Plasmonic nanoparticles and their characterization in physiological fluids

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Nanoparticles possess unique properties beyond that of classical materials, and while these properties can be used for designing a dedicated functionality, they may also pose a problem to living organisms, to human health and the environment. The specific primary routes by which nanoparticles may interact with the human body include inhalation, injection, ingestion and application to the skin. Independent of the entry route, the particles inevitably encounter a complex physiological fluid populated with *e.g.* proteins, vitamins, lipids and salts/ions. Different consequences of such an encounter may include formation of a surface-bound protein layer, particle dissolution or aggregation, which are expected to have a crucial impact on cellular interaction. Understanding cellular responses to nanoparticle interactions starts with understanding particle behavior in physiological fluids. Nanoparticles are now available in practically any size, shape and functionalization, to promote distinct optical, magnetic, and physico-chemical properties, making the prediction of their behavior, in physiological fluids, not a trivial task. Characterization has therefore become of paramount importance. In this review, we give an overview about the diversity of physiological fluids as well as present an inventory of the most relevant experimental techniques used to study plasmonic nanoparticles.

1. Introduction

The development of "nano" started over 20 years ago, with nanoscale science and technology now having an increasing impact on many aspects of our daily lives. The enormous potential and rapid expansion of nanotechnology has resulted in an exploding array of engineered nanomaterials, which are increasingly being used for commercial purposes [1] but with clear differentiation across industry sectors. Typically, materials applications are reaching the markets first, with electronics and IT applications following closely behind [2]. Nanobiotechnology and nanomedicine have the potential to revolutionize diagnostic tools and treatment strategies, which are more personalized, efficient, or easier to administer. However, healthcare and life sciences applications have the longest time-to-market: approval by the regulatory agencies, technological adjustments in manufacturing [3], and potential risks associated with engineered nanomaterials are just some of the reasons for the comparatively slow development of nanomedicines.

Nanoparticles (NPs) or sources of NPs can be classified as natural or intentional/engineered and unintentional anthropogenic activities [4]. Humans can come in contact with engineered NPs in many different ways such as through the use of consumer products containing NPs (*i.e.* food and cosmetic products), at the working place (*i.e.* occupational exposure), during disposal of the products (*i.e.* incineration) or by the intended use of NPs in biomedical applications. The possible portals where those NPs may enter the human body are the lungs *via* inhalation, the gastro-intestinal tract *via* digestion, the skin, and blood vessels *via* intravenous injection [5]. No matter if the exposure is intended or occupational, NPs will interact at a certain time with the cells of the human body. One particular fraction of engineered NPs may be defined as 'designer' particles, having a desired functionality. These particles are synthesized mostly within the walls of academic and research centers laboratories generally aiming at obtaining uniform particles. In recent years, a lot of progress was made in the synthesis of such designer NPs, which have been designed in many sizes and morphologies [6–8] to produce distinct optical, magnetic, and physico-chemical properties.

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Over the past two decades, much research has been dedicated to the study of NP-cell interactions in order to advance nanomedical applications, while determining if the potential benefits of nanotechnology could be achieved without any adverse impact upon human health. More specifically, significant effort has been dedicated to elucidate the impact of physicochemical properties of NPs, such as their size, surface charge, hydrophobicity or shape, on their interaction with cells. This interaction is routinely assessed *in vitro* to reveal mechanistic insights [9–11]. Despite the overwhelming use of *in vitro* systems to evaluate cellular responses toward NPs, the impact of an omnipresent complex physiological fluid on the material is still insufficiently taken into consideration [12–14].

2. Physiological fluids

Upon contact with physiological fluids, NPs can interact with a wide range of biomacromolecules [15], with various consequences. In order to understand the behavior of NPs in physiological fluids it is imperative to summarize the composition of the non-cellular compartments, such as mucus (gastro-intestinal (GI) or respiratory tract) or aqueous lining layer covered by surfactant (lung parenchyma), the blood or the lymphatic fluid, as well as available methods to investigate the possible interactions.

2.1. Mucus

Mucus is a biopolymer-based hydrogel produced by specific cells such as goblet cells and its main function is the protection of epithelium against infectious agents. Mucus is rich in water (>95%) and glycoproteins but also contains enzymes, immunoglobulins, proteins and salts, with the composition varying between each mucosal surface [16,17]. If particles are ingested or inhaled, they can be deposited onto the mucus, and through subsequent displacement entrapped in mucin fiber mesh, depending on their size.

The interaction of NPs with the mucus can cause a change in particle size and zeta potential. The majority of experiments to study these parameters are usually performed using isolated mucus collected from animals [18]. Recently it has been shown that small-angle neutron scattering (SANS) is a powerful technique to investigate the interaction of mucus proteins/enzymes with NPs and enables to correlate the changes in comparison to the bare NPs with mucus permeation [19]. Recently, a combination of tools, such as cryo-scanning electron microscopy, capillary penetration, and optical tweezers, was applied to assess particle mobility in mucus and model hydrogels, and penetration of particles on various length scales. It has been shown that particle mobility is dependent on the highly rigid structures within the mucus mesh but also on the adhesive properties of the particles [20]. In addition to the physicochemical parameters, the muco-adhesive properties of NPs can be determined by applying rheological measurements [21]. Another possibility is to use the Ussing chamber. With it, it has been demonstrated that the diffusion efficiency of neutral NPs was higher in comparison to uncharged particles [22]. In addition, the pH of mucus varies according to the location within the body, and the interaction of NPs with the mucin polymers can be tuned by buffer conditions such as pH and ionic strength [23].

The respiratory tract is covered by pulmonary surfactant, which consists 85–90% of phospholipids and specific surfactant proteins (SP) [24]. The binding of those constituents onto the surface of a NP can be determined by thin layer chromatography for lipids and gel electrophoresis (SDS-Page) combined with liquid chromatography/tandem mass spectrometry (LC/MS/MS). By using a specific fixation method to stabilize the phospholipids and liquid lining layer, Raemy et al. have shown that it is possible to visualize NPs in the aqueous lining layer (Fig. 1), indicating that this is an important compartment where particle properties can change, depending on the environment [25].

The airway mucus varies in composition and amount along the airway tree. The airway epithelial goblet cells and submucosal glands secrete mucus, forming a two-layer mucus blanket over the ciliated epithelium, *i.e.* a low-viscosity sol layer covered by a high-viscosity gel layer and a glycocalyx [26]. Insoluble particles are trapped in the gel layer and are moved toward the pharynx (and ultimately to the GI tract) by the upward movement of mucus generated *via* metachronous beating of the cilia also known as mucociliary clearance. The aqueous lining layer height in the lung parenchyma is very thin, *i.e.* about 5 μm as reported for primary human epithelial type II cells in culture [27], therefore, the volume is also very small and makes it difficult to collect it *in vitro* or *in vivo* to determine the colloidal behavior of NP in this liquid. An alternative approach is to use artificial alveolar fluid, and it has been shown that incubation of this liquid with silver NPs induces aggregation
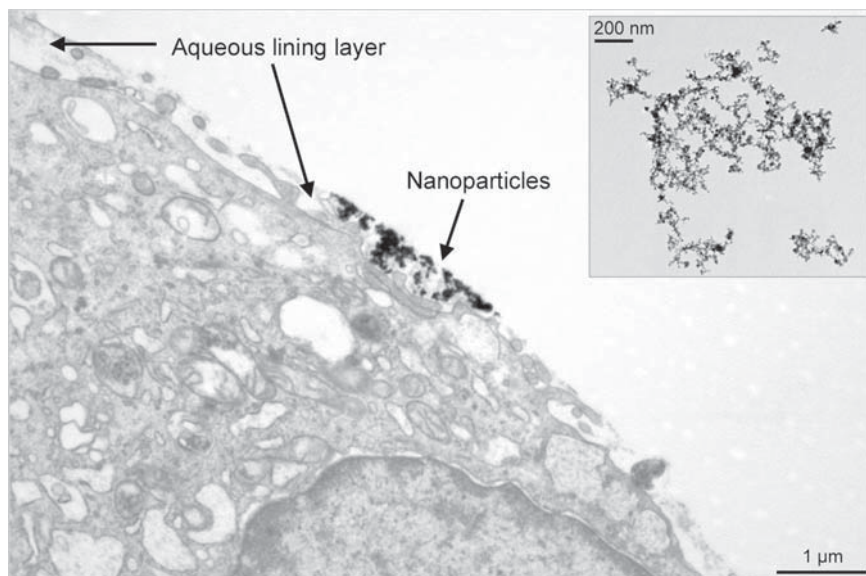

Fig. 1. TEM micrograph of a human epithelial II cell *in vitro* (A549 cells) incubated at the air–liquid interface and exposed to aerosolized NPs (inset). After 10 min of incubation the NPs are mainly localized in the aqueous lining layer formed by the cells before they are internalized.

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which was confirmed by transmission electron microscopy and dynamic light scattering experiments [28].

2.2. Blood

The main administration route up to date for biomedical NPs such as theranostic NPs is *via* intravenous injection [29], where the NPs encounter a complex cellular and molecular milieu of the human blood [30]. The human blood is composed of a cellular fraction (∼45%), *i.e.* red blood cells, white cells and platelets, and the blood plasma (∼55%) with proteins, glucose, amino acids, fatty acids and others such as clotting proteins, *i.e.* fibrinogens. The term "blood serum" refers to plasma from which fibrinogen has been removed. Blood contains more than 1000 types of proteins and over 50 of them have been identified in various experiments on the surface of NPs [15,31]. Recent findings have also shown that incubating NPs with human plasma, from subjects with different diseases and medical conditions, results in protein coronas of different composition [32]. Since blood composition is highly complex, the analysis of the NP behavior in complete (human) blood is very challenging and the studies are usually done *in vitro* using defined suspension conditions and/or *in vivo* experiments (for a review see Hall et al. [33]). Indeed, most research is focused on the identification of surface adsorbed proteins on NPs with respect to their physicochemical properties [34].

One approach to characterize aggregation and the proteins adsorbed onto the surface of magnetic NPs is to separate the NP-protein complex after injection into the blood stream of rats by a high magnetic field gradient magnetic reactor [35]. It was reported that the protein composition depends on the initial surface charge of different functionalized NPs [36]. By using a similar approach, *i.e.* incubation of magnetic NPs with 10% fetal bovine serum and a subsequent isolation using a magnetic reactor, the size and surface charge were characterized using dynamic light scattering. Although the adsorbed protein profile was similar on differently charged NPs, the positively charged NPs displayed significantly lower colloidal stability [37].

2.3. Lymph fluid

Lymph fluid is formed by interstitial fluid collection through the lymph capillaries and is then transported *via* the lymph vessels to the lymph nodes. The composition of lymph is similar to blood plasma but contains only white blood cells. Since NPs have been observed in the lymph nodes, a transport from the primary barriers into the lymph capillaries must have occurred [38,39]. This transport might occur *via* single NPs, small aggregates, or NPs inside immune cells, *i.e.* dendritic cells. Although it has been shown recently *in vivo* that the transport of quantum dots from the vaginal lumen to the lymph nodes can be explained by convective diffusion from the interstitial fluid into the lymph capillaries [40], the colloidal characteristics of NPs in this physiological fluid are still unknown and more research in this area is needed.

2.4. Cell culture media

Since the mucus and blood fluids are incredibly complex and the vast majority of studies in the field are done *in vitro*, most research to assess the colloidal behavior of NPs in physiological fluids are done in complete, *i.e.* serum supplemented cell culture media. Depending on cell type, a specific medium is used to culture cells *in vitro*. We refer the reader to specialized reviews [41] that describe the composition between the most common cell culture media.

Fig. 2 schematically shows the crowded environment of a NP (represented as 40 nm Au sphere) in a standard complete cell culture medium (*e.g.* Dulbecco/Vogt modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS)). The
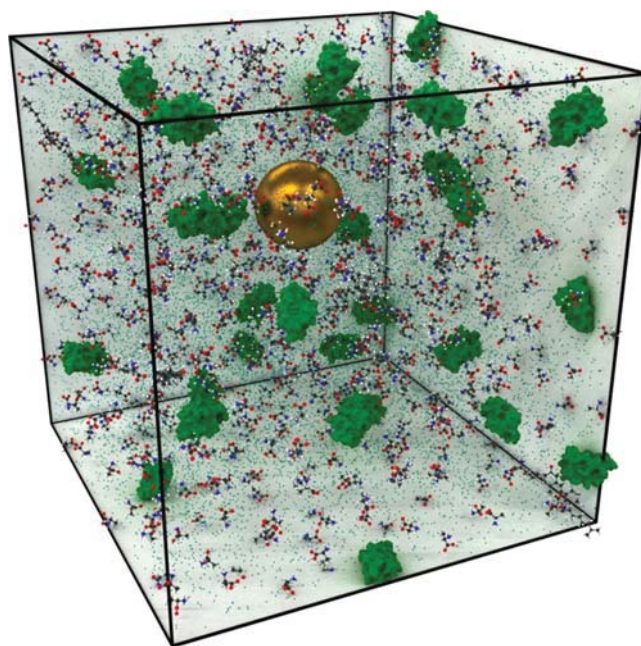

Fig. 2. Scaled illustration of an Au NP incubated into 10% serum-supplemented cell culture medium. Scale ratio $1:2.5 \times 10^{18}$ based on the volume occupied of 250 μL in a 96-well plate.

medium can be described as a buffered solution containing proteins such as serum albumin, globulins, other biomolecules such as vitamins and amino acids, and ionic salts. The box (cube with 100 nm side length) represents the volume of medium in which only one NP (at an incubation concentration of 20 μg/mL) is present. The environment of the NP in this box includes roughly 6400 amino acids, almost 100 vitamin molecules and more than 240,000 ions (cations and anions), represented as green dots, in addition to roughly 34 proteins.

Thus, NP behavior in such an environment will be dictated by the interaction with these components. This interaction can have major consequences, some of which are schematically shown in Fig. 3.

First, it can result in a tightly bound immobile protein layer formed on the particle surface (the so-called hard corona) and possibly a weakly associated mobile layer (the soft corona) [42]. Many studies have focused on identifying the major protein constituents, with the protein corona having shown to be determined by one or more characteristics of the NP, such as material composition, size, hydrophobicity, or charge [37,42]. Much of the information regarding the interaction of proteins and NPs have been summarized in several excellent reviews, which the reader is referred to [15,43].

Second, the interaction of NPs with physiological fluids can induce NP aggregation, which is a common phenomenon in this complex environment and should therefore be taken into account [44]. Aggregation refers to the, usually irreversible, inter-particular adherence, leading to the formation of large and irregularly shaped clusters [45]. This aggregation can lead to misrepresentative results and hamper experimental reproducibility [12]. For example, Wick et al. observed a more pronounced cytotoxic effect for aggregated carbon nanotubes compared to bundled carbon nanotubes, confirming the hypothesis that aggregation phenomena occur and can modify the cytotoxicity of a nanomaterial [46]. Cho and colleagues [9] showed, by using an upright and an inverted cell culture configuration, that the cellular uptake of gold NPs was generally higher in the upright configuration and that cellular uptake between the two configurations was most prominent for gold NPs with higher sedimentation rates. This additionally highlights the necessity of
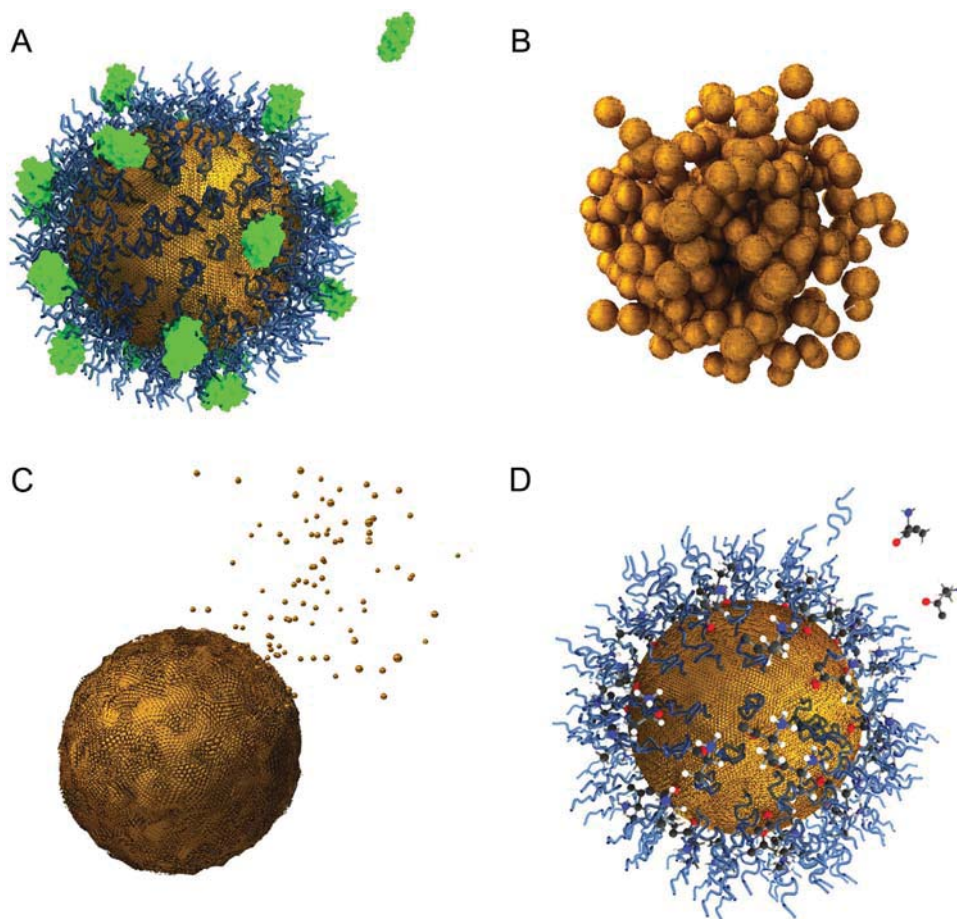
Fig. 3. Schematic illustration of possible consequences arising from NP incubation in physiological fluids. (A) Formation of protein corona; (B) NP aggregation; (C) NP dissolution; (D) removal/exchange of anchoring ligands.

taking into account sedimentation and diffusion, which can vary wildly for aggregates. In a recent review, we have described the current state-of-the art, the theory behind it, current bottlenecks in this field [41].

Third, some NPs, in particular silver (Ag) and copper (Cu), have been reported to undergo oxidative dissolution. Ion release was found to correlate with intrinsic NP properties, such as particle size, shape and surface coating, but also with matrix factors, such as dissolved oxygen, temperature, pH, ionic strength and the presence of proteins [47–49]. For example, Ostermeyer et al. have shown that increased concentrations of bovine serum albumin (BSA) result in increased dissolution rates of Ag NPs due to the high affinity of BSA toward Ag^+ ions chemisorbed on the Ag NP surface [48].

Finally, the presence of biomolecules was reported to be responsible for the removal of anchoring ligands from NP surfaces. Several studies have shown that physiological concentrations of thiol-containing molecules such as cysteine easily displace, for example, thiolated Polyethylene-glycol (PEG) from the surface of Au NPs [50,51]. The (partial) loss of the ligand, however, has far-reaching consequences as it impacts colloidal stability of the core NP, changes the adsorbed protein profile, and may lead to the complete loss of any functional grafted ligands such as fluorescent dyes, antibodies, or drug molecules.

3. Characterization of NPs in physiological fluids

The characterization of NPs in physiological fluids and the assessment of their colloidal behavior are challenging due to the complex physical and chemical forces involved, the multitude of different, highly complex physiological fluids, and the variety and complexity of analytical methods and theories upon which these methods are based. However, there are some techniques that are regularly used to study the behavior of NPs in complex fluids, as well as a growing number of novel methods that have emerged in recent years. Here we discuss the most commonly used experimental techniques and comment on considerations that must be made when characterizing such complex systems. It is important to mention that most of the research in this area is performed with NPs in serum completed cell culture media since the composition of this fluid can be controlled.

In this review, we focus on metallic NPs, most frequently silver (Ag) and gold (Au), which exhibit outstanding physical and chemical properties that are markedly different from the bulk metal [52]. The most important property of these NPs is the so-called *localized surface plasmon resonance* (LSPR) which is generated by the collective oscillation of conduction band electrons upon excitation with the appropriate electromagnetic radiation (*e.g.* a laser beam), resulting in a characteristic light scattering and absorption spectrum, which is a function of the particle size, morphology, and the local dielectric environment [53,54]. This consequently makes plasmonic NPs excellent probes for many analytical techniques that rely on light scattering and absorption. Due to their outstanding optical properties, an increasing number of studies have focused on Au for a wide range of biological and biomedical applications such as biosensing, biomedical imaging, gene and drug delivery, or disease detection [55,56].

Tables 1 and 2 summarize typical NPs, organized by the main analytical method used to characterize them in a variety of

Table 1
Dynamic light scattering of typical plasmonic NPs in a variety of physiological fluids.

Reference	NP type	Fluid(s)	Content
Albanese (2011) [57]	Au NPs and TF coated Au aggregates	RPMI	Effect of aggregation on cellular uptake and cytotoxicity
Balog (2015) [58]	Au NPs (citrate, COOH-PEG, NH$_2$-PEG and methoxy-PEG coated)	DMEM, DMEM containing 10%FBS	Characterizing state of aggregation in physiological fluid
Braydich-Stolle (2014) [28]	Ag NPs (Bare and polysaccharide coated)	Alveolar and lysosomal fluid	Stability and aggregation
Casals (2010) [59]	Au NPs	Complete cell media	Evolution of protein corona
Casals (2011) [60]	Au and Ag NPs	Complete cell media	Protein corona formation and biological implications
Cho (2011) [9]	Au NPs (bare and PEG coated)	Complete cell media	Influence of sedimentation and diffusion on cellular uptake
De Paoli Lacerda (2010) [61]	Au NPs	Protein solutions	Interaction of NPs with human blood proteins
Dominguez-Medina (2013) [62]	Au NPs (citrate coated)	PBS and BSA solutions	Synthesis of advanced NPs to prevent aggregation
Fatisson (2012) [63]	Au NPs	Complete cell media	Impact of cell culture media components on stability
Hühn (2013) [64]	Au NPs (negatively and positively charged polymers)	Complete cell media with increasing complexity	Charge-dependent interactions with proteins and cells
Kittler (2010) [65]	Ag NPs (PVP or citrate coated)	Complete cell media	Effect of proteins on dispersability and biological activity of NPs
Lankoff (2012) [66]	Ag NPs	Complete cell media (5% FBS)	Impact of aggregation on cellular response
Li (2015) [67]	Cu NPs	Complete cell media	Dissolution of NPs in cell culture media
Liu (2012) [68]	Au nanorods (CTAB coated)	BSA, HSA, IgG	Interaction with proteins
McCuspie (2011) [69]	Ag NPs (BSA coated)	Synthetic lung fluid	Dispersion stabilization of NPs and effect on colloidal stability
Mahl (2010) [70]	Au NPs (TPPTS and PVP coated)	Complete cell media (FBS up to 10%)	Stability and aggregation; Impact on cellular response
Montes-Burgos (2010) [71]	Au NPs	Human blood plasma	Protein adsorption and stability
Moyano (2014) [72]	Au NPs (sulfobetain headgroups)	Diluted human serum	Synthesis of corona-free NPs
Mukherjee (2014) [73]	Ag NPs	Complete cell media	Impact of physiological interactions on cellular dosimetry
Murdock (2008) [13]	Cu and Ag NPs	Complete cell media	Characterization of NP dispersion in cell media
Pal (2014) [74]	Ni NPs	Complete cell media	Characterization of NP dispersion in complex media and dosimetry
Pyshnaya (2014) [75]	Au nanorods and spheres (PEI and BSA coated)	Complete cell	Impact of physiological fluids on size and stability
Soliman (2015) [76]	Au nanorods	Complete cell media	Method development to improve stability in media

BSA, bovine serum albumin; PVP, polyvinyl pyrrolidone; PEI, polyethyleneimine; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RPMI, Roswell Park Memorial Institute medium; LSPR, localized surface plasmon resonance; HSA, human serum albumin; TF, transferrin; TPPTS, tris(sodium-m-sulfonatophenyl)phosphine.

physiological fluids. Approaches described in more detail include: dynamic light scattering (DLS) (Table 1), optical spectroscopies, in particular UV–is and microscopy, with a special focus on dark field hyperspectral imaging (DF-HSI) (Table 2), in addition to an overview of less commonly used techniques. It should be noted, however, that there is rarely one technique that can fully characterize a NP in a physiological fluid and often the most reliable conclusions will be drawn from an array of complementary measurements.

3.1. Scattering

Due to easy sample preparation and a simple experimental procedure as well as the availability of high-quality instruments, DLS is usually one of the first techniques to probe (plasmonic) NPs. It is also the most abundantly used in particular in the context of physiological fluids (Table 1).

Theoretical results are available for many particle morphologies as well as for aggregates and agglomerates of spherical particles, which are however most frequently attributed with the features of statistically self-similar random mass fractals. Light scattering builds on the experience that an interaction between the NPs and biomolecules will be accompanied by changes in the hydrodynamic size of the particles. The constituent of the fluids may have an impact on stability [63], and therefore, the most frequent use is to answer whether the system remains stable or undergoes aggregation [13,28,59,60,65,69,75]. It has been repeatedly shown that the degree of aggregation has an impact on cellular response [70,77] and much effort is dedicated to improve the stability of NPs in such media [76]. Although it has been demonstrated that protein adsorption can generally stabilize the system [62,68,71], surface-charge dependent interactions must be also considered [64]; characterizing the extent and time evolution of protein adsorption may bring further insights [60,72]. Other studies have used the method to study kinetics of aggregation and dissolution of e.g. Ag and Cu particles [67]. Two very important factors to be considered are particle polydispersity [78] and the variety of the physiological fluids. Since DLS is an ensemble technique where billions of scattering centers are probed simultaneously, proteins, which are highly abundant in complex fluids, contribute to scattering and if this contribution is not negligible compared to the NPs, e.g. at low concentrations, the analysis and interpretation must allow distinction between scattering from NPs and those proteins. This may be achieved by characterizing the physiological fluid alone without NPs, the NPs in an 'empty background' e.g. in water, and then finally the NPs in the fluid. Nonetheless, this approach is valid only if the interaction between NPs and the proteins is moderate, in the sense that the overall number of 'free' proteins does not change considerably. However, as soon as NP-protein complexation occurs, this approach should not be followed, as the 'reference scattering' from the protein alone is not valid anymore. In this regard depolarized scattering provides a promising alternative to characterize NP in physiological fluids, which relies on the optical anisotropy of even spherical plasmonic NPs [79], which either stems from shape and/or from internal

Table 2
Studies on plasmonic NPs in physiological fluids using spectroscopic or microscopy methods.

Reference	NP type	Fluid(s)	Content
Dark field microscopy & hyperspectral imaging (DF-HSI)			
Badireddy (2012) [83]	Ag, Au but also CNT, CeO, TiO$_2$ NPs (citrate and PVP coated)	Water and simulated waste water	Detection and characterization of NPs in complex (waste) water
Hu (2008) [84]	Au nanorods and shells	Water	Understanding factors that influence LSPR of NPs
Huang (2006) [85]	Au nanorods and spheres (antibody coated)	Complete cell media and intracellular	Au nanorods for photothermal therapy
Jenkins (2015) [81]	Au NPs (citrate coated)	Blood	Monitoring LSPR for aggregation studies
Leclerc (2014) [86]	Ag NPs (bare and cysteine coated)	Complete cell media and intracellular	Dissolution and reformation of silver NPs
Mortimer (2014) [87]	Ag, Au, CuO, TiO$_2$, NPs and QDs	Intracellular and 10 mM HEPES buffer	Semi-quantitative analysis of cellular uptake
Rosman (2012) [88]	Au nanorods and spheres (NH$_2$- or COOH-PEG coated)	Intracellular	Assessment of NP uptake and aggregation inside cells
Stacy (2013) [89]	Au nanorods (tannic acid coated)	Water and cell culture media	Detection of nanorods in cells
Transmission electron microscopy (TEM)			
Hirsch (2014) [77]	Au NPs	Water	Cryo-TEM; Effect of surface charge on colloidal stability and cell interaction
Hondow (2012) [90]	Au NPs and QDs	McCoy's 5A (modified) Media containing 10% FBS	Cryo-TEM; Quantitative characterization of NP aggregation
Rosman (2012) [88]	Au nanorods and spheres (NH$_2$- or COOH-PEG coated)	Intracellular	Assessment of NP uptake and aggregation inside cells
Zhang (2014) [91]	Au spheres (Citrate coated)	Water	Interaction between biomolecules and Au
Fluorescence spectroscopy			
De Paoli Lacerda (2010) [61]	Au NPs	Protein solutions	Interaction of NPs with human blood proteins
Huang (2014) [92]	Au NPs (thiol group containing coatings)	PBS and BSA solutions	Conformational changes of adsorbed proteins on NPs
Maffre (2011) [93]	FePt NPs (amphiphilic polymer coated, fluorescently labeled	Protein solutions	Characterization of protein adsorption on NPs
UV–Vis			
Albanese (2011) [57]	Au NPs (TF coated Au aggregates)	RPMI	Effect of aggregation on cell uptake and toxicity
Balog (2015) [58]	Au NPs (citrate, COOH-PEG, NH$_2$-PEG and methoxy-PEG coated)	DMEM, complete DMEM	Characterizing NPs in physiological fluids
Chanana (2013) [94]	Au NPs (protein coated)	PBS protease solution	Properties of protein-coated NPs before and after proteolytic digestion
De Paoli Lacerda (20100 [61]	Au NPs	Protein solutions	Interaction of NPs with human blood proteins
Dominguez-Medina (2013) [62]	Au NPs (citrate coated)	PBS and BSA solutions	Synthesis of advanced NPs to prevent aggregation
Garcia (2015) [95]	Au nanorods and spheres (glycan and PEG-coated)	10% FBS in PBS and DMEM	Stability and targeting in protein rich media
Pyshnaya (2014) [75]	Au nanorods and spheres (PEI and BSA coated)	DMEM containing 10% FBS	Impact of physiological fluids on size and stability
McCuspie (2011) [96]	Ag NPs (BSA coated)	Synthetic lung fluid	Dispersion stabilization of NPs and effect on colloidal stability
Jenkins (2015) [81]	Au NPs (citrate coated)	Blood	Monitoring LSPR for aggregation studies
Kah (2014) [97]	Au nanorods (amphi-philic ligands coating)	RPMI	Effect of NP coating on cellular response
Mahmoudi (2011) [98]	Magnetic NPs (Au coated)	FBS solution	Enhancement of uptake *via* carbon nanotubes
Oster-meyer (2013) [48]	BSA coated Ag NPs and Alginate-coated Au NPs	HEPES buffer and wastewater	Dissolution through LSPR changes
Soliman (2015) [76]	Ag prisms, Au spheres and nanorods (amphi-philic polymer coated)	DMEM and DMEM containing 10% FBS	Method development to improve stability in media
Tebbe (2015) [99]	Au nanorods (protein coated)	DMEM and DMEM containing 10% FBS	Stability of protein-coated NPs in media
Zhang (2014) [91]	Au spheres (citrate coated)	Water	Interaction modes of biomolecules with NPs
Zook (2011) [82]	Ag NPs (PEG coated)	DMEM containing BSA	Studying the dissolution rate of Ag NP in media
Surface-enhanced Raman spectroscopy			
Fu (2015) [100]	AuNPs (HSA coated)	Water	Overtime structural changes of serum albumin and NPs interactions
Jenkins (2015) [81]	Au NPs (citrate coated)	Blood	Monitoring LSPR for aggregation studies

BSA, bovine serum albumin; PVP, polyvinyl pyrrolidone; CNTs, carbon nanotubes; QDs: quantum dots; CTAB, cetyl trimethylammonium bromide; PEG, polyethylene glycol; PEI, polyethyleneimine; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RPMI, Roswell Park Memorial Institute medium; LSPR, localized surface plasmon resonance; MS: mass spectrometry; OES: optical emission spectrometry; HSA, human serum albumin; TRIS: tris(hydroxymethyl)aminomethane; TF: transferrin.

anisotropy. It has been shown that, compared to plasmonic NPs, scattering of depolarized light from the physiological fluid is weak, and thus, excellent 'contrast' with an essentially zero background can be obtained [58].

3.2. Spectroscopic and microscopy methods (Table 2)

Compared to the previously described scattering methods, optical spectroscopy is a rapid and facile technique to monitor the
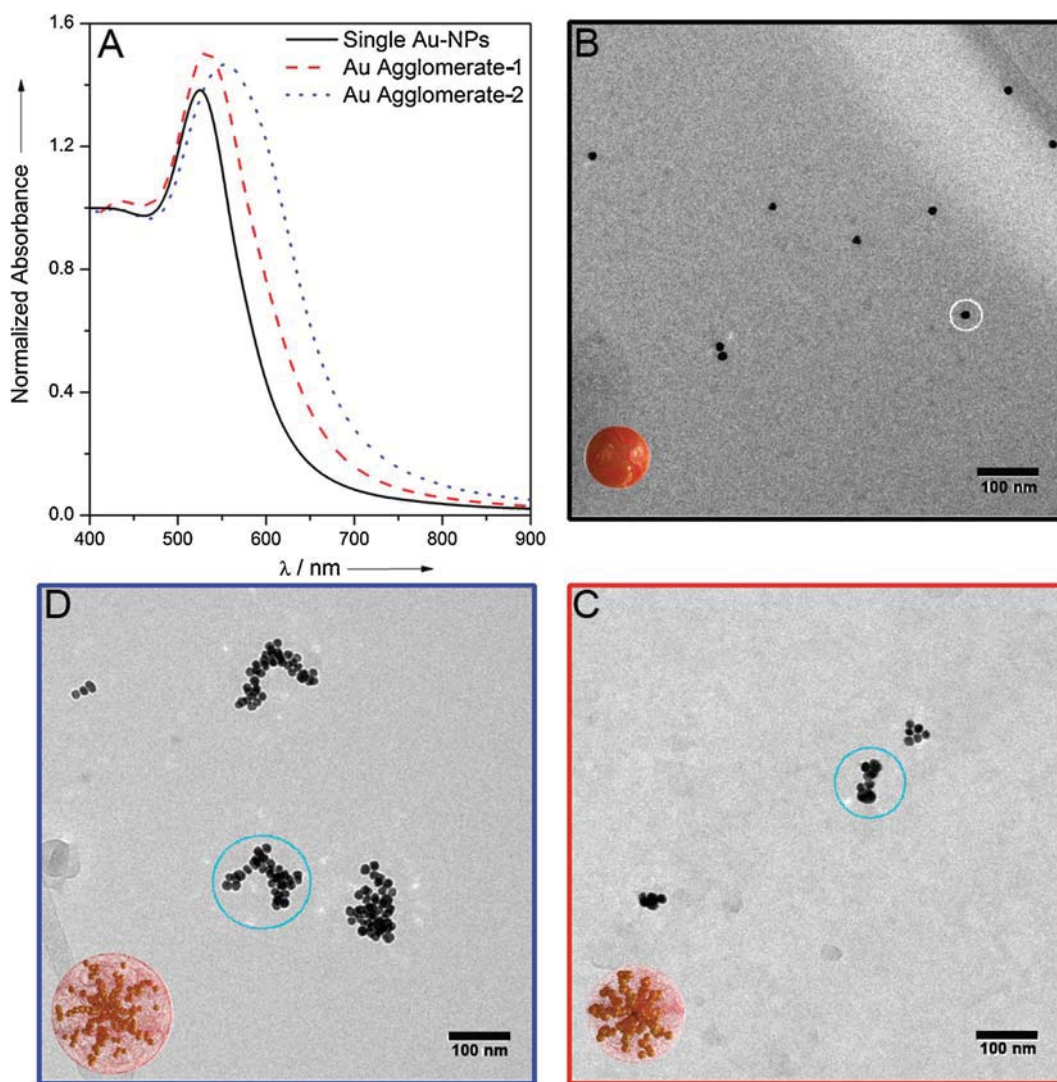
Fig. 4. (A) Extinction spectra of the single Au NPs and two different sizes of Au agglomerates. Cryo-TEM images of (B) single Au NPs and the two types of Au agglomerates (C, D). Reproduced by permission of The Royal Society of Chemistry (http://pubs.rsc.org/en/Content/ArticleLanding/2014/NR/c4nr00460d#!divAbstract) [77].

colloidal behavior of plasmonic NPs. As explained before, these particles present LSPRs, which are strongly sensitive toward aggregation: individual LSPRs can couple with each other *via* near-field interactions [53,54], resulting in changes to the position, width, and intensity of the LSPR band. As a consequence, particle aggregation typically results in a red-shift and a broadening of the LSPR absorption peak [77,80].

These changes indicate a decrease in the concentration of single NPs, confirmed by a color shift of the colloid, *e.g.* from deep red to purple for Au nanospheres and from yellow to black for Ag nanospheres. Therefore, many studies have reported the colloidal behavior of plasmonic NPs in biological media with UV–Vis Balog et al. [58] demonstrated that citrate coated Au NPs aggregated in 10% FBS supplemented cell culture media, while PEG-coated Au NPs were completely stable. Plasmonic NPs functionalized with various surface ligands, such as Au and Ag NPs with dodecyl grafted poly(isobutylene-alt-maleic-anhydride) or glycan-coated Au nanorods [95], have been recently prepared by various groups in order to improve colloidal stability in protein-rich environments. Albanese and Chan [57] developed a simple technique to produce transferrin coated Au NP aggregates of different sizes and characterized their colloidal stability through the changes of the LSPR band. Although UV–Vis can readily distinguish primary NPs from

agglomerated plasmonic NPs, the signal can be compromised by the extremely high optical density and opacity of some physiological fluids, *e.g.* blood [81] (Fig. 4).

UV–Vis spectroscopy has also been used to study, and even quantify, the dissolution of plasmonic NPs. Zook et al. [82] and Ostermeyer et al. [48] quantified Ag NP dissolution by measuring the LSPR intensity over time and comparing the sample's intensity against the LSPR intensity of a Ag NP standard curve. Unlike most existing methods, the estimation of Ag dissolution by UV–Vis allows the measurement of the concentration of Ag^0 within the NPs even in complex fluids containing chloride anions at very high concentration, since dissolved Ag^+ or AgCl particles do not have an associated LSPR band. In addition, no separation step is required to separate the particles from the dissolved species. However, this method only applies for single Ag NPs since the aggregation process will also affect the LSPR intensity.

In certain cases, UV–Vis is suitable for monitoring particle-protein interactions as the LSPR is sensitive to the local dielectric environment [54]. For this, plasmonic NPs are incubated in media of interest and, due to the formation of a protein corona, their LSPR exhibits a slight red shift and an increase in intensity over time. Many papers have used the technique for this purpose, as shown in Table 2. Protein adsorption on Au and Ag NPs has been investigated
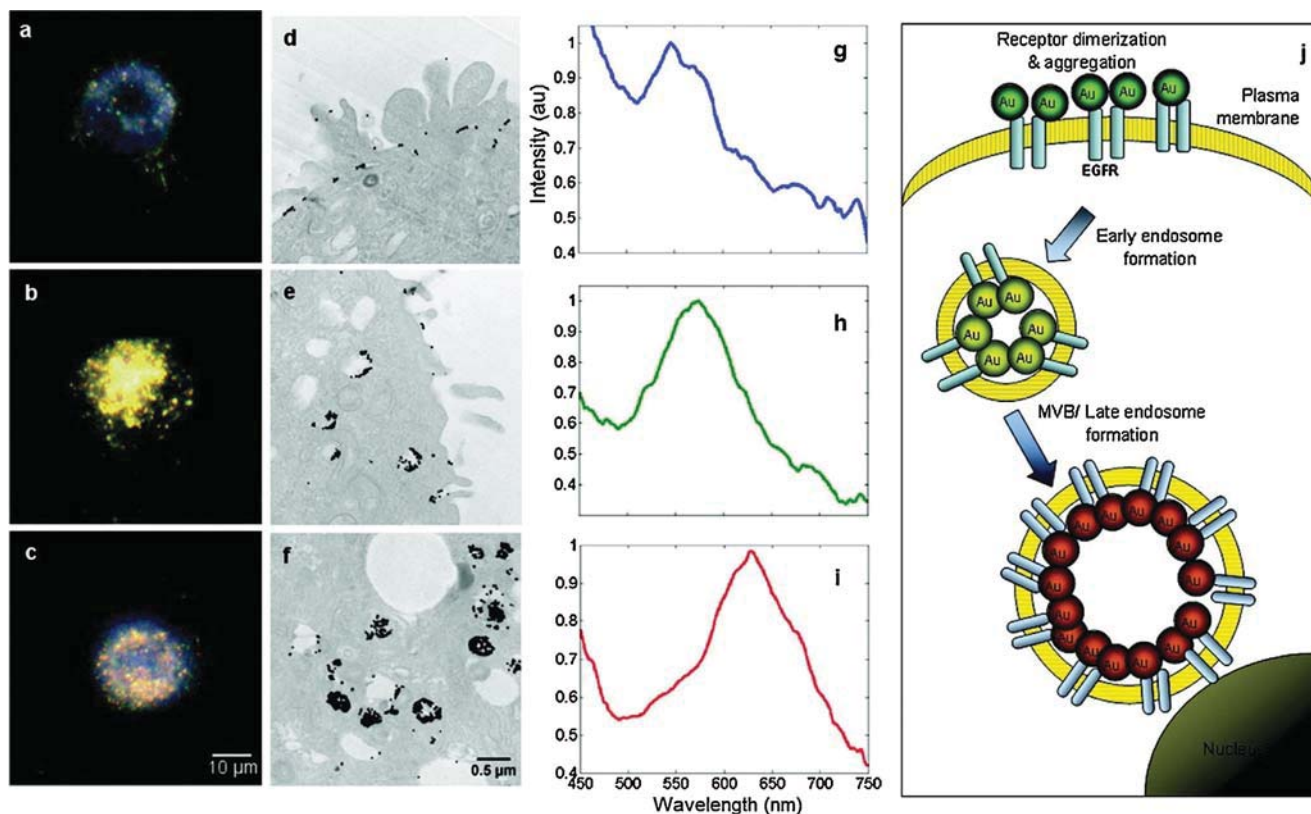
Fig. 5. Dark field micrographs of A431 cells incubated with 25 nm anti-epidermal growth factor receptor Au NP conjugates at 4 °C (a) 25 °C (b) and 37 °C (c) with the corresponding TEM of identical samples (d, 4 °C; e, 25 °C; and f, 37 °C) and mean scattering spectra per cell (g, 4 °C; h, 25 °C; and i, 37 °C). (j) Schematic of the correlation between epidermal growth factor receptor regulation state and color of scattered light. Reprinted with permission from Nano Letters 2009, 9 (10), pp. 3612–3618. Copyright 2009 American Chemical Society [105].

for different types of proteins, such as BSA [96], HSA, globulins, fibrinogen, histones, insulin [61] as well as for serum [98]. While the approach works well for bare or small molecule coated NPs such as citrate coated Au and Ag NPs, care should be taken when particles are coated by polymers, a very common situation. The polymer shell may act as a dielectric spacer and thus decrease the signal upon protein binding, *i.e.* the increase in refractive index due to protein binding should be close to the plasmonic NP surface in order to have a detectable shift in the LSPR [101]. Balog and colleagues [58] showed that BSA adsorption can be detected for citrate coated Au NPs by UV–Vis as a red-shift of the LSPR, from 519 to 524 nm. However, coating of the Au NPs with PEG did not result in any change of the LSPR band. Therefore, UV–Vis can become practically insensitive under certain conditions and monitoring the protein adsorption of such surface functionalized NPs in biological media only by UV–Vis is not necessarily conclusive and complementary techniques should be used. An alternative technique to study protein adsorption is fluorescence spectroscopy, exploiting the plasmonic NPs' ability to quench the fluorescence of molecules near the particle surface. The quenching efficiency for metallic NPs depends on the distance between the quencher and the chromophore, therefore measurements of quenching by proteins can reveal information about the NP-protein interaction. Moreover, the change in the fluorescence emission maxima arises from a change of protein conformation, which provides additional information on the protein–particle interaction. In addition, protein adsorption can be studied by fluorescence correlation spectroscopy through a decrease in the diffusion coefficient of the chromophore corresponding to an increase in particle size upon protein binding [93]. However, the technique is restricted to fluorescent compounds or fluorophore-functionalized NPs, which often bear other challenges [102].

In contrast to the ensemble characterization methods of UV–Vis and fluorescence spectroscopy, it is now possible to obtain spectral scattering information on a single NP level with high spatial resolution through the use of dark field microscopy (DFM) [103]. In a nutshell, DFM is when white light is focused obliquely onto the sample, using a specialized condenser, and only scattered light is collected by the objective. This light can then be analyzed to give a hyperspectral image, *i.e.* every pixel of the recorded image has an associated extinction spectra. Given the incredible scattering cross-section of plasmonic NPs (*e.g.* a 50 nm gold sphere has a scattering cross-section up to 10^6 fold larger than a fluorescent dye or quantum dot [104], their unique spectrum, and their sensitivity to changes in the local dielectric environment, it is possible to detect and characterize plasmonic NPs in a variety of complex biological fluids and tissues. An example of this is shown in Fig. 5 where DF hyperspectral imaging was used to follow the journey of Au NPs upon endocytosis: small clusters of NPs that scatter green light are present in early endosomes, before they are trafficked to late endosomes that scatter red light due to the formation of larger clusters [105].

One of the earliest applications of DFM was demonstrated by Schultz et al. who could detect and spectrally characterize different sized Ag NPs, as well as locate their specific binding location on a *Drosophila* chromosome [106]. In a similarly pioneering study, Huang et al. used DFM to show the preferential binding of Au nanospheres and nanorods to cancer cells over healthy cells after functionalization of the NPs with a targeting moiety [85]. Both these studies, among others [107], have applied DFM mostly as a qualitative technique to confirm the presence, and approximate abundance, of the NP of interest. However, as is often the case with microscopy, extracting quantitative characterization data about the state of NPs in biological fluids can be challenging.

The complexity of interpreting hyperspectral images of plasmonic NPs is most evident by considering the simplified case of a single NP, where the group of Gregory Hartland, among others, has explained the factors contributing to the observed linewidth of a single NP's LSPR [84]. While the NP shape and surface area influence their scattering spectra, the presence of surface bound proteins and other nearby plasmonic NPs, as for the case of agglomerates, can have a drastic, and often challenging to predict, influence on the spectra–as described in the previous section for the case of UV–Vis spectroscopy. Badireddy et al. chose to consider only single Ag NPs with spectra that matched reference spectra, of Ag NPs in water, in order to count their relative abundance in simulated-wetland ecosystem water and wastewater depending on their surface functionality, despite the presence of aggregates [83]. Conversely, Mortimer et al. used reference spectra of NPs dispersed in a cell culture buffer containing extracellular substances, thereby taking into account any fluid-dependent aggregation, resulting in higher numbers of NPs inside the protozoa found compared with when a reference spectra of NPs in water alone was used [87]. Furthermore, they indicated the applicability of semi-quantitative DFM to Au, Ag, Cu oxide, and QDs; however, as in other cases an orthogonal quantification technique, such as ICP-MS or TEM, would have helped validate their approach. A noteworthy example of how DFM can be combined with other techniques was demonstrated by Rosman et al. who used DFM as a low resolution semi-quantitative method to count the number of aggregates present, which was then combined with TEM to identify the number of particles per aggregate [88].

Contrasting with DFM, TEM is likely the most widely applied tool for NP characterization. However, in the case of physiological fluids it is not usually possible due to the high concentration of salts and proteins present which scatter and absorb the electron beam. In addition, drying artifacts [108] are a well-known phenomenon when preparing TEM grids with NPs. One of the few reliable ways to image NPs in, diluted or simulated, physiological fluids is through cryo-TEM, as shown by Hondow et al. who, after plunge freezing could measure the size and packing fraction of quantum dot agglomerates even in the presence of FBS [90]. One of the major drawbacks, however, of cryo-TEM is the expertise and time required to obtain well-prepared samples and perform reliable imaging.

3.3. Other analytical techniques

As shown in previous sections, each analytical method has its own advantages and disadvantages, which is why researchers typically resort to a multifaceted approach. Not only new methods are being developed to help characterize NPs but also methods used in different fields of science have begun to find their way into the field and help assess the question of accurate NP characterization.

Thanks to advancements in recent years in the instrumental setup and data acquisition techniques analytical ultracentrifugation (AUC) has become an interesting alternative to measure the size and shape distributions of NPs, and an increasing number of groups have applied this method [82,86]. The theory behind the AUC is using a centrifuge at high rotational speeds, which causes sedimentation in a colloidal suspension. By tracking the different sedimentation velocities of the NPs, estimations can be made about the size and shape distribution of a given sample [109–111]. Zook et al. [82] used this method to measure the size distribution of Au NP agglomerates *in situ*. They were able to distinguish between monomers, dimers, and trimmers, which is non-trivial with DLS. Although AUC proves to be able to bypass problems of *in situ* measurements, certain assumptions about *e.g.* coating densities, NPs' and agglomerate's morphology, or absorbance behavior, are required for analysis [82].

Gel electrophoresis is an established method for protein separation and analysis in biochemistry, but can also be used to analyze the interaction between NPs and proteins. Gel electrophoresis is based on the fact that charged molecules or particles move at different velocities, according to their charge to size ratio, when in an electric field. This enables the separation and quantification of different species. Moyano et al. [72] used gel electrophoresis to determine the interaction between NPs and proteins in diluted human serum. They observed that the NP mobility was affected due to protein corona formation. The limitation of the methodology was that no definite results could be achieved due to the low mobility of NPs in the agarose matrix. A possibility to circumvent this low mobility is to switch to a liquid separation medium. Le Saux et al. [112], Ibrahim et al. [113] and Liu et al. [114] have shown that capillary electrophoresis might be a suitable alternative to separate and/or determine properties of larger analytes.

In addition to the previous mentioned methods several other methods deserve to be mentioned. With isothermal titration calorimetry Huang et al. [92] were able to study the interaction between NPs and adsorbed proteins. The same group also used circular dichroism spectroscopy (CD) to analyze the structure of BSA adsorbed to the surface of Au NPs. By measuring bound an unbound BSA they determined that the secondary structure of BSA adsorbed to the NP remained intact. Tunable nanopore resistive pulse sensing is an interesting new technique for measuring NP size distributions. NPs pass one by one through a nanopore inducing a change in current, and by tuning the size of the nanopore, Pal et al. have shown that accurate size distributions of nanoparticles could be determined [74]. Taylor dispersion analysis is also an alternative to traditional characterization techniques. Oukacine et al. [115], Le Saux et al. [112] and others [116] have shown that Taylor dispersion, although much less sensitive to polydispersity than *e.g.* DLS, is advantageous in certain cases and estimates particle size accurately.

The most common methods used to measure the concentration of NPs, as well as dissolution ratios, are inductively coupled plasma-optical emission spectroscopy (ICP-OES), and mass spectrometry (ICP-MS). ICP is typically rapid, high-throughput, quantitative and extremely sensitive [117]. In this respect, ICP spectroscopies have been utilized to quantify the total content and dissolution rate of a wide range of plasmonic NPs, as shown in Table 2. The potential of ICP-MS in single particle mode has been explored as a technique to simultaneously determine size and ultra-trace concentration of NPs in suspension. If a sufficiently dilute suspension of NPs is nebulized into the plasma without previous digestion, a burst of ions is generated when each discrete particle is vaporized, atomized, and ionized. The ions of the element of interest are subsequently counted with a very high frequency, similar to the time scale of a particle event [118]. Particle aggregation can also be detected by monitoring the increase in signal intensity [81]. By collecting a large number of data points, particle size distributions can also be determined [119].

For determining changes in NP surface charge, Laser Doppler Electrophoresis and similar techniques have proven to be effective methods. Many groups have shown that zeta potential analysis is a useful complementary method for measuring not only the efficiency of particle functionalization, but also protein adsorption onto NP surfaces [58,75,91].

4. Conclusion and outlook

Understanding the behavior of NPs in physiological fluids is essential in order to study their cellular uptake, intracellular trafficking or fate. Extensive research in the bio-nano field has been dedicated to NP synthesis, surface derivatization and possible cellular reactions. However, in order to translate nanomedicine from

bench to bedside and to evaluate possible risks, a comprehensive characterization of the particles is needed not only after synthesis, but in the respective physiological fluid that the NPs are going to encounter. Since plasmonic NPs are excellent probes for many analytical techniques that rely on light scattering and absorption, research is often carried out with Au or Ag NPs. In this review, we have presented different methods that are nowadays used for the detection and quantification of plasmonic NPs in physiological fluids. In order to understand NP behavior in physiological environments, it is not enough to only supply robust and universal characterization methods but also to be aware of their limitations, and apply, whenever possible, more than one technique [41].

Looking forward, it is apparent that the field merits further investigation. It has to be noted that the majority of published studies are performed with NPs incubated in serum completed cell culture media. However, NPs for biomedical applications will be systemically injected, ingested or inhaled. Therefore, a particular emphasis on more realistic physiological fluids such as blood, mucus *etc.* should be given in future studies.

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