Nanoscale

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REVIEW

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Protein fibrillation and nanoparticle interactions: opportunities and challenges

Cite this: *Nanoscale*, 2013, **5**, 2570

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Due to their ultra-small size, nanoparticles (NPs) have distinct properties compared with the bulk form of the same materials. These properties are rapidly revolutionizing many areas of medicine and technology. NPs are recognized as promising and powerful tools to fight against the human brain diseases such as multiple sclerosis or Alzheimer's disease. In this review, after an introductory part on the nature of protein fibrillation and the existing approaches for its investigations, the effects of NPs on the fibrillation process have been considered. More specifically, the role of biophysicochemical properties of NPs, which define their affinity for protein monomers, unfolded monomers, oligomers, critical nuclei, and other prefibrillar states, together with their influence on protein fibrillation kinetics has been described in detail. In addition, current and possible-future strategies for controlling the desired effect of NPs and their corresponding effects on the conformational changes of the proteins, which have significant roles in the fibrillation process, have been presented.

Received 16th October 2012
Accepted 22nd January 2013

DOI: 10.1039/c3nr33193h

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1 Introduction

Protein fibrillation is defined as the process by which misfolded proteins form large linear aggregates or amyloid fibrils.[1] There exist a number of proteins and peptides that form amyloid fibrils during disease progression, including amyloid beta peptide,[2–5] prion protein,[6] α-synuclein,[7] polyglutamine protein,[8] glucagon,[9] and β2-microglobulin.[10,11] Among the various human amyloidogenic proteins, amyloid beta (Aβ) peptides are widely used as model peptides to investigate the effect of nanoparticles (NPs) on fibrillogenesis;[12,13] the Aβ peptide, which has a molecular weight of around 4 kDa, is an amphipathic peptide prone to self-association and formation of fibrils.[14] Monomeric Aβ is soluble under physiological conditions and has been shown to be unstructured;[15] however, the fibrillar form has a characteristic cross-β structure with stacking of β strands perpendicular to the long axis of the fiber.[16–18] The process leading from free monomeric Aβ

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to its fibrillar state involves a number of intermediate states including nucleation, oligomerization, and fibril formation. In the nucleation phase, no aggregates are formed but the non-moneric Aβ proteins interact with each other to form small nucleating units which can be used as templates. In the next step, soluble oligomers of various forms are produced. These oligomers lead to protofibrils and prefibrillar aggregates that ultimately result in the fibrillation of Aβ.[19–21]

Fibril formation occurs by nucleation dependent kinetics, wherein formation of a nucleus is the key rate-determining step, after which fibrillation proceeds rapidly; however as yet, the assembly forms of Aβ that mediate disease remain unknown,[22] although evidence suggests that it may be the oligomeric form that is neurotoxic, and that fibrillation is a defense mechanism to remove the toxic oligomers.[23,24]

It is well recognized that NPs can interact with Aβ and affect the fibrillation process.[25,26] Depending on their physicochemical properties, NPs can have various effects on the kinetics of the Aβ fibrillation process;[27–29] for instance, positively charged superparamagnetic iron oxide NPs are capable of promoting fibrillation compared with negatively charged or uncharged NPs, at the same particle concentrations.[30] Despite the huge number of publications in the field, the detailed mechanism(s) of NP interactions with amyloid proteins is(are) poorly understood; in addition, there are still severe challenges regarding how to determine the effects of various NPs on the fibrillation process in complex media (*e.g.* under *in vivo* conditions); thus, this review focuses on the well documented acceleratory/inhibitory mechanisms of NPs on the Aβ fibrillation process. We also provide an overview of "ignored" parameters (*e.g.* slight temperature changes and competitive protein interactions) on the NP–Aβ interfaces. Finally, the opportunities and risks of using different NP materials as anti-fibrillation agents are discussed.

2 Formation of amyloid fibrils and their (unwanted) effects

Since protein fibrillation is implicated in several serious diseases, many related to old-age, the onset-time of protein

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fibrillation (or at least the physical manifestation as deposited protein plaques) is typically many years. In many cases, some degree of partial protein unfolding, or denaturation, is required before proteins can form amyloid fibrils. In the body, this can result from long term age-related damage, or over-expression of the proteins resulting in crowding of amyloidogenic monomers. Until recently, the two most widely accepted molecular-hypotheses believed to explain the pathology of Alzheimer's diseases (AD) were the misfolding of some brain proteins/peptides (*e.g.* Aβ) and the effects of multivalent cations (*e.g.* Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, and Al^{3+}) in modulating the formation of plaques and tangles in the brain.[31] The misfolding of Aβ monomers causes the exposure of the core hydrophobic side chains of Aβ monomers for interactions;[32] however, multivalent cations can bind two soluble Aβ monomers *via* electrostatic interactions (the Aβ monomer has net negative charge).[13] The most important mechanism of protein fibrillation is referred to as "nucleated growth".[27] As the protein unfolding (or existence of multivalent cations) begins, there are no protein fibrils present but many non-fibrous short peptide sequences, called oligomers, which are the first step in the fibril formation.

Aggregation of insoluble protein fibrils can contribute to the progression of diseases due to toxicity or interference with the normal functioning of cells.[33–35] Hereditary and environmental factors have been identified contributing to the development of amyloid diseases.[36] For example, beta-2-microglobulin (β2m), an amyloid protein linked to the disease dialysis-related amyloidosis (DRA) that builds up in the bloodstream of patients on dialysis for kidney malfunction,[37,38] undergoes protein fibrillation and finally deposits in tissues causing joint stiffness, pain and occasionally bone fractures.[39,40] The formation of protein fibrils is not always associated with a disease. In fact, some amyloid proteins exhibit useful functional properties. For example, one of these proteins is involved in the production of melanin, which provides skin with protection against sun damage.[41]

3 Kinetics of Aβ fibrillation

The kinetics of protein fibril formation has been intensely investigated.[42–46] Through such studies scientists have been able to characterize intermediates and pinpoint pathways of protein fibrillation, and the kinetic studies also enable investigators to search for inhibitors or promoters affecting protein fibrillation. Two general models have been proposed to account for the observed sigmoid kinetics of protein aggregation–nucleation-dependent polymerization and diffusion-limited aggregation.[47] The majority of the reported data support the nucleation-growth model[48] which consists of three major phases, including: the lag, the polymerization, and the saturation phases, displaying cooperative or sigmoid behavior (Fig. 1). Each phase of separation is detected and characterized using specific methods. In the lag phase, which may differ for each protein, nucleation occurs; this is a time consuming period since making the initial nucleolus is energetically difficult and requires a minimal self-assembly unit to be formed. It is notable

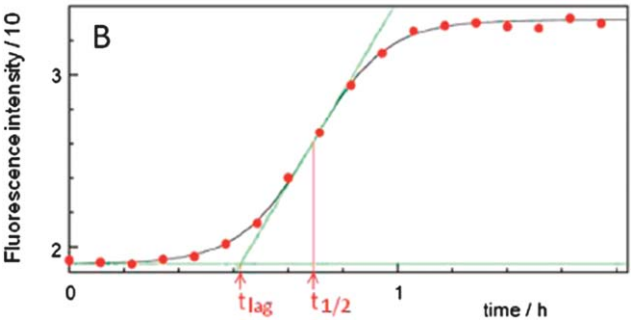

Fig. 1 Representative fibrillation curve for the amyloid beta protein as determined using the Thioflavin T assay. The figure was adapted with permission from ref. 14.

that the fibrillation of some proteins (*e.g.* serum albumin) occurs without a lag phase.[49] After formation of the nucleus, self-assembly is rapid and displays an exponential phase;[50] it is in this phase that the soluble oligomers are formed. In the last stage, where the binding of fibril-specific dyes (*e.g.* Thioflavin T (ThT) and Congo red) is no longer increasing, the fibril formation is at equilibrium (although TEM has shown that fibrils continue to mature for several hours after this in some cases, *e.g.* for superparamagnetic iron oxide nanoparticles[30]). The mature fibril consists of three to five of proto-filaments (*i.e.* the simplest fibrillar species in a fibril system, usually 2–3 nm in diameter) twisted around each other.[7]

In order to obtain the various kinetic parameters, the sigmoid function (eqn (1))[51,52] is fit to experimental data (see Fig. 1 for example).

$$F(t) = F_0 + \frac{A}{1 + e^{-k\left(t - t_{1/2}\right)}} \quad (1)$$

where $F(t)$ is the fluorescence intensity at time t, F_0 is the initial fluorescence intensity, A is the amplitude, k is the elongation rate constant, and $t_{1/2}$ is the time required to reach half the maximum intensity. The fluorescence signal, obtained using various staining fluorescence dyes (*e.g.* ThT) which only emit fluorescence upon intercalation into the cavities of the fibrils, yields information regarding the extent of amyloid formation.[53]

Here, the lag time is defined as the intercept between the time axis and the tangent with a slope k from the midpoint of the fitted sigmoid curve; in this case, the lag time could be calculated using the obtained fitted parameters as:

$$t_{\text{lag}} = t_{1/2} - \frac{2}{k} \quad (2)$$

Due to the stochastic nature of nucleation events, it is necessary to generate a large amount of experimental data (perform multiple experimental replicates) in order to ensure the validity of the obtained kinetic data. In the absence of statistically significant amounts of experimental data, the molecular mechanisms underlying the fibrillation process could not be thoroughly understood due to the low reproducibility of kinetic data.[54] The severe complexity of the system,

with multiple soluble/insoluble states, several conformational structures, various intermediate states, kinetic competition among numerous pathways, competition between fibrillation and amorphous aggregation, the competition between initial fibril formation and fibril growth (nucleation and growth), and multiple filamentous states (*e.g.* protofilaments, and protofibrils),[20,21,55] are among the major reasons for the low reproducibility of the data.[55] Besides the system complexity, a common problem faced by researchers in generating sufficient replication of Aβ fibrillation processes is the very high costs of generating synthetic Aβ. In order to overcome this shortcoming, Walsh *et al.*[56] used a recombinant expression system for production of large quantities of highly pure Aβ, which enabled scientists to do multiple replicates in a much more cost-effective manner.

It is noteworthy that, in many cases, a secondary nucleation mechanism could be involved, causing the formation of new growth centers from the existing fibrils;[57,58] in this case, a different mechanism should be considered for the nucleation and growth states.[58,59]

3.1 The effect of macromolecular crowding on fibrillation kinetics

One quarter of the intracellular environment volume of all normal cells is occupied by proteins, of which about 10% form cytoskeletal filaments and 90% are soluble globular proteins.[55,60–62] The addition of extra macromolecules to the intracellular environment or other equilibrium biological milieu, affects the normal balance of the cells/medium and could potentially result in thermodynamic or kinetic consequences for either the existing or added macromolecules (the so-called crowding effect).[55] One has to consider several factors (*e.g.* net energy changes, water activity, excluded volume, and viscosity changes) resulting from the entrance of the foreign/excess macromolecules into the biological milieu, which may cause altered protein association behavior.[55] Changes in the free energy of the biological system, a decrease of the water activity, and an increase of the viscosity of the solution are recognized as significant changes resulting from the addition of macromolecules to the biological milieu.[55] These events can, in turn, induce the formation of the most compact protein states, thereby decreasing the protein solubility and promoting protein self-association, and decreasing the diffusion rates and kinetics of diffusion-controlled reactions, sequentially.[55] Thus, amyloid formation is a type of protein self-assembly that ultimately leads to protein fibrillation. Any condition/parameter that affects the self-assembly of proteins could most likely influence amyloid formation also.

The net effect of these predetermined factors on protein aggregation and fibrillation defines the fate of the macromolecules in a crowded medium. Based on *in vitro*[63,64] and *in vivo*[65] results, it has been proposed that the amount of excess macromolecules in a biological medium can modulate the rate and extent of amyloid formation.[63,64] In addition, the effect of the composition and molecular weight of model 'crowding agents', such as polyethylene glycols (PEG) or polysaccharides (*e.g.* dextrans and ficols), on protein fibrillation has been investigated.[66] The results confirmed the significant effect of higher molecular weight macromolecules on the reduction of the fibrillation lag time in comparison with the same amount of lower molecular weight macromolecules of identical composition.[66] The reason for this observation is the enhanced capability of higher molecular weight macromolecules to alter the medium viscosity relative to the same amount of lower molecular weight macromolecules.[66] The composition of the macromolecules may have specific effects on the fibrillation process, as a result of their possible interactions with the amyloid protein. Inert macromolecules, such as polyglycols and dextrans, may be preferred as crowding agents, due to their similarities to the cellular environment.

Hellstrand *et al.*[14] studied the concentration dependence of Aβ (M1-42) aggregation kinetics and equilibrium data by means of fluorescence staining of the fibrils using ThT, and an enzyme-linked immune-sorbent assay, respectively. They found that the fibrillation kinetics arise from a sequence of events in a highly predictable manner; more specifically, the fibrillation process varies strongly with peptide concentration (crowding effect). In addition, the free Aβ concentration in equilibrium with fibrils was found to vary with the total peptide concentration in a manner expected for a two-phase system.

Although the crowding effect from the majority of macromolecules was observed to accelerate the fibrillation process,[66] there are also several reports confirming the opposite (anti)-crowding effect of some agents (*e.g.* including some NPs)[67] which can slow down or prevent nucleation dependent amyloid formation.[68] Ghahghaei *et al.*[69] probed the crowding effect of dextran on destabilization of α-lactalbumin in the presence of α-casein. The results confirmed that dextran crowding increases the rate of amyloid fibril formation in α-lactalbumin, as expected. Moreover, it was observed that α-casein was more efficient in preventing amyloid formation of α-lactalbumin in the absence of dextran than in its presence.[69] The authors claimed that dextran has the capability to induce structural changes in α-lactalbumin while it shows lower potential for inducing changes in the α-casein structure.

3.2 Stop and go kinetics in fibrillation processes

One of the problems preventing a deeper understanding of the growth kinetics of amyloid assembly is the current practice of investigating amyloid aggregation/growth in bulk assays that often hide details of the stochastic process.[70] In order to overcome this problem, and to monitor the fibrillation process in detail in real-time, single molecular spectroscopy methods such as atomic force microscopy (AFM) and fluorescence microscopy (*e.g.* total internal reflection fluorescence microscopy (TIRFM)) were recently employed.[71–74] TIRFM is based on an optical phenomenon, such that when an excitation light is totally internally reflected in a transparent solid such as a glass slide at its interface with liquid, an electromagnetic field (evanescent wave) is produced at the liquid–solid boundary. The generated wave exponentially decays in a distance-dependent manner and those fluorescent molecules (or fluorescent labels) that are in a

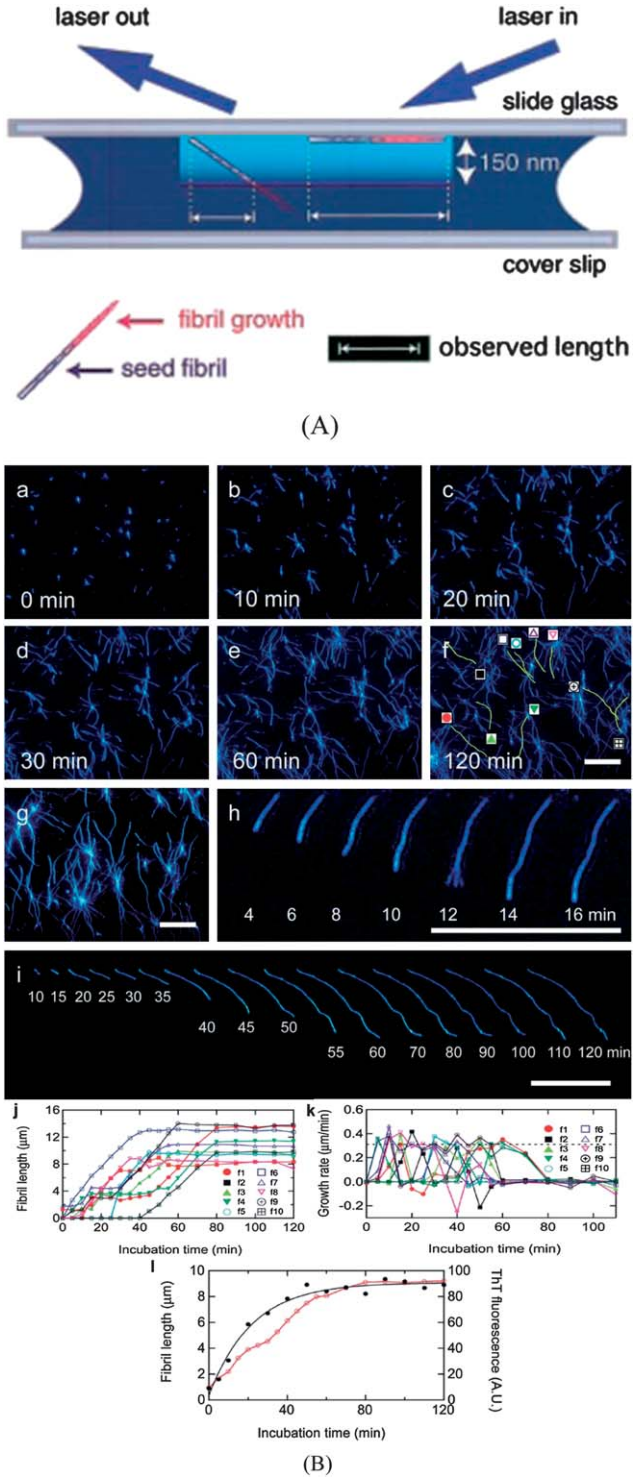

Fig. 2 (A) Schematic representation of the detection of amyloid fibrils by total internal reflection fluorescence microscopy (TIRFM). The penetration depth of the evanescent field formed by the total internal reflection of laser light is 150 nm for laser light at 455 nm, so that only amyloid fibrils lying in parallel with the slide glass surface were observed. (B) Direct observation of Aβ(1–40) amyloid fibril growth by TIRFM; (a–f) real-time monitoring of fibril growth on glass slides. In (f) the fibrils used for the kinetic analysis shown in (j and k) are indicated; (g) vertically aligned image of fibrils; (h) growth with transient fraying of the growing end at 12 minutes; (i) growth with swinging head producing rugged fibril; (j) fibril length plotted against incubation time; (k) growth rate plotted against incubation time; (l) the comparison of time-courses of amyloid fibril growth on the glass surface observed by TIRFM (○) with the growth measured in solution (●). Concentrations of the Aβ(1–40) monomer, seed, and ThT were 50 mM, 5 mg ml^{-1}, and 5 mM, respectively, and the reaction was carried out at pH 7.5 and 37 °C. The scale bar represents 10 mm. The figure was adapted with permission from ref. 74.

close proximity (a couple hundreds of nanometers) to the solid surface can be largely excited (Fig. 2A).[75]

In some single molecular spectroscopy experiments, a stop-and-go phenomenon was observed, during which the fibril (*e.g.* Aβ-peptides,[70,74,76–79] α-synuclein,[80] and glucagon[9]) growth was provisionally halted[74,80] before resuming again at a later point, whereas other experiments pointed to a constant growth rate.[73,74] Ferkinghoff-Borg *et al.*[9] proposed that the existence of approximately isoenergetic states in protein fibrillation could cause the appearance of stop-and-go kinetics; in this case, they proposed the consideration of an additional dimension in the fibrillation energy landscape. However, this energy cannot be identified easily by conventional techniques. Since deep understanding of the stop-and-go kinetics are vital, together with the fact that the various conformations of a protein can differ significantly in their energy,[81] new sophisticated techniques should be developed to probe the exact isoenergetic states during protein fibrillation.

Kellermayer *et al.*[70] employed AFM to monitor the growth of individual Aβ(25–35) fibrils. Their results showed that the fibril assembly was polarized and discontinuous. They observed that bursts of rapid (up to 300 nm^{-1}) growth phases, which could increase the length of fibrils by approximately 7 nm or its integer multiples, were interrupted by pauses (*i.e.* stop phases). The authors claimed that the amyloid assembly may involve fluctuation between a fast-growing (*i.e.* go state) state and a blocked state (*i.e.* stop state) in which the fibril is kinetically trapped due to the intrinsic structural features.[70]

In order to clarify the mechanism(s) of fibril formation, and thereby to enable the development of fibrillation inhibitors, Ban *et al.*[74] applied real-time monitoring of fibril growth using TIRFM combined with the binding of ThT, an amyloid-specific fluorescence dye (see Fig. 2B). In this case, seed dependent amyloid fibril growth of Aβ(1–40) was visualized in real-time at the single fibril level, under various conditions (no shaking, shaking, *etc.*) and compared to the fibrillation kinetics in solution (Fig. 2B).[74]

It has been found that Aβ amyloid formation is a stereo-specific reaction and that the reaction stability is affected by L/D-amino acid replacement.[74] The authors designed several analogues of Aβ(25–35), which is a toxic fragment of Aβ(1–40), consisting of L and D-amino acid residues, and their inhibitory effects were investigated using TIRFM, which revealed that some chimeric Aβ(25–35) peptides inhibited the fibril growth of Aβ(25–35) strongly; however, it is notable that the observed inhibitory effect could not inhibit the growth of Aβ(1–40).[74]

4 Amyloid detection during the various phases of amyloid formation

There are a number of ways to detect amyloids at the different stages (*i.e.* early phase (lag phase) and growth/late phase) of

amyloid formation. For more detailed and comprehensive analyses of the various detection methods, readers are referred to recent reviews.[82–86]

(a) Detecting the lag phase of protein fibrillation – the lag phase is known for the formation of nucleation species that are thermodynamically expensive to form. In addition, soluble oligomers are also present at this stage of fibril formation. Characterization of the species present in the lag phase is crucial since some of the most toxic species may be pre-fibrillar. Additionally, characterization of the lag phase can provide valuable information on the kinetic and biochemical pathway(s) leading to amyloid formation. However, identification of entities during the lag phase can be challenging due to the heterogeneity, low concentration and instability of the precursor species involved. Therefore, the lag phase is dominated by the lack of fibril formation and a slow formation of nuclei from the non-native protein precursor. The followings are some of the methods that are used to study the early phase in the protein fibrillation pathway(s).

(i) Microscopy – one approach to study the early phase of protein fibrillation is using transmission electron microscopy (TEM) or atomic force microscopy. While fibrils are easily detected using TEM, oligomers can also be seen as spherical entities in the early stages of amyloid formation. For instance, the early stage oligomerization of transthyretin, an amyloidogenic protein linked to senile systematic amyloidosis and familial amyloidotic polyneuropathy, can be detected by TEM.[87] In the transthyretin aggregation pathway, during the first 2 h, monodisperse oligomers were formed which subsequently matured into large spherical clusters (30–50 nm) over 20 h, which subsequently turned into larger oligomers. As the aggregation continued, the spherical clusters began to shrink, indicative of conformational change of the constituent proteins, and the fibrils grew in length.[87]

Using AFM can also be quite informative in detecting amyloid in the early stage.[88] For the yeast prion protein Ure2, which forms amyloid like filaments *in vivo* and *in vitro*, AFM analysis showed the Ure2 dimer and granular particles corresponding to higher oligomers in the early phase of the prion aggregation.[89]

(ii) Multiple fluorescence probes – amyloid binding dyes (*e.g.* ThT and Congo red) can be used to detect the presence of protein fibrils.[90] In particular, ThT is shown to rapidly bind to amyloid fibrils and upon binding, its fluorescence increases substantially at 480 nm when excited at 450 nm. However, these dyes are not informative in the early phase of amyloid formation since in this phase no fibril is formed and thus ThT is not fluorescent. It is also believed that the ThT signal may depend on the microscopic organization and division of the fibrils, although no effort has been made to assess this as yet.

One way to monitor the species formed during the early phases of protein fibrillation is using fluorescent spectroscopy applying several fluorescent dyes including 1-anilinonaphthalene-8-sulfate (ANS) and 4-(dicyanovinyl)-julolidine (DCVJ). As a number of reports have previously shown, ANS binding increases as the protein fibrillation increases, due to the elevation of hydrophobicity that results from the misfolding of a protein, as internal hydrophobic residues normally hidden in the core become exposed.[91,92] DCVJ, acting as a molecular motor, has recently been demonstrated as a useful probe for reporting on the early stage of oligomerization. Indeed, DCVJ showed a high fluorescence after 1 min and reached a plateau level quicker than ANS or ThT, indicating its better performance for detection of oligomers than the other probes.[87] Interestingly, DCVJ did not bind to the native transthyretin protein, giving it an additional advantage over other commonly used dyes.[93]

(iii) Photo-induced cross-linking – to detect the early stage of protein fibrillation, cross-linking can be used. Cross-linking agents can covalently connect subunits that are spatially close to one another, even from different proteins. This procedure has been used extensively to get valuable information on oligomers in the early phase of protein fibrillation.[94] Photo-induced cross-linking of unmodified protein (PICUP) has been a valuable method due to its short reaction time which renders the identification of short lived and metastable oligomers possible.[94] When PICUP is used, the protein does not need any modification or the use of an exogenous cross-linking molecule. The cross-linking reaction in PICUP is initiated by visible light photolysis of a tris-bipyridyl Ru(II) complex, in an extremely rapid reaction. Upon irradiation, a Ru(III) species is generated which can abstract an electron from the polypeptide to generate a carbon radical within the backbone of the protein. This radical can quickly react with appropriate groups that are spatially close to generate a carbon–carbon bond.[95] As shown in Fig. 3, the PICUP reaction was used to identify oligomers of TTR.[94]

(iv) Agarose gel electrophoresis – a better picture of how oligomers of varying sizes are accumulated during the early stage of a protein fibrillation pathway can be obtained using

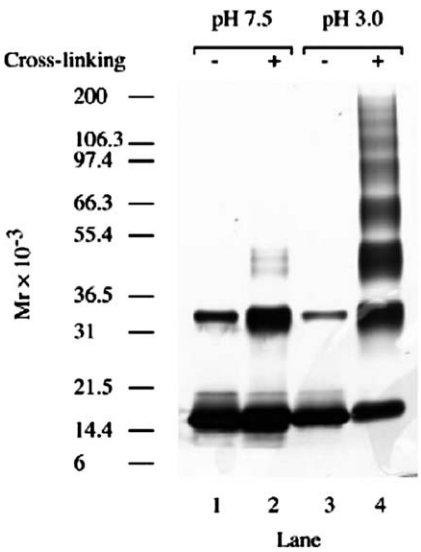

Fig. 3 Cross-linking of TTR using PICUP. TTR was incubated at 37 °C for 24 h either at pH 7.5 in the absence of zwitterionic detergent (*Z* 3–14): lanes 1 and 2, or at pH 3.0 in the presence of the zwitterionic detergent (*Z* 3–14): lanes 3 and 4. The protein was then cross-linked, and the resulting products were analyzed by SDS-PAGE. Non-cross-linked controls are shown in lanes 1 and 3. Cross-linked oligomers are shown in lanes 2 and 4. The figure was adapted with permission from ref. 94.

electrophoresis with agarose gel beads. Since gel sieves made of agarose are much bigger than the pores in polyacrylamide gel electrophoresis (PAGE), oligomers of various lengths are better detected. In one such study (Fig. 4), where the effect of ionic liquids as inhibitors of lyzozyme fibrillation was examined, agarose gel analysis clearly showed how, and at what stage during the fibrillation process, the chemical inhibitors (ionic liquids) inhibited the lysozyme fibrillation.[96] To better visualize the oligomers, the aggregates that are resolved on the agarose gels are transferred onto a semi-dry membrane and examined using a monoclonal antibody against the monomer of the amyloidogenic protein. Using this method, aggregates of low molecular weight (up to 5 MDa) can be detected.[97]

(b) Detecting amyloid during the growth phase – after the lag phase in which nucleation units are first made, the next step is generation of soluble oligomers; during the exponential growth phase, much larger clusters of oligomers are turned into prefibrillar aggregates, adding to both the length and the size of the protein aggregates. In this phase, a number of methods can be used to provide information on the structure and morphology of the fibrils.

(i) Atomic structure of fibrils – the best method to get information on the atomic structure of the fibrils to date has been solid state NMR (SS-NMR). Using SS-NMR, detailed structural information on non-crystalline solid molecules is obtained.[98] This powerful technique has been used to establish full structural models of amyloid fibrils, and to obtain valuable information on the super-molecular organization of the amyloid insoluble materials. In solid state NMR the components do not tumble isotropically; the absence of tumbling allows study of the effects of anisotropic interaction or orientation-dependent interaction.[99] To gain a high-resolution signal the solid sample is prepared by cross-polarization (CP), enabling the amplification of signals of less abundant nuclei such as C^{13} and N^{15}. Using SS-NMR the inter-nuclei distances are determined, allowing determination of the 3D structure of amyloid fibrils. In addition, the molecular packing can be deduced, from which it can be determined if the fibril structure is parallel-β-sheet or antiparallel-β-sheet. Several structures of amyloid peptides and proteins have been determined using SS-NMR.[98] In recent years, SS-NMR has also been used to study the interaction of membranes and amyloid.[100]

(ii) Secondary structure of fibrils – to obtain the secondary structure of amyloid fibrils several methods have been applied. One of the most common assays is the ThT assay which shows increased fluorescence upon binding to the β-sheet structure.[101,102] Congo red (CR) has been used for detection of amyloid in both tissue and solution.[103] When CR binds to fibrils, it causes a shift in the maximum absorbance from 490 to 540 nm. This property of CR has been used as a spectroscopic detection method for fibril formation in solution. However, there have been some issues regarding the specificity of CR for amyloid detection.[104]

Another usage of CR is its birefringence. Under cross-polarized light CR displays a unique blue-green colour that can be used for amyloid detection.[105] The birefringence of amyloid fibrils has been considered the ultimate test for their presence since only amyloid fibrils have been shown to display birefringence.[106] The typical apple-green colour of the amyloid material is shown in Fig. 5.[107]

(iii) Morphology of the fibrils – there are a number of ways to detect the morphology of fibrils, including AFM and TEM, which were introduced earlier in the section. Another method which has recently been used to look into the process and morphology of growing fibrils is TIRFM.[75,108] In TIRFM, amyloid fibrils can be visualized without covalent fluorescent labelling, using ThT which specifically binds to fibrils whereupon its fluorescence strongly increases. The advantage of this method relates to its exact measurement of fibril growth directly on the glass slide.[75]

Ban and Goto[108] were able to monitor the kinetics of β2-microglobulin fibrillation using TIRFM determining that the fibril extension was unidirectional. In order to see if TIRFM can be applied to other amyloidogenic proteins, they also examined several other fibrils (Fig. 6); it appeared that the technique was well suited for other amyloid forming proteins and peptides such as medC (corresponding to the C-terminal octapeptide of medin (medin is a 50 amino acid cleavage peptide of lactadherin)).

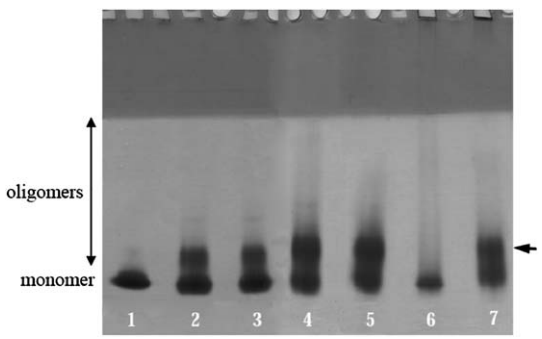

Fig. 4 Native PAGE of HEWL (hen egg white lysozyme) fibril formation at various time points; (1) native HEWL; (2) HEWL at 48 h; (3) HEWL in the presence of IL-2 (ionic liquid 2) at 48 h; (4) HEWL at 120 h; (5) HEWL in the presence of IL-2 at 120 h; (6) HEWL at 240 h and (7) HEWL in the presence of IL-2 at 240 h. The small arrow represents possible stabilized oligomeric intermediates. The figure was adapted with permission from ref. 96.

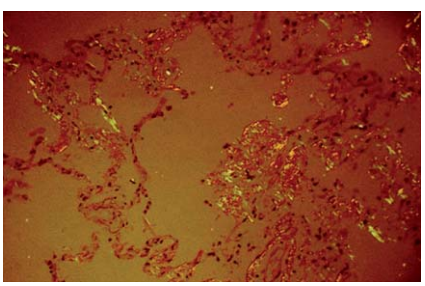

Fig. 5 Photomicrograph of Congo red-stained lung tissue, viewed under cross-polarized light. Apple green birefringence is amply displayed in the structure. The figure was adapted with permission from ref. 107.

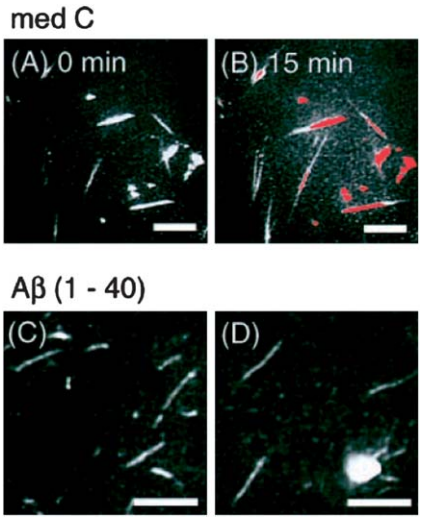

Fig. 6 Applicability of the ThT detection method *via* the TIRFM method to other amyloid fibrils. (A and B) seed-dependent fibril growth of medC. In B, the locations of seed fibrils are indicated in *red*. Aβ-(1–40) amyloid fibrils were prepared in a test tube (C) or on a glass slide (D). The figure was adapted with permission from ref. 75.

5 Molecular dynamics as an effective tool for studying protein fibrillation

One of the novel approaches for gaining molecular details regarding the mechanism(s) of protein fibrillation is molecular dynamics simulation. Although protein fibrillation is a lengthy process and thus requires immense computational capability to simulate the actual events in the process, the early events in the fibrillation pathway can be well-studied using molecular dynamics.

Following a brief introduction to molecular dynamics (MD), some of the interesting works using MD, which have provided insights regarding protein fibrillation, are presented and discussed.

Although protein crystallography has helped immensely in understanding the function of enzymes and proteins, unfortunately it has rendered a static view of proteins. By making the protein a crystal, the protein is removed from its natural environment, which is mainly aqueous, and the biomolecule is instead studied as a rigid structure where fluctuation and flexibility of atoms are mainly ignored.[109] However, the atoms in proteins in their native environment are quite flexible and show considerable fluctuation. One effective way of looking at fluctuation, flexibility, and even motion at the molecular scale is *via* molecular dynamics.

Molecular dynamics is an excellent approach for understanding the details associated with protein fibrillation at the molecular level. There are two types of MD; continuous molecular MD and discontinuous MD. The first is based on an all atom protein model and the second is based on intermediate protein models.[110] In MD the trajectories of atoms are computed by solving Newton's equation of motion, exploiting classical physics to solve the motion of molecules at regularly spaced time intervals or time steps. At the beginning of each step, the net force acting upon molecules is computed; having the force, the acceleration of the molecules can be determined using Newton's second law ($F = ma$), and subsequently the positional velocity can be predicted. In addition, at each step, the thermodynamics of each step is calculated.[110]

There are several widely available software programs that have been used for MD studies of proteins including CHARMM (Chemistry at HARvard Molecular Mechanics) and GROMACS (GROningen MAchine for Chemical Simulation).[111,112] Although each software may use some specific parameters, they often begin from the same starting molecular model and have various steps that are pretty similar. In all-atom MD, where counting of every atom in the molecule and the solvent is intended, a molecular model which represents a physical description of the protein is needed. A protein molecular model can be composed of the amino acid sequence and the residue topology. The residue topology can be obtained from the protein database (pdb) and may include data on the various amino acids making up the protein and their order or sequence, the value of the bond and torsion angles, the charge on each residue, and identification of those atoms that are capable of H-bonding. Thus, an X-ray crystal of a protein with a good to a great resolution is a good starting model.

There could be several steps in a typical MD simulation: (A) initial step (preliminary): generating protein structure files, known as PSF. As explained above, the protein structural data is a good starting point for making the PSF. (B) Energy minimization step: this step involves finding a set of coordinates where the potential energy is minimized. This step is used to omit structural defects resulting from overlapping atoms and distorted bonds and torsion angles. There are several techniques that can be used for energy minimization including steepest

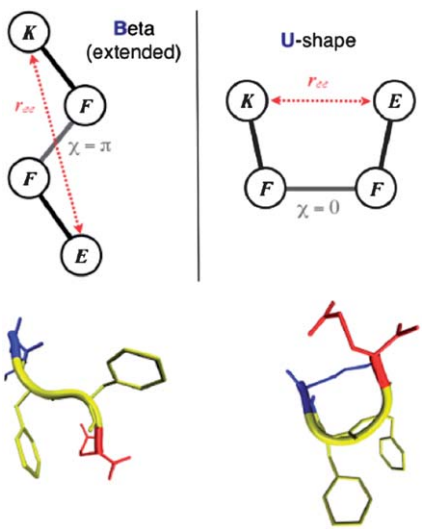

Fig. 7 Ideal, planar structure for the extended, β-strand conformation of the Aβ peptide (top left). Representative snapshot of the β-strand monomer conformation obtained from the simulations (bottom left). Ideal, planar structure for the compact, U-shape conformation of the Aβ peptide (top right). Representative snapshot of the U-shape monomer conformation obtained from the simulations (bottom right). The figure was adapted with permission from ref. 120.

descent (SD), Newton–Raphson (NRP), and Powell (POWE). (C) Heating step: the velocities are increased until the stable temperature and structure is obtained. (D) Equilibration step: the structure, temperature, pressure, and energy are all stabilized with respect to time. If, for example temperature is changed substantially, the velocity can be modified in a way that brings the temperature to what was requested. (E) Production step: the thermodynamic parameters are calculated in this phase.

An MD simulation provides molecular snapshots of how proteins arrange themselves, which of the residues are interacting, and the H-bond distance between key amino acids. MD also provides the protein gyration, which is a structural property, and the internal energy that allows determination of the melting temperature, the free energy, and the heat capacity of the protein.

5.1 Amyloid formation and molecular dynamics

MD studies have helped greatly in the elucidation of the molecular mechanism(s) of protein aggregation. Indeed, the exploitation of MD has been essential in understanding protein fibrillation, when the intermediate protein aggregates have shown metastability and low structural orders which make

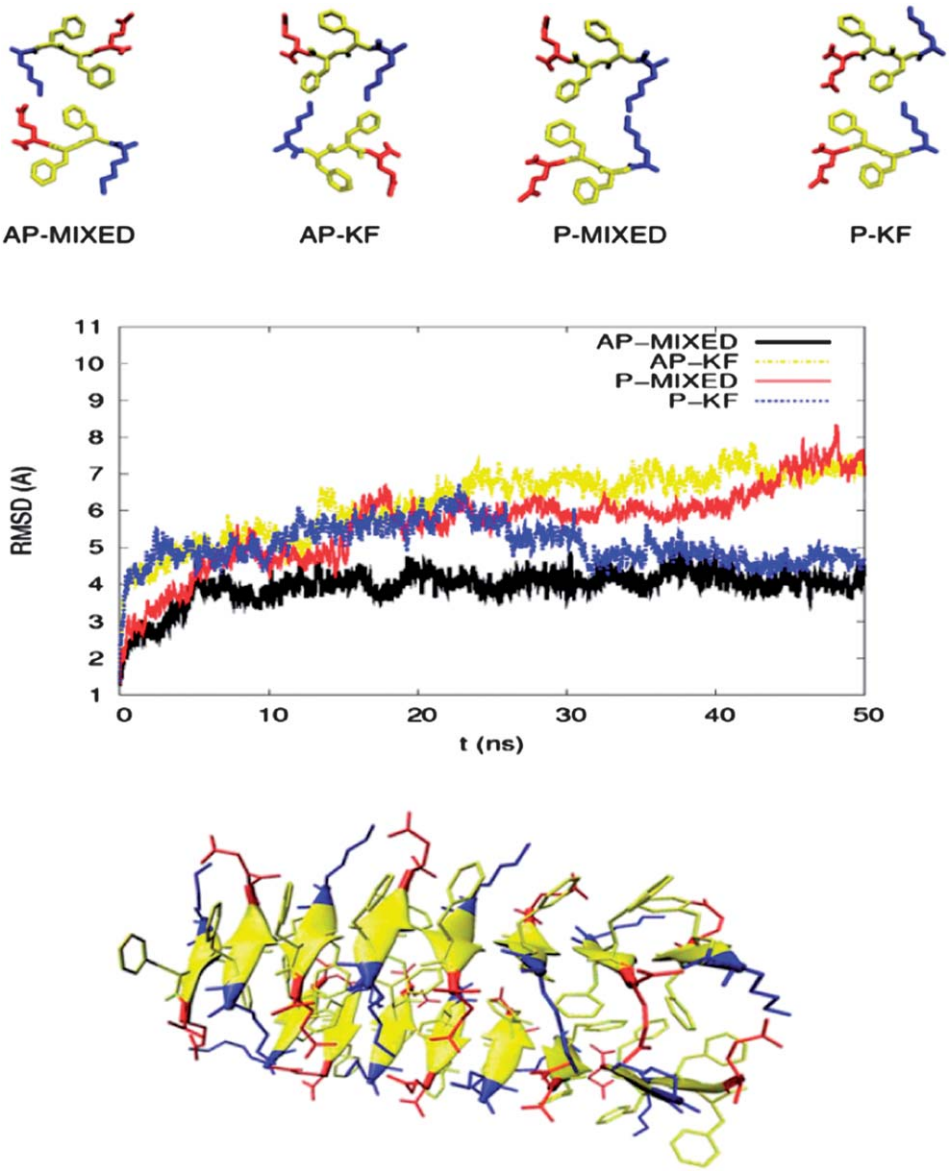

Fig. 8 (Top) The four different protofibril interface positions in the simulations. The protofibrils labeled AP-KF and P-KF have at least one close contact between lysine (K) and phenylalanine (F) at the interface level, whereas the protofibrils AP-MIXED and P-MIXED have close contact between two phenylalanine residues at the interface level; (middle) time series for the backbone root-mean-square displacement (RMSD) obtained from the single temperature simulations of the four different KFFE protofibrils. The RMSD time series eventually show that the structure labeled as "AP-MIXED" was the most stable over the simulation time length and (bottom) representative/average structure of the stable AP-MIXED protofibril. The average dimension of the stable AP-MIXED protofibril is in agreement with the values of the fibril dimension (12–16 Å) calculated from electron micrographs. The figure was adapted with permission from ref. 120.

them difficult to study experimentally.[113,114] Additionally, MD has been used to predict the amyloid propensity of peptide and proteins.[115,116]

Several MD studies have concentrated on determining the molecular mechanism of protein fibrillation, and have explored the conformational changes that the native and the intermediate molecules undergo.[117–119] In one study, a long simulation time (6.2 μs) of a region of Aβ (10–35) generated an important structure: a strand-loop-structure (SLS); this structure contained a salt bridge D23–K28 and a hydrophobic core.[118] Knowledge of the presence of this type of intermediate in the fibrillation of Aβ, enables explanation of how monomers can bind to the fibril ends through a specific conformation.

In another recent study, simulation of a model peptide, KFFE, which is known as the minimal system for aggregation, was performed to examine the effect of different forces involved in amyloid formation.[120] The tetra peptide contains an aromatic hydrophobic core (phenylalanine residues) and two oppositely charged amino acids (K and E) at the ends. Aromatic (or hydrophobic) and electrostatic effects have been previously considered as the two main forces acting in protein fibrillation pathways.[121,122] Additionally, phenylalanine had been rated as a highly amyloidogenic amino acid residue among the 20 naturally occurring amino acids.[123] However, how these two forces (aromatic/hydrophobic and electrostatic) act upon a peptide or protein causing amyloid formation was not completely understood. Using replica exchange MD, Bellisia and Shea were able to investigate the various conformations of the KFFE peptide as a monomer, dimer, and fibril.[120] As shown in Fig. 7, the simulation resulted in two distinct structures: the extended-beta structure and a more compact U-form. In the early stage of aggregation, the two forces are competing with each other; that is, the peptide in the monomer form is in equilibrium between the beta-conformation and non-beta conformation. Based on competition between the electrostatic force (due the terminal charges) which favors the non-beta conformation and the aromatic force (due to F residues) which stabilizes the extended beta structure, the specific structure is populated. In the dimer formation, the structures are stabilized by oppositely charged side chains and the charge–charge interaction between the termini, whereas the aromatic residues play a relatively minor effect in the dimer stabilization.[120] To simulate the protofibrils, four double layers were considered at the interface; two structures with anti-parallel (AP) interlayer orientation and two structures with parallel (P) interlayer orientation, as shown in Fig. 8. The root square deviation data confirmed that the AP-MIXED protofibril structure was the most stable, and that this was the protofibril structure containing almost a locked contact between lysine and glutamic acid and phenylalanine at the interface.

In a very recent study, the importance of nonlocal backbone contact was demonstrated in the aggregation of Aβ. A helical structure of Aβ, representing a membrane bound conformation was used for simulation in explicit water[124] in order to examine the most probable aggregation-prone structure (APS). Using all-atom molecular simulation of the entire Aβ (42 residue) peptide, the early phase of fibrillation was probed, and it was realized that the non-local backbone H-bond formation correlated well with the exposure of hydrophobic residues in the peptide. As shown in Fig. 9, three clusters were found during the simulations, labeled A through C. These structures all showed changes in gyration and helicity compared to the initial conformation (Fig. 10a). In the first cluster (populated between 4 and 18 ns), the dynamicity of the C-terminal region and the formation of a loop (residues 25–29) were the distinctive features, resulting in a reduction in the gyration and a disruption in the native helical structure. However, no significant nonlocal backbone interaction was seen (Fig. 10b). The second cluster appeared between 18 and 43 ns and showed two remarkable changes; the presence of nonlocal backbone contact and variation in the hydrophobic solvent accessible surface area (SASA). The nonlocal backbone interaction between regions $L_{17}V_{18}$ and $V_{39}V_{40}$ was significant due to the nonlocal contacts. In cluster C (between 45 and 60 ns), the number of nonlocal backbone contacts increased (up to 60) and H-bonding between some residues were detected. In addition, in cluster C, the hydrophobic SASA variation increased to nearly 25 percent.[124] Based on their simulation data, Lee and Ham[124] proposed a model signifying the important role that

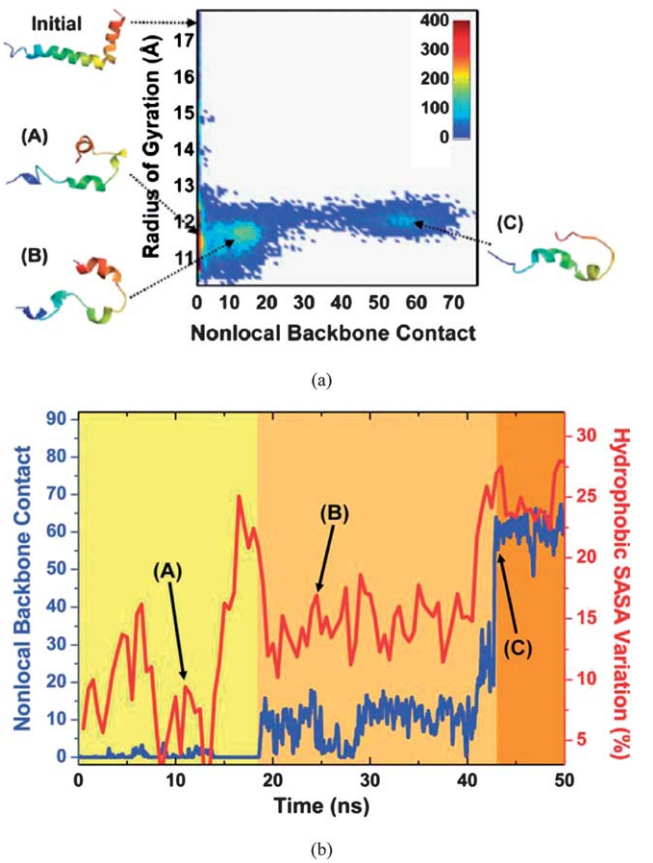

Fig. 9 (a) Link between the number of nonlocal backbone contacts and the radius of gyration (Å). Representative structures of each cluster are shown: (A) 11 ns, (B) 25 ns, and (C) 43 ns. The structures are color-coded based on the sequence, ranging from blue to red at the N- and C-termini, respectively; (b) hydrophobic solvent accessible surface area (SASA) variation (red line) of the starting structure and nonlocal backbone contacts (blue line) are plotted against simulation time. The figure was adapted with permission from ref. 124.

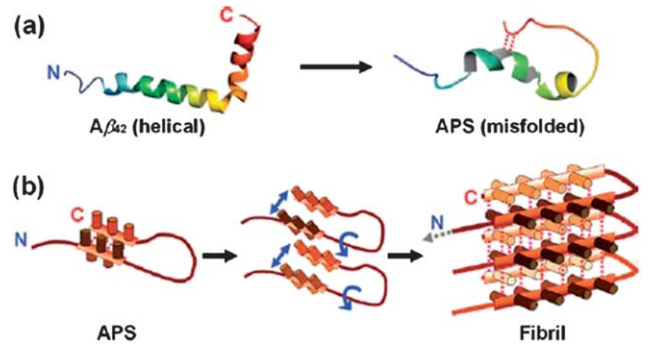

Fig. 10 (a) Production of an aggregation-prone structure (APS) from a helical Aβ42 monomer. Nonlocal backbone H-bonding is displayed as a red dotted line. (b) Possible aggregation model from the APS/misfolded state of the Aβ42 monomer to the fibril. The side-chain is portrayed as having a cylindrical shape and H-bonding is displayed as red dotted lines. In the APS, side-chains are vertically aligned to the direction of the intra-molecular H-bonds, and the blue-straight arrows represent the breakage of nonlocal intra-molecular H-bonds. The blue-curved arrows show how the side-chains turn over after the nonlocal H-bond breakage. In the fibril, intermolecular H-bonds are shown as red dotted lines, and side-chains are placed orthogonal to the direction of fibril growth. The figure was adapted with permission from ref. 124.

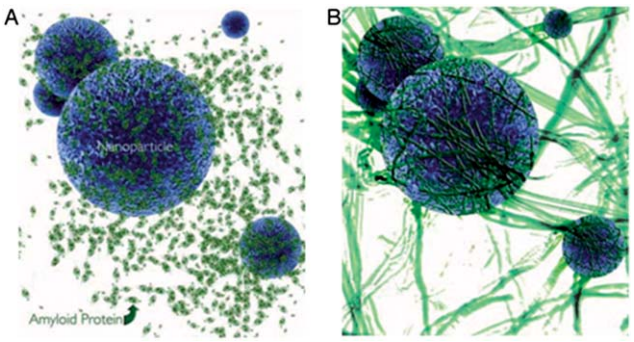

Fig. 11 Artistic rendering of amyloid protein fibrillation in the presence of NPs. (A) Large NPs (blue) and an amyloid protein (green) in its monomeric, folded state and (B) association of the amyloid protein with the NP surfaces, resulting in the generation of small oligomers, which are the precursors to fibrils. In solution, larger protein fibrils appear as their growth is enhanced by the surface association of proteins. The figure was reprinted with permission from ref. 162.

nonlocal contacts play in the protein fibrillation process (Fig. 10).

6 Effect of nanoparticles on the fibrillation process

Due to the presence of a high proportion of atoms at the surface, nanoparticles (NPs) have distinct properties compared with the bulk form of the same material; specifically, the surface properties at nanoscale, are dominant over the bulk properties.[125–127] These properties are quickly revolutionizing many industrial areas; one of the important enhancements of nanoscience is in the medical arena, leading to the emergence of the field of "nanomedicine".[128] NPs are recognized as promising and powerful tools in the fight against human brain diseases such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease.[129,130] One potential application of NPs in the treatment/diagnosis of brain diseases is to harness their effects on protein fibrillation for therapeutic advantage. It has been found that diverse substances, such as small molecules and peptides, have the capability to interfere with the fibrillation process.[131–134] Due to the tendency of many amyloidogenic proteins to accumulate at phase boundaries, the fibrillation process is also largely affected by the presence of biological macromolecules (*e.g.* amyloid precursor protein,[135–138] antibodies through passive immunization with anti-Aβ antibodies[139–141] and non-antibody-based natural mechanisms[142,143]), small organic molecules or non-peptides (*e.g.* inositols, phenols, and indoles),[136,144,145] and peptides[146] or other foreign surfaces (*e.g.* NPs, dialysis tubing, *etc.*).[67,147,148]

Linse and co-authors[149] have described a series of studies to understand the role of fundamental intermolecular interactions in protein folding, protein binding and the role of foreign surfaces (including NPs) in protein fibrillation. This work highlighted the potential for NPs to promote assembly of proteins into amyloid fibrils *in vitro* by assisting the nucleation process (see Fig. 11). NPs could thereby potentially open the door to new routes of controlling biological self-assembly for use in nanomedicine, arising from a novel but general surface-mediated nucleation mechanism. It is well known that NPs are coated with proteins in a biological fluid,[128,150–157] with potential for structural and functional perturbations of the surface-bound state of the protein.[158] Proteins may be bound in a native-like or denatured form, depending on the protein surface properties (charge, hydrophobicity, stability), and on the NP characteristics (hydrophobicity, size, coating).[154,159–161]

The potentially high concentration of proteins adsorbed at the NP surface can enhance the probability of partially unfolded proteins coming into frequent contact, leading to faster clustering.[163,164] These properties of NPs can influence protein self-assembly reactions, and they can potentially perturb important biological processes; finally they could enhance diseases involving protein misfolding and assembly, or where the oligomeric form is the toxic agent, they can potentially promote the removal of the oligomers by accelerating the fibrillation process as a therapeutic route. However, as shown in Table 1, below, the interactions between NPs and proteins are case-specific, and universal trends have yet to be discovered, suggesting the need for caution and careful testing of proposed nano-medicines with regard to their interactions with different amyloidogenic proteins.

NPs can be used to diagnose or better understand amyloid diseases. In most examples thus far, this detection occurs *ex vivo*, *i.e.*, by testing a sample of blood or cerebral spinal fluid. Thus, the patient is not exposed to the NPs. The basic concept is that a NP binds to a particular biomolecule, producing a characteristic signal that is absent or weaker without the protein–NP interaction. An early preclinical work includes the use of gold NPs to detect a marker for Alzheimer's disease in the blood, and the use of quantum dots to provide better image contrast to improve understanding of the Alzheimer's amyloid fibril structure.[165–168]

Table 1 Summary of the effects of different NPs on the fibrillation of different amyloidogenic proteins in solution. Note that solution conditions may not have been identical across studies, but two main mechanisms appear to be emerging: protein binding at the surface in a fibrillation-competent conformation leading to accelerated formation of critical nuclei and fibrillation and protein binding at the surface leading to reduced solution concentration/fibrillation incompetent conformation and delayed formation of the critical nuclei and consequently delayed fibrillation[a]

Classification of NPs	NPs composition	Protein	Remarks	Reference
Metal oxides	Maghemite (γ-Fe_2O_3)	Insulin	A significant direct slow transition from α-helix to β-sheets was observed during insulin fibrillation in the presence of the NPs; NPs showed an inhibitory effect	194 and 195
	Titanium oxide	Aβ	Showed strong adsorption capacity, leading to a high local concentration of the Aβ peptide on the NP surface, resulting in a shortened lag-time for Aβ assembly into fibrils	12,196
	Zinc oxide	Aβ	No discernible effect on fibril formation	196
	Silica	Aβ	No discernible effect on fibril formation	196
	Cerium oxide	β2-Microglobulin	Enhanced the probability of appearance of a critical nucleus for nucleation of protein fibrils; hence, the lag time decreased	181
Semiconductors	Quantum dots (QDs)	β2-Microglobulin; α-synuclein	QDs accelerated the fibrillation process by enhancing the probability of formation of the critical nucleus, which is required for fibrillation of both proteins	181 and 197
	N-Acetyl-L-cysteine capped QDs	Aβ	The capped QDs inhibited fibrillation due to intermolecular attractive interactions such as hydrogen bonding between the NPs and the amyloid fibrils resulting in blockage of the active elongation sites on the fibrils	198
Carbon based	Carbon nanotubes	β2-Microglobulin	Carbon nanotubes accelerated the fibrillation process. No mechanistic information provided	181
	Fullerene	Aβ	Fullerene was found to significantly inhibit fibril formation by specifically binding to the central hydrophobic motif of Aβ	134
	C_{60} and C_{70}	Aβ	C_{60} and C_{70} had no significant effect on the fibril formation	196
	Fluorinated carbon NPs	Aβ	By induction of a helical structure in Aβ, fluorinated NPs inhibited peptide aggregation	199
Polymers	Teflon	Aβ	Transferring Aβ from solution to the Teflon surface strongly promoted α-helix formation; increasing the degree of coverage of the Teflon by Aβ lead to a conformational change toward a more enriched β-sheet structure	200
	PEGylated phospholipid nanomicelles	Aβ	PEGylated phospholipid nanomicelles showed an inhibitory effect on the fibrillation process. Mechanism not discussed	201
	NIPAM/BAM particles	β2-Microglobulin; Aβ; islet amyloid polypeptide (IAPP)	Using NIPAM/BAM NPs, a high local protein concentration was achieved at the particle surface leading to the formation of critical nuclei and subsequently amyloid fibrils; the NPs had an acceleratory effect on the fibrillation process. With Aβ and IAPP, strong interactions of the proteins with the NPs reduced the solution concentration and thereby delayed the formation of the critical nuclei and the onset of fibrillation	181 and 202
	Amine-modified polystyrene NPs	Aβ	Dual effect observed, depending on the protein to NP surface area ratio. Kinetic effects varied from acceleration of the fibrillation process by reducing the lag phase at low particle surface area, to inhibition of the fibrillation process at high particle surface area	25
	Nanogels composed of a polysaccharide pullulan backbone with hydrophobic cholesterol moieties	Aβ	The nanogels showed an inhibitory effect on Aβ fibril formation by induction of a change in the conformation of Aβ from a random coil to an α-helix- or a β-sheet-rich structure	

[a] PHFBA: poly (2,2,3,3,4,4,4-heptafluorobutyl acrylate) and NIPAM/BAM: *N*-isopropylacrylamide/*N*-*tert*-butylacrylamide.

Several studies have shown the potential for NPs to inhibit the formation of amyloid protein fibrils associated with Alzheimer's disease (with a potential application in disease prevention) or to slow down the progress of fibrillation reaction, although to date these studies have been in solution, under controlled conditions, and thus the connection to the actual

disease progression is slight as yet, and significantly more work is needed.[67,169] NPs are being investigated as novel agents for penetrating the blood–brain barrier to deliver drugs to diseased brains and more specially for targeting Alzheimer's disease. At this point, none of the treatments have been approved for use in humans as they are still in the research phase.[170–180]

Linse *et al.*[181] suggested that NPs can provide novel mechanisms for the moderation of amyloid diseases. They observed that several kinds of NPs (copolymer particles, cerium oxide particles, quantum dots, and carbon nanotubes) can significantly enhance the rate of a protein fibrillation process, using the example of β2m (the protein involved in dialysis related amyloidosis), which results in the monomeric proteins assembling into fibrous strands (fibrils). They argued that the potential to induce nucleation of fibrillation is linked to the attachment of proteins to NP surfaces resulting in increased local protein concentrations and thus an enhanced probability of appearance of a critical nucleus for fibrillation. Surface-bound proteins may have structural and functional perturbations (although in the case of the in-solution fibrillation experiments, the solution conditions are established such that the protein is in a slightly unfolded state, *i.e.* the molten globule state, so that the fibrillation will occur on a relevant/measurable timescale); these alterations in protein conformation can promote their aggregation into oligomers which are the precursors of larger and longer fibrils. The observed shorter lag nucleation phase depends on the amount and coating of particle. There is exchange of protein between the solution and the NP surface, and β2m forms multiple layers on the NP surface, providing a locally increased protein concentration, and promoting oligomer formation. This suggests a mechanism involving surface-assisted nucleation that can increase the risk for toxic cluster and amyloid formation, but that also promotes the removal of these clusters by accelerating the formation of the non-toxic insoluble plaques.

Small changes in the physicochemical properties of NPs can also have significant effects on the fibrillation process of Aβ;[30] for example, superparamagnetic iron oxide NPs with various surface chemistries (*i.e.* positive, plain, and negative dextran coatings) and different sizes (*i.e.* single- and double-dextran layer coated NPs) were incubated with Aβ monomers. The results showed that the thickness and surface charge of the coating layer of the NPs affect the kinetics of fibrillation of Aβ in aqueous solution; more specifically, a size and surface area dependent "dual" effect on Aβ fibrillation was observed.[30]

Fei and Perrett[12] have also examined the effect of NPs on protein folding and aggregation, providing insight both into the mechanisms of these processes and how they may be controlled. The unfolding kinetics of lysozyme or β-lactoglobulin when adsorbed onto silica NPs shows that upon adsorption, the proteins show a rapid conformational change at secondary and tertiary structure levels.[182–184]

Bovine serum albumin (BSA) undergoes conformational changes at different pH and provides a good model for investigating the effect of NPs on protein conformational change. In the presence of gold NPs, BSA shows a decrease in the α-helical structure (as detected by circular dichroism), and a significant increase in β-sheet and turn structures (as detected by infrared spectroscopy).[182] Even if the native structure of the protein is retained after adsorption onto the NP surface, the thermodynamic stability of the protein may be decreased.[183] Hence, the application of NPs in drug delivery is challenged by the potential for destruction/modulation of the protein function when NPs enter the human body. Therefore, it is essential to understand in detail the effects of protein–NP adsorption, including identifying which properties of NPs determine their tendency to perturb protein conformation.

Although there are extensive reports on the effect of NPs on the kinetics of amyloid fibrillation, there are several "ignored factors" which remain poorly understood and need to be extensively considered in future. These include: (i) the effect of competitive binding from non-fibrillogenic proteins present in the "corona" that forms around NPs immediately upon contact with living systems;[185] and (ii) the effect of slight temperature changes (*i.e.*, in the physiological range for example related to inflammation or disease) on the amyloid fibrillation process.

It is increasingly being accepted that the surface of NPs is covered by various proteins, *i.e.* the protein "corona", upon entrance into or contact with a biological medium.[185,186] Thus, what the amyloidic proteins actually "see" *in vivo* is the corona coated NPs rather than the pristine or bare NPs. In order to probe the effect of corona coated NPs on the kinetics of Aβ fibrillation process, various types of corona coated nanomaterials (*e.g.* silica NPs, polystyrene NPs, and carbon nanotubes) were incubated with Aβ monomers;[187] it was demonstrated that the "hard corona" (*i.e.* the long lived protein shell at the surface of NPs) coated NPs, regardless to their physicochemical properties, can increase the lag time and slow down the fibrillation rate of Aβ compared to the fibrillation rate in the presence of the bare NPs The presence of the protein corona resulted in reduced access of Aβ to the NP surface and consequently reduced levels of Aβ fibril formation compared to the bare NPs, and the degree of effectiveness depended on how effectively the NP surface was covered by proteins.[187]

Regarding the other ignored factor mentioned above, *i.e.* slight temperature changes such as those that can occur during inflammation or disease, it was shown that changing the incubation temperature (*i.e.* in the physiologically relevant range), can have "dual" effects (*i.e.*, acceleratory and/or inhibitory depending on the NP and protein properties) on the amyloid fibrillation process, in the presence of hydrophobic (polystyrene) NPs, but only acceleratory effects in the case of hydrophilic NPs (*i.e.*, silica).[188] Increasing the temperature from 37 to 42 °C results in the core hydrophobic backbone of the Aβ monomers being potentially more available for interaction with NPs (see Fig. 12).

Cell "vision" may also be an additional currently ignored factor for consideration when evaluating the cellular impacts of amyloid oligomers/fibrils. Walsh *et al.* have suggested that it is the oligomeric Aβ entities that are toxic to neuronal cells, cell vision has been defined as the numerous detoxification strategies that any particular cell can utilize in response to toxins (*e.g.* amyloid oligomers/fibrils).[189–192] The uptake and defense mechanism could be considerably different according to the

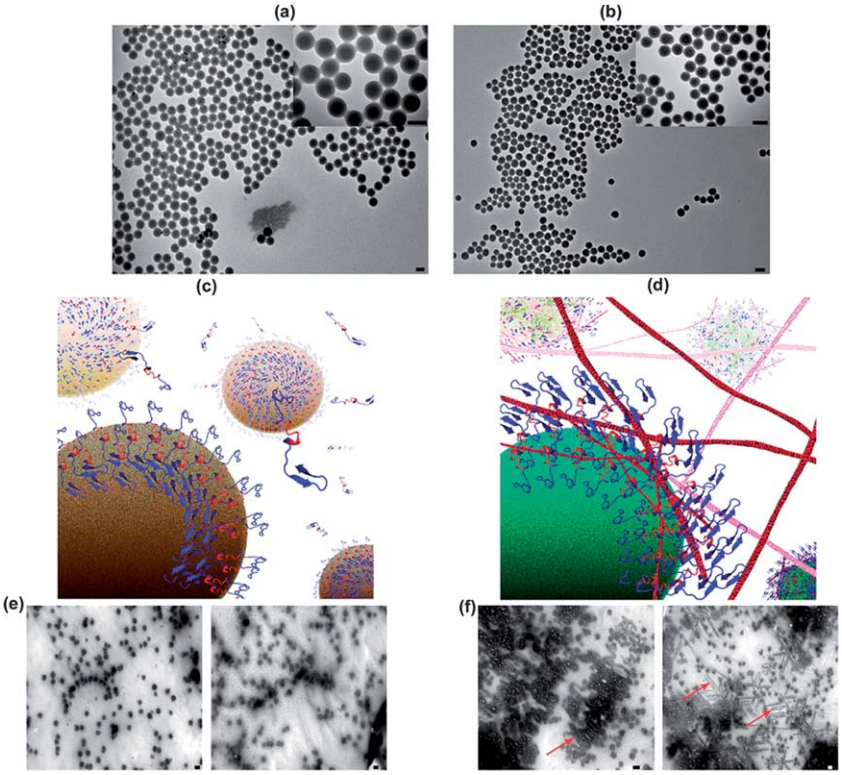

Fig. 12 TEM images of (a) polystyrene and (b) silica NPs (scale bar is 100 nm), showing the existence of spherical NPs with narrow size distribution. (c and d) Representative schemes illustrating the exposure of amyloids' hydrophilic and hydrophobic backbones to the free amyloid monomers after interaction with polystyrene and silica NPs, respectively, at 42 °C. (e and f) TEM images of the Aβ proteins following interaction with polystyrene and silica NPs, respectively, at 42 °C. As seen, there is no trace of fibrillation in (e); however, severe fibrillation (see red arrows for example) was observed in (f). In (e) and (f), left and right images correspond to interaction of Aβ with NPs at 20 and 400 min, respectively; scale bar is 100 nm; The figure was adapted with permission from ref. 188.

cell type, and indeed it has also been suggested that misfolded proteinaceous aggregates of HypF-N, a prokaryotic factor not associated with any amyloid disease, stimulate generic cellular responses as a result of the exposure of regions of the structure (such as hydrophobic residues and the polypeptide main chain) that are buried in the normally folded proteins. Thus, what the cell "sees", when it is faced with amyloid oligomers/fibrils, is most likely dependent on the cell type, which should be considered for interpretation of the toxic effects of amyloid oligomers/fibrils. The extent of the cytotoxic effects is elicited by supplementing the media of a panel of eight different cultured cell lines (murine C2C12 skeletal myoblasts, rat L6 skeletal myoblasts, human SH-SY5Y neuroblastoma cells, human HeLa adenocarcinoma cells, human IMR90 pulmonary fibroblasts, human A549 carcinoma cells, human A431 epidermoid carcinoma cells and monkey COS7 renal cells) with aggregates of HypF-N, a prokaryotic factor not associated with any amyloid disease. Cell susceptibility to damage from the aggregates was significantly related to the ability of cells to counteract early modifications of the intracellular free Ca^{2+} and redox status.

6.1 Nanocrystals are used as a FRET probe to detect protein-folding intermediates

From the single molecule viewpoint, probing the conformational changes of the Aβ structure upon interaction with NPs would be vital in order to achieve a deep understanding of the formation of the toxic structures; in this case, a number of advanced techniques are emerging to decipher the principles of protein folding. Besides AFM, CD, and fluorescence methods, Forster resonance energy transfer (FRET) assays can be used to track the conformational changes of proteins at a molecular scale. Distinguishing features and advantages of inorganic NPs for probing protein folding by FRET (compared to conventional organic dyes) include their tunable emission potential, their exceptional photostability, and their higher photoluminescence lifetime and higher quantum yield.[12] For instance, Sarkar *et al.*[193] employed a FRET assay in order to probe thermally induced conformation changes of human serum albumin using semiconductor CdS quantum dots as tags.

7 Conclusions and future perspectives

More than 30 proteins and peptides are known to cause human amyloid diseases, most of which affect the elderly when the natural processes to prevent protein aggregation fail, and all of which are currently untreatable. These diseases involve self-assembly of soluble proteins into insoluble fibrils *via* the formation of oligomeric structures that possess toxic properties.[203,204] It has been suggested that the fibrils are nature's way of removing the neurotoxic oligomeric species which are responsible for many of the cognitive and other effects

associated with amyloid diseases, certainly in the case of Alzheimer's disease. Surface interactions appear to be critical in these processes, as indicated by the fact that the presence of liquid–air, liquid–solid, or liquid–liquid interfaces have significant effects in promoting fibril formation, often by changing the protein conformation from its native state where the hydrophobic residues responsible for aggregation are hidden in the protein core, to a fibrillation-competent conformation; in this case, hydrophobic residues from neighboring proteins interact to form clusters and eventually fibrils, typically involving a cross-β structure with stacking of β strands perpendicular to the long axis of the fiber.

NPs, with their enormous surface area which act as a scaffold for protein adsorptions, and their small size which allows them unique access to tissues and cells, including the potential to cross the blood–brain barrier, offer significant potential for probing the mechanisms of protein fibrillation, and in the longer term for diagnosis or even treatment of amyloidogenic diseases. However, a key step towards achieving this is to ensure that any such diagnosis or treatment does not itself cause unintended harm. Thus a full understanding of NP interactions with, and impacts on, living systems is required before such therapies could be approved and widely implemented.

Much of the work to date involving NP interactions with amyloidogenic proteins has been *in vitro*, under conditions that promote the protein fibrillation so that it can be measured on an experimental timescale (as many of these processes can take over 60 years or more *in vivo*, which is not practical from an experimental viewpoint). Thus, any connections to disease are speculative at this point, but the data certainly suggest a role for NPs as mediators of protein fibrillation, which if it can be successfully harnessed offers significant potential for therapy for these currently intractable diseases.

From the NP–amyloidogenic protein interaction studies to date, two main mechanisms appear to be emerging: protein binding at the surface in a fibrillation-competent conformation leading to accelerated formation of critical nuclei and fibrillation, or protein binding at the surface leading to reduced solution concentration/fibrillation incompetent conformation and delayed formation of the critical nuclei and consequently delayed fibrillation. Either of these mechanisms could potentially be utilized to target different amyloidogenic processes, depending on whether the risks from the oligomeric species or the fibrils themselves are deemed to be higher. For example, interaction of the Aβ peptide with various NPs has been found to lead to both acceleration and inhibition of fibrillation. Indeed a dual effect was observed with some particles, where, depending on the ratio of protein to surface area, different effects were observed, indicating that therapies could be designed from either angle by using NPs to retain the protein in its native (fibrillation incompetent) state, or by using NPs to promote the fibrillation competent conformation and the formation of critical nuclei thereby accelerating the rate of fibrillation and potentially reducing the exposure of neurons to the toxic oligomeric species.

There are a few less studied parameters (*i.e.* the effect of competitive binding from other proteins, slight temperature changes, and differences in cellular capacities to regulate their responses to fibril challenge) on the real effects of NPs on the amyloidic proteins which need extensive *in vitro* and *in vivo* evaluation in future. Thus, to answer which therapeutic approach will prove optimal, most efficacious and safest, the solutions cannot be predicted *a priori* based on the current data; but certainly the field is wide open and significant opportunities exist for development of novel therapeutic approaches.

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