolayer in the absence and presence of metallic ions by AFM imaging and SERS analysis is presented in this paper. The topographic images of A β 42 monolayer either in the absence of metallic ions or in the presence of metallic ions (Zn²⁺, Ca²⁺ and Al³⁺) show significantly different surface structures. The obtained three-dimensional surface topography of A β 42 monolayer show significantly aggregating nanostructures in the presence of metal ions, suggesting aggregation events occurred between A β 42 molecules with the addition of metal cations. Furthermore, the effect of metal cations on the conformational change of A β 42 was studied by SERS, which resulted in good agreement with those observed by AFM. These results, therefore, suggest that the presence of metallic ions plays a crucial role in the accelerated aggregation behavior of A β 42. It

is rational to assume that to block the effects from metal cations or obstruct the interaction between A β and metal cations may have great potential in new drug design for AD.

Abbreviations

AFM: atomic force microscope; SAM: self-assembled monolayer; XPS: X-ray photoelectron spectroscopy; SERS: surface-enhanced Raman Scattering; AD: Alzheimer's disease; A β : β -amyloid peptides; MHA: 16-Mercaptohexadecanoic acid; EDC: 1-ethyl-3-(dimethylaminopropyl) carbodi-imide hydrochloride; NHS: N-Hydroxysulfosuccinimide; PBS: Phosphate buffered saline

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Experimental design and manuscript writing: Yang Xie. Data collection and analysis: Lin Yu, Yuna Fu and Heng Sun. Instrument operation: Chundong Liu. Providing overall thinking: Jianhua Wang. All authors reviewed and approved the final version of the manuscript.

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Figures

Figure 1

A schematic diagram of mercaptan self-assembly method, which shows covalent attachment of Aβ42 on the carboxyl terminal of thiol-modified gold substrate

Figure 2

(a) Binding energy spectra of bare gold; (b) Binding energy spectra of MHA; (c) Binding energy spectra of Aβ42 monolayer; (d) Binding energy spectra of S2p after MHA modification. The spectra were measured at 25 °C.

Figure 3

(A1) Topography of A β 42 monolayer imaged by AFM in PBS at the incubation time of 24h; (A2) Topography of A β 42 monolayer imaged by AFM in PBS at the incubation time of 48h; (B1) Topography of A β 42 monolayer in the presence of Zn $^{2+}$ at the incubation time of 24h; (B2) Topography of A β 42 monolayer in the presence of Zn $^{2+}$ at the incubation time of 48h; (C1) Topography of A β 42 monolayer in the presence of Ca $^{2+}$ at the incubation time of 24h; (C2) Topography of A β 42 monolayer in the presence of Ca $^{2+}$ at the incubation time of 48h; (D1) Topography of A β 42 monolayer in the presence of Al $^{3+}$ at the

incubation time of 24h; (D2) Topography of A β 42 monolayer in the presence of Al³⁺ at the incubation time of 48h. The concentration of metal ions is 10 μ M. (the scanning size is 1 $\times$ 1 μ m) (pH7.4)

Figure 4

Raman spectrum curves for A β 42 monolayer in the presence of Zn²⁺(A) Ca²⁺(B) Al³⁺(C)

Figure 5

Raman Spectra about various metallic ions effects on A β 42 (integration time for 10s), with line A as control group; (1) in the presence of Zn²⁺ (2) in the presence of Ca²⁺ (3) in the presence of Al³⁺

Figure 6

Intensity ratio I_{1375}/I_{1669} of $A\beta_{42}$ molecule in the absence and presence of metal cations (integration time for 10s)