NOVEL APPLICATION OF INSULIN AMYLOID SUPERSTRUCTURES AS TEMPLATES FOR SURFACE-ENHANCED RAMAN SCATTERING

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Introduction

Merging biostructures with nonbiological components, such as metallic layers, may result in novel hybrid materials with exceptional characteristics. Surfaces of certain metals are particularly fascinating because of their unique optical properties utilized for example in surface-enhanced Raman scattering (SERS) [1]. Consequently, these properties make them especially useful parts of systems with applications in fields like microelectronics, plasmonics or clinical diagnostics.

The unparalleled diversity of polypeptide chain conformations makes proteins and polypeptides unusually versatile components for coupling with other materials. A special class of proteinaceous structures - amyloid fibrils, which are nanometric β-sheet-rich aggregates composed of orderly-stacked misfolded protein molecules – thanks to their unique thermodynamic stability [2] seems to be one of the most promising.

In our previous works we have shown that hydrodynamic forces, such as shear flow, align immature insulin fibrils into highly-organized superstructures with remarkable chiroptical properties termed –ICD/+ICD after the strong extrinsic Cotton effect detected through induced circular dichroism (ICD) in amyloid-bound achiral dye - thioflavin T [3]. These structures retain their stability and geometry upon transferring onto solid substrates and do not change properties even after prolonged storage at room temperature. Moreover, similarly to other amyloids, –ICD fibrils are resistant to proteases and chemical denaturants. The special microarchitecture of the –ICD insulin aggregates has inspired us to investigate their application as a nanoscaffold for metallic SERS-active surfaces using 4-mercaptobenzoic acid (4-MBA) as a model inelastic light scatterer often used in Raman studies [4].

Experimental

Preparation of cross-linked –ICD insulin amyloid superstructures

–ICD fibrils were prepared by vortexing 1 wt. % solution of bovine insulin (from Sigma, USA) in 0.1 M NaCl, pH 1.9 for 24 h at 60 ^{o}C / 1400 rpm using Eppendorf Thermomixer Comfort accessory. Once fibrillation of insulin was complete, a small aliquot was collected for Raman measurements, while the remaining suspension of –ICD fibrils was mixed with an aqueous solution of glutaraldehyde (GA) to the final concentration of GA 2.5 v/v %. Meanwhile, 2.5 v/v % solution of GA in saturated disodium phosphate (Na_2HPO_4) buffer in 0.1 M NaCl, pH 9 was prepared. The buffered GA solution was poured slowly to vigorously stirred suspension of –ICD fibrils in 2.5% GA, until pH reached 7.2. After 1 h of agitation at room temperature –ICD fibrils were centrifuged and washed repeatedly with excess of deionized water for removal traces of the cross-linker and salts.

Scanning electron microscopy measurements

For scanning electron microscopy (SEM), droplets of aqueous suspensions of cross-linked –ICD fibrils were deposited on silicon wafers and vacuum-dried. Dry films of fibrils were sputtered with 5 nm layer of Au/Pd alloy. The images were collected on a Zeiss Leo 1530 microscope.

SERS and Raman measurements.

In order to obtain amyloid scaffolds for SERS, aqueous suspensions of cross-linked –ICD fibrils were dropped onto microscopic glass slides and allowed to dry up in vacuum. Protein films were then sputtered with a 100 nm layer of Au. Subsequently, the metallic surface was covered with fresh 2 wt. % solution of 4-MBA in tetrahydrofuran (THF). After a few minutes, excess of unbound 4-MBA was rinsed out with pure THF. Dried samples were subjected to SERS measurements in a 180^{o} backscattering mode (Fig. 1a). All Raman measurements were carried out with excitation at 1064 nm with 0.5 W laser power. Other details are described elsewhere [5].

Estimation of the Enhancement Factor in SERS spectra

The Enhancement Factor (*EF*) is defined as:

$$EF = (I_{SERS}/I_{bulk}) \times (N_{bulk}/N_{ads}),$$

where N_{bulk} is the number of molecules sampled in the bulk, N_{ads} is the number of molecules adsorbed and sampled on the SERS-active substrate, I_{SERS} is the intensity of a vibrational mode in the surface-enhanced spectrum, and I_{bulk} is the intensity of the same mode in the Raman spectrum. All the intensities were obtained directly from the experiment. We calculated the *EF* value according to intensity of the $\nu(C\text{-}C)_{ring}$ ring-breathing mode at 1073 cm^{-1} (SERS) / 1093 cm^{-1} (bulk). When determining N_{ads} in the laser spot, it was assumed that 4-MBA molecules are adsorbed as a monolayer on Au surface with the density of 0.5×10^{-9} mol/cm^2 [6]. For our experimental setup the penetration depth of the laser beam was estimated to be ca. 25 μm, which, given the density of 4-MBA (1.35 g/cm^3) yielded N_{bulk} equal to 2×10^{-5} mol/cm^2.

Results and Discussion

The insulin amyloid fibrils were prepared in typical conditions, i.e. at low pH, in the NaCl presence and under strong agitation. The positive net charge of insulin at pH 1.9 hampers – through electrostatic repulsion –aggregation and prevents self-assembly of higher superstructures. However, in the presence of chloride counterions, screening of Coulombic forces is provided and precipitation of –ICD fibrils is facilitated. Nevertheless, it is necessary to remove NaCl, which when crystallizing on the fibrils, may become a competing artifact scaffold for metallic layer. Because elution of the salt from –ICD fibrils promotes their disassembly [3], this was prevented by cross-linking with GA. The degree of stability of –ICD superstructures was confirmed by ICD measurements of amyloid-bound ThT molecules [5].

The SEM image in Figure 1b present how individual insulin fibrils of ca. 2 nm in diameter align into larger domains covering the surface of –ICD insulin aggregate with variety of spikes and fissures of different sizes, and roughening Au film deposited directly on the protein film, as indicated in Figure 1a.

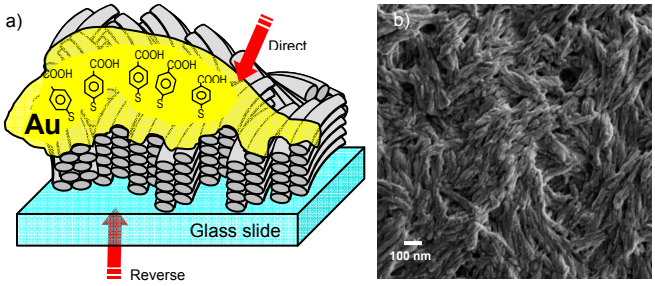

Fig. 1 a) Preparation of glass slides with sandwiched 4-MBA/Au/cross-linked -ICD amyloid layers for SERS measurements. The red arrows indicate two directions of the backscattered incident laser beam examined in this study: direct and reverse. b) The corresponding SEM image obtained after sputtering amyloid fibrils with Au/Pd.

In Figure 2, a Raman spectrum of 4-MBA prepared on the Au/–ICD sandwich system (a) is juxtaposed with spectra of bulk 4-MBA (b), and intact insulin fibrils (c). The spectrum of the Au/–ICD-bound 4-MBA film has two major peaks at 1584 and 1073 cm^{-1} assigned to $\nu(C\text{-}C)_{ring}$ ring-stretching and $\nu(C\text{-}C)_{ring}$ ring-breathing modes, respectively [4], but lacks other bands present in the Raman spectrum of 4-MBA (Fig. 2a and 2b). The absence of the 2564 cm^{-1} band corresponding to S-H stretching vibrations could be easily explained as the evidence of binding thiol molecules to the substrate via sulfur atom. However, the absence of the C-H stretching band at 3065 cm^{-1} must be considered as a slight alteration of the selection rules when the Raman scattering is enhanced through the SERS phenomenon. Indeed, the spectrum shown in Figure 2a is very similar to SERS spectra of 4-MBA presented earlier [4]. The red-shifting of the $\nu(C\text{-}C)_{ring}$ ring-stretching and ring-breathing peaks has also been described therein. To conclude, the spectrum of 4-MBA on the Au/–ICD sandwich shows all the features of SERS rather than of bulk Raman scattering.

We have estimated the *EF* to be about 10^4 (Experimental) by comparing intensities of the $\nu(C\text{-}C)_{ring}$ ring-breathing modes for bulk and surface-bound 4-MBA molecules. Notably, the SERS spectrum of 4-MBA seems not to be affected by contributions neither from bulk 4-MBA, nor the protein scaffold, as the comparison with the Figure 2c proves. This observation is quite similar to those presented in previous SERS studies where protein molecules, despite contiguity of the metallic surface, remained absent from the SERS spectrum [7]. Although the lack of protein bands in SERS spectra is beneficial for scaffolds selected on the basis of being spectrally invisibe, it nevertheless remains intriguing. More detailed discussion of this concern is provided elsewhere [5].

Conclusion

In conclusion, we have shown that newly-synthesized insulin amyloid superstructures may serve as nanotemplates for Au films becoming an effective substrate for SERS of 4-MBA. Obtained composite biomaterial may prove beneficial in areas such as photonics and material chemistry.

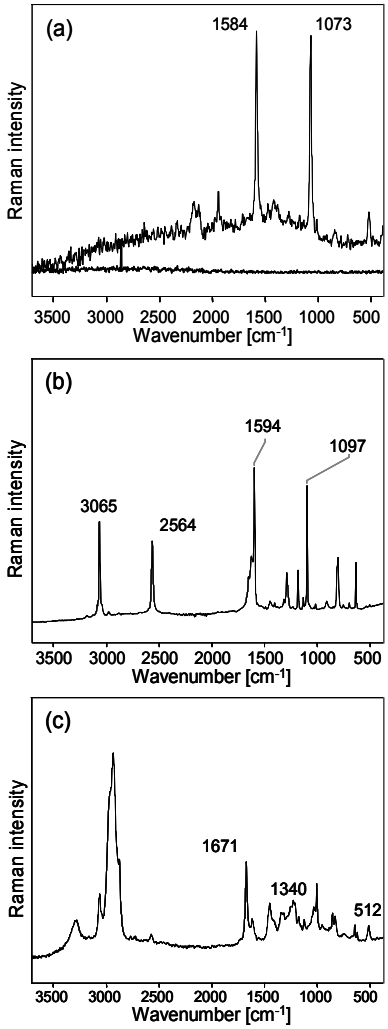

Fig. 2 (a) SERS spectra (direct scattering) of 4-MBA bound to Au/cross-linked -ICD amyloid superstructures (top), bound to smooth Au film on a clean glass slide (bottom). (b, c) Raman spectra of bulk 4-MBA and insulin fibrils, respectively.

References

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