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Modulating protein amyloid aggregation with nanomaterials

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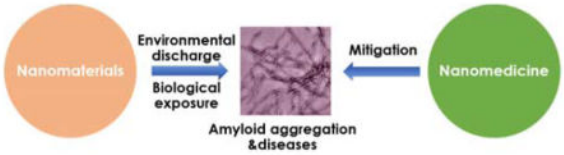
Abstract

Direct exposure or intake of nanopaticles (NPs) to the human body can invoke a series of biological responses, some of which are deleterious, and as such the role of NPs *in vivo* requires thorough examination. Over the past decade, it has been established that biomolecules such as proteins can bind NPs to form a ‘corona’, where the structures and dynamics of NP-associated proteins can assign new functionality, systemic distribution and toxicity. However, the behavior and fate of NPs in biological systems are still far from being fully understood. Growing evidence has shown that some natural or artificial NPs could either up- or down-regulate protein amyloid aggregation, which is associated with neurodegenerative diseases like Alzheimer’s and Parkinson’s diseases, as well as metabolic diseases such as type 2 diabetes. These effects can be either indirect (e.g., through a crowding effect) or direct, depending on the NP composition, size, shape and surface chemistry. However, efforts to design anti-amyloid NPs for biomedical applications have been largely hindered by insufficient understanding of the complex processes, even though proof-of-concept experiments have been conducted. Therefore, exploring the general mechanisms of NP-meditated protein aggregation marks an emerging field in bio-nano research and a new stage of handling nanotechnology that not only aids in elucidating the origin of nanotoxicity, but also provides a foundation for engineering *de novo* anti-amyloid nanomedicines. In this review, we summarize research on NP-mediated protein amyloid aggregation, with the goal of contributing to sustained nanotechnology and safe nanomedicine against amyloid diseases.

Graphical Abstract



Understanding nanoparticle-mediated protein amyloid aggregation is essential for sustainable nanotechnology and safe nanomedicines

Introduction

We are living in an era wherein access to nanotechnology is commonplace. The consumption of engineered nanomaterials for a variety of commercial products continues to grow steadily by year. However, there are still challenges and gaps in current knowledge regarding the potential hazards of nanomaterials towards the “safe handling of nanotechnology”.[1] Engineered nanomaterials, in particular NPs, enter the environment via the routes of air, water or land,[2] either resulting from purposeful application or as constituents of waste products, and have the capacity to circulate within a given ecosystem. The overarching impact of nanomaterials within the environment continues to be investigated even as we move forward in refining their production, sophistication and overall efficacy (Fig. 1). In 2015, *Environmental Science: Nano* presented a special issue under the theme of “Nanotoxicology in environment”, which collected notable studies investigating biological responses within model tissues or organisms to different engineered NPs.[3–11] Until now, practical techniques and models have been implemented for identifying the distribution and environmental fate of NPs.[12,13] Evaluations for ecological hazards and proper risk assessments of engineered nanomaterials have also been gradually completed.[14,15] However, further research is required to establish the role of NPs *in vivo*, in order to assess their biocompatibility and elucidate the origin of cytotoxicity.[1,3]

NPs can enter a given biological system via different routes, yet the immune system is always the first barrier for them to overcome.[16] Generally speaking, due to their large surface-to-volume ratio, it is expected that NPs possess the capacity to interact with a myriad of biomolecules, especially with proteins.[17] After more than a decade of research concerning NP-protein systems, it is well established that the physicochemical properties of NPs, including their chemical composition, size, surface property, and shape, are critical parameters for NP-protein interactions.[18–28] The NP-protein complex, or the NP-protein ‘corona’, determines the biological identity including the fate and transformation of the NP ‘core’ in intracellular and extracellular environments.[20,29–31] Comprehensive reviews on the topic of protein corona can be found elsewhere.[19,20,31,32] Briefly, owing to the capability of NPs in systemically circulating and passing through multiple organ systems prior to bodily clearance, the composition of the protein corona, in particular the transient ‘soft corona’ in the outer layer of the NP-protein complex, can evolve dynamically. Although protein concentrations may be important for their initial contact with NPs, affinities between the two species determine final NP-protein complex according to the Vroman effect.[33–35] Proteins with high binding affinity for NPs render a ‘hard corona’, which remains stable throughout circulation of the NPs within the body. However, the propensity for certain proteins to form a ‘soft’ or ‘hard’ corona on the same NP may be modulated by the environment. For example, using spectroscopic techniques Shang *et al.* found that pH altered the conformation of bovine serum albumin (BSA) bound to gold NP (AuNP) bioconjugates.[36] This behavior can prove problematic for NPs designed to change their structure or propert in response to environmental conditions such as temperature and pH, a strategy frequently employed for delivery of chemotherapeutics. The shift in corona composition in biological environments of neutral to acidic pH could impede the delivery capacity of these NPs and attribute to the low success rates of NP therapeutics.[37] In addition, NPs designed for drug delivery and

bioimaging may be off-target or prematurely cleared due to non-specific protein interactions.[38,39] Therefore, reducing or avoiding the interactions between NPs and environmental proteins is a common strategy employed through the generation of 'anti-fouling' NPs. This can be achieved through the grafting of polyethylene glycol (PEG)[40] as the 'gold standard' of anti-fouling, but additionally utilizing more biologically relevant constructs such as phosphorylcholine and nucleic acids.[41,42] In brief, protein anti-fouling can be advantageous, as NP-protein interactions can alter protein conformation, disabling their native function and even conferring cytotoxicity.[43] Certain NPs, in particular those that are highly positively charged, can associate with membranes[44] potentially resulting in cell death; protein binding can alter the net surface charge of NPs and reduce NP-membrane contact, thereby ameliorating the cytotoxic properties of the NP.

In reality, the complexity of biological systems is such that most biological consequences of NP introduction are actually unknown, aside from immediate notable responses from the immune system. There is, however, growing evidence demonstrating the links between ambient particulate matter and neurodegenerative diseases and neurological disorders.[45–48] Microparticles from air pollution, introduced to the human body through inhalation, are capable of systemic circulation and accumulation within extracellular and intracellular environments.[46] Maher *et al.* reported that magnetite NPs could cross the blood-brain barrier (BBB) and penetrate brain tissue, causing damage to neuron cells and potentially involved in the development of Alzheimer's disease (AD), a neurodegenerative disease strongly linked to cytotoxic protein aggregation.[49] Other degenerative diseases, such as type 2 diabetes (T2D) and Parkinson's disease (PD), are also correlated with the cytotoxic aggregation of a particular 'amyloidogenic' protein or peptide. Collectively, these pathologies are referred to as amyloid diseases.[50] Therefore, it is critical to explore intake of both environmental NPs and NP-based biomedical products as well as their association with protein aggregation. Beyond nanotoxicity, NPs with the capacity to strongly inhibit protein aggregation could be ideal candidates for the next generation of biomedicines to combat amyloid diseases. This review explores the growing research area of amyloid aggregation inhibition through the application of nanomaterials, and highlights potential opportunities for the wider research community.

An overview of NP-mediated protein amyloid aggregation

Protein misfolding and amyloid aggregation

A protein's structure and stability play an important role in its amyloid aggregation. In the case of globular proteins, protein misfolding is a necessary step of exposing their otherwise buried hydrophobic cores and backbone hydrogen-bond donors and acceptors, and constitutes a significant energy barrier toward the subsequent aggregation and fibrillization (Fig. 2A).[54] Intrinsically disorder proteins (IDP),[55–58] on the other hand, bypass such initial destabilization[56,71] where the major free energy barrier corresponds to nucleation of β-sheet rich aggregates before down-hill elongation and saturation of amyloid fibrils. Despite variations in primary, secondary and tertiary structures of amyloid-forming proteins, the kinetic process of amyloid aggregation and overall core structure of the final fibrils have been shown to be generally conserved. Mahmoudi *et al.* recently presented a comprehensive

review on protein amyloid aggregation, with special emphases on protein fibrillization kinetics and amyloid detection during the different phases of amyloid formation.[59] According to the widely accepted nucleation-growth model, the fibrillization process typically consists of a lag phase, a polymerization/elongation phase and a saturation phase, following a sigmoidal trend that can be visualized over time by employing amyloidophilic fluorescent dyes such as thioflavin T (ThT) (Fig. 2B).[60] Of the three phases, the lag phase usually represents the major rate-limiting step as it requires crossing one or more free energy barriers (Fig. 2A). In terms of atomic and mesoscopic morphologies, Sunde *et al.* demonstrated using X-ray that core cross-beta-sheet structures are a ubiquitous trait of amyloid fibrils; wherein continuous beta-sheets are formed with beta-strands running perpendicular to the long axis of the fibrils.[61] To date, only one amyloidogenic species, the peptide PSMα3 from *Staphylococcus aureus*, has been reported to form alpha-helical amyloid-like structures.[62]

Although the kinetic and mechanistic details have been explored for some model systems like amyloid beta (Aβ), the amyloidogenic peptide implicated in AD, detailed structural evolution and corresponding roles of key residues during the aggregation process are largely unknown.[59,63,64] Assumptions made on the behavior and toxicity of one type of amyloid protein may not be transferrable to another due to the differences in protein structure, their associated chaperones as well as their cellular and tissue environments. A pertinent example is of Aβ and human islet amyloid polypeptide (hIAPP, also known as amylin), an amyloidogenic peptide implicated in T2D pathology.[65,66] Though the two peptides have a similar number of residues and are even capable of co-aggregation,[66] they are oppositely charged and display markedly different aggregation kinetics – namely, hIAPP reaches the saturation phase 1–2 orders of magnitude faster than Aβ.[66–69] Therefore, for any amyloid protein system, each individual step in the aggregation pathway, from monomers to fibrils, must be identified. In addition, there is also a need to specify the structures and functions of the intermediate states that link each step in the amyloid fibrillization pathway, so that the critical conformations for either cytotoxicity or aggregation can be identified.

Impact of nanoparticles in different phases of protein amyloid aggregation

With increasing knowledge of NP-protein interactions and established mechanisms of protein amyloid aggregation, the possible scenarios in which NPs interfere with protein aggregation pathways can be explored.[59,70] Additionally, while NPs may participate in the entire process of protein misfolding and aggregation, the exact mediating effect of NPs likely varies depending on each specific aggregation phase, where the relevant concentrations of intermediate protein species as well as corresponding equilibria between them could change significantly[71] (Fig. 2). For instance, Linse *et al.* investigated the effect of copolymeric NPs on the fibrillization of β2-microglobulin (β2m) and concluded that the NPs accelerated the protein fibrillization by promoting the nucleation process, one of the vital steps for amyloid fibrillization.[72]

NPs are able to affect protein stability, which is closely related to the aggregation propensity.[73] Upon binding to NPs, changes in both secondary and tertiary structures can be induced within the native protein, causing partial unfolding or protein melting—

conformations essential for aggregation of globular amyloidogenic proteins, such as α-synuclein.[61,54,74] Even globular proteins not considered endogenously amyloidogenic have been investigated for their aggregation behavior under destabilizing conditions with the presence of NPs.[75–81] An example is BSA, a highly inert serum protein whose amyloid aggregation was observed after being exposed to polystyrene NPs or AuNPs.[36,82] Additional structural analysis indicates that the NPs could induce irreversible conformational changes in BSA. Interactions with certain NPs drive a decrease in α-helical content and promote a significant increase in β-sheets and turns, with the abundance in β-sheets suggesting a high probability of amyloid-like aggregation.

Guzzi *et al.* investigated the mediation effect of Cu^{2+} and Fe^{3+} cations on the amyloid fibrillization of β–lactoglobulin, a major proteinaceous component of milk, at high temperature and acid pH.[75] He found that Cu^{2+} played a catalytic role and stimulated β–lactoglobulin fibrillization without binding to the protein, while Fe^{3+} permanently bound to some fibrillization-related residues and inhibited fibril production by interfering with the nucleation phase. Though not natively amyloidogenic, lysozyme has been linked with hereditary systemic amyloidosis.[74,76,78–81,83] Small molecules such as polyphenols, cysteine, zinc ions and various dyes have been found to inhibit lysozyme aggregation,[78–81] while several metallic NPs have been shown to induce or promote lysozyme amyloids.[76,83] Zhang *et al.* investigated the interaction between lysozyme and AuNPs, and found that AuNPs rendered protein aggregates.[83] Cheng *et al.* explored the effects of metal oxide NPs on the structure and activity of lysozyme. While CeO_2 NPs triggered the transition of lysozyme secondary structure from alpha-helices to beta-sheets, inducing the hydrophobic region to become exposed to the solvent, ZnO NPs had little effect on lysozyme structure.[76] The formation of toxic species resulting from NP-induced aggregation of non-amyloidogenic proteins represents another cause of nanotoxicity, besides the toxic effects induced by immune response and ion dissolution.

NPs can also target the late stages of protein amyloid aggregation, namely the elongation and saturation phases. Xiao *et al.* investigated the inhibition of $A\beta_{1–40}$ amyloid aggregation with N-acetyl-L-cysteine capped quantum dots (NAC-QDs) and proposed that the hydrogen bonding between NAC-QDs and amyloid fibrils resulted in blockage of the active elongation sites on the fibrils (i.e. fibril-end capping).[84] Using molecular dynamics simulations Yang *et al.* revealed that graphene nanosheets could penetrate and extract peptides from pre-formed amyloid fibrils.[52] Given extensive library of proteins identified within human circulation, current research related to NP-mediated regulation of amyloid fibrillization lacks data concerning the structure and aggregation of plasma proteins upon protein-NP interactions.[25,59,63,70,85]

NPs may also function as ‘crowding agents’, providing a nanoscale interface that enhances local protein concentration, thus facilitating protein aggregation.[86,87] Using coarse-grained simulations, Auer *et al.* proposed a condensation-ordering mechanism in NP-catalyzed peptide aggregation.[88] Mahmoudi *et al.* recently demonstrated the concept of surface-assisted nucleation of Aβ fibrillization in the presence of NPs.[59] Another relevant review on this topic is provided by Kunznetsova *et al.*[87] The authors implicated macromolecular crowding as a factor affecting protein structure, folding, shape, conformational stability,

binding of small molecules, enzymatic activity, protein-protein interaction, protein-nucleic acid interaction, and pathological aggregation. PEG and polysaccharides are common 'crowding agents', with their capacity to act in this role determined by several parameters, including chemical composition and molecular weight.[59] Protein aggregation is also sensitive to changes in the physiological environment, e.g., temperature, pH and ion concentration. Levine *et al.* have explored the roles of osmolytes in regulating the conformation and aggregation propensities of the R2/wt peptide, a fragment of tau containing the aggregating paired helical filament (PHF6).[89] They found that by shifting the population of IDP monomer structures, osmolytes in urea halted aggregation and N-oxide (TMAO) promoted peptide oligomerization. They subsequently proposed a 'superposition of ensembles' theorem to rationalize how IDP structure and aggregation were regulated in the cell.

Challenges in predicting the effects of nanoparticles on protein aggregation

With increasing knowledge, we have been gradually building up libraries pertaining to amyloidogenic proteins and peptides, in addition to how these species interact with various NPs.[74,59,90] Although such resources are extremely valuable for providing first-hand references for the study of nanotoxicology and NP design, there are two major bottlenecks to overcome.

First, interactions of NPs and different amyloidogenic species must be considered on a case-by-case basis, as predicting the behaviors of new NP-protein systems based on data from known systems can frequently prove erroneous. Cabaleiro-Lago *et al.* altered the stability and intrinsic aggregation rate of single-chain monellin by introducing a series of five mutants and found that the anti-aggregation effect of NPs differed significantly between the mutant species. Specifically, N-isopropylacrylamide: N-tert-butylacrylamide (NiPAM/BAM) copolymer NPs promoted the aggregation of mutants with high intrinsic stability and low intrinsic aggregation rate, while inhibiting the aggregation of mutants with low intrinsic stability and high intrinsic aggregation rate.[91] In another example, copolymeric NPs demonstrated opposing effects between different systems, showing promotion of β2m fibrillization yet inhibition of Aβ fibrillization.[72] Variations in NP composition, size, surface chemistry and charge can also induce different behaviors in the same given amyloidogenic peptide or protein. For instance, Vácha *et al.* and Radic *et al.* both reported that NPs either up- or down-regulated protein aggregation, depending on the binding affinity between the proteins and the NPs.[94,90] It has been found that altering polymer hydrophobicity and net surface charge can modulate their action on protein amyloid aggregation.[59,71,92,93] As synthetic polymers such as NiPAM/BAM and dendrimers are utilized for drug delivery, the fact that their design may potentially have a downstream impact on amyloid aggregation *in vivo* should be taken into consideration.[92,93] A similar phenomenon has also been observed for superparamagnetic iron oxide NPs (SIONPs), where positively charged SIONPs were capable of promoting amyloid fibrillization compared with negatively charged or uncharged SIONPs at the same concentration.[59] With amino-modified polystyrene NPs, Cabaleiro-Lago *et al.* observed acceleration and inhibition of Aβ fibrillization in the presence of NPs with small and large surface areas, respectively.

Secondly, though numerous types of NPs show potential as anti-amyloid agents, the likelihood of these candidates successfully advancing to clinical trials is very slim. The cost of blind screening is significant, even from a computational standpoint, and there is no guarantee that NPs will significantly impact amyloid aggregation. Therefore, certain elements of NP design should be considered in order to predict the effect of specific NPs on protein aggregation. To ultimately establish a practical and conclusive model system for the screening of potential anti-amyloid NPs, further research is needed to determine how parameters such as NP surface chemistry and factors in the surrounding environment (global charge, ionic strength) are effective against a given amyloidogenic protein or peptide.

Amyloid-mediated cytotoxicity and mitigation with nanomaterials

Amyloid-based diseases, including AD, PD and T2D, each have a specific amyloidogenic peptide or protein implicated in their pathology. More than 20 amyloidogenic peptides and proteins are implicated in human diseases, with notable examples including Aβ[67], hIAPP[68], prion protein[95], α-synuclein[96], pTau[97], serum amyloid protein[98] and β2-microglobulin[64]. Aβ and hIAPP are two of the most frequently used model proteins for the study of protein aggregation. Aβ peptides are produced through sequential cleavage of the amyloid precursor protein by both β- and γ-secretase, which then vary from 39 to 43 amino acid residues in length.[69] hIAPP is a 37-residue peptide hormone co-synthesized, co-stored, and co-secreted with insulin by pancreatic β-cell islets, with an endogenous role in glycemic control.[68] The fibrillization of each of these peptides involves a complex multistep process, transitioning from monomeric form to soluble oligomers and protofibrils, or amyloid ‘seeds’, until finally forming large, hydrophobic mature fibrils. Current research favours low molecular weight soluble oligomers, presented as intermediate species within the aggregation pathway and serving as major contributors to amyloid-mediated cell death.[99,100] As discussed in the previous section, the kinetics of amyloid protein aggregation include multiple processes such as lag, elongation, and saturation phases displaying a cooperative or sigmoidal behavior (Fig. 2B).[101]

hIAPP is considered one of the most amyloidogenic peptides, as it readily forms amyloid aggregates *in vitro* at micromolar concentrations and reaches the saturation phase within several hours of incubation.[102] Surprisingly, though natively stored within β-cell granules at millimolar concentrations, intracellular amyloid aggregation rarely occurs in healthy individuals.[103] It has been proposed that components of the granular environment, including the presence of insulin, zinc ions and C-peptide in high abundance, as well as unique physiological conditions, such as low pH, aid in the stabilization of hIAPP in its native form. Although some predictions based on computational techniques have been proposed, how those components coordinate is largely unknown.[104]

In this review our discussion on amyloid aggregation regulation by NPs is dually on Aβ and hIAPP for the following reasons: firstly, though they possess no sequence homology, these two peptides share a similar number of residues; secondly, these two peptides demonstrate similar aggregation pathways and, potentially, a similar mechanism of toxicity. Lastly, and perhaps most intriguingly, many anti-amyloid agents are reported effective on both Aβ and hIAPP.[105] However, compared to research on Aβ, studies of hIAPP aggregation inhibition

have been lacking. In this light, knowledge and techniques gleaned from Aβ research could be implemented into studies on hIAPP. Multiple nanomaterials, including NPs of various composition and plant-based polyphenols, have been identified as effective anti-amyloid agents.[106] Typically there are two different ways to categorize anti-amyloid agents. One category pertains to the NP type, which is more suitable for single amyloid protein model systems such as Aβ.[59] The other format of categorization is based on different mechanisms of amyloid inhibition, which is more focussed on mechanistic similarity. In an early review, Härd and Lendel introduced general mechanisms on the inhibition of amyloid formation including native-state stabilization, sequestering of monomers, and use of small-molecules, peptides and antibody-mediated inhibition, as well as nature's regulation.[107] Here we utilize the second method, presenting what can be learned from current anti-amyloid strategies against amyloidosis, and additionally provide useful information about the pathologies of AD and T2D in particular.

Protected amyloidogenic region regulation

For a given amyloidogenic protein or peptide, the 'amyloidogenic region(s)' corresponds to one or more sequence segments that are critical for the formation of amyloid fibrils.[74] Usually, the amyloidogenic regions constitute the beta-sheet rich core of the fibril. Self-association of these regions and subsequent inter-chain beta-sheet formation result in the formation of cross-beta fibrils.[108] In the case of Aβ, nuclear magnetic resonance and hydrogen/deuterium exchange studies have identified the amyloidogenic region to be present within residues $A\beta_{16–22}$ ($_{16}$KLVFFAE$_{22}$) and $A\beta_{31–36}$ ($_{31}$IIGLMV$_{35}$) of the full length peptide.[109–112] Considering mixed antiparallel-parallel beta-strands to have the capacity as amyloid seeds, several studies have reported that fragments $A\beta_{16–22}$, $A\beta_{25–35}$, $A\beta_{35–42}$, and $A\beta_{37–42}$ could also be assembled into beta-sheet rich Aβ oligomers.[113–119] Amyloidogenic regions of a similar sequence length have also been identified in hIAPP, wherein the hexamer $hIAPP_{22–27}$ ($_{22}$NFGAIL$_{27}$)[61] – or, in some literature, the pentamer $hIAPP_{23–27}$ ($_{23}$FGAIL$_{27}$) – have been found critical for amyloid formation and cytotoxicity.[120,121] Indeed, the importance of the amyloidogenic region in determining amyloid-mediated cytotoxicity has been validated through sequence comparison with murine or rat IAPP, which contain five to six variations in residue regions 20–29 compared to hIAPP yet do not aggregate to form amyloid structures, and are found non-toxic to pancreatic β-cells.[121]

Accordingly, inhibition of amyloid contact at these key residues has been widely adopted as an anti-amyloid strategy. Fullerenes as well as their derivatives have demonstrated a strong inhibition effect on Aβ aggregation, and further studies indicated that this was due to their targeting of the amyloidogenic regions. Kim *et al.* utilized ThT fluorescence studies to assess the effect of 1,2-(dimethoxylmethano) fullerene (DMF) on the fibrillization of full length $A\beta_{1–40}$ and fragment $A\beta_{11–25}$. They found that DMF can inhibit initial peptide aggregation through binding to the central hydrophobic motif KLVFF.[122] Xie *et al.* studied the octomerization of $A\beta_{16–22}$ fragments in the presence of C_{60} NPs by performing extensive replica-exchange molecular dynamics (REMD) simulations. It was reported that strong interactions between the fullerene NPs and $A\beta_{16–22}$ fragments significantly weakened peptide-peptide contact, thus limiting amyloid fibrillization.[123] An additional study conducted by Sun *et al.* investigated the effect of DMF on the conformation of $A\beta_{1–42}$

dimer. Computational simulations indicated that the interaction of DMFs with Aβ peptides also greatly impeded the formation of β-hairpins and inter-peptide β-sheets.[124] As such, it was shown that fullerene could either directly bind to the central hydrophobic motif LVFFA or C-terminal hydrophobic region $A\beta_{31–40}(_{31}IIGLMVGGVV_{40})$, both of which are considered amyloidogenic regions for Aβ.[124,125] Polymers have additionally been found to effectively target the amyloidogenic region. Poly(amidoamine) (PAMAM) dendrimers have been extensively investigated as delivery vehicles for various therapeutics. Gurzov *et al.* utilized PAMAM dendrimers with terminal hydroxyl groups (Fig. 2B), and discovered that hIAPP fibrillization could be completely inhibited in their presence, consequently demonstrating a significant reduction in amyloid-mediated cytotoxicity *ex vivo*. Further computational studies indicated that PAMAM dendrimers could encapsulate one or two hIAPP monomers within their core subsequently forming a unimolecular micelle that shielded amyloid contact from free hIAPP, thus terminating the amyloid fibrillization pathway.[53]

Competing self-assembly regulation

It is known that protein-protein binding can act as a mutual stabilizing agent, inhibiting the unfolding and aggregation of each protein's native state.[107] Despite the fact that hIAPP and Aβ are intrinsically disordered, binding of these peptides to native proteins found in the intra- or extracellular milieu could act to inhibit amyloid aggregation *in vivo* (Fig. 2B). Indeed, we have previously discussed the stabilization of highly concentrated hIAPP within pancreatic β-cell granules by native components, and it stands to reason that similar phenomena can occur extracellularly. For instance, human serum albumin (HSA), the most commonly found protein in the plasma, is also one of the most potent inhibitors of Aβ self-association outside of the brain; it acts as an "external sink", and does not need to cross the BBB. HSA selectively binds with Aβ oligomers but does not interact appreciably with the monomers.[126,127] This strategy of amyloid inhibition through a "monomer-competitor" mechanism was also observed in the case of GA (thioglycolic acid)-stabilized CdTe NPs and their effect on Aβ self-association. Sequestering of both Aβ oligomers and Aβ monomers by CdTe NPs effectively shifted the state of equilibrium in the local population, thus inhibiting amyloid fibrillization of Aβ.[128] Experimental results also indicated that the fibrillization inhibition capacity of GA CdTe NPs is of analogous or improved efficacy to HSA.[128] As previously mentioned, Mahmoudi *et al.* incubated silica NPs with plasma and found that the protein corona was involved in the retardation of Aβ fibrillization.[70] Therefore, smart design of novel nanomaterials as anti-amyloid agents should take into account of the identity profile of a protein corona formed on a given agent, and engineer it to form a corona most suited to inhibit amyloid aggregation.

Inhibition by stabilizing off-pathway species

While aggregation inhibition by protecting the amyloidogenic region and competing self-assembly are effective strategies targeting the early phase of amyloid aggregation, there are alternative strategies redirecting the aggregation pathway to reduce cytotoxicity. Small molecules have been intensively screened for wide-ranging beneficial properties, especially related to pharmaceutical design or nutrition supplements. In previous sections, we introduced several small molecules capable of inhibiting amyloid aggregation. A large

number of small molecules can also impact amyloid fibrillization through non-specific binding. Polyphenolic compounds, including epigallocatechin gallate (EGCG, from green tea), curcumin (from turmeric) and resveratrol (from red wine), are well-known fibrillization inhibitors. Nedumpully-Govindan *et al.* applied molecular dynamics simulations to investigate polyphenol-mediated disruption of hIAPP aggregation at the molecular level. They found that hIAPP-polyphenol hydrogen bonds and pi-pi stacking combined with hydrophobic interactions were responsible for the stabilization of the oligomers. These polyphenols were shown to associate with the oligomers and form unique morphologies distinct from on-pathway oligomers, depending on the molecular ratio of the small molecules to the peptide.[105] Indeed, the exact inhibition effect of polyphenols as well as other natural products on amyloid aggregation can often be seen in a concentration-dependent manner.[106,129]

Amyloid inhibitors complexed with nanoparticles

Naturally occurring inhibitors have been considered promising anti-amyloid candidates due to their high biocompatibility while biomimetic molecules have also been explored. For instance, inspired by natural inhibition of hIAPP aggregation by insulin and other non-amyloidogenic variants such as rat IAPP, peptide inhibitors as well as peptide-mimetics have been developed to directly compete with peptide self-assembly, or act as 'beta-sheet breakers' to inhibit nucleation of amyloid fibrils.[107,130–133,134–136] However, application of those amyloid inhibitors may be limited by some of their intrinsic physicochemical properties – e.g., peptides have a tendency to aggregate; small molecules often have low water solubility; and some of the naturally occurring anti-amyloid inhibitors lack binding specificity for targets. Significant efforts have been made to apply nanotechnology to overcome the issues mentioned above. Xiong *et al.* proposed conjugation of peptide inhibitors to AuNPs. With the hybridization of two peptide inhibitors, VVIA and LPFFD, onto AuNPs (VCD10@AuNP), the authors reported that the cytotoxicity mediated by Aβ aggregation species was significantly reduced in the presence of the hybrid AuNPs, even at a low dosage.[137] Palmal *et al.* also reported that curcumin-functionalized AuNPs were well dispersed in aqueous conditions, which offered an enhanced performance against amyloid fibrillization and in dissolving amyloid fibrils.[138] Once specific targeting capabilities can be introduced to overcome the molecular 'promiscuity' of plant-based polyphenols, conjugation with NPs may represent a powerful tool to inhibit aggregation of a range of amyloidogenic proteins and peptides.[139] As discussed previously, NPs with drug delivery capabilities such as NiPAM/BAM and dendrimers possess anti-amyloid properties themselves.[92,93] It is a promising strategy to load anti-amyloid small-molecule inhibitors by utilizing their encapsulation ability or directly conjugating natural amyloid inhibitors around their surface to achieve a boosted anti-amyloid effect on designated targets.

Functional amyloids and nanotechnology

In human biology, amyloid formation is almost universally a pathogenic process. A notable exception is the amyloidogenic peptide Pmel17: its fibrillization plays a vital role in the production of melanin, and yet does not elicit cytotoxicity *in vivo.*[140] So-called 'functional' amyloids are frequently observed in bacteria (Fig. 3A) and perform a variety of roles, including aiding in biofilm formation (Fig. 3B), pathogen-host interactions, evasion of the

host's immune system, and even as antimicrobial agents against competing bacteria.[141,142] Overall, amyloid formation enhances the pathogenicity of bacteria, leading to infections that are highly difficult to treat and fouling in agriculture and food packaging. Though many NPs, in particular silver NPs (AgNPs) have been utilized as antimicrobials, most strategies to specifically combat bacterial amyloid formation have utilized small molecules.[143–146] Cegelski *et al.* identified two small molecules with anti-amyloid capabilities against *Escherichia coli* curli amyloids, known as curlicides, with the capacity to inhibit *E. coli* amyloid-dependent biofilm formation.[143] Andersson *et al.* and Chorell *et al.* screened libraries of small molecule curlicides and also demonstrated an inhibitory effect on biofilm formation in *E. coli.*[145,146] Romero *et al.* utilized *Bacillus subtilis* as a model bacterium to screen anti-amyloid agents, and found several small molecules capable of inhibiting biofilm formation in *B. subtilis* and additionally in *E. coli* and *B. cereus.*[144] Functional amyloids have, however, found further use in nanotechnology due to their structural properties, lack of toxicity, and robust nature. Chen *et al.* utilized AuNPs and QDs with *E. coli* curli amyloids to engineer a number of nanomaterials, including a platform for gold nanowire and nanorod production, a biofilm-based electrical switch, and the creation and modulation of fluorescent lifetime of different QDs.[147] Biocompatible amyloids generated through high temperatures and acidic conditions from β-lactoglobulin can be hybridized with activated porous carbon to form an effective filtration system for wastewater, capable of reducing heavy metal ion concentration by 3–5 orders of magnitude per passage (Fig. 3C).[148] Remarkably, functional β-lactoglobulin amyloids have recently also been used as a transporter for *in vivo* iron fortification.[149]

Challenges and prospective

In the above sections we have introduced various types of NPs that interact with amyloidogenic peptides and proteins to mediate anti-amyloid properties. However, many challenges remain before nanotechnology can be fully implemented as a potent resource for amyloid regulation.

First, it is an ultimate goal in the pharmaceutical industry to design functional nanostructures with minimal adverse effects within the circulation and maximum efficacy at the target. The design of NPs to suit a biological system is complicated by the changeability of the environment within the body, for example the pH and ionic strength can vary greatly in different compartments and between intracellular and extracellular milieu. As a result, NPs may underperform or be rapidly cleared from the body when conditions beyond their limits of stability. This has been found relatively common for metal-based NPs.[148,151] Neupane *et al.* discussed the presence of a 'bridging' mechanism wherein NPs could be connected by proteins, thus forming larger aggregates and reducing their dispersity.[151] Therefore, surface coating chemistries that enable robust functionality of a given NP within a dynamic environment are required for biomedical applications.

Secondly, growing evidence supports the understanding that deformation of the cell membrane by amyloid aggregates is a major mechanism of amyloid-mediated cytotoxicity.[152] Therefore, the roles of biological interfaces, such as membranes/lipid bilayers or microtubules, are undoubtedly critical to consider when investigating amyloid

aggregation. In a recent study, Terakawa *et al.* reported that liposomes are able to regulate the fibrillization of Aβ, and more specifically, small liposomes (≤ 50 nm) significantly accelerated the nucleation step of the peptide. Larger liposomes, however, decreased the amount of fibrils formed but did not notably affect the lag time. The morphologies of Aβ fibrils became shorter and the amount of amorphous aggregates became larger as liposomes increased in size.[153] The presence of a large variety of membrane interfaces *in vivo*, from nanoscale micelles to cell plasma membranes, further complicates the nature of amyloid aggregation. How NP mediation of amyloid aggregation is affected by surface seeding of amyloids, NPs, or a given NP-amyloid complex is vital to elucidating how each factor comes into play *in vivo*. In a recent study, Kim *et al.* systematically investigated how the size, charge and shape of AuNPs affected Aβ aggregation on the brain lipid bilayer.[154] In general, there is no consensus as how NPs and interfaces co-regulate amyloid aggregation, and how they affect cell function.

Thirdly, the mechanisms of amyloid aggregation-related diseases are still far from being well understood. In some cases, more than one misfolded protein can be implicated in a single disease, such as the simultaneous accumulation of Aβ and pTau aggregation in AD.[66] There are also cases of amyloidogenic proteins with close correlations; namely, there is a substantial overlap of pathological abnormalities leading to relatively frequent appearance of mixed pathologies, characterized by the presence of multiple protein aggregates in the same tissue. For instance, it is common to find in the brains of AD patients accumulation of α-synuclein and TDP43, which are major factors implicated in PD and amyotrophic lateral sclerosis respectively.[155–160] This so-called ‘cross-seeding’ phenomenon often occurs in the brain concerning local peptides like Aβ and pTau, but can also be found in the pancreas with the formation of co-amyloids of hIAPP.[161,162] Those new findings present multiple challenges to existing paradigms since, for the purpose of simplicity, we have only considered the effect of NPs on a single species of amyloidogenic peptide. In the case that multiple amyloid peptides are concurrently present, the functionality of existing inhibitors might be lost or reversed given the changed relative binding affinity either between NPs and proteins or between the proteins themselves.

Finally, even assuming NPs have achieved their designated function, the subsequent biodegradation and elimination of NPs is a vital consideration. For example, despite the fact that some NPs like colloidal inhibitors significantly decease cytotoxicity mediated by amyloid fibrillization, aggregates of the particles themselves can have deleterious consequences within a biological system, such as inducing blood clots. It is even more dangerous for ion-based NPs, where ion leakage may impact local ion balance, with the full scale of which not yet known.

Conclusion

We have presented an overview of current progress in NP-mediated inhibition of amyloid aggregation and associated cytotoxicity. Utilizing hIAPP and Aβ as model pathogenic amyloids, we have demonstrated the strategies available to inhibit amyloid aggregation and to further combat amyloid-related diseases, such as AD and T2D. There are still many challenges to be considered before anti-amyloid NP agents can be developed for application

in humans. However, regulating protein amyloid aggregation with NPs is a new and promising interdiscipline, and presents tremendous opportunities for implementing nanotechnologies in biomedical applications. In that regard, learning from functional amyloids and their environmental and *in vivo* applications may prove beneficial.[147,149]

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Environmental significance

One potential adverse effect of nanoparticles (NPs) at large is NP binding-induced protein aggregation, which could consequently elicit nanotoxicity. Mounting evidence shows that NPs, depending on their core composition, size, shape and surface chemistry as well as the intrinsic properties of the protein, could either promote or inhibit amyloid aggregation. Understanding the physicochemical properties of NPs, proteins, and environmental conditions that govern NP-mediated protein aggregation may facilitate "safe-by-design" nanotechnology for biocompatibility and sustainability. Research in this new frontier can help design novel anti-amyloid nanomedicine, and guide exploration of functional amyloids. This new phase of nano-bio research is critical to revealing the relationships between NPs and their surrounding biological environments from bottom up, thus ensuring the effective use of nanomaterials/nanotechnology for human health and environmental sustainability.

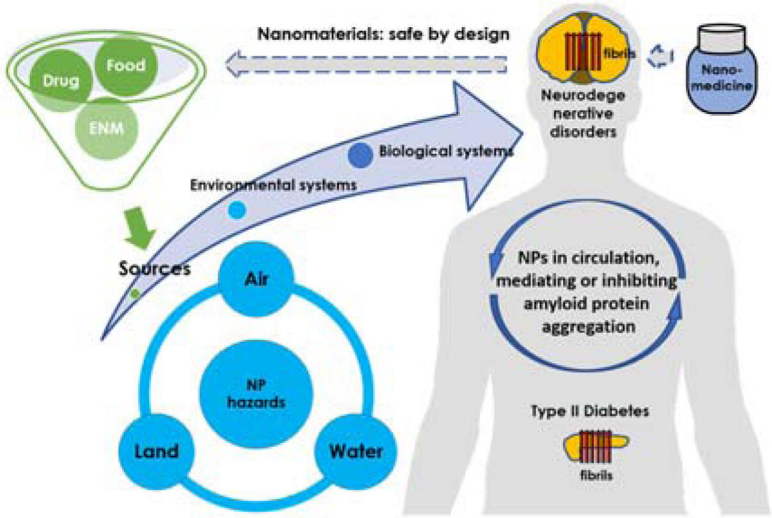

Figure 1.

Nanomaterials enter environmental systems as potential bio- hazards and spread via the routes of land, air and water. They come into contact with biological systems, including humans, and may trigger various amyloid diseases due to protein aggregation. Understanding the effects of nanomaterials from the perspective of protein aggregation may facilitate the design of environmentally responsible nanomaterials and the development of nanomedicine against amyloid diseases. ENM: engineered nanomaterials. NP: nanoparticle.

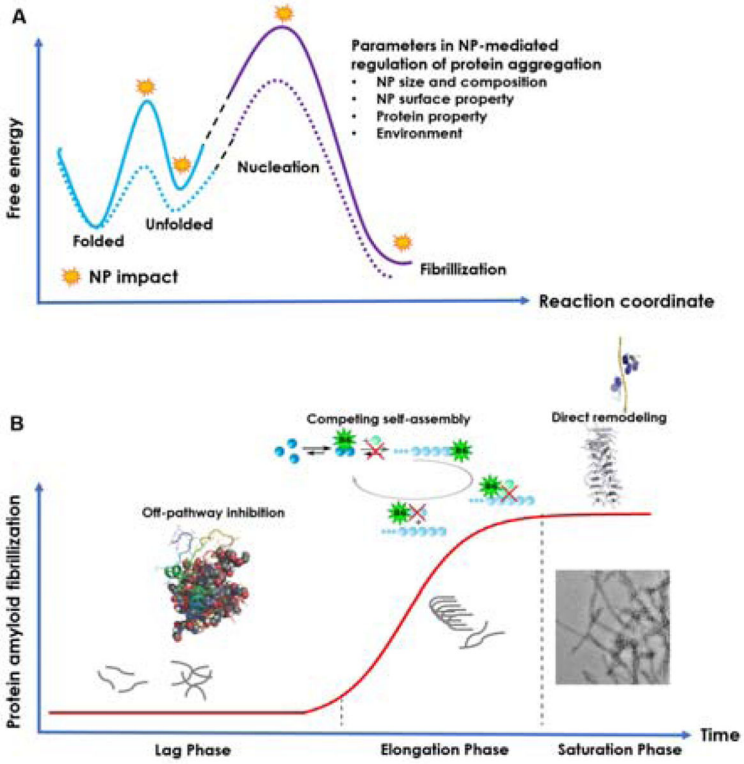

Figure 2.

(A) Schematic free-energy landscape of protein misfolding and amyloid aggregation (solid curve). The shift (dashed line) occurs in the presence of NPs, which modulates the energy basins of various states as well as major energy barriers separating them, such as unfolding and aggregation nucleation. (B) The kinetic process of protein amyloid fibrillization and mitigation with PAMAM-OH dendrimer, DNAB6 and graphene sheets during the lag, elongation and saturation phase, respectively. Adapted from *J. Biol. Chem.* 2014; 289, 31066-31076[51]; *Nanoscale* 2015, **7**, 18725–18737[52]; *Small*, 2016, **12**, 1615–1626,[53] copyright 2016 John Wiley & Sons.

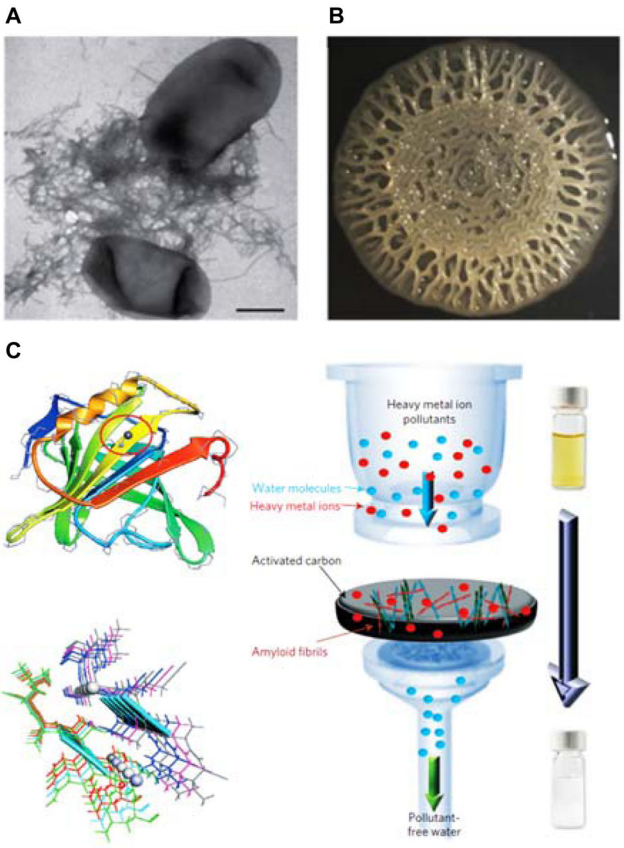

Figure 3.

(A–B) Curli amyloid formation in *Escherichia coli*. A: TEM image of curli amyloid plaques formed by two *E. coli* bacteria. Scale = 500 nm. B: *E. coli* biofilm formation aided by generation of curli amyloids. (C) β-lactoglobulin amyloids for wastewater purification through metal coordination of the 121-cys-containing amyloidogenic fragment (LACQCL) of the protein. The metal ions are indicated in the red circle and shown as spheres in the left panels. A–B: Adapted from *Biochim. Biophys. Acta BBA - Mol. Cell Res.*, 2014, **1843**, 1551–1558,[150] copyright 2014 Elsevier. C: Adapted from *Nat. Nanotechnol.*, 2016, **11**, 365–371,[147] copyright 2016 Nature Publishing Group.