Single-molecule studies on individual peptides and peptide assemblies on surfaces

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This review is intended to reflect the recent progress in single-molecule studies of individual peptides and peptide assemblies on surfaces. The structures and the mechanism of peptide assembly are discussed in detail. The contents include the following topics: structural analysis of single peptide molecules, adsorption and assembly of peptides on surfaces, folding structures of the amyloid peptides, interaction between amyloid peptides and dye or drug molecules, and modulation of peptide assemblies by small molecules. The explorations of peptide adsorption and assembly will benefit the understanding of the mechanisms for protein–protein interactions, protein–drug interactions and the pathogenesis of amyloidoses. The investigations on peptide assembly and its modulations could also provide a potential approach towards the treatment of the amyloidoses.

1. Introduction

Peptide assembly has been drawing considerable interest because of its significance in biological recognition associated with interactions between proteins and interactions between peptide drugs and protein targets, novel materials based on peptide assembly and amyloid aggregation associated with many diseases. It is significant and valuable to explore the peptide assembly mechanism via non-covalent molecule–molecule interactions. There are many reports exploring the peptide assembly structures in bulk systems. Different structures such as nanotubes, nanofibres and vesicles have been observed for various potential or practical applications, such as biomineralization materials, gene and drug delivery, three-dimensional cell culture and antimicrobial reagents. The Stupp, Zhang and Gazit groups have done pioneering works in this field, and readers interested in it could refer to several review papers for detailed discussion [1–3].

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The assembly of peptides on solid surfaces is considered not only as a reflection of biological processes, but also for heterogeneous biocatalysis, which has drawn much attention in recent years. Peptide assembly on surfaces has been investigated from various aspects, especially molecular-level studies by scanning tunnelling microscopy (STM). Peptide assembly is characteristic of hierarchical molecular assembly with multi-level processes involved, which is initiated by the assembly of building blocks into clusters via non-covalent interactions, followed by the assembly of clusters into more complicated superstructures [4]. For instance, many designed peptides and amyloid peptides commonly go through hierarchical assembly from nanoparticles to protofibrils, and then twist together into nanofibres. The detailed structural information and the associated assembly mechanism of the hierarchical peptide assembly would be the key to control and modulate it for biological and material applications.

STM has become a powerful tool for studying organic and biological molecules owing to its high structural resolution and adaptability to various environments, including liquid–solid interfaces and ultrahigh vacuum (UHV) conditions. It has been successfully demonstrated to probe the structures of a variety of molecular monolayers with submolecular-level resolutions. The investigations on the assembly structures of organic functional molecules by using STM have deepened our understanding of molecular interactions and molecule–substrate interactions, especially hydrogen-bond-involved molecular interactions. Many unique molecular assembly structures, including host–guest complexes, multi-component hydrogen-bonded networks and chiral assemblies, have been fabricated in a controlled way. Readers who are interested in assemblies of organic functional molecules are referred to previous reviews [5–9]. The applications of the STM method in studies of biomolecules also have fascinated many researchers for its high structural resolution without complicated sample preparation, for instance single crystal growth, and for its sensitivity to electronic structure of the molecules. Kawai group has demonstrated the partial sequencing of single DNA molecules using a UHV STM system. The submolecular image of long DNA and the spectroscopic investigations on different bases gave rise to possible recognition of DNA bases along the DNA chain [10]. Recently, more and more works have been focused on peptide assemblies ranging from dipeptides, tripeptides, polyamino acids to model peptides with blocked sequence, all the way to disease-related peptides with complicated sequences. These works have opened a window for the surface sciences into the biological world and vice versa.

We distinguish the subject of this review from the traditional two-dimensional crystalline assembly of proteins on mica surface for structural analysis by high-resolution atomic force microscopy (AFM) imaging and biomolecular adsorption on silicon or oxide surface for life origin and catalysis. For these subjects, the reader is referred to recent review papers [11, 12]. In this review, we present the recent progress on the molecular-level investigation of peptide assembly as well as the interactions between peptides and functional molecules. The diverse interactions, such as intermolecular and intramolecular hydrogen-bond interactions, hydrophobic interaction, electrostatic interaction and so on have been considered to underline the various assembly behaviours of peptides observed in STM studies. The molecular structures of various peptides are reviewed from individual peptide molecules to peptide assemblies, from simple peptides, such as dipeptides, tripeptides, tetrapeptides and blocked polyamino acids, to relatively complicated peptides with biological functions. Furthermore, the modulator effects on the aggregate morphology and aggregation behaviours of amyloid peptides in solution are discussed as well.

2. Structural analysis of single peptide molecules

Single-molecule studies on individual peptide molecules are essential to the understanding of the biological functions and the assembly mechanisms of peptides. For its capability of high-resolution structural analysis and environmental adaptability, STM could be applied to study
peptide structures at the molecular level. Lingenfelder et al. [13] and Kalashnyk et al. [14] have demonstrated the structural analysis of single peptide molecules and their assemblies by UHV-STM, which will be discussed in detail later. In most of the cases, it is very challenging to immobilize the individual peptide molecules on a surface stably enough for STM imaging. A molecular matrix is applied in several recent reports for accommodation of peptide molecules inside the cavity of the host network, or for stabilization of the folding conformation and assembly of the peptide in a lipid bilayer.

Some organic molecules are capable of forming a range of nanoporous molecular networks via hydrogen bonds, metal–ligand coordination bonds and van der Waals interactions. Such networks with well-defined cavity and geometry could be used to trap guest species. Short linear peptides have been reported to be included in the molecular matrix with elongated cavities [15, 16]. Single-molecule study on a cyclic peptide, an immunosuppressant cyclosporine A (CsA), has also been reported with molecular matrix of a star-shaped oligofluorene derivative (StOF-COOH3) [17]. Hexagonal porous network structures are formed by StOF-COOH3 molecules at the liquid–solid interface with an inner cavity size of 4.7 nm (figure 1). The individual CsA molecules are dispersed within the hydrophobic cavity of the network formed by StOF-COOH3 and the high-resolution STM images of CsA show polygon-like characteristics. As this cyclic peptide has ion channel function through complexation with ions, such as Ce$^{3+}$, Mg$^{2+}$ and Ca$^{2+}$, the CsA–Mg$^{2+}$ complex is also investigated at the molecular level. Two adsorption characteristics observed in the CsA–Mg$^{2+}$ complex suggest its asymmetrical configurations and the difference in their binding energies is estimated to be 1.88 kJ mol$^{-1}$.

Direct visualization of pores formed by alamethicin (Alm) and gramicidin D (gD) in a matrix of phospholipids using electrochemical STM (EC-STM) (figure 2) validates the barrel-stave model [18,19]. Two-dimensional crystalline structures of Alm are formed with the assistance of the lipid. The STM images of Alm/lipid system suggest that every channel consists of six Alm molecules and each Alm is shared between the two adjacent channels [18]. The STM images of gD/lipid system show triangular pores corresponding to the molecular structures of gD from the protein data bank [19]. The latter study also proved that the peptide affects only the properties of the lipids next to it, while not the ordering of the lipids in the bulk matrix [19]. These investigations provided direct evidence for the peptide aggregation and peptide–lipid interactions, which is beneficial for the understanding of their biological activities.
3. Adsorption and assembly of peptide on surface

(a) Structural effects on the adsorption and assembly of peptide

In the early studies on adsorption of amino acids, such as glycine and alanine, on metal surfaces, it was demonstrated that hydrogen-bond formation is the driving force for the surface superstructure formation [20–23]. Glycine or alanine molecules lie approximately parallel to the surface and the molecules bond to the surface through the two oxygen atoms of the carboxylate group and the nitrogen atom of the amino group. The adsorption symmetry and
Figure 3. (a,b) Two STM images of di-L-alanine on Cu(110) at low coverage. The molecules were evaporated at a sample temperature of 248 K and scanning took place at 208 K. Two islands in (b) showing parallel (P) or antiparallel (A) di-L-alanine molecules in adjacent rows. Image sizes of (a,b) are 16 × 16 nm and 9 × 9 nm, respectively. (c) STM image of dialanine islands on Cu(110) (25 × 25 nm). All molecules in an island are oriented parallel or antiparallel to the [332] direction as indicated by the two directions of the arrows. The dialanine was evaporated at a sample temperature of 363 K and imaged at 198 K. (Adapted from [27], Copyright 2003, Elsevier.)

the molecule–substrate interactions of the amino acids have provided valuable information for the understanding of the peptide adsorption and assembly structures on surfaces.

As the first report on peptide adsorption on a surface, Barlow et al. [24] reported the bonding, organization and dynamical growth behaviours of the two homo-tripeptides tri-L-alanine and tri-L-leucine on Cu(110) by using reflection-absorption infrared spectroscopy. Long-range-ordered surface structures with molecular axes perpendicular to the surface were observed for tri-L-alanine peptide under high flux conditions. The saturated bilayer phase at higher coverage was very similar to an antiparallel β-sheet conformation characteristic. Under low flux conditions, a more flat-lying molecular orientation was predicted owing to the chelation of amide C=O with the copper surface. Under all conditions, the main driving force for the tripeptide assembly structures on the surface was ascribed to hydrogen-bond formation. The intact molecular structures of tripeptides on the metal surface were also confirmed, which encouraged the peptide assembly studies on surfaces [24].

A dipeptide is the smallest possible peptide molecule consisting of two amino acids. Diphenylalanine (Phe-Phe) has been found to assemble into functional nanostructured materials [25,26], so adsorption and assembly of dipeptide on surface also attracted much attention. Di-L-alanine molecules [27] were observed to nucleate into small islands along (332) direction on Cu(110) surface, consisting of single or double rows of molecules (figure 3). In most cases, the STM images show that the dialanine molecules appear as single bumps, while under certain undefined tip conditions the molecule is imaged as a pair of protrusions, a smaller one and a larger one. The paired images give rise to the identification of the peptide organization in the assembly structure. The dipeptide molecules are always oriented in the same direction within the same row, whereas molecules in adjacent rows may be either parallel or antiparallel. The alignment of molecules within a row in the same direction indicated hydrogen-bond formation between the terminal carboxylic group of one molecule and the terminal amino group of another molecule. The dependence of the molecular directions on the thermal history of the surface also revealed the presence of an energy barrier between parallel and antiparallel adsorption directions.

Recently, the supramolecular structures of Phe-Phe peptide were investigated by UHV-STM [13]. STM images of Phe-Phe assembly revealed that the dipeptide molecules could be identified by characteristic two neighbouring bright spots. The Phe-Phe molecules are capable of forming ordered supramolecular assembly structures via hydrogen-bond and hydrophobic interactions between the dipeptides. The video recording of STM observations of two isolated
molecules suggests that they experience the first change in the adsorption geometry by forming an initial pair, and then undergo several further rearrangements until a stable pair is formed as final state. This result implements a single-molecule-level dynamic picture of enantioselective molecular interactions for possible mechanism of conformational adjustments.

The molecular-level investigation of peptide assemblies for the simplest dipeptides with different side chains and chiralities would lead to a deeper understanding of the interaction mechanisms of peptide assemblies and provide hints and information for the assembly structures and mechanisms of longer peptides with biological functions.

**b) Odd–even effects on the adsorption and assembly of peptide**

Odd–even effect on assembly of alkanes and alkane derivatives has been widely studied, which could be described as assembly structure changes associated with the odd or even number of structural units, such as CH₂ group or other moieties [28,29]. Recently, we reported the odd–even effect of assembly structures of polyglutamine (PolyQ) on graphite surface [30]. The statistics of the apparent peptide strand length revealed different length distributions for PolyQ7 and PolyQ8, in which one predominant peptide length was observed for PolyQ7, but three major apparent strand lengths for PolyQ8 (figure 4). This phenomenon could be considered as a reflection of one predominant peptide conformation for PolyQ7 and three coexisting ones for PolyQ8. This asymmetric adsorption effect of peptides reflected the odd–even effect of residue numbers on peptide adsorption conformation and assembly structures.

**c) Sequence effect on the adsorption and assembly of peptide**

As STM image contrast is a convolution of the topographic structure and the electronic structures and influenced by stability and the adsorption conformation of the molecules, the sequence recognition of peptide should be very challenging. If we consider the contribution from the peptide backbone is nearly the same, then the contribution to apparent brightness from the side groups would be dominant which means that the recognition of peptide sequence would be possible in principle.

We have recently endeavoured to study the correlation between the brightness contrast in STM images and specific residue groups for understanding the sequence effect on peptide adsorption and assembly. Two synthetic model peptides, R₄G₄H₈ and F₄G₄H₈, containing four kinds of residue groups (Phe-benzyl, Arg-carbamidine, Gly-hydrogen and His-imidazole) representing typical types of interactions between residues are investigated. The results (figure 5) [31] show that the observed brightness contrast in STM images follows the order Phe > His > Arg > Gly, which could help distinguish the residue groups in R₄G₄H₈ and F₄G₄H₈. Consistent with STM results, molecular dynamics (MD) simulations show that the interaction energy of the four different residues with graphite surfaces follows the same order, indicating that the brightness contrast of STM images is correlated with the interaction energies of the different residues with graphite surfaces. This work could be beneficial for studying peptide-related interfacial processes such as site-specific interactions between molecular species or surface atoms and peptides.

Kalashnyk *et al.* [14] have also made great contribution in sequence effect on the peptide assembly. Highly ordered adsorption structures were observed for the two tetrapeptides KFFE and KVVE, which have been reported as the minimal peptide units forming amyloid fibrils *in vitro*. The same terminal K and E residues with different central F and V residues could facilitate the investigation of the impact of π–π interactions on the assembly process. The high-resolution STM results (figure 6) reveal two distinct patterns with parallel arrangement for KFFE and antiparallel one for KVVE, which is similar to the arrangements in fibrillar structures. The individual residues on the peptide chains and dynamic changes between residue conformations are also identified by STM investigations at molecular level. From a thorough statistical analysis of the STM images, the intrapeptide correlations are weaker than the interpeptide correlations, which suggest that π–π interactions do not seem to be dominant in peptide ordering.
Figure 4. (a,d) High-resolution STM images of PolyQ7 and PolyQ8 coassembled with 4Bpy, respectively. (b,e) Length distribution histograms of PolyQ7 and PolyQ8 measured from the STM images, respectively. The step size of the chart is 0.325 nm. (c,f,g) Proposed models of PolyQ7 and PolyQ8. C-terminus of PolyQ forms hydrogen bond with 4Bpy and N-terminus of PolyQ prefers to assume a head-to-head configuration on HOPG surface. (Adapted from [30], Copyright SIOC, CAS, Shanghai, and Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.) (Online version in colour.)

(d) Substrate effect on peptide assembly

The peptide assembly structures are dominated by the intermolecular interactions and molecule–substrate interactions, so the substrate effect on peptide conformation and peptide assembly is one of the important topics in peptide assembly on surfaces. In recent reports, conformational change and peptide assembly dynamics have been found when some peptides adsorbed and assembled on graphite surfaces.
A two heptad-repeat peptide, DELERRIRELEARIK, with mostly $\alpha$-helical secondary structures was studied with X-ray photoelectron spectroscopy, circular dichroism (CD) spectroscopy and STM [32]. STM observations revealed that the adsorbed polypeptides formed lamella-structured assemblies on the graphite surface, with neighbouring peptide separation of 4.7 Å, which suggested $\beta$-sheet-like secondary structures distinctively different from the typical $\alpha$-helical structure in solution (figure 7). The CD results with and without addition of graphite particles provided supporting evidence of the structural transformation from $\alpha$-helical to $\beta$-sheet secondary structures. Based on the above experimental results, the Wei group [33] performed all-atom MD simulations, to investigate the dynamics of early stage conformational transition in the absence and presence of a graphene sheet. The results confirmed the unfolding and assembly of the peptide chains into an amorphous dimer at graphene surface. The adsorption and unfolding of the peptide at graphene surface were initiated from the C-terminal region owing to strong interactions between RIK segments and graphene surface.

In other related studies, the fragment of accessory viral protein R (Vpr) in human immunodeficiency virus (HIV), Vpr13–33, was observed to undergo conformation change from $\alpha$-helix to $\beta$-sheet structures in the presence of graphene oxide (GO) (figure 8) [34]. AFM and CD experiments revealed the referential adsorption of Vpr13–33 on GO accompanied by the conformation change. The submolecular structures of the Vpr13–33 peptide assembly on graphite surface by STM confirmed the $\beta$-sheet structures of Vpr13–33 on GO surface. These conformation change and accelerated aggregation on GO surface are believed to be related to the hydrophobic interactions. The reduced cytotoxicity of Vpr13–33 to neuroblastoma cells and T cells by GO is proved by fluorescent leakage assay to be associated with the inhibited ‘pore forming’ function of Vpr13–33 by conformation change, preferential adsorption and accelerated aggregation. Such
Figure 6. (a) STM image of row structure for KFFE on the Au(111) surface and the representative model with Phe side chains highlighted in green. (b) The side view of the model and the oval indicates putative surface binding. (c) STM image of antiparallel assembly structure for KVVE on the Au(111) surface with overlaid top view model. The oval indicates antiparallel organization of the Lys side chain. (d) Side view model with Au surface indicated by the horizontal bar. Val side chain carbons highlighted by transparent spheres for (c) and (d). (Adapted from [14], Copyright 2012, American Chemical Society.) (Online version in colour.)

Figure 7. Schematic model of the structural transformation of the polypeptide DELERRIRELARIK. In the top left corner, the polypeptide shows α-helical structure in solution. The polypeptide structural transformation induced by the surface is proposed to be α-helical in solution to a β-sheet-like structure on the HOPG surface. (Adapted from [32], Copyright 2009, American Chemical Society.) (Online version in colour.)

conformational rearrangements upon adsorption on a hydrophobic surface could benefit the studies on protein–surface interactions.

Surface reconstruction upon adsorption of small molecules has been intensively studied in the surface science field, whereas peptide-induced surface reconstruction is rarely reported.
Figure 8. (a) STM image of Vpr13–33 assembly on graphite surface. The length of the red arrow including six peptide molecules is 2.65 nm. (b) Tapping mode AFM image of 0.1 mg ml\(^{-1}\) Vpr13–33 on mica with previously deposited graphene oxide. (c,d) Corresponding height distributions from (b) for peptide fibrils on exposed mica surface without GO coverage and peptide fibrils on GO surface, respectively. (Adapted from [34], Copyright 2013, Elsevier.)(Online version in colour.)

Drastic reconstruction of the Au(111) surface was reported by Humblot et al. [35], driven by the adsorption of insulin growth factor tripeptide molecules. STM images show that the surface reconstruction can occur at room temperature without annealing, and the reconstruction dynamically changes with the time extension. Finger-like structures with width around 10 nm and length up to 45 nm are formed upon surface reconstruction with the same running direction. The authors believe that the occurrence of the reconstruction process is owing to the strong ‘chelating’ interactions between the terminal \(-\text{COOH}\) and \(-\text{NH}_2\) groups with the gold atoms.

4. Folding structures of the amyloid peptides studied by scanning tunnelling microscopy

Amyloid assembly on surfaces or at interfaces has been drawing increasing interest in the past several years owing to its association with many degenerative diseases. A series of amyloidal peptides (beta-amyloid peptides (A\(\beta\)), human islet amyloid polypeptide (hIAPP), prion protein and TAR DNA-binding protein 43 (TDP-43) are intensively studied in biochemistry and biophysical fields [36–41] because of their correlation with significant diseases, such as Alzheimer’s disease, type II diabetes mellitus, transmissible spongiform encephalopathy (TSE), frontotemporal lobar degeneration (FTLD), amyotrophic lateral sclerosis (ALS), etc. The
aggregation of Aβ peptides to oligomers, fibrils and senile plaques is reported to be toxic to neurons [36,37,42,43]. Abnormal accumulation of amyloid deposits of hIAPP is toxic to β cells. Amyloid-like fibrils resulting from prion protein aggregation are reported to impair neuronal cell function leading to TSEs [44–46]. Hyperphosphorylated, cleaved, ubiquitinated, mislocalized and aggregated TDP-43 protein is reported to correlate to nearly half of the FTLD cases and most of the ALS cases and some other neurodegenerative diseases [41,47]. However, the underlying molecular mechanisms for their cytotoxicity remain to be elucidated. Recently, it has been reported that diverse amyloidal peptide fragments could interact with phospholipid molecules to form nanopore structures [48]. The formation of these nanopore structures might possibly wreck the ion channel of cell membranes, imparting cytotoxicity to the neuron [48]. Many efforts have been made for studying the structure and the mechanism of the amyloidal peptide assembly by various experimental and theoretical methods. Till now, it is still challenging to fully understand the assembly mechanism of the amyloid peptides and also the neuron toxicity mechanism of the peptide aggregates.

The assembly behaviours of peptides with hairpin structures are an important category of topics for STM studies owing to their relevance to neurodegenerative diseases. As an example, the assembly structures of Aβ42 have been investigated by STM [49], as presented in figure 9. The high-resolution STM images reveal the lamella characteristics. The measured length of the peptides from STM images is appreciably less than the value for fully extended peptide structures and can be attributed to the formation of the hairpin structures. This result is consistent with the previous reports of Aβ42 explored by solid-state NMR [50]. A distribution of core region length of Aβ42 has also been observed, which could be related to the folding multiplicity of the peptide hairpin structures. The assembly structure of another typical amyloidal peptide, hIAPP related to type II diabetes mellitus, was also studied by STM [51] The lamella characteristics of hIAPP assemblies are revealed and multiple molecular folding structures with an average of 11 amino acid residues for the core regions are also explored [51]. These studies mentioned above demonstrated the feasibility of the STM technique in investigating the assembly structures and aggregation behaviours of amyloidal peptides.

![Figure 9](image-url)
The above studies have shown very informative data on the hairpin structures of amyloid peptides, Aβ42 and hIAPP, while the lack of sequence recognition at the present stage would not allow for the assignment of the folding sites directly. In order to gain further insights into the folding structures of hIAPP, a labelling molecule, 4,4’-bipyridyl (4Bpy), was introduced to coassemble with the hIAPP8–37 peptide [52]. The identification of the key sites for the β-structure motifs of the hIAPP was facilitated by the measurement of the peptide length from the C-terminal labelled with 4Bpy molecules (figure 10) [52]. According to the hairpin structures of hIAPP peptide, the C-terminal labelling molecule 4Bpy could only provide the information of C-terminal peptide length. In order to obtain the key sites for the N-terminal β-structure motif, STM investigations on hIAPP37–8 were also performed, which had the identical sequence of hIAPP8–37 but with reversed N and C termini. From the systematic investigations, it is evident that the IAPP8–37 analogues, including different mutations, share common structure motifs of IAPP8–17 and IAPP26–37 with the most probable key sites at positions around Ser19/Ser20 and Gly24. These observations reveal the similar amyloid formation tendency in the C and N terminal segments because of the sequence similarity, whereas the differences in specific key sites could be associated with the sequence variations. The results could be beneficial for studying structural polymorphism of amyloidical peptides with multiple β-structure motifs.

In another related study, the lengths of the core β-motifs in an amyloidogenic TDP-43 C-terminal domain and their mutations were measured and statistically analysed using STM. The correlation between the aggregation propensities with the β-structure length for wild-type (Wt) and mutant TDP-43 peptides is confirmed by both STM imaging on surface and thioflavin T (ThT) fluorescence assay in buffer solution (figure 11) [53]. Comparison of the Wt TDP-43 peptide with A315T mutant, phosphorylated mutant A315T(p) and A315E mutant TDP-43 peptides reveals the longer β-domain of A315E and A315T(p). The stronger aggregation capacities of A315E and A315T(p) than those of Wt and A315T peptides are also evident from the ThT fluorescence results.
Figure 11. STM experiments show that the Wt, A315T, A315T(p) and A315E TDP-43 peptide form a different length β-sheet at the carboxyl terminus. STM images of two-dimensional assemblies of 4Bpy-labelled Wt, A315T, A315T(p) and A315E peptides are shown in (a), (c), (e), and (g), respectively. Number in the histogram means the most probable residue number of the core β-domain. Histograms with Gaussian fitting of the length distribution of core β-domain for (b) Wt, (d) A315T, (f) A315T(p) and (h) A315E peptides. (Adapted from [53], Copyright 2013, Elsevier.) (Online version in colour.)

The positive correlation between β-domain length and the aggregation propensity indicates the feasibility of understanding the aggregation and finally the cytotoxicity of the amyloid peptides by analysing the structural characteristics of β-motifs at molecular level.

5. Interaction between amyloid peptide and dye or drug molecules

It is well known that amyloid aggregation can be detected by labelling with dye molecules, such as Congo red and ThT [54,55]. Some dye molecules have also shown capability of inhibiting the neuronal toxicity efficiently [56]. However, the mechanism is still ambiguous as to how these species interact with the amyloid peptides to reduce cell toxicity.

As STM has the capability to resolve single molecules and single peptides at the same time, the real-space imaging of adsorption sites and configurations of labelling molecules on target peptides would lead to the feasible design of possible drugs. The interaction between labelling molecule, multi-functional phthalocyanine (Pc) derivative PcCu(SO3Na)4 and four representative polyamino acids was reported recently [57]. Octaphenylalanine (polyF8) with a hydrophobic aromatic group, octahistidine (polyH8) with a positively charged group, octatyrosine (polyY8) with a hydrophilic aromatic group and heptaglutamine (polyQ7) with a hydrophilic group are selected as model peptides. The peptide–4Bpy coassemblies are prepared and the binding of PcCu(SO3Na)4 on peptide and 4Bpy molecules can be statistically analysed by frequency counting (figure 12) [57]. As the binding affinity of PcCu(SO3Na)4 on 4Bpy molecules can be considered as a constant, the relative binding affinities of PcCu(SO3Na)4 on four different polyamino acids could be deduced with normalization. The relative binding affinities of PcCu(SO3Na)4 on the four model peptides are nearly one order of magnitude different. The binding of functional labelling or drug molecules on different polyamino acids could provide a window to understand protein–drug interactions.

The binding behaviours of labelling molecules on biologically associated peptides are reported on prion segments and Aβ42 [49,58]. The high-resolution STM images facilitate the identification of the binding sites of ThT molecules on prion segment, GNNQQNY peptide [58]. ThT molecules are apt to adsorb onto the peptide assembly and orient parallel with the peptide strands. Four different binding modes are identified, in which the ThT molecules with parallel configuration binding next to the C-terminus and on top of peptide strands is the preferential binding mode. The preferential binding of the benzothiazole moiety of ThT molecules to the C-termini of GNNQQNY
peptides could be explained as the electrostatic attraction between positively charged moieties on ThT molecules and negatively charged carboxyl groups on peptides. From the studies on the interaction between amyloid peptide and dye or drug molecules, one may further understand the binding site, binding affinity and mechanism of the molecules on targeted peptides or proteins.

**Figure 12.** Binding modes of dye molecules with specific amino acid motifs identified with STM technique. (a) The STM images (upper) and proposed models (bottom) of ThT binding on GNNQQNY/4Bpy coassembly structures, between peptide strands and near C termini (CB, (i)), atop peptide strands and near C termini (CA, (ii)), between peptide strands and near N termini (NB, (iii)), and atop peptide strands and near N termini (NA, (iv)). (b) Relative binding affinities of PcCu(SO$_3$Na)$_4$ with different amino acid residues. The STM images of PcCu(SO$_3$Na)$_4$ binding on PolyQ7 peptide–4Bpy coassembly structures (i) atop peptide and (ii) atop 4Bpy, and (iii) the proposed model. (iv) Statistical results of relative binding affinities of dye molecule PcCu(SO$_3$Na)$_4$ on different poly(amino acids). ((a) Adapted from [58], copyright 2011, American Chemical Society; (b) adapted from [57], copyright 2011, Royal Society of Chemistry.) (Online version in colour.)
6. Modulation of peptide assemblies by small molecules

Peptides consist of various amino acids; thus, peptide assemblies will inherently involve diverse interactions and could be molecularly tuned by other molecules introducing interactions, promoting or inhibiting the peptide assembly. Such efforts are reflected in the studies on variations in assembly structure and aggregation process by small molecular modulators interacting with the peptide terminals and peptide side chains. Based on the prevalent interactions between the peptides, it is feasible to modulate the peptide aggregations via hydrogen-bond interactions. It may be proposed that three classes of modulators could be considered to regulate assembly structures of peptides. The first one is the main chain modulator for interrupting the $\beta$-sheet extension along the fibril axis. The second one is the terminal modulator for interrupting the $\beta$-sheet extension along the peptide strands by interacting with the N-termini [59] or C-termini of the peptides [60,61]. The third one is the molecular modulator that can interact with the side groups of the peptides to interrupt the $\beta$-sheet stacking and twisting. All these modulations could be beneficial for identification of peptide structures and inhibition of amyloidal peptide assembly. Several present examples are listed below.

(a) Modulation of peptide assembly structures with N-terminus modulators

Phe-Phe molecules are capable of forming ordered supramolecular assembly structures via hydrogen-bond and hydrophobic interactions between the dipeptides [25,26]. However, they could not form extended assembling structures, most possibly owing to chain–chain repulsion and kink defects [13,59]. Upon addition of organic molecules terephthalic acids (TPAs), the coassembly of peptides and organic molecules was explored by STM [59] as shown in figure 13. The ellipse and dumbbell features are attributed to TPA and Phe-Phe molecules, respectively. The introduction of TPA favours more orderly and extended supramolecular assemblies in comparison with the assemblies of Phe-Phe alone. The measured spacing among the molecules and their orientation indicate that intermolecular hydrogen bonding between carboxylic groups of TPA and nitrogen atom of Phe-Phe might be the main driving force to the heterogeneous lamella. Moreover, the integrity of the Phe-Phe chain motifs indicates
that the intrachain binding is stronger than the TPA–chain interaction. However, this latter interaction is evidently strong enough to overcome the original chain–chain repulsion and to link the diphenylalanines.

(b) Modulation of peptide assembly structures with C-terminus modulators

Another modulation approach with carboxyl terminus modulators can be seen by using organic molecules containing heterocyclic rings with nitrogen atoms, such as 4Bpy and 1,2-di(4-pyridyl)ethylene (DPE). As examples, modulation of Aβ10–20 and Aβ33–42 (which are vital fragments for the Aβ core regions of the hairpin structures) with terminal modulators 4Bpy and DPE was explored by STM in ambient conditions [60, 61]. Taking Aβ10–20 as an example (figure 14) [61], in the presence of 4Bpy molecules, the lamella characteristics of coassemblies of Aβ10–20 and modulators are retained with 4Bpy molecules embedded between Aβ10–20 stripes. The N−H−O hydrogen-bond interaction between the nitrogen atoms of 4Bpy or DPE and the −COOH groups of peptide C-termini is deterministic to the modulated peptide assemblies. In principle, the modulation of the assembly structures reported for Aβ10–20 could also be applied to other amyloid peptides.

The above STM observations demonstrate that modulators could regulate the assembly process of the peptide Aβ on the molecular level. As the aggregation of the Aβ peptides into oligomers, protofilaments, fibrils and plaques is a hierarchical assembly process, it is plausible that the assembly modulation on the molecular level could affect the higher level assemblies, such as the morphology of peptide aggregates [61]. The AFM images of Aβ10–20 aggregates in the presence of the modulators, 4Bpy and DPE molecules, demonstrate reduced fibril formation compared with the aggregates of pristine Aβ10–20. The aggregation behaviours of peptides in solution tracked by turbidity measurements show that the modulators could appreciably accelerate the aggregation of peptide Aβ10–20 in aqueous solution.
It should be noted that in recent years, more and more reports support the neuron toxicity of the oligomers, rather than the mature fibrils and the senior aggregate amyloid peptides [42,43]. The introduction of modulators could accelerate the peptide aggregation via interactions between the peptides and modulators and reduce the concentration of the peptide oligomers in solution. It could be beneficial for exploring ways of reducing the neuron toxicity by accelerating the aggregation process of amyloid peptides relating to neurodegenerative diseases. The appreciably reduced cytotoxicity of amloidogenic peptides was observed in association with the accelerated aggregation process by chaperone-like molecular species (figure 14) [61]. These observations could be beneficial for designing new functional molecules as therapeutic agents for amyloidoses based on a novel accelerating aggregation approach.

(c) Modulation of peptide assembly structures with side group modulators

Terminus modulators demonstrated dramatic effects on peptide assembly characteristics. The dominant driving forces in the modulation process of peptide assembly with terminus modulators are the hydrogen-bond interactions between the N-terminus and C-terminus, which are common to all peptide sequences and lead to little sequence specificity. The specific interactions between the side groups of peptides and organic molecules could provide possible avenues for sequence recognition of the peptides based on structural analysis. The preliminary results for labelling the Aβ33–42 assembly were reported recently via the hydrophobic interactions between Aβ33–42 and Pc molecules [60]. Pc molecules were observed to adsorb on top of the coassemblies of Aβ33–42 and DPE. More Pc molecules preferred to stack on the peptide chains rather than the DPE molecules. These results indicate that labelling molecules with hydrophobic moieties could help to identify the hydrophobic residues of peptides for future therapeutic agents for amyloidoses.

7. Conclusion and perspectives

This review has summarized the efforts on gaining molecular-level insights into adsorption and assembling of peptides on surfaces by STM. Various factors influencing the peptide assembly have been discussed, such as sequence effect, structural effect, odd–even effect, substrate effect, etc. The identification of folding sites in amyloid aggregation would shed light on the structural analysis for amyloid peptides, which is notoriously difficult. The diversity of interactions, such as hydrogen-bond and hydrophobic interactions, is explored for regulating the peptide assembly structures with various molecular modulators. Recent works on the assembly structures and aggregate modulation of peptides have demonstrated the advantages of STM for studying the aggregation behaviours of peptides and molecular design for labelling molecules. It is a promising aspect to make full use of diverse molecular modulators to inhibit or accelerate the hierarchical assembly process of peptides and possibly enable peptides to form distinct assembly structures. In order to realize the controlled assembly structure and assembly process, more endeavours should be made for possible identification of peptide residues, determining peptide secondary structures and exploiting new molecular modulators. These challenges will also provide rich ground for the development of STM techniques by combining the structural resolution with chemical recognition capability.

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