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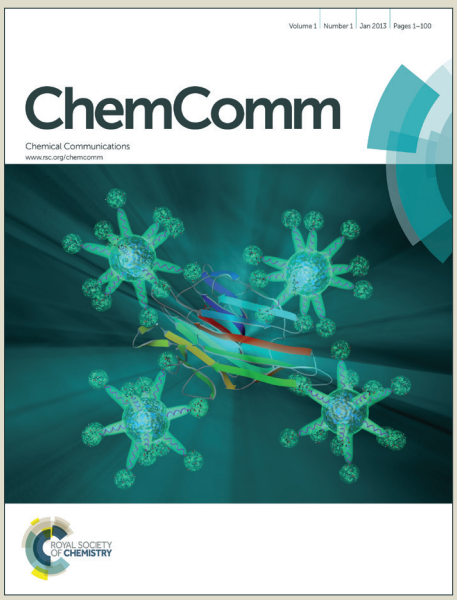

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ARTICLE TYPE

Label-Free Electrical Assay of Fibrous Amyloid β Based on Semiconductor Biosensing

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We propose, as an alternative to conventional spectroscopic assays, a simple method for discriminating fibrous amyloid proteins by using a label-free semiconductor-based biosensor. The highly sensitive assay is expected to be useful for accelerating amyloid related research.

Alzheimer's disease (AD) is a neurodegenerative disease, characterized by severe cognitive dysfunction and memory impairment.[1] Amyloid β protein (Aβ) is a major component of senile plaques, which is one of the pathological hallmarks of AD.[2, 3] According to the amyloid hypothesis of AD, Aβ aggregation and the formation of senile plaques precedes neuronal death in the AD brain. Therefore, Aβ is believed to play a significant role in the onset of AD.[4] Aβ40 and Aβ42 are two major species found in senile plaques of AD patients.[4] The difference between both species is the length of amino acid residues. However, Aβ42 is considered to play a greater role in the formation of amyloid compared with Aβ40, based on findings that Aβ42 is more prone to *in vitro* aggregation[5, 6] and that it is initially deposited under *in vivo* conditions.[7, 8] To understand the mechanisms underlying AD for screening potential inhibitors of Aβ aggregation to prevent its onset, the detection of aggregated Aβ is thus required.[9] In general, Thioflavin T (ThT) and Congo red (CR) bind specifically to fibrous Aβ, and are thus used to detect the aggregation of Aβ via a standard spectroscopic assay, which has been established to frequently distinguish β-sheet-rich fibrous Aβ.[10-13] However, this assay may be inefficient and labour intensive because it requires extrinsic labelling, multiple-stage procedures, and specific optical equipment. To eradicate these issues, we proposed a new semiconductor biosensor to detect Aβ aggregation without employing any labels. The semiconductor biosensing devices, which are expected to be manufactured in large quantities at low cost, detect potential changes on the gate surface in terms of the intrinsic charge of adsorbed species specifically interacting with probe molecules.[14-17] Among the various amyloid binding compounds,[18] CR possesses two amino moieties in its molecular structure, thus it is easily immobilized on the sensing surface by a conventional cross-linking reaction.[19] By using CR as a probe, we demonstrated in the present study that CR-immobilized surface of the FET gate was successfully constructed and utilized to monitor the pathologically important Aβ fibril formation ranging from fM to μM levels.

We examined the specificity of the assay device to fibrous Aβ

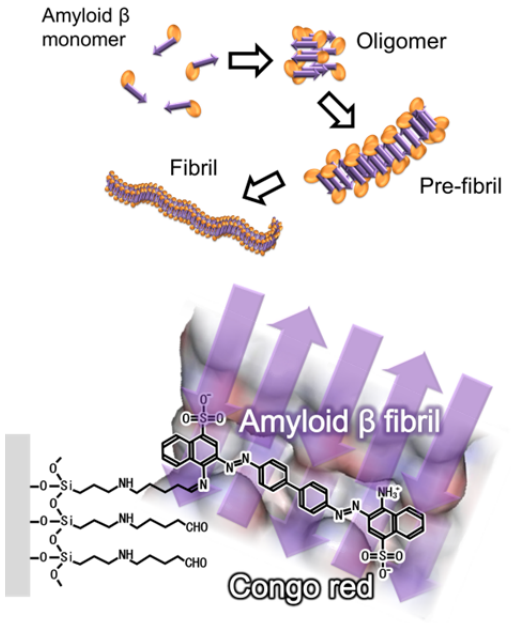

Figure 1. Schematic illustration of the label-free electrical assay for discriminating fibrous Aβ proteins. The assay detects intrinsic charges of fibrous Aβ proteins, which possess the interaction with Congo red molecules immobilized on the sensing surface.

by comparing the responses associated with the addition of fibrous Aβ42 and non-fibrous Aβ40. The rate of fibril formation of Aβ42 is reported to be faster than that of Aβ40 because the residues of Aβ42 are longer than those of Aβ40 by two hydrophobic amino acids.[5] First, the structural changes of Aβ42 and Aβ40 during the growth were observed by using atomic force microscopy (AFM) in an adsorbed state on the CR-immobilized substrate. Here, immobilization of CR molecules on the substrate was characterized by using X-ray photoelectron spectroscopy (Fig. S1, ESI†). For the specimens reacted with Aβ42, which was incubated for 1 day and 2 days after dropping its solution on the CR-immobilized surface, only a small number of molecules were observed (Fig. 2a, b), suggesting that monomeric Aβ42 has no interaction with the CR molecules. As anticipated, mature fibrils consisting of long and smooth structures were observed when its incubation period reached up to 3 days (Fig. 2c). The fibrous Aβ42 possesses a cross-β structure, which interacts strongly with the CR molecules, resulting in its specific adsorption on the

surface. Conversely, for Aβ40, little aggregates were observed for the incubation period reached up to even 3 days (Fig. 2d–f). These results show that the rate of aggregation of Aβ40 in this study is in good agreement with the result reported in the previous literature on amyloid formation.[5] It should be noted here that the Aβ fibrils were grown in the incubation tube, not on the CR-immobilized surface, without any distraction. (ESI†). Thus, the present AFM observation also confirmed that the Aβ42 protein changes its structure with the duration of incubation.

Subsequently, the electrical detection of the fibril Aβ proteins was examined by using the FET-based assay device (Fig. 2g). Here, the Aβ is considered to have a negative charge under the measurement conditions (pH = 7.4) because of its isoelectric point (pI) calculated equal to 5.5.[20] The significant response of the assay device was observed when the Aβ42 protein, which had been incubated for 3 days was added, while little response was observed for Aβ42 protein incubated for 1 day or 2 days. This result demonstrates that the Aβ42 incubated for 3 days, the specific adsorption of which on the CR-immobilized surface was discussed based on AFM results in the preceding section, can be electrically detected by the present FET device. The charge-detectable region for FET-based detection in terms of Debye length was calculated to be equal to ca. 7.5 nm in this system (0.01× PBS),[14] suggesting that the binding sites between the probe molecule (its length: 2.1 nm)[21] and target protein were located within the Debye length. The height of the fibril was measured by AFM to be equal to 5–10 nm, suggesting that the specifically-adsorbed fibrils occupied the charge-detectable region, whereas some fibrils were accumulated up to 20–40 nm. In contrast, no significant signals were detected when the surface of devices was exposed to the solution of Aβ40 proteins which had been incubated for the period between 1 day and 3 days. The Aβ40 proteins rarely aggregated with each other, suggesting that the Aβ40 did not possess the cross-β structure. Therefore, the label-free assay device discriminated between the fibrous and non-fibrous Aβ protein with high selectivity.

Surprisingly, it was revealed that the FET-based assay allowed quantitative sensing of fibrous Aβ42 (incubated for 3 days) ranging from 100 fM to 100 μM. Fig. 3 shows the sensor responses of three different FET devices displaying CR molecules with respect to fibrous Aβ42 concentration. Increasing the concentration of Aβ42 molecules increased the negative charge associated with the number of adsorbed Aβ42 within the Debye length, resulting in the increase in the response. It should be noted that, as anticipated, essentially no shift was observed when non-related protein, human serum albumin solution (100 μM), was used instead of Aβ42 solution (Fig. 3). In this study, the small probe immobilized on the device surface, which makes effective use of the charge-detectable region for the FET-based detection in terms of Debye length,[16] achieves the highly sensitive detection of fibrous Aβ proteins. In addition, the broad range of detection in nine orders of magnitude is responsible for the target Aβ fibril interacting with CR molecules at many points. The number of the binding sites between Aβ fibril and CR molecule increases with the growth of the fibril, suggesting that the dissociation constant (K_d) for the fibrils and CR molecules may change because of the polyvalent interactions.[22] Compared with the conventional ThT assay and CR assay, the present assay has an advantage of improving the sensitivity. To our best knowledge, the sensitivity of the above-mentioned conventional assays for the amyloid fibrils corresponds to no less than 0.1 μM levels.[10, 23, 24] Also, we believe that the assay will acquire additional advantage in minimizing loss of the measurement time compared with the conventional methods after accomplishing the decrease in reaction time to less than 15 min.

To date, label-free measurements, based on quartz crystal microbalance (QCM) and surface plasmon resonance (SPR), have clarified the mechanism of amyloid fibril elongation.[25] For such reported measurements, small pre-formed seed fibrils were immobilized on the sensor surface to monitor the amyloid growth, in other words, to intensively investigate how the amyloid aggregates grow. In the present study, the amyloidophilic molecule, Congo red, was immobilized as a probe to capture the amyloid fibrils, demonstrating that the electrical assay provides fruitful information regardless of whether the amyloid aggregates exist or not without depending on any large controlling instruments and close adjustments before measurements. Thus, we succeeded for the first time in detecting the amyloid fibrils at femtomolar levels and propose an alternative analytical method to quantify the total amount of aggregated Aβ *in vitro*.

The aim of this study is to establish a simple method for discriminating fibrous Aβ, as an alternative to conventional spectroscopic assays used in the amyloid related research. The FET biosensor developed in this study exhibited a strong

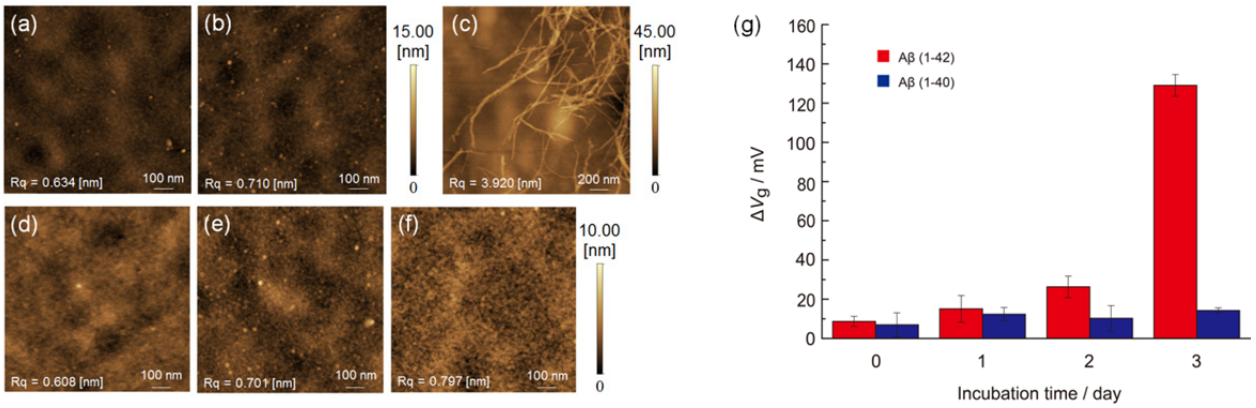

Figure 2. Comparison of Amyloid growth between Aβ42 and Aβ40. Atomic force microscopy images of surfaces of the assay device treated with (a–c) Aβ42 and (d–f) Aβ40. The incubation duration of the amyloid were (a, d) 1 day, (b, e) 2 days, and (c, f) 3 days, respectively. (g) Relation between incubation time and the sensor responses caused by Aβ adsorption for the assay devices.

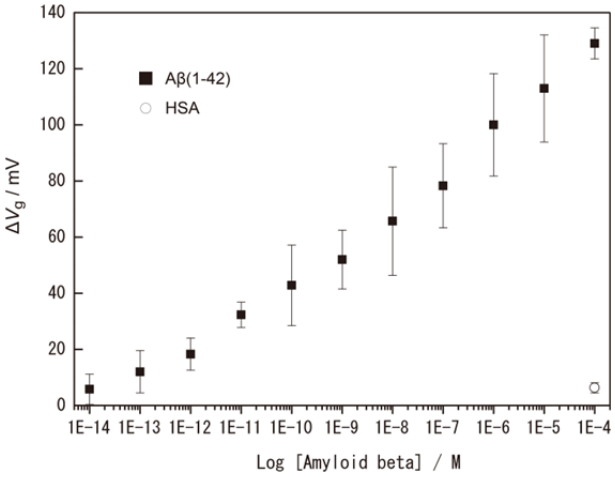

Figure 3. Quantitative determination of fibrous Aβ42 (incubated for 3 days) (closed symbols) ranging from 10 fM to 100 μM using the FET-based assay. The open symbol indicates the sensor response for non-related protein (HSA, 100 μM).

response on 3 days-incubated Aβ, which showed that it detected Aβ fibers. This type of highly sensitive assay will be useful for promoting the amyloid related research such as screening drugs to inhibit Aβ aggregation.[26] Recently, protofibrils and Aβ oligomers observed at the early onset of disease preceding fibril formation has been recognized as additional markers of Alzheimer's disease.[27] As reported previously, Aβ oligomers are small and metastable compared to fibrous Aβ, making them difficult to identify in biological specimens.[28, 29] An oligomer specific compound to construct Aβ oligomer specific FET biosensor will be used in the future.

Conclusions

We proposed a simple electrical assay to detect fibrous Aβ proteins without using any label. The assay is based on the semiconductor biosensing which detects the intrinsic charge of Aβ proteins adsorbed on the surface. It was demonstrated that the assay discriminated between fibrous and non-fibrous Aβ proteins, and determined the fibrous Aβ concentration at the femtomolar sensitivity. The present assay is a promising protocol to provide valuable information for ever-improving amyloid related research.

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Notes and references

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