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Nanoparticle colloidal stability in cell culture media and impact on cellular interactions

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Nanomaterials are finding increasing use for biomedical applications such as imaging, diagnostics, and drug delivery. While it is well understood that nanoparticle (NP) physico-chemical properties can dictate biological responses and interactions, it has been difficult to outline a unifying framework to directly link NP properties to expected *in vitro* and *in vivo* outcomes. When introduced to complex biological media containing electrolytes, proteins, lipids, etc., nanoparticles (NPs) are subjected to a range of forces which determine their behavior in this environment. One aspect of NP behavior in biological systems that is often understated or overlooked is aggregation. NP aggregation will significantly alter *in vitro* behavior (dosimetry, NP uptake, cytotoxicity), as well as *in vivo* fate (pharmacokinetics, toxicity, biodistribution). Thus, understanding the factors driving NP colloidal stability and aggregation is paramount. Furthermore, studying biological interactions with NP at the nanoscale level requires an interdisciplinary effort with a robust understanding of multiple characterization techniques. This review examines the factors that determine NP colloidal stability, the various efforts to stabilize NP in biological media, the methods to characterize NP colloidal stability *in situ*, and provides a discussion regarding NP interactions with cells.

1. Introduction

The turn of the century saw a dramatic rise in the amount of research reporting the use of nanotechnology for biomedical purposes such as implant coatings, imaging agents, diagnostics, drug delivery systems, and other therapies.^{1,2} Nanoparticles (NPs) used in medicine, nanomedicine, are appealing not only because the nano-size results in unique physico-chemical properties, which can mediate biological response (*e.g.* the enhanced permeability and retention (EPR) effect can improve uptake of drug delivery NPs into tumors, nano-textured surfaces can improve implant performance, superparamagnetic NPs can enhance MRI contrast), but NPs also offer distinct advantages for technology commercialization (*e.g.* enabling new indications for drugs, rediscovering drugs that may have previously failed clinically, providing stronger intellectual property protection).^{1,3,4} The development of the field nanomedicine also saw a concurrent growth in new analytical techniques and instruments for characterization. Thus, the excitement surrounding nanotechnology and nanomedicine resulted in large global investments into the field. The U.S. are predicted to invest \$1.5 billion to various agencies through the National Nanotechnology Initiative (NNI) in 2015, and the NNI annual budget grew from \$0.5 billion in 2001 to almost \$2.0 billion in 2010. Under the Seventh Framework Programme funding initiative (FP7), the European Commission invested €2.5 billion (approximately \$3.4 billion) from 2007-2011 and investment into nanotechnologies is explicitly outlined in the European Union's largest ever research endeavor (€80 billion over 7 years), the Horizon 2020 program. However despite the

surge in funding and the significant investigation into NPs used for biomedical applications, their translation into clinical use has encountered variable success.^{5,6} There still exists a wide gap in the number of investigational reports, and those formulations that enter clinical trials. This gap is of course a complex matter influenced by many factors. However, it may in part be due to difficulty in understanding and controlling the most fundamental nanoparticle (NP) interactions with the biological environment. As more nanomedicines enter clinical trials, regulatory agencies are requiring more physico-chemical characterization and fundamental understanding in order to determine how the physico-chemical properties influence biological behavior.⁷ To this point, it is critical to fully understand how NP properties and more importantly colloidal behavior in biological solutions such as cell culture medium or body fluids, impact cellular interactions and cell responses.

Upon contact with physiological fluids, NPs will interface with a wide range of biomacromolecules.⁸ In the past many studies have focused on the interplay between the intrinsic properties of the NPs and the components of the physiological environment, and the subsequent cellular responses towards the NPs *in vitro*.⁹⁻¹² One factor that continues to be understated is the colloidal stability of NPs in biological media. In this complex environment NP aggregation is a common phenomenon.¹³ Aggregation refers to the usually irreversible inter-particle adherence, which leads to the formation of large and irregularly shaped clusters.^{14,15} NP aggregation can lead to misrepresentative results and impedes experimental reproducibility by changing the cellular uptake and toxicity profile of the particles.^{11,16,17} Despite the increasing use of *in*

in vitro systems to evaluate cellular responses towards NPs, the possible change of their colloidal properties upon suspension in physiological fluids, is rarely taken into consideration.^{16,18-20} This is even more surprising as aggregation of NPs has been shown to directly (*i.e.* due to their increased size) and indirectly (*i.e.* due to their altered diffusion and sedimentation velocities) influence the *in vitro* cellular response.

In a broader context, understanding the factors that influence NP colloidal stability and aggregation when introduced into biological media is important for the development of safe and effective nano-therapeutics for clinical use. NP colloidal stability, or lack thereof, will determine their biodistribution, pharmacokinetics, and systemic toxicity *in vivo*.^{21,22} For example Aoki *et al.*²³ found in an *in vivo* toxicity study of nano-hydroxyapatite administered at high doses intravenously to Wistar rats that the cause of morbidity was likely NP aggregation and subsequent capillary blockage in the lungs. Similar studies have shown that NP aggregation and pulmonary capillary blockage can lead to morbidity *in vivo*.²⁴⁻²⁶ Furthermore, studies have shown that NPs which are stable in blood have prolonged circulation half-life, and are less likely to be rapidly cleared by the body's reticuloendothelial system (RES).²⁷⁻²⁹ In most cases the *in situ* formation of aggregates for intravenously administered nano-therapeutics is undesirable as this will lead to rapid clearance in the liver and RES, thereby limiting the probability that the NPs reach their therapeutic targets. Thus, a fundamental understanding of NP aggregation is important to rationally design NP whereby it can be understood how they may behave at molecular, cellular, and systemic levels within biological systems.

The purpose of this review is to draw the attention of the nano-community to the impact of colloidal interactions of NPs in physiological media used for cell cultures: What are the factors affecting NP colloidal stability, which methodologies are used for characterization, and what are key challenges when undertaking any *in vitro* studies?

2. Colloids – theoretical considerations

Colloidal materials are composed of at least two phases, where one solid phase is dispersed in the second, frequently liquid, phase. The dimensions corresponding to the dispersed phase are considered to be in the sub-microscopic region, yet well above the atomic size range. At these sizes NPs are able to interact with biological systems on a sub-cellular level.

2.1 Colloidal behavior in water

Aqueous solutions, containing simple electrolytes, are the only systems where colloidal stability of NPs can be effectively quantified. Colloidal stability is determined by inter-particle behavior resulting from intermolecular and surface forces (*e.g.* van der Waals (vdW) forces, the repulsive electrostatic double layer (EDL), and structural forces such as depletion attraction). The balance of these forces determines the colloidal stability of NPs in an aqueous suspension. The attractive vdW forces result

from the interaction of induced, instantaneous, or permanent dipoles in the interatomic bonds of NPs, which destabilize a colloidal system (Figure 1a).³⁰⁻³² The attractive vdW forces are repulsed by the EDL of the NPs, which stabilizes the dispersion. Most NPs carry some surface charge in aqueous environments due to ionization/dissociation of surface groups, or due to the adsorption of charged molecules or ions to the particle surface. The net charge of the system is balanced by the formation of a cloud of counterions around the particle. This cloud is composed of the Stern layer, which consists of counterions adsorbed on the charged surface of the NP, and the diffuse layer, an atmosphere of ions of opposite net charge surrounding the NP. Characterization of nanoparticles surface charge is commonly done in terms of the particle zeta potential, which is the electrostatic potential of the particles measured at the so-called shear plane, *i.e.* at the distance from the surface where ions are not bound to the particle. Overlapping EDLs of two like-particles induces a repulsive force caused by the osmotic interactions between counterions (Figure 1b).³²⁻³⁷

A classical theoretical approach to determine the state of a colloid can be found in the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. The theory uses the superposition of attractive vdW and repulsive forces caused by the EDL to predict the stability of a colloidal system.^{33,38} According to the classical DLVO theory, colloidal stability increases with increasing net surface charge. For particles with amphoteric surfaces, *e.g.* metal oxides, the surface charge of a particle is dependent on the pH of the suspension. At pH values near the isoelectric point or point of zero charge of NPs the overall surface charge of the particles tend to be neutral. As a consequence the repulsion of the NPs, caused by the EDL, decreases in favor of attractive vdW forces, promoting aggregation.³⁹ The EDL can also be influenced by the ionic strength of the suspension. High ion concentrations result in a compression of the EDL, which may further induce the collapse of colloidal systems.⁴⁰

In addition to the aforementioned forces, depending on the composition of the colloidal material, other contributions to the stability of the system have to be included. The adsorption of macromolecules, such as synthetic polymers or biopolymers, onto the surface of NPs can lead to a steric stabilization of the colloid. Steric stabilization provides a powerful tool to enhance the dispersion state of NPs under otherwise harsh conditions by preventing two particles from forming attractive vdW interactions due to osmotic pressure and elastic recoil effects (Figure 1c). Osmotic pressure acts at longer distances, whereas elastic recoil effects dominate at shorter distances. The magnitude of these interactions further depends on the grafting density of the macromolecules to the surface of the particle and its interaction with the dispersion media.⁴¹⁻⁴³ It has been shown that by using charged macromolecules or proteins, the stabilization of colloidal systems could be improved due to the combined electrosteric repulsion.⁴⁴⁻⁴⁶ However, steric stabilization is only effective if a uniform polymer coating is present on the particles surface. In the case of a patchy polymer layer, steric stabilization is not effective. In fact, it is worth

noting that macromolecules can also introduce aggregation of NPs due to bridging effects, where a polymer chain of high molecular weight interacts with more than one NP and therefore leads to the formation of aggregates. Bridging flocculation is based on the strong interaction of, for example, a polymer with the NP surface, and occurs in the presence of very high molecular weight and not too high concentrations of macromolecules in the suspension where the amount of polymer chains is not sufficient to completely cover the surface of each particle.⁴⁷

Beside electrosteric stabilization, other repulsive forces (e.g. hydration forces), as well as specific attractive forces (e.g. depletion, hydrophobic or magnetic forces) contribute to the stabilization or destabilization of a colloidal system.⁴⁸ The resulting extended DLVO (X-DLVO) theory is the addition of these individual intermolecular and surface forces assuming that each of these interactions is totally independent from the other. By superimposing these various influences into an energy-distance curve, it is possible to understand and predict the behavior of colloids. An example of such an energy-distance plot is shown in Figure 1d. Depending on the influencing forces and their magnitude, primary minima, secondary minima as well as energy barriers (maxima) can be predicted. Minima depict particle distances where flocculation or aggregation will occur, whereas maxima represent energy barriers, which prevent particles from coming into contact.^{49,50}

The current theories might fail to describe all systems in general, be it due to the incomplete description of the colloidal systems, the difficulty in obtaining the correct parameters required for the calculations, or due to the fact that the models neglect certain physical phenomena.⁵¹ However, the DLVO theory and extended versions of it have significantly advanced our understanding of colloidal systems, and have been applied in a number of studies of different research fields, such as ceramic processing,⁵² industrial separation processes, microbial adhesion,⁵³ virus adsorption to surfaces,^{54,55} transport of colloids in groundwater,⁵⁶ and aquatic environments.^{57,58}

When it comes to evaluating NP interactions with cellular systems the colloidal behavior of the respective materials in idealized, simplified aqueous solutions does not fit these complex physiological environments. Due to the high ionic strength and presence of macromolecules (e.g. proteins), understanding the effect of physiological fluid composition on colloidal stability is paramount.^{59,60}

2.2 Colloidal interactions in cell culture medium

The influence of physico-chemical properties of NPs on cellular interaction is routinely assessed using *in vitro* systems, where NPs interact with the components of the present cell culture medium prior to any cellular contact.^{61,62} The complexity of the environment a NP is confronted with once suspended in the protein-supplemented and electrolyte rich cell culture medium (CCM) is illustrated in Figure 2.

The environment that the NPs, represented as a 40 nm gold sphere, are found in when delivered into cell culture media is shown. Media is a buffered solution that is principally comprised of proteins such as serum albumin or globulins (violet spheres), and a number of biomolecules including amino acids (blue spheres), and ionic salts (red dots). These constituents influence the hydrodynamic behavior of NPs. Stabilized NPs can also become unstable through molecule/protein adsorption or loss of surface functionality, resulting in the formation of aggregates. These processes can further influence the *in vitro* behavior and significantly alter NP mobility.

The impact of this complex biological fluid on the colloidal behavior of the NPs is, however, rarely or insufficiently taken into consideration despite the fact that changes in the dispersion state of NPs have been shown to influence the ensuing cellular interaction and response.^{11,16,18,20,63} Admittedly, experimental assessment of the colloidal stability in the biological environment is challenging and the multitude of different CCM with a range of different components further contributes to the complexity. When NPs are delivered into cell culture media, their colloidal and chemical properties are not only altered due to the presence of proteins but also because of the high ion content (Figure 2). Studies addressing the influence of electrolyte ionic strength on colloidal stability recognized quite early that stability can collapse due to screening of the electrostatic interactions.⁶⁴ This screening can result in aggregation, and multivalent electrolytes were found to be more efficient than monovalent ions at suppressing the stabilizing effect of the electric double layer (EDL).⁶⁵ Furthermore, the ionic strength and nature of electrolytes was reported to define the morphology and rate of aggregate formation.⁶⁶ In the case of polyelectrolytes adsorbed onto nanoparticles, the pH-induced conformational change was found to be significant for colloidal stability.⁶⁷ Considering chemical changes, it has been shown that the surface charge of carboxyl and amine terminated NPs depends on pH due to the equilibrium of protonation (COOH or NH₃⁺)-deprotonation (COO⁻ or NH₂) of these functional groups.⁶⁸ Alternatively, several studies have demonstrated that NPs, such as Ag, quantum dots or ZnO NPs, can release ions and that the rate of ion release is influenced by particle size, particle functionalization, and the local environment.⁶⁹⁻⁷² Moreover this degradation has been correlated with their cytotoxicity (e.g., the ability to generate reactive oxygen species).⁷³

While this review focuses on NP colloidal stability in CCM, the effect of the intracellular environment cannot be discounted. The majority of NPs are taken up via endocytotic mechanisms (for reviews see ⁷⁴⁻⁷⁶) during which they are exposed to varying conditions, such as pH changes from 7.4 (in the extracellular medium), to 5.5 (in late endosomes), and finally to 4.5 (in lysosomes).⁷⁷ This altered environment can have several consequences, for example it was shown that bound or adsorbed proteins can be rapidly degraded.⁷⁸ Ag NPs, one of the most widely studied engineered NPs, were shown to dissolve in endosomes and lysosomes.⁷⁹ In addition to their

acidic pH, lysosomes also contain high levels of hydrolytic enzymes that can result in the degradation of the entire NPs or their coating.⁸⁰ Thus, the dynamic nature of the cellular environment, with electrolytes, proteins, and enzymes affecting NP stability and dissolution, must be kept in mind.

A brief analysis of the composition of different CCM, as well as comparison to human plasma, shows the difference in biological media composition (Table 1). This can help explain the effect these differences have on the colloidal stability of NP.

Overview of media. Depending on cell type, a specific medium is used to culture cells *in vitro*. CCM serves the express purpose of supplying *in vitro* cultures with nutrients required for cell survival, growth, and differentiation, acts as a source of energy for cellular metabolism, and mediates an optimal extracellular environment (*i.e.* pH and osmolarity).^{81,82} It follows that media composition can vary based on the metabolic and nutritional needs of different cell types, and this may in turn impact the colloidal stability of engineered NPs. Furthermore, CCM is different in composition compared to human plasma or other biological fluids.^{83,84} Table 1 compares the composition between some of the most common CCM (*i.e.* Dulbecco/Vogt modified Eagle's medium, DMEM; minimal essential medium, MEM; or Roswell Park Memorial Institute medium, RPMI),^{81,85,86} with human plasma (See Supplementary Information for full formulations).^{83,87} As discussed previously, ion concentration and pH factor heavily into the stability of colloid suspensions. The table shows differences in ion concentrations and the levels of free amino acids between the different CCM and human plasma. It is apparent that CCM is a complex aqueous environment, significantly different from the ideal suspension of NPs in pure H₂O, and is also a step-up in complexity compared to phosphate buffered saline (PBS), a commonly used isotonic buffered salt solution.

For example, RPMI contains approximately 5-fold more phosphate ions (5.63 mM) compared to DMEM (0.92 mM), MEM (1.01 mM), and human plasma (1.00 mM). PBS has twice as many phosphate ions (10 mM) as RPMI, and therefore contains approximately 10-fold more phosphate compared to DMEM, MEM, and human plasma. RPMI also contains half the concentration of magnesium ions (0.41 mM) compared to both DMEM (0.81 mM) and MEM (0.81 mM), and approximately a quarter of the magnesium ions in human plasma (1.5 mM). Aside from the complexity of ions within CCM, the presence of macromolecules (*e.g.* proteins, lipids) further complicates the interaction of NPs with their *in vitro* environment.

Notably, the total protein content of a CCM, which is commonly supplemented with 10% fetal bovine serum (FBS), is near 3.0–4.5 g/L. This is approximately 20 times lower than the protein levels in human plasma (65–80 g/L). Considering that numerous studies have shown protein adsorption plays a significant, albeit conflicted, role in NP stability and interaction with biological systems, the presence of proteins in CCM is a non-trivial issue.^{88–90} Depending on the species of the serum source, sera further differ in the levels of fatty acids. Human

serum for example is 7.5 times richer in lipids (especially polyunsaturated fatty acids) than FBS.⁹¹ Furthermore, since FBS suppliers only analyze certain components of the sera due to financial restraints, the lipid content is generally not assessed. Neither is the amount of many small molecules (*e.g.* growth factors) or specific proteins. CCM can therefore be characterized as a heterogeneous environment, and considering the variable composition of supplemented CCM, results obtained by characterizing NPs in water or buffered solutions do not represent the colloidal system under *in vitro* conditions.

2.3 Approaches to stabilize NPs in cell culture media

Surface modification of NPs is one of the most widely accepted methods to improve colloidal stability.^{11,92} Therefore, it is important to develop a robust understanding of the mechanisms governing NP stability, and to apply these methodologies in biological applications. Table 2 summarizes different types of NPs, organized by the fundamental approach used to stabilize them in cell culture media. Approaches designed to avoid the formation of aggregates in complex biological media include: electrostatic, steric, or electrosteric stabilization.^{61,93}

Electrostatic stabilization. When NPs are added into cell medium, the EDL has a charge that reflects not only the particle surface but also the surrounding medium. The ionic strength of the media also determines the radial size of the diffuse layer at the NP surface. Low ionic strength enables a large ion cloud extending far from the particle surface, which repels particle-particle interaction. Meanwhile, high ionic strength compresses the EDL, inducing NP aggregation due to van der Waals forces.^{40,51} Electrostatically stabilized NPs have generally shown poor stability in cellular media. In fact, formation of a stable adsorbed protein layer upon incubation seems to be the singular factor for NPs originally stabilized electrostatically.

Mahl *et al.*⁶⁰ reported gold NPs electrostatically stabilized with a tris(sodium-*m*-sulfonatophenyl) phosphine (TPPTS) coating. The hydrodynamic diameter (d_H) of TPPTS-stabilized gold NPs in H₂O was 25 nm but dispersion in RPMI-1640 cell culture medium without serum caused aggregation to a d_H of 750 nm. Dispersion of these NPs in RPMI-1640 supplemented with either fetal calf serum (FCS), or bovine serum albumin (BSA), resulted in d_H of 50 nm. Similar results have been reported for other nanomaterials. Chen *et al.*⁹⁴ demonstrated that 2,3-dimercaptosuccinic acid (DMSA)-modified iron oxide (Fe₂O₃) NPs aggregated in media without FCS, but were stable in RPMI with 10% v/v FCS. Graf *et al.*⁹⁵ studied the influence of surface modifying silica particles (SiO₂) and their colloidal stability in CCM. They demonstrated that particles stabilized by L-arginine, L-lysine, and amino silane with short alkyl chains undergo aggregation in un-supplemented, and FCS-supplemented cellular media.

Table 1. Main components and parameters of commonly used cell culture media compared to human plasma.*

Classification	Component-Details	DMEM + 10% FBS	MEM + 10% FBS	RPMI-1640 + 10% FBS	Human Plasma
Amino acids	Total (mM)	10.65	5.43	6.44	2.32 - 4.05
	Vitamins	Total (mM)	0.15	0.04	0.24
Cations	Sodium, Na^+ (mM)	155.31	144.44	124.27	142.00
	Potassium, K^+ (mM)	5.33	5.33	5.33	4.00
	Calcium, Ca^{2+} (mM)	1.80	1.80	0.42	2.50
Anions	Magnesium, Mg^{2+} (mM)	0.81	0.81	0.41	1.50
	Iron, Fe^{3+} (mM)	0.25	n/a	n/a	10.00 - 27.00
	Chloride, Cl^- (mM)	117.47	124.37	100.16	103.00
Proteins	Bicarbonate, HCO_3^- (mM)	44.05	26.19	23.81	27.00
	Sulfate, SO_4^{2-} (mM)	0.81	0.81	0.41	0.50
	Nitrate, NO_3^- (mM)	0.74	n/a	0.85	20.00
Parameters	Phosphate, PO_4^{3-} (mM)	0.92	1.01	5.63	1.00
	Total (g/L)	3.00 - 4.50	3.00 - 4.50	3.00 - 4.50	65.00 - 80.00
	Serum albumin (mM)	0.05	0.05	0.05	0.58
	α -globulins (g/L)	0.30	0.30	0.30	8.10
	β -globulins (g/L)	0.27	0.27	0.27	11.50
	γ -globulins (g/L)	0.07	0.07	0.07	15.60
Parameters	IgG (mM)	3.56 E-0.05	3.56 E-0.05	3.56 E-0.05	0.08
	pH range	7.00 - 7.40	7.00 - 7.40	7.00 - 7.40	7.34 - 7.42
Parameters	Osmolality (mOsm/kg)	320 - 360	280 - 320	270 - 310	276 - 295

*Recently, our understanding of the composition of human plasma and serum has been greatly enlarged by the and the Human Plasma Proteome Project (HPPP).^{83,87}

Ji *et al.*⁹⁶ reported that titanium dioxide (TiO₂) NPs increased their d_H in the presence of CCM without serum. However, addition of BSA or fetal bovine serum (FBS) was shown to somewhat stabilize NPs, as there was little change in size following addition of proteins. Similar findings were reported with a library of NPs including SiO₂, ceria (CeO₂), Fe₂O₃, and TiO₂.⁹⁷ NPs were characterized in H₂O, RPMI without serum, RPMI supplemented with 10% FBS, and RPMI supplemented with 1% BSA. In general, NP which had a negative zeta potential in H₂O tended to form larger aggregates in media with 10% FBS compared to un-supplemented RPMI or RPMI with 1% BSA. Conversely, NPs which had a positive zeta potential in H₂O tended to form large aggregates in un-supplemented RPMI, and then were stabilized by the presence of FBS or BSA. Here the addition of serum proteins in media was shown to decrease aggregation in certain situations, likely due to protein adsorption. The adsorption of proteins on the NP surface starts as soon as the NPs are dispersed in medium,

which then provides electrosteric repulsion that occurs faster than NP agglomeration. These trends were not observed across comparable studies, and these differences may be attributed to NP material, experimental method, or characterization. However, this clearly demonstrates that electrostatic stabilization is not sufficient to avoid aggregation in biological media. More advanced methods to stabilize NP go beyond electrostatic interactions at the particle surface.

Steric stabilization. Steric stabilization is the most commonly used approach to increase the stability of NP in suspension, and is generally accomplished via natural (*e.g.* dextran, alginate, chitosan) or a synthetic polymer coating (*e.g.* poly(ethylene glycol), PEG; poly(vinyl alcohol), PVA; poly(vinyl pyrrolidone), PVP).^{27,98,99} As discussed previously, destabilization of colloidal NP systems is significantly related to protein-NP interaction. There are two approaches to mitigate the complex and variable effect of protein adsorption on NP

colloidal stability: either to ensure that a uniform monolayer of proteins adsorbs on the particles surface, or to reduce the adsorption of proteins on NPs. Given the difficulty in pursuing the former approach, the latter has been much more commonly utilized. It has been demonstrated that protein adsorption is decreased, or not observed, when the NPs are coated with hydrophilic and neutral surfactants and polymers (*e.g.* PEG, Pluronic F-127, or Poloxamine 908).¹⁰⁰ Polystyrene nanospheres coated with Poloxamine 908 showed a reduction of fibronectin adsorption,¹⁰¹ and other studies have shown that coating SiO₂ NPs with Pluronic F-127 reduced serum protein adsorption.¹⁰² Apart from the chemical composition of the coating, grafting density and polymer conformation were shown to play major roles in reducing the adsorption of serum proteins.¹⁰³

PEG is a well investigated and frequently used polymer in the field since it has been shown to decrease protein adsorption, and *in vivo* pharmacokinetic studies have revealed that PEGylation of NPs can result in prolonged circulation half-life. This is generally explained by reduced adsorption of opsonizing/serum proteins and increased stability of NPs in blood/serum.^{27,28,104} Gref *et al.*²⁷ showed that adsorption of proteins onto NP surfaces was dependent on PEG coating molecular weight, and PEG has also been shown to improve colloidal stability.^{105,106} Thus, functionalization of the NPs' surface with a non-ionic surfactant,¹⁰⁷ or neutral polymers can improve colloidal stability.¹⁰⁸⁻¹¹¹ Many studies have also reported the formation of aggregates when sterically stabilized NPs were introduced to CCM. Mason *et al.*¹⁰⁸ and Hirsch *et al.*¹¹¹ demonstrated the formation of aggregates in cellular media of PEG-coated gold NPs and PVA-coated superparamagnetic iron oxide NPs, respectively. Stebounova *et al.*¹⁰⁹ also reported PVP-coated silver NPs that were not stable in *in vitro* conditions. This poor stability is often explained by polymer-medium interactions. In some systems the polymer coating can become unstable and dissociate from the NP surface in the high ionic strength fluids. Zhang *et al.*¹¹² showed that gold NPs functionalized by thiol terminated-PEG (PEG-SH) could be de-stabilized via ligand exchange by the small molecule dithiothreitol (DTT), whereby DTT dissociates the Au-S bond of PEG-SH, and releases the PEG coating. However, gold NPs functionalized with PEG-thioctic acid (PEG-TA), which contained multiple thiol groups that act as anchoring ligands, were shown to be more stable. Larson *et al.*⁹⁰ reported similar results where PEG-SH can be displaced by cysteine and cystine, two amino acids present in physiological media, resulting in gold NPs being subsequently destabilized by serum proteins. To mitigate this issue, gold NPs were functionalized with PEG terminated by a thiolated alkyl group. This end group provides an alkyl linker that acts a hydrophobic shield between the PEG molecule and NP surface, thus reducing competitive displacement of PEG by other small, thiol-containing molecules.

Steric stabilization has proven to be an effective method to improve NP colloidal stability. It can reduce protein adsorption,

and polymeric coatings can act as a physical barrier to prevent NP aggregation.

Electrosteric stabilization. One approach to further stabilize NPs in suspension is to augment steric stabilization to include electrostatic-repulsive forces, resulting in electrosteric stabilization. This is generally accomplished via a charged polymer coating on the NP surface. This approach takes into consideration changes to NP surface due to media with high ion concentrations as well as the possible interaction between particle systems and other molecules present in solution. Chanteau *et al.*¹¹³ reported poly(acrylic acid)-functionalized sub-10 nm Fe₂O₃ and CeO₂ NPs that were stable in cell culture media for 1 week. However, the same NPs modified using phosphonate-terminated poly(ethylene oxide), instead of poly(acrylic acid), were not completely stable in cellular media. Similar results using different ligands have been reported by others.^{107,111,114}

Interestingly, exceptional stability of chitosan-based nanocapsules in cellular media has been reported.¹¹⁵ Here, it was demonstrated that colloidal stability cannot solely be explained by electrosteric repulsion. The stability was attributed to the hydrophilic nature of chitosan nanocapsules which demonstrates another stabilization mechanism based on short-range repulsive hydration forces.

Thus, additional mechanisms of stabilization are being explored to improve colloidal stability in biological media. In order to design new generations of NPs, the investigation is being extended to not only particle-media interactions, but also particle-stabilizer interactions.¹¹⁶⁻¹¹⁸ However, these previously discussed studies show that surface functionalization alone may not be sufficient to totally stabilize particles in CCM.

2.4 The role of proteins

The presence of proteins in CCM results in complex and highly variable effects on NP stability. Proteins have been shown to sterically stabilize or de-stabilize NPs upon their suspension in CCM. For example, citrate capped gold NPs aggregate in PBS, but are stable in the presence of proteins.⁶⁰ The ability of proteins to stabilize colloidal systems has been shown to strongly depend on both the protein concentration and the composition of the NPs. SiO₂ NPs, for example, aggregate in PBS supplemented with low protein concentrations, but are well-dispersed in PBS containing high protein concentrations.^{95,119} Polystyrene NPs, on the other hand, form large aggregates upon suspension in plasma independent of the protein concentration, which was explained by the slow protein adsorption on these NPs hindering steric stabilization.^{120,121} Hondow *et al.*¹²² concluded that streptavidin-coated quantum dots undergo aggregation in both pure cellular medium and cellular medium plus serum, but the number of QDs per aggregate was less in serum.

When NPs are added to CCM, they enter in contact first with albumin and other serum proteins, forming the so-called protein corona,^{19,88} and BSA has been widely explored for

dispersing NPs.^{60,93,96} Mahl *et al.*⁶⁰ reported that the presence of BSA in the cellular medium efficiently prevented gold NP aggregation. They also observed the diameter of gold NPs was 2-fold larger for tris(sodium-m-sulfonatophenyl)phosphine(TPPTS)-gold NPs and 1.4-fold larger for PVP-gold NPs in the presence of the proteins, indicating a surface layer of adsorbed protein which offered additional stability. By applying fluorescence correlation spectroscopy it has been demonstrated that the HAS corona on polymer-coated FePt and CdSe/ZnS NPs consisted of a single protein monolayer.¹²³ Several reports have demonstrated that FBS at a typical concentration of 10%, can improve NP colloidal stability in various cellular media.^{59,60,94,96} Chen *et al.*⁹⁴ reported that surface functionalized magnetic NPs dispersed in cellular medium (RPMI-1640) with FCS exhibited increased stability due to extra electrosteric repulsion provided by proteins adsorbed on the particle surface. However, many reports have demonstrated that the formation of a protein layer destabilizes the NPs. When NP dispersions are added to serum, protein adsorption strongly occurs, which in turn affects the surface charge distribution.¹²¹

Thus, the effect of protein adsorption on aggregation has not been totally established. It is clear that proteins and molecules present in cellular media can significantly alter the aggregation behavior of NPs. The reasons why NP-protein interactions have been shown to cause the collapse of a colloidal system is, however, still under discussion. For example, strongly positive charged chitosan latex particles underwent charge neutralization and electrostatic screening upon interaction with BSA, resulting in low colloidal stability.¹²⁴ On the other hand it was shown that the zeta potential of NPs is shifted to comparable negative values after incubation with supplemented CCM, independent of their intrinsic surface charge.^{16,63,125} The low colloidal stability of positively charged latex NPs could therefore also be explained by bridging flocculation caused by the favorable interaction of the reactive amine groups on the particle surface with the negative surface charge of the protein.^{124,126}

Whether NP-protein interaction leads to the collapse or stabilization of colloidal systems has been shown to depend on an array of different parameters, both with the components of the media and the physico-chemical NP properties. It therefore remains difficult to predict how protein adsorption affects the colloidal stability of NPs. The difference in protein concentration of supplemented CCM and human plasma further impedes the direct extrapolation of data obtained in *in vitro* experiments to colloidal behavior of NPs *in vivo*. However, probing and taking possible aggregation of NPs under physiological conditions into consideration is a first step towards accurate interpretation of cellular responses *in vitro*, which in turn helps to better predict subsequent biological effects *in vivo*.

2.5 Fundamental interactions between proteins and surfaces

Due to the complexity of NP interactions in complex sera, some groups have addressed surface-protein interactions in a much more idealized situation. As it is well known that proteins have a strong tendency to adsorb on surfaces, researchers have investigated the interactions between surfaces coated with proteins.^{127,128} Most part of the published works have used flat surfaces coated by different types of proteins and have used either a surface force apparatus or an atomic force microscope (AFM) to measure the force between surfaces. The interactions have generally not only been measured between highly flat surfaces, such as mica,¹²⁹⁻¹³¹ silicon wafers,¹³² or silica,¹³³⁻¹³⁵ but also polystyrene¹³⁶ coated by uniform layers of different proteins under different pH conditions and ionic strengths. The proteins investigated include human apolipoprotein AII,¹³¹ lactoferrin,¹³² Protein A,¹³⁰ *b*-casein,¹²⁹ BSA,^{133,136} and apoferritin.¹³⁴

Some general conclusion can be drawn from these investigations. For not too high ionic strengths and not too small separation distances, DLVO theory seems to provide a reasonably good quantitative description of the interactions.^{127,131,132,135} At short separation distances, instead, a strong repulsive interaction is typically observed.^{129,130,132,134-136} This is usually a result of steric interactions, and/or hydration interactions.^{127-129,135} The results are difficult to model since short range interactions are strongly dependent on the detailed structure of the adsorbed proteins. There is also a debate on whether hydration forces rather than steric interactions should be held responsible for the strong repulsive potential.^{127,135} In some cases, it was verified that proteins were not adsorbed in a monolayer, but rather in a multilayer, thus making it even more difficult to precisely model what happens at short separation distances. At higher ionic strengths, when electrostatic repulsions are strongly shielded, DLVO theory is no longer able to describe the interactions.¹³¹ Once again, the behavior is strongly protein dependent, and difficult to rationalize. In an interesting work, Dickinson *et al.*¹²⁸ used a more advanced self-consistent field modeling approach, originally developed for polymers, to describe the structure of adsorbed casein proteins on hydrophobic proteins. Their findings were able to explain the different stabilization ability of *a₁*-casein and *b*-casein, based on the different amino acid sequences of the proteins. *b*-casein was shown to be able to provide strong electrosteric repulsion; while *a₁*-casein lead to bridging flocculation among oppositely charged patches. This shows that strong differences are observed even among proteins belonging to the same family.

Table 2. Colloidal stability of NPs in cell culture media as a function of stabilization method.

Stabilization Approach	Nanostructure	Stabilizing Molecule	Medium	Result	
Electrostatic	Al ₂ O ₃ ¹⁸	Bare oxide surface	RPMI-1640 with 10% FBS	Unstable	
	Au ¹¹⁰	Bare Au surface	Lysozyme solution		
	Au ¹³⁷	Carboxylic-acid functionalized hydrocarbon	DMEM with 10% FBS		
	CeO ₂ ¹⁶	Bare oxide surface	RPMI-1640 with Glutamax and 10% FBS		
	CeO ₂ ¹³⁸	Bare oxide surface	DMEM + 10% FBS		
	CeO ₂ ¹³⁹	Tetramethylammonium hydroxide	DMEM + 10% FBS		
	CoO ¹³⁹	Tetramethylammonium hydroxide	DMEM + 10% FBS		
	γ-Fe ₂ O ₃ ¹⁴⁰	Citrate coating	RPMI		
	γ-Fe ₂ O ₃ ¹⁴⁰	Citrate coating	RPMI + 10% FBS		
	γ-Fe ₂ O ₃ ¹¹³	Citrate coating	DMEM with 10% FBS		
	Fe ₃ O ₄ ¹³⁹	Tetramethylammonium hydroxide	DMEM + 10% FBS		
	Iron/Graphite ¹⁴¹	Bare graphite surface	RPMI-1640 with 10% FBS		
	SiO ₂ -coated magnetite ¹⁴¹	Bare SiO ₂ surface	RPMI-1640 with 10% FBS		
	SiO ₂ ¹⁴¹	Bare SiO ₂ surface	RPMI-1640 with 10% FBS		
	SiO ₂ ^{89, 142}	Bare SiO ₂ surface	MEM with 10% FBS		
	SiO ₂ ¹⁴³	(3-aminopropyl)-trimethoxysilane	PBS		
	TiO ₂ ⁹⁶	Bare oxide surface	DMEM		
	TiO ₂ ¹⁴⁴	Bare oxide surface	Keratinocyte media		
	TiO ₂ ¹⁴⁴	Bare oxide surface	Keratinocyte media + 10% FBS		
	Au ⁶⁰	Tris(3-sulphophenyl) phosphine trisodium salt	RPMI-1640		
	SiO ₂ ⁹⁵	L-Lysine	DMEM + 10% FBS		
	SiO ₂ ⁹⁵	L-Arginine	DMEM + 10% FBS		
	SiO ₂ ⁹⁵	(3-aminopropyl) trimethoxysilane	DMEM + 10% FBS		
	Ag ¹³⁹	Citrate coating	DMEM + 10% FBS		Stable
	Au ¹³⁷	Carboxylic-acid functionalized hydrocarbon	deionized H ₂ O		
	Au ¹³⁹	Citrate coating	DMEM + 10% FBS		
	Au ⁶⁰	Tris(3-sulphophenyl) phosphine trisodium salt	RPMI-1640 + 10% FBS		
	Au ¹⁰⁰	Citrate coating	Human plasma		
γ-Fe ₂ O ₃ ¹⁴⁰	Citrate coating	PBS			
SiO ₂ ⁹⁷	Bare SiO ₂ surface	RPMI-1640			
SiO ₂ ⁹⁷	Bare SiO ₂ surface	RPMI-1640 with 10% FBS			
SiO ₂ ⁹⁵	N-(6-aminohexyl)-aminopropyltrimethoxysilane	DMEM + 10% FBS			
TiO ₂ ¹⁴⁵	Bare TiO ₂ surface	RPMI-1640 with 10% FBS			
<ul style="list-style-type: none"> • EDL repulsion is not great enough to stabilize particles. • Destabilization occurs due to: <ul style="list-style-type: none"> ○ The difference in ionic strength when changing from water to CCM. ○ Desorption of the stabilizing molecule through the competitive exchange with other CCM components. ○ Inter-particle attractive forces, e.g. magnetic attraction from IONP, hydrogen bonding between silica NP surface hydroxyl groups. • In general, serum decreases aggregation because proteins adsorb onto NP surfaces and provide electrosteric repulsion. 					
Steric	Surface-adsorbed molecules	Fe ₂ O ₃ ¹¹⁴	Polyvinyl alcohol	DMEM; RPMI	Unstable
		CeO ₂ ¹¹³	Phosphonate terminated poly(ethylene glycol)	DMEM with 10% FBS	
		Ag ⁵⁹	Polyvinylpyrrolidone	RPMI	
		Au ⁶⁰	Polyvinylpyrrolidone	RPMI	
		Polystyrene ¹⁰⁷	Lutensol AT50	DMEM with 10% FBS	Stable
		Fe ₂ O ₃ ⁶³	Polyvinyl alcohol	DMEM with 10% FBS	
		Au ⁶⁰	Polyvinylpyrrolidone	RPMI-1640 with 10% FBS	
		Ag ⁵⁹	Polyvinylpyrrolidone	RPMI-1640 with 10% FBS	
Fe ₂ O ₃ ¹¹⁴	Polyvinyl alcohol	DMEM with 10% FBS or RPMI-1640 with 10% FBS			

Covalently bound surface molecules	SiO ₂ ¹⁴¹	Poly(ethylene glycol)	RPMI-1640 with 10% FBS	Unstable
	Magnetite ¹¹⁷	Poly(ethylene oxide)-DOPA	PBS, PBS with 10% FBS	
	Au ⁹⁰	Thiolated poly(ethylene glycol)	DMEM with 5% FBS	
	PLGA ¹⁴⁶	Chitosan modified poly(lactide-co-glycolide)	RPMI-1640	Stable
	Au ¹¹²	Thiolated poly(ethylene glycol)	Phosphate Buffer with lysozyme	
	Fe ₂ O ₃ ¹⁴⁷	Poly(lactide)-poly(ethylene glycol) micelles	Human blood plasma	
	Au ⁹⁰	Thiolated-mercaptopdodecanoic acid-poly(ethylene glycol)	DMEM with 5% FBS	
Magnetite ¹¹⁷	Poly(ethylene oxide)-triNDOPA	PBS, PBS with 10% FBS		
<p>Molecules adsorbed onto NP surface (neutral polymers, oligomers, non-ionic surfactant):</p> <ul style="list-style-type: none"> • Steric repulsion forces improve colloidal stability. • NP stable independent of solution's ionic strength. • Stabilizing molecule may be desorbed through competitive exchange with other components in media. • Proteins either stabilize NPs through adsorption onto particle surface, or destabilize through competitive exchange of stabilizing molecules. <p>Molecules covalently bound to NP surface (e.g. chitosan, PEG, PLA-PEG):</p> <ul style="list-style-type: none"> • Generally enhanced colloidal stability. • Coating can be improved through multipoint attachments between polymer and NP. • Robust polymer coating reduces protein adsorption. 				
Electrosteric	Au ¹⁴⁸	Phosphonate amphiphilic diblock-copolymer coating	DMEM, PBS + 1% L-Glu + 1% P/S and DMEM + 1% L-Glu + 1% P/S	Unstable
	Fe ₂ O ₃ ⁶³	Vinylalcohol/vinylamine copolymer coating	DMEM with 10% FBS	
	Fe ₂ O ₃ ¹¹⁴	Vinylalcohol/vinylamine copolymer coating	DMEM	
	Fe ₂ O ₃ ¹¹⁴	Vinylalcohol/vinylamine copolymer coating	RPMI-1640	
	Fe ₂ O ₃ ¹¹⁴	Vinylalcohol/vinylamine copolymer coating	RPMI-1640 with 10% FCS	
	Fe ₂ O ₃ ¹¹⁴	Polyethylenimine	DMEM	
	Fe ₂ O ₃ ¹¹⁴	Polyethylenimine	DMEM with 10% FCS	
	Fe ₂ O ₃ ¹¹⁴	Polyethylenimine	RPMI-1640 with 10% FCS	
	Poly(L-lactide) cationic ¹⁰⁷	Cetyltrimethylammonium chloride	DMEM with 10 % FCS	
	Polystyrene cationic ¹⁰⁷	Cetyltrimethylammonium chloride	DMEM with 10 % FCS	
	Polystyrene-carboxyl ⁶³	Native anionic polymer charge	DMEM with 10% FBS	
	Polystyrene-amine ⁶³	Native cationic polymer charge	DMEM with 10% FBS	
	SiO _x ⁹³	HSA coating	PBS	
	SiO _x ⁹³	Tween 80 coating	PBS	
	TiO ₂ ⁹³	Tween 80 coating	PBS	
	TiO ₂ ⁹⁶	BSA coating (2 mg/mL)	Bronchial Epithelial Growth Medium, DMEM, Luria-Bertani Broth, Tryptic Soy Broth	
	ZnO ⁹³	HSA coating	PBS	Stable
	ZnO ⁹³	Tween 80 coating	PBS	
	Ag ⁹³	HSA coating	PBS	
	Ag ⁹³	Tween 80 coating	PBS	
Ag ⁹³	Mouse serum coating	PBS		
Au ¹⁴⁸	Phosphonate amphiphilic diblock-copolymer coating	PBS, PBS + BSA, DMEM + BSA and DMEM + 10% FBS		
Au ¹⁴⁸	Trimethylammonium amphiphilic diblock-copolymer coating	PBS, DMEM, PBS + 1% L-Glu + 1% P/S, DMEM + 1% L-Glu + 1% P/S, PBS + BSA, DMEM + BSA, and DMEM + 10% FBS		
Au ¹⁴⁹	Cysteine-functionalized alginate-derived polymer covalent bonding	PBS		

Au ¹⁵⁰	SH-C11alkyl-PEG-NH ₂ covalent bonding	DMEM
Au ¹⁵⁰	SH-C11alkyl-PEG-COOH covalent bonding	DMEM
Au ¹⁵⁰	SH-PEG-NH ₂ covalent bonding	DMEM
Au ¹⁵⁰	SH-PEG-COOH covalent bonding	DMEM
Au ⁶⁸	Vinylalcohol/vinylamine copolymer coating	RPMI-1640 with 10% FBS
Au ⁶⁸	SH-PEG-NH ₂ covalent bonding	RPMI-1640 with 10% FBS
Au ⁶⁸	Mixture of Vinylalcohol/vinylamine copolymer coating and SH-PEG-NH ₂ coating	RPMI-1640 with 10% FBS
Au ⁶⁸	Carboxylated polyvinyl alcohol coating	RPMI-1640 with 10% FBS
Au ⁶⁸	SH-PEG-COOH covalent bonding	RPMI-1640 with 10% FBS
Au ⁶⁸	Mixture of carboxylated polyvinyl alcohol and SH-PEG-COOH coating	RPMI-1640 with 10% FBS
CeO ₂ ¹¹³	Poly(acrylic acid)	DMEM with 10% FBS
Fe ₂ O ₃ ¹¹³	Poly(acrylic acid)	DMEM with 10% FBS
Fe ₂ O ₃ ⁶³	Carboxylated polyvinyl alcohol coating	DMEM with 10% FBS
Fe ₂ O ₃ ¹¹⁴	Vinylalcohol/vinylamine copolymer coating	DMEM with 10% FBS
Fe ₂ O ₃ ¹¹⁴	Polyethylenimine	RPMI-1640
Fe ₂ O ₃ ¹⁴⁰	Poly(acrylic acid) coating	RPMI-1640
Fe ₂ O ₃ ¹⁴⁰	Poly(acrylic acid) coating	RPMI-1640 with 10% FBS
Fe ₂ O ₃ ¹⁵¹	FBS coating	RPMI-1640 with 4% FBS
N-acetylated-Chitosan nanocapsules ¹¹⁵	Native hydrophilic acetylated aminopolysaccharide surface	MEM with 10% FBS
N-acetylated-Chitosan nanocapsules ¹¹⁵	Native hydrophilic acetylated aminopolysaccharide surface	RPMI-1640 with 6% FBS
N-acetylated-Chitosan nanocapsules ¹¹⁵	Native hydrophilic acetylated aminopolysaccharide surface	Endothelial cell growth medium (ECGM) with 5% FCS
NaYF ₄ ¹¹⁶	Cross-linked poly(maleic anhydride-alt-1-octadecene)-bis(hexamethylene)triamine coating	RPMI-1640 with 10% FBS
Poly(L-lactide) anionic ¹⁰⁷	Sodium dodecylsulfate	DMEM with 10 % FBS
Polystyrene anionic ¹⁰⁷	Sodium dodecylsulfate	DMEM with 10 % FBS
Polystyrene-amine ⁹³	Native charged polymeric nature	PBS
Polystyrene-carboxylated ¹⁵²	Transferrin coating	Human blood plasma
Polystyrene-carboxylated ¹⁵²	Transferrin covalent bonding	Human blood plasma
Polystyrene-carboxylated ⁹³	Native charged polymeric nature	PBS
Polystyrene-sulfonated ¹⁵²	Transferrin coating	Human blood plasma
SiO _x ⁹³	Mouse serum coating	PBS
TiO ₂ ⁹³	Human serum albumin (HSA) coating	PBS
TiO ₂ ⁹³	Mouse serum coating	PBS, and RPMI-1640 with 10% FBS
TiO ₂ ⁹⁶	FBS coating (1 – 5%)	Bronchial Epithelial Growth Medium, DMEM, Luria-Bertani Broth, Tryptic Soy Broth, Synthetic Defined medium, Yeast Extract Peptone Dextrose medium
TiO ₂ ⁹⁶	BSA coating (2 mg/mL)	DMEM, Synthetic Defined medium, Yeast Extract

			Peptone Dextrose medium
	ZnO ⁹³	Mouse serum coating	PBS
<ul style="list-style-type: none"> • Efficacy varies between different media due to different solution chemistries. • Strongly adsorbed/bound macromolecules act as a physical barrier against aggregation. Charged macromolecules provide extra electrosteric repulsion. • Strong binding of macromolecule reduces protein adsorption. • In general, positively charged NPs/coatings aggregate more due to bridging flocculation between negatively charged proteins and positively charged NPs. • Negatively charged NPs are generally stable in serum media. • Hydration forces provide additional stability. 			

Most of the aforementioned studies treat the ideal case of uniformly coated surfaces. Some work has also addressed the effect of partial surface coverage. Ramos *et al.*,¹³¹ for example, have also measured the forces between mica surfaces not fully covered by apolipoprotein AII. Their results indicate that the force is fully repulsive only in the case of fully covered surfaces. When only partially covered surfaces approach one another, attractive interactions are measured. This phenomenon is likely driven by bridging and intercalation. The experiments on flat surfaces covered by proteins certainly provide insightful information, which however cannot be always immediately applied to particles. More realistic experiments involve the use of colloidal probe techniques, where a colloidal particle is attached to the tip of an AFM, and used to probe interactions with either a flat surface or other particles positioned on a surface.^{132,133} Due to the large size of the particles used in these systems, no significant differences are observed in comparison with measurements done between flat surfaces

The effect of protein adsorption on the stability of particles has also been probed directly by a few groups using scattering techniques.¹⁵³⁻¹⁶¹ Two major findings have emerged from all these investigations. Contrary to what was expected for charge-stabilized particles from traditional DLVO theory, which foresees a progressive destabilization of particles as the ionic strength increases, protein coated particles show a restabilization at high ionic strengths.^{157,159} At low ionic strengths a progressive destabilization is measured with an increase in ion concentration, at high ionic strength the particles regain stability. The effect has been primarily observed in the case of particles coated by various antibodies and antigens, and is referred to as anomalous colloidal stability. It has been explained in terms of hydration forces generated by counterion adsorption on the proteins.¹⁶⁰ Very often this effect is accompanied by a change in overall surface charge of the particles, as electrophoretic mobility measurements indicate. Attempts to provide more physically sound explanation using the extension of DLVO theory introduced by Ruckenstein *et al.* have proved to be quite unsuccessful.^{155,162} The second consequence of the presence of a protein layer on the surface of the NPs is the peculiar mechanical weakness of the aggregates formed.¹⁵⁹ This has been explained by looking at the estimated interaction energy profiles of particles. The presence of short range repulsion at short separation distances promotes aggregation of protein coated particles in secondary energy minimum. Secondary energy minima are much shallower than primary energy minima, thus leading to much weaker bonds

than those formed when particles aggregate in a primary energy minimum. This leads to difficulties in characterization of the structure of these aggregates, which tend to be very prone to restructuring.

An important consideration can be drawn from all these investigations. When particles are exposed to complex media, the kinetics of adsorption of proteins and other biomolecules is likely to play a crucial role. Even though full coverage of surfaces can provide additional stabilization, at least under certain circumstances, the presence of only a few patches of organic molecules is most likely to induce destabilization and promote bridging flocculation effects, as some investigations indicate. Furthermore, the modeling of colloidal stability using traditional DLVO theory is only expected to provide reasonably accurate results under ideal conditions, which are typically very far from those encountered in physiological solutions. These idealized studies provide controlled examination of NP protein interactions. Conversely, a more developed understanding of NP aggregation can emphasize the relevance of this phenomenon when considering NP behavior *in vitro*.

3. Aggregation

Until this point, our discussion has crudely treated NPs as “dispersed” or “aggregated.” However, NP aggregation behavior can be elegantly modeled and described in certain cases. Heterogeneity (*e.g.* high ionic strength, salt content, or the presence of biomacromolecules) is an innate feature of CCM, and forces defining inter-particle interactions will determine the fate of the colloidal suspensions. Briefly, a repulsive net force ensures stability, while an unbalanced attractive net force will cause the stability to quickly vanish. Mixing otherwise stable NP colloids with CCM can unbalance the inter-particle interactions. Consequently, the destabilized suspension will rapidly collapse and the NPs will aggregate.^{13, 18, 20} Upon aggregation, NP transport dynamics, such as diffusion and sedimentation, will drastically alter compared to stable, dispersed NP. The large size will result in a larger hydrodynamic radius, and therefore aggregates may be rendered susceptible to sedimentation, whereas single small NP will typically exhibit only Brownian motion. However, the aggregate will not behave the same as a larger single particle of an equally large hydrodynamic radius, as aggregates will have a variable packing density which will further impact particokinetics.¹⁶³ The specific surface area of aggregates will also drastically change compared to monodispersed NPs, which

may heavily influence the interface between NP aggregates and cells. Thus understanding aggregation formation is crucial to understanding the impact of NP aggregation on biological systems.

In the absence of external forces, aggregates of NPs are irregular and exhibit an arbitrary random shape and a disordered structure. Such aggregates have a so-called mass-fractal structure and exhibit a striking feature called self-similarity, where the characteristic features of randomness and disorder can remain invariant over a large range of length scales (Figure 3).¹⁶⁴

Fractal clusters are composed of loosely aggregated NP and also empty voids, which is clearly visible on TEM micrographs.^{165,166} A fundamental concept to describe the structure of mass fractals is the fractal dimension, d_f , which can be related to the typical packing density of particles within an aggregate.¹⁶⁷ Owing to the arbitrary shape and random structure, aggregates are ill-defined in nature; yet, one may still quantify properties relevant for the study of NP-cell interactions via the fractal dimension. In fluid, the relevant characteristic size is the hydrodynamic radius, R , whose value is close to the cluster radius of gyration.¹⁶⁸ Both the hydrodynamic radius and the effective mass density are functions of the d_f and aggregation number, N . These values therefore influence the transport of aggregates via diffusion and sedimentation.¹⁶⁹ In solution the motion of aggregated and clustered NPs is generally different from that of the primary NPs, and this results in differences in the amount of NP which will reach a cell's surface *in vitro*.^{163,170}

The d_f therefore accounts for the nonlinear relationship between the number of particles, and the corresponding size and mass of the aggregation cluster.¹⁶⁹ Such power-law relationship is very characteristic for self-similar structures. The formation of aggregates and clusters can be reversible or irreversible. The rate and nature of the aggregate formation are complex functions of the now-imbalanced inter-particle forces and NPs dynamics. The fractal dimension is a function of the probability that NPs collide and bind together. The rate of collisions is proportional to the particle concentration and the Brownian dynamics, and the chance of binding upon colliding is a function of the inter-particle forces. Recently a novel experimental approach has been presented which accurately measures the effective density of agglomerates in suspension based on the volume of the pellet obtained by centrifugation of nanomaterials in suspension.¹⁷¹

When the inter-particle interactions are dominated by attraction, the rate and structure of aggregate formation is influenced solely by the Brownian motion because the NPs bind as soon as they collide. This limiting case is diffusion-limited cluster aggregation (DLCA), and the fractal dimension of DLCA clusters is close to 1.8.^{166,172-174} Interestingly, the fractal dimension of diffusion-limited clusters is universal and independent of the NP size as long as the polydispersity of the NPs is moderate.¹⁷³ The opposite case is when the NPs must collide more than once before becoming bound. The kinetics of this process, reaction-limited cluster aggregation (RLCA), may

be very slow, and the fractal dimension of RLCA clusters is near to 2.1.^{14,173} A higher fractal dimension indicates a more closely, densely packed structure. As a function of time, the average cluster size has been observed to grow exponentially for RLCA, while a power-law behavior has been observed for DLCA. However, most colloidal systems display a complex behavior including the features of both DLCA and RLCA. For example, Limbach *et al.*¹⁶ demonstrated that small variations in the surface chemistry of the particle may have crucial impact on the stability, and therefore, may significantly slow down the aggregation rate. Similarly, electrolyte concentration, pH, temperature, shear rate, and particle concentration all influence the rate and type of aggregation.^{175,176} Furthermore, if the bonds among the particles are either very weak or partially reversible it allows for movement of the particles in the aggregate and restructuring, thus resulting in a higher fractal dimension value. Understanding how these numerous factors interact can provide a more clear idea of how NP aggregate, and understanding aggregation provides more predictive abilities for how the NP will behave *in vitro*. To this point, thorough NP characterization provides a means for understanding NP properties, aggregation, and ultimately behavior and interaction with cells.

4. Nanoparticle characterization

The assessment of the colloidal stability in physiological fluids is non-trivial. The variety of physical and chemical forces involved, the complexity of analytical methods and theories upon which these analytical methods are based all compound the difficulty in accurately evaluating the dispersion state of NPs in biological environments. These aspects are further complicated by the multitude of different, highly complex physiological fluids NPs interact with. However, there are a number of techniques that are commonly used to evaluate NP physico-chemical properties and dispersion state, as well as a growing number of state-of-the-art methods. Here we discuss the most commonly used characterization approaches, and comment on considerations that must be made when characterizing NP colloidal stability.

Particle size, stability, the onset of aggregation and its evolution in time should ideally always be probed *in situ*, since *ex situ* methods require deposition onto a substrate, which may bring in uncontrollable, and hence, unpredictable artifacts. Three major categories dominate the currently applied experimental approaches: scattering, optical spectroscopy, and microscopy. Light scattering techniques are by far the most frequently used *in situ* methods when describing size and polydispersity of suspended particles. Dynamic light scattering (DLS) is a technique where particle size and polydispersity is determined by monitoring the time-dependent fluctuations in the intensity of the scattered light due to particle motion, which, in dilute samples, can be directly related to the particle's diffusion coefficient. In suspension, due to the Brownian motion of NPs, the scattering intensity exhibits temporal

fluctuations from which particle size can be recovered from the diffusion coefficient, which is inversely proportional to the size according to the Stokes-Einstein equation.¹⁷⁷ DLS is also relatively straightforward to perform and highly quantitative. Commercial cutting-edge instruments are widely available and affordable. However, the subtleties of the theory behind DLS can be obscured by the experimental simplicity, especially when dealing with polydispersity. An inherent characteristic feature of scattering is that the measured size is intensity weighted, *i.e.* the light scattered by a particle is proportional to the sixth power of its size. In the obvious case of polydisperse samples, this implies that large particles or aggregates produce very strong signals that can dominate over that produced by small particles. Unfortunately, the deconvolution of DLS data is mathematically an ill-posed problem and prone to generate artifacts, even more so because the intensity-weighted statistical distribution should be transformed into number-weighted statistical distribution in order to diminish the effect of aggregated particles and better report the size of smaller, dispersed NP. Furthermore, these techniques generally require a dilute suspension to ensure single scattering events and to mitigate inter-particle interactions. Depolarized DLS (DDLDS) extends the technique for particles possessing optical or shape anisotropy, *e.g.* nanorods.¹⁷⁸ The experimental procedure of static light scattering (SLS) is very similar, however, instead of analyzing the time-dependent fluctuation of the scattered intensity, the angle-dependence of the scattered intensity is analyzed. The scattering intensity as a function of the momentum transfer, q , relates directly to the size and form of the particles, via Fourier transformation. The scattered intensity of a fractal system follows a power law as a function of the momentum transfer, which renders scattering to be essential in the assessment of fractal properties.¹⁶⁸

Compared to static scattering, optical spectroscopy is a rapid technique to monitor the colloidal stability of noble metal NPs (*i.e.* gold, silver). These particles display unique and strongly enhanced optical properties known as localized surface plasmon resonances (LSPRs). The basis of LSPRs is the coherent collective oscillations of conduction electrons upon interaction with the incident light (an electromagnetic radiation).¹⁷⁹ The LSPR gives rise to a pronounced extinction band in the visible-near infrared (NIR) region of the electromagnetic spectrum, and the position and width of the LSPR band is highly affected by particle aggregation. When plasmonic NPs are close, individual plasmon oscillation can couple with each other via near-field interactions, resulting in coupled LSPR modes,¹⁸⁰ which impact the distribution of the electric field around the nanostructure.¹⁸¹ As a consequence, particle aggregation results in a spectra red shift and broadening of the peak.¹⁸² These changes indicate a decrease in the concentration of single NPs, which is confirmed by a color shift of the colloid from deep red to purple for gold NPs or from yellow to black for silver NPs.^{11,183}

In certain cases LSPR is suitable for monitoring particle-protein interaction in biological media, as LSPR is sensitive to the local dielectric environment.¹⁷⁹ Metal NPs incubated in

biological media exhibit a red shift in their LSPR spectra and an increase in band intensity over time due to the formation of a stable protein corona on the NPs metal surface.^{139,184} This approach may be valid for “bare” particles, *e.g.* citrate-coated gold or silver NPs, but should be carefully revised when particles are coated, with for example a polymer shell. The polymer may act as a dielectric spacer and insulator, thus decreasing the sensitivity to refractive index changes.¹⁸⁵ As a consequence UV-Vis can become practically insensitive, and monitoring the protein adsorption of surface-functionalized NPs in biological media cannot be conclusive. Therefore complementary techniques should be used.

In contrast with scattering and optical spectroscopy, NPs can be directly visualized by microscopic imaging. These methods of choice allowing resolving NPs in the range of Ångstrom to nanometer include electron microscopy such as transmission (TEM) or scanning electron microscopy (SEM), and atomic force microscopy (AFM). TEM is a microscopy technique whereby a beam of coherent electrons (the primary beam) is transmitted through a sufficiently thin specimen (< 500 nm) thereby offering a convenient method for measuring the diameter of electron dense NP, and resolving structures at the Ångstrom level.¹⁸⁶ TEM is however limited because samples must be dried onto a grid for imaging, as TEM is performed under vacuum. This can result in drying artifacts (*e.g.* aggregation of NP), and it is therefore difficult to gain information about the NP dispersion state *in situ*. SEM works by scanning an electron beam over the sample, which scatters secondary electrons from the sample to detectors.¹⁸⁶ This method provides detailed information about the NPs morphology, topology, and composition, and can resolve structures at the nanometer level. Conventional SEM is also performed under vacuum, and therefore is limited by drying artifacts of samples. Furthermore, depending on the NP material, some samples need to be prepared with an ultrathin electrically conducting material (*e.g.* gold, palladium, platinum). However, environmental SEM (ESEM) provides a means of imaging samples in a hydrated, non-desiccated state. This allows for samples to be imaged in a somewhat aqueous environment and can be done without coating with an electrically conducting material.^{187,188}

AFM is a technique whereby a fine tip is mounted on a highly sensitive cantilever and scanned across a surface. Measuring the deflection of the cantilever provides a quantitative image of the surface's topography. Thus, AFM can be used to quantitatively measure the size and morphology of NPs, and can resolve structures on the angstrom scale.¹⁸⁹ AFM has the advantage of operating in an aqueous environment. However can still be limited by either aggregation artifacts based on NP interactions on the surface, or NP-AFM tip interactions.³⁹

Characterization of NP is critical for not only obtaining physico-chemical information regarding the NP, but also to fully understand how NP behavior is tied to these properties. To that end, it is of utmost importance to not only robustly

characterize NP with multiple techniques, but to also understand the limitations of each characterization method.

5. Conclusions

The above discussion attempts to summarize the factors which dictate NP stability in physiological fluids such as cell culture media. However, it can be seen that NP aggregation is highly system dependent and one must consider a number of factors: material, NP physico-chemical properties, surface coatings, solution properties and components, the presence of macromolecules, *et al.* Thus providing a comprehensive framework to predict NP stability in media or physiological solutions (based on experimental or theoretical data) is exceedingly difficult. General, albeit well-established, trends indicate that the presence of a stabilizing coating (*e.g.* PEG) improves NP stability and reduces the adsorption of proteins to the NP surface. Likewise the strength or stability of the interaction between the stabilizing coating and the NP is also important to maintain colloidal stability, as constituents of the solution have been shown to displace stabilizing molecules and initiate aggregation. One must also consider the inter-molecular interactions of coatings, and inter-particle forces. Conversely, the composition of the physiological solution must be considered. Thus, the complicated question of NP aggregation depends on the system presented.

It is apparent that NP stability and the propensity of NPs to aggregate in complex physiological fluids merits further investigation. In order to more easily comprehend NP behavior in biological experiments, it is necessary for nanomedicine research to (1) heavily rely on interdisciplinary research between physicists, materials scientists, biologists, nanotoxicologists, medicine, etc. (2) supply robust and universal characterization methods with regards to physico-chemical properties and be aware of their limitations, (3) use, if possible, more than one technique to study the materials, and (4) characterize NPs in an environment, which is as close to the experimental environment as possible. Currently the issue of NP aggregation is understated in the literature, which may in part lead to the difficulty in translating nanomedicines from the bench top to the clinic. However, with a concentrated interdisciplinary effort with a heavy focus on both analytics and understanding fundamental behavior of NPs in solution, it may be possible to more successfully advance the use NPs for biomedical applications.

As of today, theoretical models such as the extended DLVO theory cannot be used to provide reliable predictions of the stability of NPs in complex biological media. This is partially due to the theory inability to correctly describe the behavior of the interface between particles and media, but also to the intrinsic experimental inaccuracy in the values of parameters, such as surface charge density, ligand density, adsorption of biomolecules, etc. which need to be supplied to the theory. While more research efforts on the theoretical side are certainly necessary, researchers currently have to primarily rely on multiple characterization techniques to probe colloidal stability

of NPs in complex media and to engineer better NPs. Characterization include non-invasive scattering techniques, which are the most reliable tools to spot aggregation, but also analytical techniques, such as titration, calorimetry, spectroscopy, *etc.* to gain a deeper understanding of how the biological environment impacts NP colloidal stability.

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