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# Construction of a protein array on amyloid-like fibrils using co-assembly of designed peptides

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**Amyloid-like fibrils formed from *de novo* designed short peptides, made up a nanoscale scaffold on which streptavidin was arranged in a regular spacing, potentially allowing the development into an array technology utilizing bio-nanoconstructs.**

The aggregation of proteins and peptides into amyloid fibrils is most commonly associated with a variety of fatal disease such as Alzheimer's disease and the transmissible spongiform encephalopathies (prion diseases).[1] Amyloid fibril is an undesirable state for proteins as biomolecules, however, it is a fascinating nanoconstruct because of its highly ordered quaternary structure in which numerous β-stranded polypeptide chains align regularly.[2] Thus, this type of fibril has the potential to be engineered into novel proteinous nanoscale materials, and many fibrous proteins and peptides including of both natural and artificial origin have been investigated.[3–7] We have previously demonstrated that *de novo* designed peptides undergo self-initiated structural transition and fibril formation, equipped with representative properties of amyloid.[3a–d] Cofibril formation from two, three or four peptide species with well-designed amino acid sequences was achieved, so that the charged residues within the β-strands were complementary to each other.[3c,d] This indicates a possibility of functionalising the fibrils by co-assembling of peptides with various elements to develop a fibrillar peptide material as a well-ordered nanoconstruct (Fig. 1). Here, toward fabrication of a nanoscale array utilizing designed peptides, we report a protein (streptavidin) array immobilized on a fibrillar peptide assembly. The biotinylated peptides were incorporated into the fibrils co-assembled with non-biotinylated peptides, allowing regular immobilization of streptavidin onto the fibrils. The avidin-immobilized fibrils possibly make a nanoscaffold onto which a variety of functional groups are arranged. The engineered fibrous peptides can be applied to develop arrays of chemical and biological molecules on the nanoscale construct.

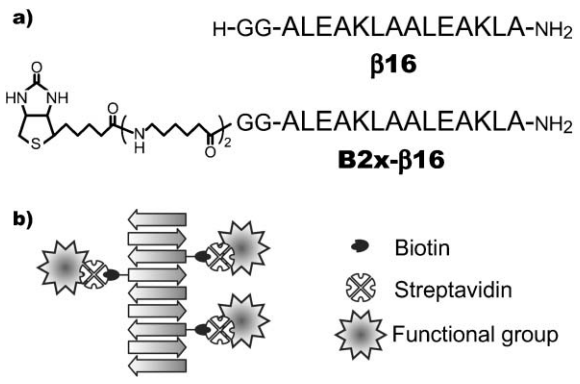


**Fig. 1** (a) Amino acid sequences of the designed peptides. (b) Illustration of the cofibrils containing biotinylated peptides and introduction of streptavidin with a functional group.

We designed β16 and biotinylated B2x-β16 peptides[8] that could assemble into amyloid-like fibrils according to the previous studies (Fig. 1).[3a–d] B2x-β16 peptide had a biotin group at the N-terminus as a streptavidin-binding domain. When the peptide sequence was drawn as a β-sheet model, hydrophobic Leu residues and hydrophilic Glu and Lys residues were separated on the different faces, and the amphiphilic faces were inverted at the centre of β-strand.

Cofibril formation and the morphology of the designed peptides were analyzed by circular dichroism (CD) measurement, thioflavin T (ThT) binding assay, and transmission electron microscopy (TEM). Biotinylated peptide (B2x-β16) was mixed with non-biotinylated peptide (β16) in a non-aggregated state in trifluoroethanol (in the molar ratio of 0–1% B2x-β16), and the peptide secondary structure was analyzed by CD measurements. Both β16 alone and mixed peptides containing B2x-β16 formed a slightly α-helical ($[\theta]_{222} = -6500$ deg cm$^2$ dmol$^{-1}$ for mixed peptide containing 1% B2x-β16), but predominantly a random coil structure shortly after dilution in 20 mM Tris·HCl buffer (pH 7.4). Through the incubation at 50 °C, transformation to the β-sheet structure occurred time-dependently, as indicated by CD spectra with a negative peak at 219 nm and a positive peak at 203 nm ($[\theta]_{219} = -13\,900$ deg cm$^2$ dmol$^{-1}$ and $[\theta]_{203} = 54\,200$ deg cm$^2$ dmol$^{-1}$ for the mixed peptide containing 1% B2x-β16). After the transition, ThT added to the peptide solutions exhibited strong fluorescence emission at 480 nm (excited at 440 nm). ThT bound to amyloid fibrils is known to show characteristic fluorescence emission, and is widely used as a probe for amyloid fibrils. The result of ThT binding assay indicates that the β-structural peptides form amyloid-like fibrils. Direct observation of the peptide aggregates by TEM revealed that the peptides formed fibrillar structures (Fig. 2(a)). The fibrils were ~20 nm in width and of varying length (several hundred nanometers to several micrometers).

Specific introduction of streptavidin onto the matured fibrils was achieved using co-assembling fibrils with biotinylated peptides. To pick out streptavidin under TEM observation, the streptavidin modified with colloidal gold (Au-Av: diameter of colloidal gold is 20 nm) was added to the matured fibrils (0–1% B2x-β16).[9] No Au-Av was observed on the fibrils formed by β16 alone (containing no biotin), while attached Au-Av particles were observed on the fibrils containing B2x-β16 (Fig. 2(a)). These results showed that biotinylated peptides were incorporated into fibrils, and streptavidin molecules attached to the biotin groups on the fibril. Comparing with the cofibrils containing different ratios of B2x-β16 (1–0.01%), the quantity of Au-Av particles accumulated on the fibrils was inclined to become fewer, as the ratio of B2x-β16 became lower. This suggests that there is a correlation between the mixing ratio of two peptides at a random coil state and the existence ratio of biotin in the formed fibrils.

The distance between successive Au-Av particles bound to the fibrils containing 1% B2x-β16 indicated that the gold particles were distributed preferably on average every 50, 100, 150 nm (Fig. 2(b)), suggesting a periodic binding of Au-Av particles on the fibrils at

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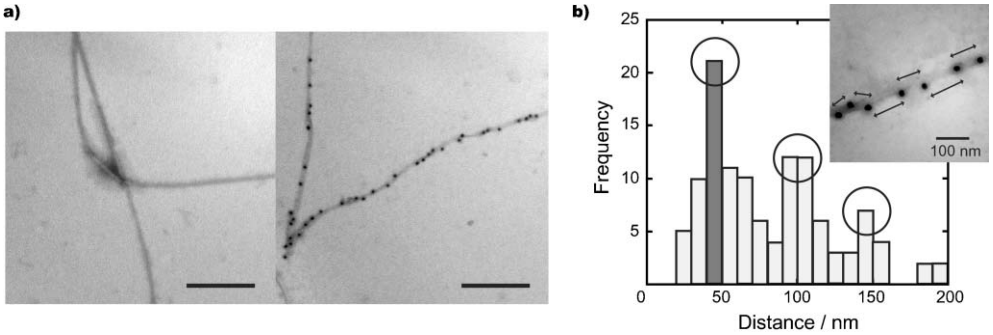


**Fig. 2** (a) TEM images of β16 fibrils (left) and the cofibrils containing 1% B2x-β16 (right), onto which gold-labelled streptavidin was added. Scale bars, 500 nm. (b) Histogram showing the distance between gold particles bound to β16 fibrils containing 1% B2x-β16.

every *ca.* 50 nm. The distance of hydrogen bonds between peptide strands is 4.7 Å along the fibril direction in the cross-β-sheet structure,[2] and in the fibrils of our designed peptide with homologous sequence it was found to be 4.1 Å as elucidated by X-ray reflection.[3c] The average 40–50 nm intervals between Au-Av particles represent the distance about 100–120 peptides aligned along the fibril axis. Hence, it can be ideally considered that one biotinylated peptide is preferably incorporated in every 100–120 peptides during fibrillogenesis. However, the observed fibrils with ~20 nm width are supposed to consist of several β-sheet filaments bundling regularly,[2,5,6] because the 5–6 nm-length peptide of β16 (16 amino acid residues) makes a single filament of about 5 nm width, if the peptides align perpendicularly to the fibril axis. The assumed structure suggests that distances between biotin groups along the fibril direction should be shorter than 50 nm if biotinylated peptides were incorporated every 100 peptides. Despite this, Au-Av particles were bound every 50 nm in our experiment. One of the possible explanations is that streptavidin would bind with plural biotin groups. Sterptavidin has four binding sites for biotin, and would bind to plural biotin groups closely arranged in the fibrils, probably resulting in no observation of intervals less than 20 nm and in broad peaks in the histogram. Another speculation is that biotin groups could be exposed enough to bind streptavidin every 50 nm. Dobson *et al.* reported that periodic binding of anti-fluorescein IgG to cofibrils assembled from insulin and a partial short peptide from other protein labelled with fluorescein.[7] The insulin cofibrils showed a regular helical twist every 60 nm along the fibril axis, and the antibodies bound to fluorescein exposed according to the structural periodicity of fibrils such as helical twist of the fibrils. However, the binding of the antibody to other cofibrils made from two types of other short peptides with native sequences (with no distinct design in their sequences) did not show high periodicity. On the other hand, in our experiments the periodic binding of avidin to the fibrils was demonstrated, although the fibrils were formed from short peptides. Our results may arise from the purposefully designed peptide sequences considering the side-chain interactions to affect regular fibril formation. We have previously demonstrated using *de novo* designed peptides that the intermolecular side-chain interactions such as ionic pairing, hydrogen bonding, and hydrophobic interaction were constitutive for the fibril formation.[3] The peptides used in this study were also well designed considering the matching of the side-chain interactions between antiparallel β-strands to regulate the fibril formation. It is conceivable that the formed fibrils have a regularity or periodicity in the fine structure, although the direct evidence such as helical twist could not be found in TEM images. In our speculation, the biotin groups in the fibrils were exposed to the aqueous environment according to the structural periodicity of fibrils determined by the side-chain interactions, leading to the periodic binding of avidin onto the fibrils. The biotinylated peptides were quantitatively and randomly incorporated into the cofibrils. Therefore, the frequency of bound Au-Av particles on the fibrils consequently reflects the structural periodicity of fibrils owing to the regular peptide alignment. It is suggested that the arrangement of streptavidin on the peptide fibrils can be controlled using well-designed peptides.

In conclusion, we have succeeded in the arrayed immobilization of natural protein onto the fibrillar nanoconstruct formed from designed peptides. Streptavidin was disposed regularly at intervals of 50 nm on the fibrils containing biotinylated peptides. Other functional molecules can be arrayed onto the fibrils used in this study by adding functionalized streptavidin to biotinylated fibrils and/or adding functionalized biotin to streptavidin-immobilized fibrils. In fact, the fluorophore-labelled avidin bound to the fibrils has enabled single fiber observation (data not shown). Furthermore, engineered peptide fibrils formed from small peptides is of advantage, because the peptide element constructing fibrils can be easily modulated and modified by its amino acid composition,[3e] chain length, and incorporation of functional groups. The present study implies that a variety of functional molecules can be immobilized on peptide nanofibrils in controlled distance and amount, thus indicating the possibility to design nanoscale bioconstructs with functionalities.

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8 The peptides were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase peptide method, then purified by semi-preparative reversed-phase HPLC, and identified by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) and amino acid analysis.

9 Peptide solutions were diluted to 1.0 μM and mixed with Au-Av solution, then incubated for 0.5–1 h. Final concentrations were 500 nM for peptides and 7 nM for Au-Av. Mixed samples were adsorbed to a carbon-coated copper grid and then negatively stained with 0.2% (w/v) aqueous phosphotungstic acid.