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REVIEW

Nanoparticles in relation to peptide and protein aggregation

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Abstract: Over the past two decades, there has been considerable research interest in the use of nanoparticles in the study of protein and peptide aggregation, and of amyloid-related diseases. The influence of nanoparticles on amyloid formation yields great interest due to its small size and high surface area-to-volume ratio. Targeting nucleation kinetics by nanoparticles is one of the most searched for ways to control or induce this phenomenon. The observed effect of nanoparticles on the nucleation phase is determined by particle composition, as well as the amount and nature of the particle's surface. Various thermodynamic parameters influence the interaction of proteins and nanoparticles in the solution, and regulate the protein assembly into fibrils, as well as the disaggregation of preformed fibrils. Metals, organic particles, inorganic particles, amino acids, peptides, proteins, and so on are more suitable candidates for nanoparticle formulation. In the present review, we attempt to explore the effects of nanoparticles on protein and peptide fibrillation processes from both perspectives (ie, as inducers and inhibitors on nucleation kinetics and in the disaggregation of preformed fibrils). Their formulation and characterization by different techniques have been also addressed, along with their toxicological effects, both in vivo and in vitro.

Keywords: amyloid formation, inducer, inhibitor, nanoparticle, nucleation, toxicity

Introduction

Nanoparticles are nanosized materials with a dimension of 1–100 nm. These particles exhibit interesting optical, electronic, and catalytic properties, which are very different from those of the corresponding bulk materials. Nanoparticles can be naturally found in the atmosphere, primarily in natural waters, soils, and sediments. Both natural and synthetic nanoparticles have practical applications in a variety of areas, ranging from environmental remediation to an emerging multidisciplinary field that combines chemistry, engineering, physics, biology, and medicine (Figure 1).[1] Nanoparticles are expected to bring about a revolution for the inhibition of protein and peptide aggregation – a process related to several "misfolding diseases."

Proteins are important biological macromolecules that are fundamental to the proper functioning of cells and organisms; therefore, the impact of nanoparticles in living organisms at the protein level is a critical issue that is attracting increasing attention from researchers. Protein and peptide aggregation into characteristic amyloid fibrils is a major cause of various neurodegenerative diseases like Alzheimer's, Parkinson, Creutzfeldt–Jakob disease, and others.[2,3] In such diseases, there is a conversion of proteins or peptides from their soluble functional states to highly organized fibrillar aggregates.[3] Altogether, the accumulation of abnormal protein and peptide aggregates

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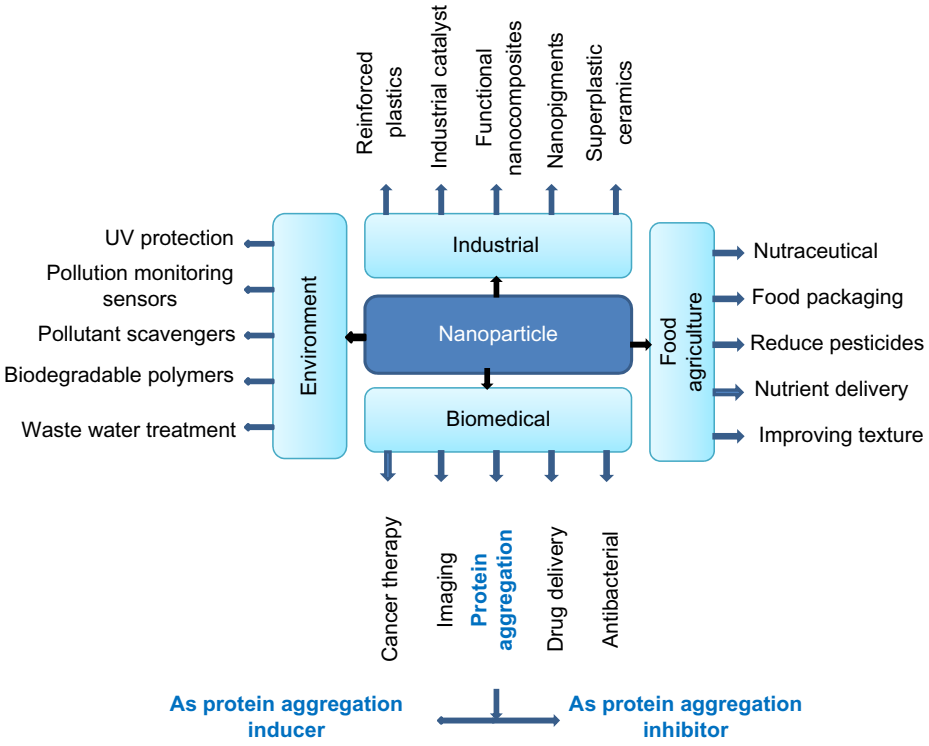

Figure 1 Application of nanoparticles in various fields such as in the biomedical, environmental, industrial, and food agriculture industries.
Abbreviation: UV, ultraviolet.

exerts toxicity by disrupting intracellular transport, overwhelming protein degradation pathways, and/or disturbing vital cell functions. In addition, the formation of inclusion bodies is known to represent a major problem in the recombinant production of therapeutic proteins and peptides.[4] On the other hand, amyloids also hold biological importance, as positive physiological activity (like human Pmel17) has an important role in the biosynthesis of pigment melanin, and factor XII protein of the homeostatic system is activated by amyloid formation.[5] Amyloid fibril formation proceeds through nucleated growth mechanism, and the conversion of a peptide or a protein into its fibrillar form (measured by different techniques) includes a lag phase followed by an exponential phase.[6,7] It is the lag phase in which nuclei form and further monomers or oligomers are associated in the fibrillation of proteins. The fibrillation behavior can be modulated by several factors including hydrophobicity, types of secondary structures, the presence of chaperons, peptide inhibitors, as well as solution properties such as ionic strength, pH, temperature, and so on.[8,9] Thermal denaturation leads to the exposure of hydrophobic residue, which increases hydrophobic attraction that overcomes electrostatic repulsion, and triggers the aggregation of amorphous aggregates. Hill et al[10] found that net attraction causes precipitation, while interaction of repulsive charges causes amyloid formation. Recently Raccosta et al,[11] working on lysozyme aggregation, clearly indicated that at low pH and low ionic strength, a lysozyme solution is thermodynamically stable in both its native and denatured state (Figure 2). Formation of amyloid occurs not only at higher temperatures, where proteins undergo partial unfolding, but also at pH levels far from their isoelectric point where proteins are electrically charged.[12,13] It has been shown that modified surfaces of lipid bilayers, collagen fibers, liquid air, liquid–solid, or liquid–liquid interfaces have specific and significant effects in promoting amyloid formation.[14,15] Nanoparticles possess an enormous surface area and are found to influence the amyloid-forming behavior of proteins very controversially. The interaction of nanoparticles with proteins can affect both protein structure and function; ie, they can inhibit or facilitate amyloid formation.

The experimental data showed that copolymer particles, cerium oxide particles, carbon nanotubes, and quantum dots enhance the rate of fibril formation from β2 microglobulin by decreasing the lag time for nucleation.[16] It was observed that the duration of the lag phase depends upon the amount and nature of the particle surface. Wu et al[17] observed that titanium oxide nanoparticles promote Aβ peptide amyloid aggregation, and in these types of cases, nanoparticles may act as catalysts for amyloid assembly. The molecular events behind the processes beginning from the native to fibrillar

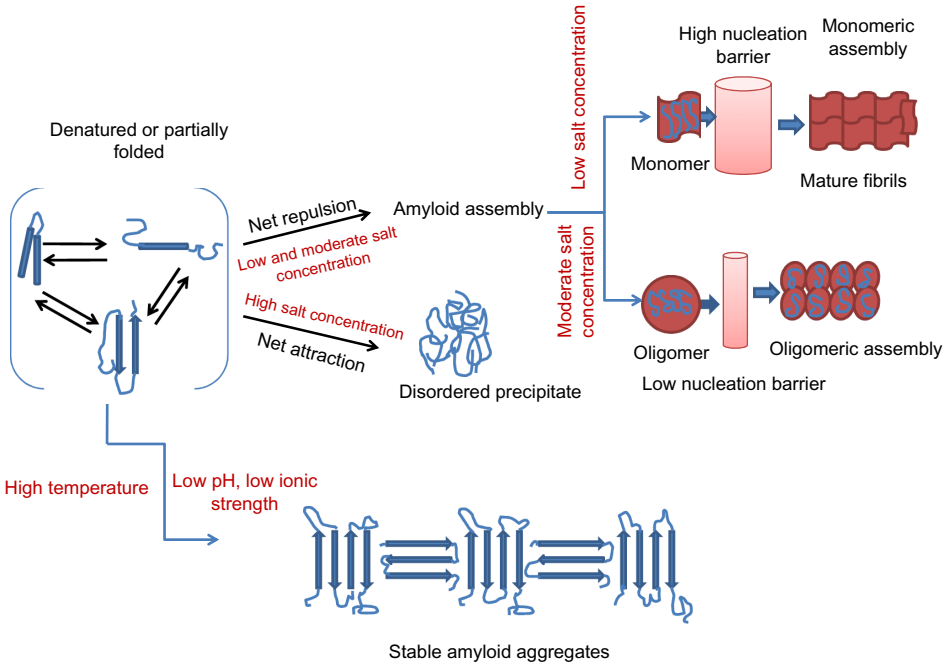

Figure 2 Effect of salt, pH, and temperature on protein aggregation.
Note: Low and moderate salt concentrations cause amyloid assembly, while a high salt concentration is responsible for disordered precipitates.

state remain elusive, but the data from many studies suggest that fibrillation involves a number of intermediate oligomeric states of different association numbers and structures.[18] Engineered nanoparticles may be an effective strategy for inhibiting the fibrillation of proteins at its various stages, while keeping in mind safety issues such as biocompatibility and biodegradability.

In the present review, we explored nanoparticle effects on protein fibrillation from both perspectives (ie, as an inducer and an inhibitor), their preparation, characterization, and toxicity (both in vivo and in vitro). We also highlighted some peptide and protein nanoparticles with their unique functionalities and potential applications.

Characteristics of nanoparticles

Synthetic nanoparticles include polymer conjugates, polymeric nanoparticles, lipid-based carriers such as liposomes and micelles, dendrimers, carbon nanotubes, peptides, and gold nanoparticles, including nanocages.[19] Owing to their high surface area-to-volume ratio, it is possible to achieve high ligand density on the surface for targeting purposes. In terms of composition, nanoparticles are comprised of mainly two parts: the core material and the surface modifier that may be engaged to change the physiochemical properties of the former.[20] Changes in protein conformation upon binding with nanoparticles, or increased local protein concentration on the nanoparticle surfaces could promote aggregation, while trapping of early aggregation intermediates may inhibit further aggregation.[21] Nanoparticles possess huge amounts of Gibb's free energy, which enhances their adsorption capacities. The conjugation of proteins to nanoparticles not only stabilizes the system, but also introduces biocompatible functionalities to these particles for further biological interactions or coupling.[22,23] Nanoparticles also offer the benefit of carrying functional groups (amino and carboxylic groups), which can be used for surface modification.[24] On the other hand, nanoparticles possess higher stability during storage, in vivo stability after administration, as well as being easily adjustable during preparation.[25]

Peptide nanoparticles and their conjugates

Peptide nanoparticles offer several unique advantages that make them attractive for therapeutic applications. Their smaller size minimizes the overall radius of the resulting peptide nanoparticle conjugate, while still affording high valance (ie, the number of peptides per nanoparticle).[26] Apart from this, their size reduces immunogenicity in vivo, and their production is economical and facile. Peptide nanoparticles are biocompatible, derived from naturally occurring protein precursors, and can be very specific; they also bind tightly to the receptors.[27] Based on the structural features of amyloid fibrils and the mechanism of their assembly, several peptides and their analogs have been designed and

synthesized towards various protein aggregation diseases. Pentapeptides, like KLVFF (Lys-Leu-Val-Phe-Phe) and LVFFA (Leu-Val-Phe-Phe-Ala) are widely used as binding elements to design inhibitors of fibrillation as they mimic some residues in the hydrophobic core of Aβ 1-40 fibrils.[28] LV (Leu-Phe) and VF (Val-Phe) peptides containing leucine and valine residues, respectively, in the hydrophobic core region provide an attractive tool for capturing the toxic Aβ 1-42 aggregates in Alzheimer's disease.[29] In this process, soluble oligomers that are responsible for this disease, and the small amounts of mature Aβ 1-42 fibrils, are transformed into peptide fibrils. However, a novel strategy was reported by Li et al[30] for inhibition of amyloid beta peptide aggregation by polyoxometalate–peptide hybrid particles.[30] The authors reported that self-assembly of these peptide hybrids is effective against Aβ inhibitors. Various small peptides called β-sheet breakers are effective against the process of beta-amyloid fibrillation by dissolving amyloid fibrils in vivo as well as in vitro. Jarmula and Stepkowski[31] reported two β-sheet breakers iAβ5 (LPFFD) and iAβ6 (LPFFFD), which have two to three consecutive Phe residues that are responsible for forming a stacked conformation. Apart from this, cyclic peptides have great potential to be therapeutic agents against many debilitating amyloid-related diseases. One example of this type of peptide was reported by Todorova et al;[32] this peptide, cyc (60–70), is derived from human apolipoprotein CII, which acts against amyloid formation by the fibrillogenic peptide, apoC-II (60–70). Liu et al[33] explored a B6 peptide in conjugation with polyethylene glycol–polylactic acid nanoparticles to enhance the delivery of neuroprotective drugs across the blood–brain barrier for the treatment of Alzheimer's disease. These modified conjugates show higher accumulation in brain capillary endothelial cells via lipid raft-mediated and clathrin-mediated endocytosis.[33]

Protein nanoparticle and their conjugates

Due to their defined structure and biodegradable nature, protein-based nanoparticles provide large possibilities for surface alterations and for the covalent attachment of drugs and ligands.[34] In addition, they are nontoxic and easy to crosslink, they can be easily modified and sterilized, are usually not contaminated with pyrogens, and generally possess low antigenicity.[35] Gelatin, albumin, gliadin, legumin, zein, and soy are some proteins that are commonly used for nanoparticle formulations. Gelatin is obtained by controlled hydrolysis of fibrous, insoluble proteins, and by collagen, which is widely found as the major component of skin, bones, tendons, and connective tissue.[36] The mechanical properties, swelling behavior, and thermal properties depend significantly on the degree to which gelatin crosslinks.[37] Gelatin has either a positive or negative charge depending upon the type of amino acids residues. Formation of type 1 and type 2 gelatin depends upon the treatment of collagen with an acid and base, respectively. Glycine, proline, and alanine are present in gelatin, and these elements are responsible for the formation of gelatin's triple-helix structure. They also provide great flexibility for modification and covalent attachments, and further modification takes place on the particle's surface or in the matrix of the particle.[38,39]

Recently, Kuntworbe and Al-Kassas[40] developed cryptolepine hydrochloride-loaded gelatin type A nanoparticles that provide a better approach in the treatment of malaria. Zein, a prolamine protein obtained from maize with three-fourths lipophilic amino acid residues and one-fourth hydrophilic amino acid residues, provides unique aqueous–alcohol solubility, as well as contributes to its film-forming property. It is widely used for nanoparticle preparation in food and nutraceutical industries, and for encapsulating lipophilic bioactive compounds. For nutraceutical and drug encapsulation, one of the most widely utilized proteins is soy protein.[41] The soy protein isolate-enriched form of soy protein possesses a balanced composition of polar, non-polar, and charged amino acids,[41] which can incorporate ligands through its various functional groups. This protein is beneficial in pharmaceutics, because it can help to design an appropriate drug against various protein aggregation diseases, which may be due to polar, non-polar, or charged amino acid residues.[41] For the formation of mucoadhesive nanoparticles, another protein, gliadin (which is obtained from the gluten of wheat and vicillin, and which is rich in lipophilic and neutral amino acids), appears to be a suitable polymer in the formulation industry.[42]

Among all of the proteins, albumin is an attractive macromolecule that has been shown to be biodegradable, nontoxic, metabolized in vivo to produce innocuous degradation products, available in pure form, non-immunogenic, and soluble in water; it makes an ideal candidate for nanoparticle formulation.[43] Albumins are of different types like ovalbumin, human serum albumin, bovine serum albumin, and so on. Due to define primary structure and high content of charged amino acid residues, albumin-based nanoparticles could allow for the electrostatic adsorption of positively- and negatively-charged molecules without the requirement of other compounds.[44,45] Apart from this, they can be easily prepared under normal environmental conditions by various methods like coacervation, controlled desolvation, and emulsification. In addition, albumin nanoparticles are well

tolerated by our body, which is supported by various clinical trials on registered human serum albumin-based particle formulation like Albunex[45] (Mallinckrodt Pharmaceuticals, Hazelwood, MO, USA) and Abraxane®[46] (Celgene Corporation, Summit, NJ, USA).

Preparation methods

Currently, there are various methods that are being used for the preparation of nanoparticles. Several comprehensive reviews are available where the preparatory methods are discussed in great detail.[47–49] The commonly employed materials and methods used for the synthesis of nanoparticles are summarized in Table 1. Protein-based nanoparticles, such as those that use albumin, gliadin, legumin, and so on, are synthesized by an emulsification method in which phase separation is required by addition of a desolvating agent, followed by modifications in temperature as well as pH.[50–54] Nanoparticles synthesized from metals such as gold, copper, and silver, are synthesized using the citrate synthetase method. Gold nanoparticles are synthesized by the reduction of chloroauric acid using trisodium citrate in the presence of a stabilizing agent; however, copper nanoparticles are synthesized by the reduction of copper salts by sodium citrate and myristic acid.[55,56] Silver nanoparticles are synthesized by the method described by Bae et al,[57] as shown in Figure 3. Among the organic compounds, poly(lactic-co-glycolic) acid is most popular in the synthesis of nanoparticles, as it controls the parameters using bottom–up and top–down techniques.[58] For the preparation of nanoparticles from different inorganic nanomaterials such as metal oxides, alloys, chalcogenides, and pinctides, chemical methods are very useful.[59] Recently, Wei et al[60] reported various inorganic nanoparticles such as terbium, erbium, yttrium, zinc, gadolinium, and others, using a facile homogeneous precipitation method, and the authors found that these particles show low toxicity even at a concentration of 5 mg/mL.[60] Dendrimers are synthesized by two commonly used approaches (ie, convergent and divergent[61]), as described in Figure 4.

Analytical techniques used for the characterization of nanoparticles

When materials are reduced at nanoscale dimensions, they show unique properties that are different from their massive counterparts. In order to characterize nanoparticles in a solution, their particle size, size distribution, morphology, composition, surface area, surface chemistry, and reactivity are important factors that need to be defined accurately. These properties make nanomaterials a suitable carrier for unique sensing applications and, at the same time, they may also create complications during the characterization process.

Table 1 Standard methods for nanoparticle formulation using various techniques

Nanoparticle material	Examples	Methods that are being used	Brief description	References
Protein	Albumin	Emulsification method	Phase separation in aqueous media by addition of a desolvating agent	53
	Gelatin/legumin	Emulsification method	Phase separation in aqueous medium with modifications in temperature and pH	54
Metals	Gold nanoparticles	Citrate synthesis method	Reduction of chloroauric acid using trisodiunm citrate	55
	Copper nanoparticles	Citrate synthesis method	Reduction of copper salt by sodium citrate and myristic acid	56
	Silver nanoparticles	Citrate synthesis method	Reduction of silver nitrate with sodium citrate and sodium borohydride	57
Organic	PLGA nanoparticles	Top–down and bottom–up techniques	Polymer is dissolved in organic phase and further emulsified with a suitable surfactant	58
Inorganic	Terbium, erbium, yttrium, zinc, gadolinium, alloys, etcetera	Facile homogeneous precipitation method	Zinc acetate dehydrate and lanthanide nitrate salts are used, as well as thiourea	59,60
Functional groups	Dendrimer nanoparticle	Divergent and convergent approaches	Reactive initiator is exposed to appropriate reagents, which results in branch assemblies	61

Abbreviation: PLGA, poly(lactic-co-glycolic acid).

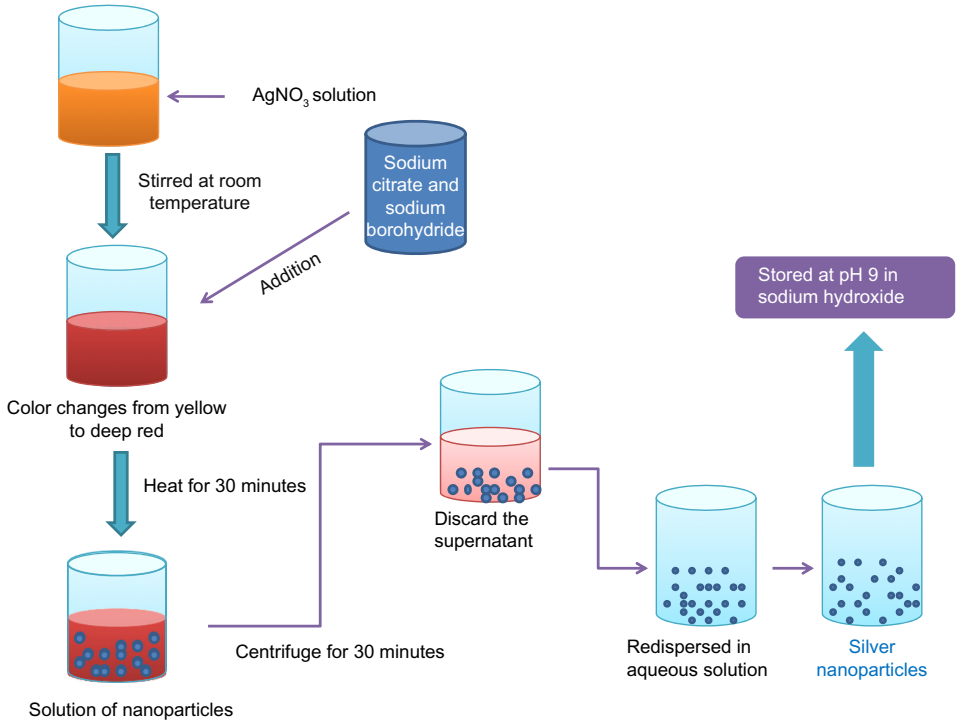

Figure 3 Preparation of silver nanoparticles using a citrate synthesis method.

Choosing the right method for the characterization of nanoparticles is a challenging task since one should be aware that each technique has its own limitations. The characterization of nanoparticles is carried out through various techniques like ultraviolet–visible spectroscopy, dynamic light scattering (DLS), scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction analysis, thermo-gravimetric analysis, X-ray photoelectron spectroscopy, and electrospray ionization mass spectroscopy (ESI-MS).

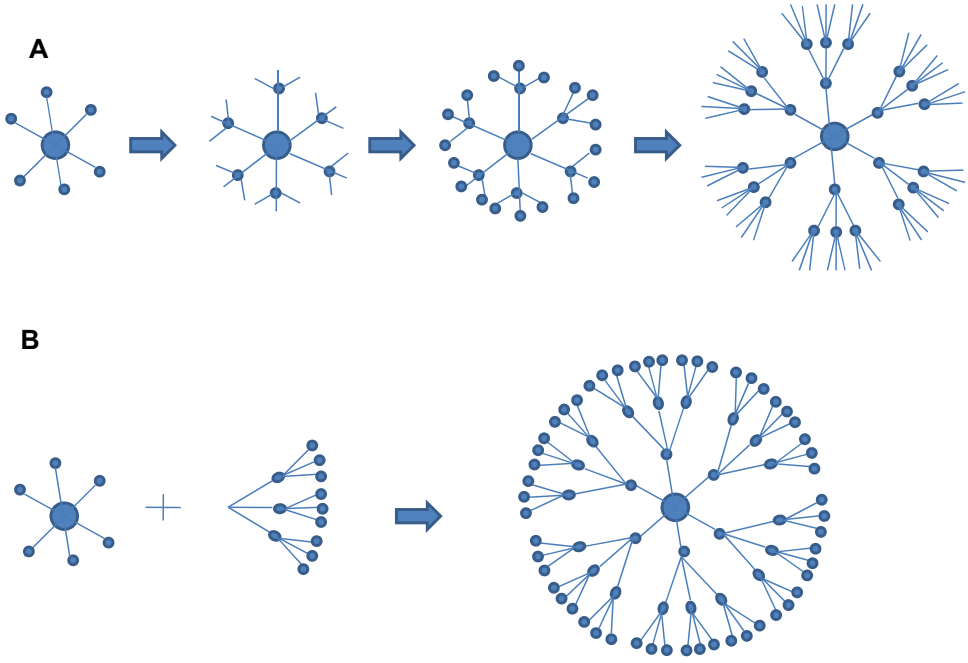

Figure 4 Different methods used in dendrimer formation. (**A**) Divergent and (**B**) convergent approaches for dendrimer preparation.
Notes: Adapted with permission from Crespo L, Sanclimens G, Pons M, Giralt E, Royo M, Albericio F. Peptide and amide bond-containing dendrimers. *Chem Rev.* 2005;105(5):1663–1681.[61] Copyright (2005) American Chemical Society.

The size of nanoparticles is one of the key parameters that influence the interaction between nanoparticles and proteins. Protein structure, function, and their adsorption patterns are also strongly influenced by the size of nanoparticles. Small sized particles have higher relative mobility in intracellular uptake, and they have a wider range of biological targets, when compared to microparticles, for disease diagnosis and treatment. Desai et al[62] and Zauner et al[63] showed that the size of nanoparticles influenced cellular uptake. Nanoparticle formulation with small size and maximum stability always pose a greater challenge, as described by Babaei et al.[64] DLS is the most suitable technique to determine the particle size of nanoparticles. It can also be used to evaluate particle size, size distribution, and zeta potential in a solution.[65] DLS is fast and easy to operate for particle characterization, especially for colloidal suspensions. There are several advantages associated with DLS: simplicity; sensitivity and selectivity to NPs; short time of measurement; and the fact that calibration is not needed. Therefore, this technique is increasingly used for nanoparticle characterization in various science and industry fields.[66,67] However, some problems are encountered when measuring samples with larger size distributions or multimodal distributions.[67] If the measured colloid is monodispersed, the mean diameter of the nanoparticles can be determined using the DLS technique. For polydispersed colloids, there is a risk during the DLS measurement, as small particles can be screened by bigger particles, since bigger particles have more scattering property.

Particle morphology is another important parameter for the characterization of nanoparticles, and this is achieved with the help of microscopic techniques like SEM and TEM. Both of these techniques produce a resolution that is a thousand times greater than the optical diffraction limit. SEM uses a beam of high-energy electrons to produce a variety of signals that contain information about the sample's surface composition, topography, and other properties like electrical conductivity. We can analyze the sample at various times because X-rays generated by SEM do not lead to a loss of volume of the sample. Magnification of approximately 10 –500,000 times can be obtained, and it is controlled by voltage supply to the x, y deflector plates, or by supply of the current to the scanning coils. However, electron microscopy creates a risk of radiation damage that is caused by the electron beam, which leads to the generation of free radicals. The diffusion of free radicals and the loss of mass may cause physical damage to the sample.[68] Also, TEM suffers from the limitations of poor contrast, especially in the event of peptide/protein nanoparticles and their conjugates.

The chemical composition of the nanoparticulate surface can be detected by X-ray photoelectron spectroscopy. The underlying principle is that X-rays strike the sample, which leads to the emission of photons at different energy levels for different elements.

The particle size of synthesized nanoparticles can also be characterized using ESI-MS. This technique is particularly suitable for analyzing the product mixtures, as it causes nearly no fragmentation of the different components. ESI uses electrical energy to assist in the transfer of ions from the solution into the gaseous phase before they are subjected to mass spectrometric analysis. Thus, ionic species, as well as neutral species (converted to ionic form in a solution or in the gaseous phase) can be studied by ESI-MS. ESI-MS also offers precise analysis of the core and shell composition of nanoparticles, as it alone gives the distribution of composition. More significantly, it also contributes to providing a deeper understanding of the nanoparticle's stability and reactivity and, thus, has obvious applications for control of the functionalization and biofunctionalization of nanoparticles.[69,70]

The most important aspect regarding nanoparticle characterization is the surface modification of the nanoparticle; its efficiency can be measured either by surface charge, an increase in surface hydrophobicity, or by the density of functional groups. The surface modification of the aqueous suspension containing the nanoparticles can be measured by determining its zeta potential. This reflects the particle electrical potential and is influenced by particle composition, as well as the medium in which the particle is dispersed.[47] The zeta potential reflects colloidal stability, and this property can be influenced by the interaction between particles. Since most of the colloidal system is stabilized by electrostatic repulsion, the larger the repulsive forces, the less the chance that aggregates form. Normally, the zeta potential of the nanoparticles lies in the range between −10 mV to +10 mV,[71] while the zeta potential of <-30 mV is considered as presenting with highly anionic character; moreover, a value $>+30$ mV depicts a highly cationic character (Table 2).

Nanoparticle as a protein and peptide aggregation inducer

Proteins are biologically important molecules in several respects. The binding of proteins in their native-like or denatured state depends on protein surface charge, hydrophobicity, intrinsic stability, and also on the particles' characteristics. When nanoparticles are introduced in a living organism, they interact with a variety of different cellular components

Table 2 Characterization of nanoparticles by different techniques

Techniques	Parameters	Properties that are analyzed
Dynamic light scattering	Hydrodynamic radius	Nanoparticle size, size distribution, and zeta potential analysis
Photon correlation spectroscopy	Velocity distribution by measuring the dynamic fluctuation of scattered light	Nanoparticle average size, polydispersity index analysis, etcetera
X-ray diffraction analysis	Diffraction pattern	Phase identification, nanoparticle structure, size, lattice parameters
X-ray photoelectron spectroscopy	Binding energy of the detected electrons	Nanoparticle composition, uniformity of composition
Laser doppler anemometry	Frequency shift and phase shift	Zeta potential and particle composition
Thermogravimetric analysis	Temperature and time as a function in mass change	Kinetic parameters, physical and chemical properties
Transmission/scanning electron microscopy	Electron scattering	Morphology of particles, distribution of particles

with largely unknown pathological consequences. Various studies have concluded that the process of aggregation may be induced by nanoparticles through different mechanisms. Nanoparticles provide surface charges to promote the adherence of proteins, and their large surface area provides the potential to induce protein aggregation. However, this requires that the particle size be <10 nm, as it is reported that particles >30 nm have no influence on protein adsorption.[72] It is well known that fibrillation is a nucleation-dependent mechanism, and that nucleation is triggered by external factors.[73] Nanoparticles act as catalysts to facilitate the subsequent assembly of peptides into stable fibril aggregates by increasing the local concentration of those peptides.[74] Nanoparticles may act like conventional catalysts by reducing the energy barrier between fibril formations due to an increase in the population of prefibrillar aggregates.[75] Nanoparticle surfaces can act as platforms for protein association, and this association induces significant changes in protein structure.

Further, low pH and high temperature lead to the unfolding of proteins, which ends with the formation of fibril like structures.[76] High protein concentrations may lead to the formation of amyloid aggregates. This is illustrated by the fact that multiple layers of β2-microglobulin, which are formed on the surface of N-isopropylacrylamide/N-tert-butylacrylamide nanoparticles, as measured by surface plasmon resonance, clearly indicates that there must be a high local protein concentration that is favorable for the amyloid assembly.[77] Particle shape also plays a significant role, as anisotropic particles (like single-walled carbon nanotubes) are well suited to anisotropic biomolecules, like deoxyribonucleic acid (DNA), as aggregation inducers.[78] Another observation by Rocha et al[79] showed that hydrogenated complexes promote β-sheet structures, leading to aggregation. The reduced zeta potential in hydrogenated complexes facilitates the interaction between peptide molecules, resulting in conversion of Aβ40 from random coils to β-sheet structures.[79] Very recently, Jones et al[80] demonstrated that fibrinogen, and other anionic blood proteins, undergo rapid and extensive aggregation by densely charged cationic G7 dendrimers through a thrombin-independent and cellular activation-free mechanism. The acidic nature of these proteins and their high concentration in blood predispose these elements to aggregation with oppositely charged dendrimers.[80]

Nanoparticles as a protein and peptide aggregation inhibitor

The primary strategies that are used to overcome neurodegenerative diseases are focused on inhibiting the self-assembly of proteins that are responsible for the fibril formation. The differences in the binding properties of oligomers and monomers can be exploited as a way to achieve this inhibition. Also, oligomers are considered to be the most neurotoxic species among Aβ agglomerates; their blocking by nanoparticles will be of great concern to the biological perspective.[81] Various techniques (thioflavin T-binding assay, TEM, and surface plasmon resonance) are used to investigate the nanoparticle's ability to interact with proteins during fibrillogenesis.[82,83] The interaction between thioflavin-T with amyloid fibrils causes a red shift in emission spectra, but no response is observed in the presence of soluble proteins, oligomers, or amorphous aggregates.[82,83] Circular dichroism and photoluminescence are other important techniques that are used to investigate the inhibition of the fibrillation process, as these techniques provide a kinetic shift towards unfolded regions and make a clear distinction between protein secondary and tertiary structures.[84] Moreover, using a crystallographic model, Heldt et al[85] provided the first evidence that insulin amyloid fibril formation occurs predominately unidirectionally, demonstrating that it may be asymmetric and propagate mostly in one direction.

In order to design an efficient inhibitor, as well as a nanomolecular species that is suitable for this purpose,

there is a need to focus on the forces that are responsible for fibrillation. The interactions that are primarily involved in fibril formation are hydrophobic, electrostatic, van der Waals, as well as hydrogen bonding.[86] Lee et al[87] have demonstrated a three-stage kinetic model for amyloid formation, highlighting the molecular conformational properties that would be needed in order for inhibitors to bind to the nucleus or to other oligomers, so as to reduce their toxic effects. Inhibition by nanoparticles could be done at the nucleation phase (by increasing the lag phase), the polymerization phase (by decreasing the elongation phase), or via diversion of the peptide from the polymerization pathway to reduce the endpoint at equilibrium. Nanoparticles slow down the rate of fibrillation by altering the amount of free monomeric peptides that are present; yet, at the same time, fibril formation could not be prevented.[88] Experimental data show that nanoparticles added at the beginning of the kinetic experiment arrest the fibrillation process in lag phase. However, nanoparticles did not show any inhibitory effect when added after the control lag time (Figure 5). This indicates that once critical nuclei are formed, the elongation process is so favorable that the addition of nanoparticles does not have any effect on monomer/oligomer interactions with nanoparticles.[88]

High particle concentrations result in a strong interaction between peptides and nanoparticles (when present at the beginning or added later); this is enough to slow down the fibrillation process, probably by adsorption of the monomeric fibrils or initially formed oligomers.[88] Once fibril formation has started, the addition of nanoparticles may reverse the process or destroy the fibrils.[88] High protein concentrations lead to the formation of amyloid aggregates, but in some cases, high local protein concentrations inhibit fibril formation, as the interaction between nanoparticles and proteins leads to the blocking of active sites for fibril formation.[89] Recently Skaat et al[90] designed novel amino acid-based polymer nanoparticles (containing hydrophobic residues, similar to the hydrophobic core sequence of Aβ) and observed that polyA-FF-ME (poly(N-acryloyl-L-phenylalanyl-L-phenylalanine methyl ester)) nanoparticles affect the kinetics of nucleation and oligomer formation prior to the formation of Aβ fibrils by increasing the fibrillation lag time. This could be explained on the basis that hydrophobic interactions, as pairs of FF residues, in these nanoparticles provide high affinity for the Aβ40 prefibril aggregates, leading to a disruption in the nuclei by decreasing the concentration of monomers/oligomers in the solution, and thus interfering with the elongation process.[90]

Milowska et al[91] investigated the effect of poly(amido amine) (PAMAM) G4 dendrimers on α-synuclein, and they compared the structural changes in the presence of nanoparticles (dendrimers) with the help of circular dichroism. The

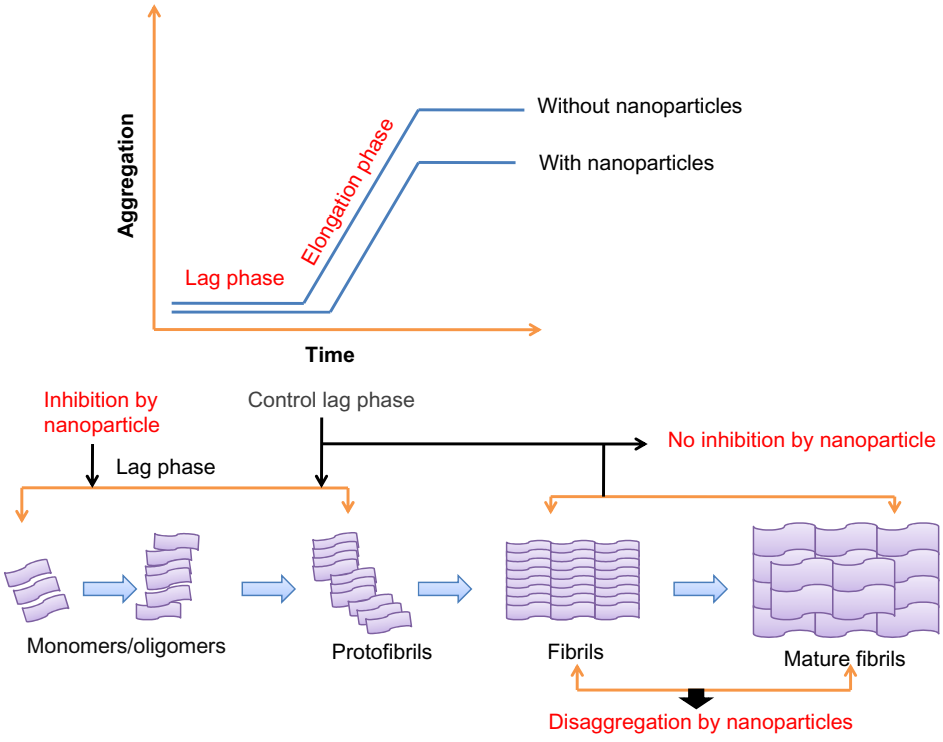

Figure 5 Effect of nanoparticles on nucleation kinetics.
Note: The addition of nanoparticles causes an increase in the lag phase and a decrease in the elongation phase.

higher percentage of α-structure, as well as the presence of a positive signal in the range of 195–206 nm after incubation with the nanoparticles, indicated that there was a reduction in the β-sheet structure.[91] Dendrimer nanoparticles can be used as thermodynamic and kinetic inhibitors based on their size and concentration.[92] In the case of kinetic inhibitors, the final amount of fibrils remained unchanged with the varying lag time, while thermodynamic inhibitors did not affect the amyloid formation rate; however, with the thermodynamic inhibitors, the final amount of fibrils was reduced. Lower generation (ie, G3) PAMAM dendrimers have a clear influence on the elongation rate of Aβ aggregation – an effect that is less pronounced for the higher generation (G4, G5). Rekas et al[93] investigated the effect of PAMAM dendrimers on α-synuclein during the fibrillation and decomposition of preformed fibrils, and found that dendrimers act as inhibitors in the formation of the β-sheet structure during fibrillation, while disrupting the existing β-sheets or their agglomerates during the decomposition process. Milowska et al[94] observed the strong inhibition of α-synuclein by phosphorus-containing dendrimers, depending on the dendrimer/protein ratio; the authors also found that these conjugates are effective quenchers of tyrosine fluoresecence as there will be a decrease in fluorescence intensity of intrinsic tyrosine. It is likely that the intermolecular interaction between the tyrosine hydroxyl and cationic end groups of the dendrimer are responsible for this process. It also suggests that the cationic groups of phosphorus dendrimers interact with the basic amino acid N-terminal region of α-synuclien, which further leads to fibril inhibition. Dendrimers used in lower concentrations are effective, while larger concentrations block the interaction of proteins and dendrimers. In addition, they interact with each other, stick together, and form larger agglomerates (dimers or oligomers).

Miura et al[95] used sulfonated dendrimers and reported that they exhibited a significant inhibitory effect against aggregated amyloid β-peptide, and they also reduced β-sheet conformation. These sulfonated dendrimers displayed saccharides for effective protein and peptide affinity, and they interacted with Aβ peptides via electrostatic interaction with the basic amino acid residues. Inhibition can also be induced by binding the nanoparticles to the hydrophobic motif of a peptide, as reported by Kim and Lee.[96] The authors reported the specific binding of fullerene to KLVFF hydrophobic patches of Aβ peptide, which is a primary target for inhibition of aggregation. Insulin fibril formation is a physical process in which non-native insulin aggregates, and further deposition of insulin fibrils causes type 2 diabetes. A significant inhibition of insulin fibrils was observed by Skaat et al[97] in the presence of γ-Fe_2O_3/PHFBA (poly(2,2,3,3,4,4,4-hept afluorobutyl acrylate)) nanoparticles. These nanoparticles slow down the transition of α-helix to β-sheets during insulin fibril formation.

Toxicity due to nanoparticles

In spite of their production and worldwide usage, there is a serious lack of information about the adverse effects of nanoparticles on human health and environment.[98] Preliminary studies show that nanoparticles can affect biological behavior at the cellular, subcellular, tissue, organ, and protein levels, but their toxicity must be overcome before the large-scale production of safe and efficient applications in the fields of pharmaceutics and in medicine can occur.[100] The undesirable effects of nanoparticles may be due to their small size, chemical composition (purity, electronic properties, or crystallinity), surface structure (surface coatings – inorganic or organic), solubility, shape, and aggregation behavior.[99] A large number of standardized methods are being addressed both in vitro and in vivo to assess the toxicity of these particles.[100] Both in vivo as well as in vitro studies have their own advantages, as in vitro studies reduce the chance of animal handling, and it is easy to perform and can be repeated. In vitro toxicity assessments have revealed that zinc-based nanoparticles have greater toxicity when compared to copper and silver nanoparticles, while iron and titanium nanoparticles possess the least amount of toxicity.[101,102] Nanoparticles possess a carcinogenic effect which is triggered by the production of reactive oxygen species (ROS) by macrophages, and also leads to DNA damage, as well as to the induction of inflammatory lesions – all of which are associated with carcinogenesis.[103] Trouiller et al[104] and Park et al[105] reported that the generation of ROS is closely related to the oxidation of DNA and proteins in the nucleus, as well as to DNA breakage, as they found that the distribution of titanium dioxide nanoparticles induces genetic instability and DNA damage, which was consistent with the site of ROS production in the cell.

The most suitable methods used to determine the cytotoxicity of nanoparticles are MTT assay (3-[4,5-dimethylthiazol-2-yl]-2-5 diphenyl tetrazolium bromide) for cell viability or cell proliferation, and lactate dehydrogenase assay, which is an indicator of cell membrane integrity.[106] As nanoparticles enter the body, they come in contact with the cell membrane, which is their primary site of interaction, and induce disturbances in the lipid phosphobilayer due to changes in the local concentration of the surface charge.[107] Nel et al[9] observed that cationic nanoparticles exhibit more toxicity than their

anionic analogs, or than the other analogs that possess a net neutral charge but are of the same size. Polymeric particles show slightly different mechanisms, as they make holes in the cell membrane and lead to cytotoxicity by reduction in the levels of protein and lipids.[108] Toxic effects have also been observed in membrane proteins, such as in ion channels that play an important role in molecular transport and transmembrane signaling.[109] Silver nanoparticles, for example, are found to alter the confirmation of membrane proteins, which affect the channel opening, thus resulting in neuronal dysfunction.[110] Nanomaterials can also exert their effects at genetic level, as they bind directly or indirectly to DNA and damage it via inflammatory response or by promoting oxidative stress.[110,111] An extensive review of the toxicological effects of nanomaterials are described in various articles that focus on nanomaterial composition and dimension, routes of exposure and administration, translocation and distribution, and clearance from the body.[112,113]

Conclusion

The emerging scenario in fibril formation can be considered as the generic behavior of peptides and proteins. This leads to intermolecular β-sheet assembly, characterized by high stability and resistance to proteolysis. Such aggregation in the central nervous system, or in any of the other organs, contributes to several diseases, resulting in an intensive search for agents that prevent or inhibit aggregation, and that break and destroy the existing protein aggregates.[114] Nanoparticles provide an attractive tool; as nanoparticles enter into biological fluid, a dense layer of proteins surround the nanoparticles' surface, modifying their physiochemical properties and thus their efficacy for their intended application. Control of amyloid formation via nanoparticles can be explained based on two factors: first, the surface chemistry of nanoparticles; and second, the protein's intrinsic stability. These factors determine the interaction between nanoparticles and proteins or peptides, which then leads to dual effects on the fibrillation process of amyloid proteins, and hence they act either as a microreactor or destabilizing agent, causing acceleration or retardation of amyloid formation. Since we know that fibrillation is concentration-dependent, nanoparticles induce amyloid formation by providing a high local concentration of peptides. These peptides either form amorphous aggregates, or show structural changes in the formed aggregates; in addition, inhibition may be due to the high-charge density and hydrophobic groups that are necessary to stabilize the α-helix structure. The study of nanomaterial and biological interfaces allows us to understand the underlying mechanisms involved in nanotoxicity, and these properties provide a model through which to distinguish cell states, while offering a new possibility to exploit these properties for nanotoxicity research. This is especially true since the effects of nanomaterials on cellular biophysical responses have only recently garnered attention. Currently, nanotechnology has reached a remarkable stage, where Aβ aggregation both in the brain and in peripheral circulation can be manipulated; however, some important questions remain to be answered before engaging in further research and clinical investigations. These include questions surrounding the efficiency of the nanoparticle system, the validation of symptom alleviation in representative Alzheimer's disease models in vivo, and the employment of US Food and Drug Administration-approved molecules for nanoparticle formulation. Moreover, the non-invasive administration of nanoparticles must be considered for repeated and prolonged therapeutic purposes. Although nanotechnology is expected to have a huge impact against various neurodegenerative diseases, as well as in drug delivery, imaging agents, cancer therapies, and in various applications in the antiseptic industry, the food industry, and pharmaceutics, a crucial gap has yet to be filled concerning the elucidation of nanotechnology etiology, for which a great deal of effort is still required.

Disclosure

The authors report no conflicts of interest in this work.

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