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REVIEW ARTICLE

Gold nanoparticles and bioconjugation: a pathway for proteomic applications

Cláudia Couto[1], Rui Vitorino[2,3,4], and Ana L. Daniel-da-Silva[1]

[1]CICECO-Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal, [2]Mass Spectrometry Center, Organic Chemistry, Natural and Agro-Food Products Research Unit (QOPNA), Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal, [3]Department of Medical Sciences, iBiMED - Institute for Biomedicine, University of Aveiro, 3810-193 Aveiro, Portugal and [4]Department of Physiology and Cardiothoracic Surgery, Faculty of Medicine, University of Porto, Porto, Portugal

Abstract

In the last few years gold nanoparticles (AuNPs) became extremely interesting materials due to their enhanced optical, chemical and electrical properties. With the intention of taking advantage of those properties, the use of AuNPs has spread into a wide variety of areas such as physics, chemistry, biology, industry and medicine. More interestingly, their ability to form robust conjugates with biomolecules has given proteomics a new tool to improve aspects where the current methods to study proteins and their interactions in living cells cannot achieve the success required. In this review we present some of the current methods for AuNPs synthesis, the tailoring of their surface with ligands to improve stability and strategies to conjugate with biomolecules. Lastly, we also discuss their application in proteomic methods and recent developments in clinical diagnosis.

Keywords

Biosensor, gold nanoparticles, synthesis, functionalization, protein, ligand, protein enrichment, mass spectrometry

History

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Introduction

Nanotechnology is a rapidly growing area of investigation that encompasses distinct research fields from sciences to engineering with multiple applications widely spread in common products, such as shampoos, detergents, cosmetic products, or with pharmaceutical applications. The nanoscale provides enhanced physical, chemical and biological features, such as fluorescence and optical,[1] magnetic,[2] and electrical properties.[3] In particular, metal nanoparticles produced from noble metals such as gold, silver and platinum, show improved characteristics that are inherent to their size and shape, in comparison to their original-sized material. Additionally, these nanoparticles have a high surface-to-volume ratio, providing a high reactive surface suitable for different applications. Bionanotechnology takes advantage of nanomaterials to build hybrids between inorganic particles and biological molecules, in order to combine their advantageous characteristics and similar size.[4] In this area, gold nanoparticles (AuNPs) distinguished themselves for their unique characteristics (Figure S1) and received great attention since the pioneer work of Faraday.[5] Characteristics like stability and the ability to conjugate with biomolecules is what attracts most attention. AuNPs have also greatly benefited the proteomic research field by enhancing several proteomic techniques already in use to study the proteome, its proteins and protein-protein interactions, with faster and more accurate results. Nowadays, AuNPs have found roles in research, medicine and industry as sensors and as diagnostic and therapeutical tools.[6]

Address for correspondence: Ana L. Daniel-da-Silva, CICECO, Department of Chemistry, Aveiro Institute of Materials, University of Aveiro, Aveiro 3810-193, Portugal. E-mail:ana.luisa@ua.pt

Gold nanoparticles

Gold nanoparticles are produced from gold precursors and have proved to have higher biocompatibility and lower toxicity in comparison with other noble metal nanoparticles. Their size can vary from 2 to 100 nm which is still 100 to 10 000 times smaller than human cells.[7] AuNPs have also drawn attention due to their enhanced and unique optical, electronic and catalytic properties. Their optical properties rise from the effects that occur due to the coherent collective oscillation of the metal conduction electrons relative to the nuclei with the oscillating electromagnetic field of incident light. This phenomenon is resonant when an incident light with a specific frequency is absorbed by the nanostructure [8] and yields a noticeable band in the optical spectrum, called localized Surface Plasmon Resonance (SPR) band, due to the scattering and absorption of photons. The wavelength of the SPR band is dependent on particle size and shape and nature of the medium.[9] Due to its sensitivity, SPR is often used in detection methods.[8,10] AuNPs optical properties have also known to improve the Raman scattering effect. This effect is the small fraction of light scattered by certain molecules that is different from the incident light (inelastically scattered at different wavelengths) due to the vibrational and rotational modes of the molecules. The scattered signal is characteristic

of each different molecule, but it is weak. The use of metal surfaces, such as AuNPs, near the molecules have shown to improve signal, thus creating the surface-enhanced Raman scattering (SERS) effect.[11] In the last few years, AuNP-based SERS spectroscopy became a powerful bioanalytical tool for the detection of molecules and proteins with diagnosis and therapy interest.[12–15]

Long-term AuNPs colloidal stability is still a major challenge considering their high surface area-to-volume ratio and tendency to aggregate in solution. This stability can be improved by charged agents that surround the gold nanocore and repel other particles in solution (electrostatic stabilization) or by the coordination of sterically bulky organic molecules that act as protective shields on the metal surface (steric stabilization). Even so, this stability can easily be perturbed. Aggregation of negatively charged AuNPs can be induced in the presence of cationic or oligocationic species in solution, or anionic species in the case of positive particles.[16] In these conditions, the repelling surface charges are disturbed and AuNPs distance decreases causing a shift in the SPR band towards higher wavelengths. However, the loss of colloidal stability might be an advantage, since many colorimetric assays have been based in this visual shift to detect targeted adsorption to the AuNPs surface.[17]

Synthesis

Physical, chemical and biological methods have been developed over the years to synthesize AuNPs.[18] Each method yields different results in terms of size, shape and characteristics of the particles.

Spherical AuNPs

Perhaps the most commonly used synthesis is the Turkevich method developed in 1951.[19] This water based synthesis uses citrate as both the reducing and stabilizing agent. Trisodium citrate dehydrate is added to a boiling chloroauric acid solution ($HAuCl_4$) under vigorous stirring. The resulting wine-red solution has negatively charged Au particles averaging 20 nm in size due to the low reducing strength of citrate. By controlling the ratio of citrate to gold it is possible to obtain particles up to 150 nm, although with broad size distribution.[20] Nevertheless, more recently Puntes and coworkers [21] reported the synthesis of highly monodispersed quasi-spherical citrate-stabilized Au nanoparticles up to 200 nm using a seed-growth strategy based on Turkevich method. On the other hand, inverting the sequence of the citrate and $HAuCl_4$ addition yields AuNPs smaller than 10 nm with improved size distribution.[22] The colloidal solution obtained from the Turkevich method shows a plasmon band around 520 nm for particles between 5 to 30 nm.[23] When the diameter increases to 100 nm the plasmon band broadens and shifts towards higher wavelengths. Above 100 nm the particles display distinct bands corresponding to dipole and quadrupole plasmon modes.[24] To overcome problems in dispersity of particle size, strategies of synthesis such as seed-mediated growth [25,26] were developed in order to obtain spherical nanoparticles with high monodispersity and better size control.

Along the years this method has been improved to include pH or temperature variations, with the use of ultrasound and fluorescent light radiation. To produce spherical AuNPs with smaller sizes, the two-phase Brust–Schiffrin method is ideal. It relies on the reduction of $HAuCl_4$ by sodium borohydride ($NaBH_4$) in the presence of an alkanethiol. Since $NaBH_4$ is a stronger reducing agent than citrate, naturally the synthesis yields particles of smaller size (average 2 nm with narrow size distribution), thiolate-stabilized in the organic phase.[27] A photocatalytic method that uses tin[IV]-porphyrin complex as photocatalyst was also reported.[28]

Non-spherical AuNPs

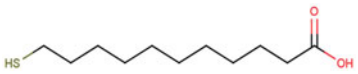
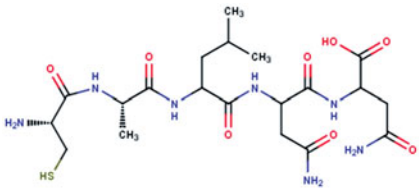
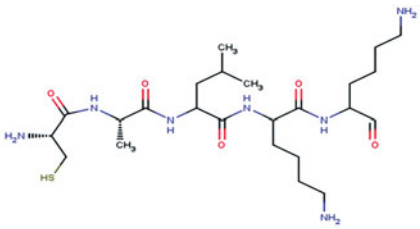
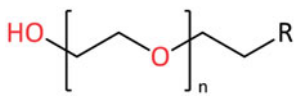
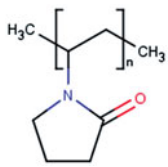
Spherical AuNPs (up to 100 nm) display a single plasmon band due to the SPR phenomenon, however, when the isotropic shape changes, the resonance bands multiply according to the new axes created. For instance, gold nanorods (AuNRs) show two bands that are related to electron oscillations across and along the axis, transverse and longitudinal.[29] The transverse plasmon band has a maximum extinction around 520 nm in the optical spectrum. Meanwhile, the longitudinal plasmon band can be shifted from the visible to the near-infrared (NIR) by tuning their aspect ratio.[29–31] These qualities were decisive when choosing AuNRs for clinical applications, as the release of heat that results from excitation is interesting for photo-thermal cancer therapies.[32] AuNRs are usually prepared using seeded growth methods.[29]

Triangular AuNPs can be obtained by chemical reduction and photochemical synthesis and their dimensions can be altered by changing the synthesis parameters.[33] The vertexes of the triangular prism contribute greatly to their enhanced properties. Nanotriangles also show SPR bands related to different resonance modes. These modes oscillate at different frequencies, which can be separated by 100 to 400 nm, and can shift along the visible and NIR spectra according to size, edge length, thickness and vertex morphology.[33] Other Au nanostructures that allow SPR band tunability in the NIR region include nanostars, nanocages and silica core-Au shell nanoparticles, also designated as Au nanoshells. Each shape requires a well-defined synthesis,[34] and produce particles with interesting optical properties for bio-applications [35] as well as the opportunity to take advantage of the new surface area available.

Biosynthesis

Biosynthesis use natural, relatively low-cost products to achieve similar AuNPs as in traditional chemical synthesis. In an overgrowing ''green'' conscious world, these sustainable methods aim to minimize the use of chemicals and their environmental impact. Plant extracts are strong candidates when choosing the material. To understand which compounds in plant extracts were responsible for reducing gold ions to nanoparticles, Santos and coworkers [36] studied the extract of *Eucalyptus globulus* Labill. The results showed that the sugars present, as well as ellagic acid, act as stabilizing agents, while galloyl derivatives are responsible for gold reduction. Microorganisms, specifically prokaryotic bacteria, are also widely used for AuNPs synthesis. Since the discovery of the formation of nanoparticles inside some bacteria, they have been used in the synthesis of metallic nanoparticles.[37] AuNPs have been obtained from *Lactobacillus* strains,

Table 1. List of thiolated and non-thiolated ligands commonly used to functionalize gold nanoparticles and their applications.

Ligand	Designation	Functional group	Application	Reference
	MUA[1]	$-COO^-$	Colorimetric sensor to detect Hg^{2+}, Cd^{2+}, Fe^{3+}, Pb^{2+}, Al^{3+}, Cu^{2+}, and Cr^{3+} in water	[43]
	CALNN[2]	$-COO^-$	Colorimetric immunoassay for ABA-GE[6] determination	[44]
	CALKK[3]	$-NH_3^+$	Used as additive in protein crystallization	[45]
	PEG[4] and derivatives	R= –OH, –SH, –COOH,$-NH_2$, $-OCH_3$	Cell incorporation of pegylated AuNPs and imaging and therapy	[46]
	PVP[5]	N^+	AuNP-PVP conjugated with curcumin to improve its solubility in water	[47]

[1]11-Mercapto undecanoic acid; [2](cysteine, alanine, leucine, asparagine, asparagine); [3](cysteine, alanine, leucine, lysine, lysine); [4]Polyethylene glycol; [5]Poly(vinylpyrrolidinone); [6]Abscisic acid glucose ester.

Shewanella algae or *Rhodopseudomonas capsulate*.[37] The reducing amino-acids in proteins/enzymes can act as both reducing and stabilizing agents in AuNPs synthesis, obtaining the nanoparticles ready for application, as in the case of laccase from the *P. variable*.[38] However, even though biosynthesis of AuNPs is a promising field of experimentation, the control over size and shape is still poor.

Surface functionalization

One of the main advantages of AuNPs is the possibility to tune their surface chemistry with functionalization strategies to enhance their stability and performance according with the application required. Functionalization enables conjugation with other molecules, but also prevents stability loss and uncontrolled aggregation. The aim is to bind functional ligands or to form a stable monolayer at the surface of AuNPs by chemisorption, electrostatic interaction, or hydrophobic interaction.

As previously discussed, capping agents are used during synthesis in order to stabilize and control particle growth. However, these agents can be exchanged by ligands with higher affinity for gold. Thiol (-SH) terminated ligands are the common choice to create Self Assembled Monolayers (SAMs) around AuNPs.[39] These ligands exchange places with the adsorbed capping agent on the AuNPs surface due to the higher affinity of sulfur for gold, leading to the formation of a strong dative bond S-to-Au atoms.[40] The successful formation of SAMs can be monitored in the UV-Visible spectrum, as was the case of SAMs produced with a pentapeptide ligand that induced a shift of approximately 2 nm in the AuNPs plasmonic band.[40] Some commonly used thiolated ligands are 11-mercaptoundecanoic acid (MUA) [41] and 3-mercaptopropionic acid (MPA), among others [42] (Table 1).

Hydrophilic ligands of thiol-terminated oligo- or polyethylene glycol (PEG) are used in order to significantly improve their *in vitro* and *in vivo* stability. This concept was proved by Manson [48] Gao [49] and respective coworkers with AuNP-PEG stable in a broader salt concentration range than AuNP-citrate.[48] It has also been shown that having more than one thiol termination on the PEG ligand to provide the anchoring, increases their pH and salt concentration stability range, when compared to monothiol-PEG or even AuNP-citrate.[50] It also takes advantage of PEG ability to bind to the cell membrane. Consequently, PEGylated AuNPs are ideal candidates to be applied in drug delivery since the PEG layer helps internalizing the AuNPs into target cells [51] and to permeate brain microvasculature.[52]

Small peptides, usually composed of five amino acids, have a thiol anchor from a cysteine, a hydrophobic center and a hydrophilic ending group.[40] It is the ending group that, depending on the pH, will be able to provide a distinguished surface charge to the nanoparticles. Negative charge occurs in the case of amino acids such as asparagine containing carboxyl groups or positive in the case of lysine which has an amino group. Moreover, since they are structurally similar to proteins, it is expected that their exposed charged groups interact through electrostatic interactions with opposite charged amino acids to create bionanoconjugates. Such has been the case where CALNN (cysteine, alanine, leucine, asparagine, asparagine) functionalized AuNPs successfully adsorbed tyrosinase [53] for phenolic compound detection, or the determination of lead (II) in aqueous medium.[54] Triangle nanoparticles have also been conjugated with peptides that successfully inhibit the function of aspartyl proteinase related to a condition caused by *Candida albicans*.[55] In the same fashion, thiol terminated oligo- or polynucleotides have been synthesized to functionalize AuNPs as well.[56]

Functionalization with lipid membrane coating is a somewhat recent strategy that embraces both AuNPs surface shielding and functional ligand incorporation. The phospholipid layer enables AuNPs to be insulated from the external environment by a layer of hydrophobic acyl chains which can be modified with lipid-tethered ligands.[57] Recently, Gao and coworkers.[57] were able to develop AuNPs-citrate functionalized with red blood cell membrane. Red blood cell membranes hold a large variety of markers, signal receptors and proteins that prevent them from phagocytic cell actions. The group successfully obtained fully enclosed AuNPs in membrane less susceptible to be eliminated from the system.[57]

While most Au nanoparticles functionalization has been done using thiolated ligands, other organic and inorganic components have been used for tailoring Au surface functionality aiming bio-applications. A variety of non-thiolated polymers was reported for AuNP stabilization and functionalization, including poly(N-vinylpyrrolidone) (PVP),[47] polyethyleneimine (PEI),[58] and chitosan [59] among others.[1,60] The polymer can act simply as a stabilizer.[59] or both as a reductant and stabilizer [58] during Au NPs synthesis, or as post-modifier of pre-formed Au NPs.[47] Among inorganic materials, silica coating has emerged as one of the most versatile strategies to impart stability and surface functionality to AuNPs. The silica shell provides a springboard for straightforward functionalization with variable functional groups via well-established silica surface chemistry with readily available silane coupling agents, for subsequent conjugation with biomolecules to form bionanoconjugates.[61]

Bionanoconjugation

Perhaps the most important application for AuNPs is the immobilization of biomolecules: peptides, enzymes or even DNA. These biomolecules are fundamental components of living organisms and their abundance and diverse functionalities provide a wide variety of possibilities to manipulate their properties and apply in medicine or industry. Since biomolecules have sizes from 2 to 20 nm they are ideal to adsorb to the surface of AuNPs.[62] It is of the upmost interest to take advantage of the high surface-to-volume ratio of AuNPs, their stability in physiological conditions and optical properties to build hybrids that combine the benefits of both materials.

Proteins are able to adsorb to the surface of AuNPs simply by the interaction of protein residues and AuNPs surface ligands. Adsorption can also occur directly in contact with the gold surface via SH bond in the case of proteins containing cysteines (Figure 1a). On the other hand, the existence of amino, carboxyl or hydroxyl groups from lysine, aspartic or glutamic acid, and serine or tyrosine residues [63] can also create AuNP–protein conjugates. AuNPs functionalized with ligands charged in certain pH conditions (e.g. carboxyl groups above pH 4 are negative), can adsorb to protein residues of opposite charge through electrostatic interactions (Figure 1c and d). However, simple adsorption is not the strongest interaction and can be reversed by changing the medium conditions. To create a more robust conjugate it is necessary to covalently bind the biomolecule to the modified surface of AuNPs. Cross-linking agents with reactive groups towards protein functional groups are ideal for this task (Figure 1b). EDC/NHS with COOH reactivity towards NH_2 in proteins is the most common agent used, nonetheless, there are several other options available with different chemistries or lengths for each purpose desired.[63] Other conjugation strategies include affinity-based systems or the use of protein co-factors.[64]

Regarding the shape of AuNPs, spherical particles have been the ones attracting major interest in application since the curvature of the particle promotes three-dimensional protein structure conservation due to less contact points between residues and the gold surface.[64] When designing a conjugate, it is important to prevent conformational changes that might lead to function loss, especially in the case of enzymes.

Even though the focus of this review is proteomic application, it is important to note that AuNPs functionalized with oligonucleotides also have important applications in bioconjugation. For example, it is possible to develop covalent or non-covalent AuNP-oligonucleotides conjugates for *in vivo* nucleic acid delivery into cells for gene silencing therapies.[65] On the other hand, these oligonucleotide-based systems have also been used for the biosensing of single DNA mutations.[66]

Gold nanoparticles in proteomics

Proteomics is an investigation field that has been the focus of considerable attention during the last decade in order to have a better understanding of cellular functions. Understanding the basic function of a cell will open vast possibilities for the diagnosis, prevention, and the development of target treatment strategies to cure diseases that have no cure or where current treatments are ineffective.

To achieve this deeper insight it is necessary to study all proteins expressed by an organism under certain conditions. To accomplish this, it is necessary to understand their structure, localization, modifications, function and how they

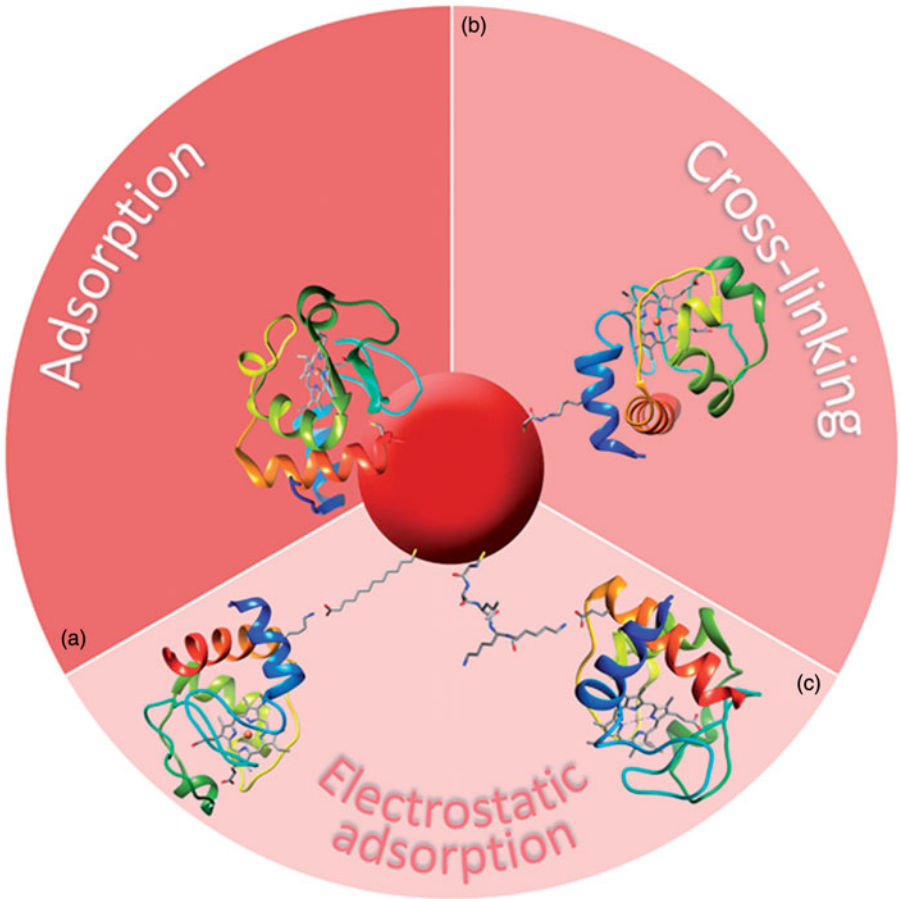


Figure 1. Proposed three-dimensional models of: (a) the bioconjugation between spherical AuNPs and a free cysteine residue of Cytochrome C from Yeast through the sulfur atom (PDB code: 1YCC); (b) the covalent bioconjugation between spherical AuNPs with EDC/NHS cross-linker and an exposed Lysine amino acid of Cytochrome C from Horse Heart (PDB code: 1HRC); (c) the electrostatic bioconjugation between spherical AuNPs functionalized with MUA and an exposed Lysine amino acid of Cytochrome C from Horse Heart (PDB code: 1HRC); d) the electrostatic bioconjugation between spherical AuNPs functionalized with CALKK and an exposed glutamic acid amino acid of Cytochrome C from Horse Heart (PDB code: 1HRC). Secondary structures of Cytochrome C represented in ribbon and Colored with the rainbow code from blue to red (N terminal in blue to C-terminal in red). Model generated with Chimera and ligands with Marvin Sketch and Marvin Space.

interact with other proteins. Additionally, detailed information about post-translation modifications (PTMs), such as, glycosylation, S-nitrosylation, phosphorylation [67] mutations in proteins associated with diseases and interactions between proteins and drugs, is of extreme importance. With this amount of information required, and due to the complexity of the proteome, it is evident that this task has not been easy over the years. Until recently, proteomics research pointed to 20,300 protein-coding human genes expressed in eukaryotic cells, of which there is limited knowledge of only two-thirds of the proteins.[68] Therefore, the complexity of the proteome poses the first challenge.

The current approaches of identification available, such as 2D electrophoresis, chromatography or mass spectrometry, are heavily impacted by the large range of proteins available in biological samples. For example, there are more than 10 000 different protein species in blood samples alone.[68]

The greatest challenge is the separation of the desired protein among the remaining proteins that constitute the medium in which it is inserted, the detection of low-concentration proteins and achieving quantifiable quantities of the protein of interest. Additionally, the variety of the existing sample preparation protocols, different techniques and equipment available largely influences data reproducibility.

The introduction of nanomaterials in proteomics with the hope to overcome these issues branched into a new path, called nanoproteomics.[69] They brought advantages that include real-time multiplexed analysis, low sample and reagent consumption, high sensitivity, and the miniaturization of assays and their quickness.[70]

The special properties of AuNPs drew attention from the proteomics investigation community to the degree that these particles emerged as a very attractive element to use in many techniques already in use. The aim is to improve parameters such as selective separation, enrichment and detectability levels. Furthermore, AuNPs brought the possibility of developing sensitive immunoassays, biosensors, biomarker detection, and drug delivery.

Protein enrichment

A particular difficulty in proteomics is obtaining the desired protein in enough concentration to be identified by the existing techniques and equipment, even though the detection levels of such equipment are constantly improving. Consequently, enrichment of low-abundance proteins is one of the points requiring fast development. The large surface-to-volume ratio of AuNPs and their ability to conjugate with peptides and proteins emerged as a solution to overcome this issue.

Simple methods were developed to take advantage of the specific isoelectric point of proteins.[71] Nonspecific adsorption of proteins was accomplished by changing the pH of the medium, considering that in certain pH conditions some proteins have a surface net charge that is mostly positive and are able to adsorb to the surface of negative nanoparticles. This affinity adsorption is achieved by using AuNPs

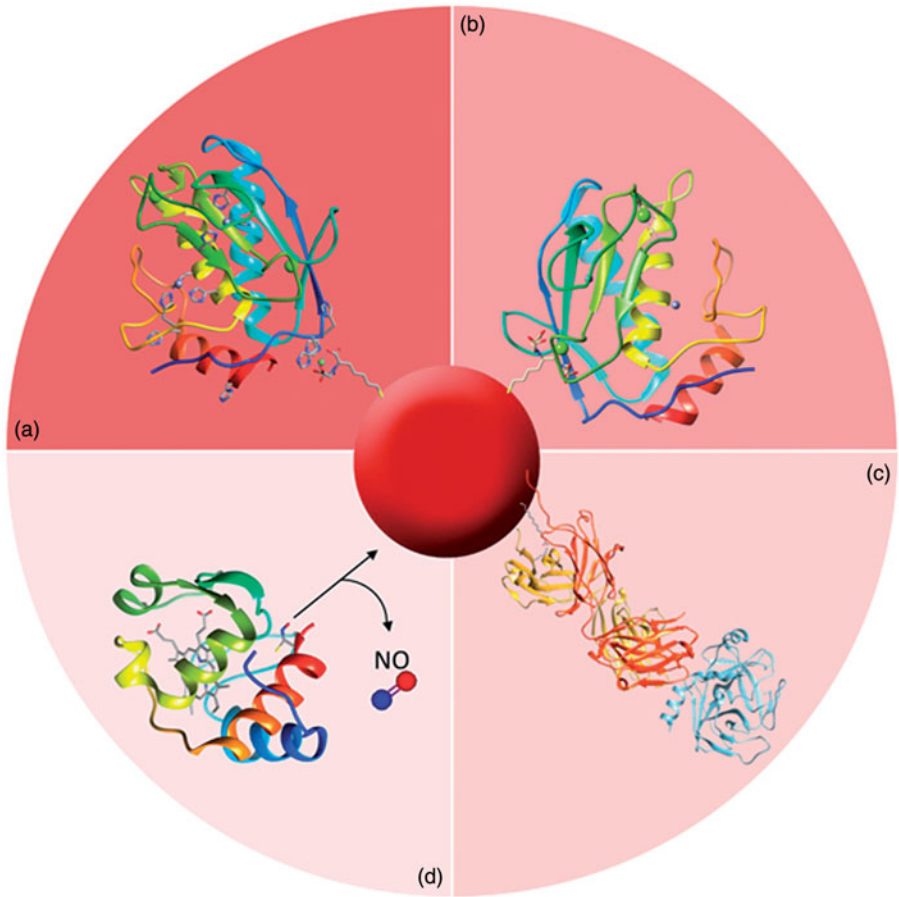


Figure 2. Proposed three-dimensional model of: (a) the affinity-based bioconjugation between spherical AuNPs functionalized with NTA-Ni(II) and exposed histidine amino acids belonging to a His-Tag in Metalloproteinase 9 (MMP-9) (PDB code: 4H2E); (b) of the affinity-based bioconjugation between spherical AuNPs functionalized with thiolated ethylenediamine tetraacetic acid (EDTA) and a calcium ion in Metalloproteinase 9 (MMP-9) (PDB code: 4H2E); (c) the bioconjugation between spherical AuNPs and a nitrosylated cysteine amino acid of Cytochrome C from Yeast (PDB code: 1YCC); (d) the bioconjugation between MUA functionalized spherical AuNPs and *Mus musculus* monoclonal antibody 8G8F5 Fab (colored in yellow and orange) complexed with *Homo sapiens* Prostate-specific antigen (Colored in blue) (PDB code: 2ZCL). Secondary structure of MMP-9 and Cytochrome represented in ribbon and Colored with the rainbow code from blue to red (N terminal in blue to C-terminal in red). Model generated with Chimera software. NTA-Ni(II), EDTA, NO and MUA ligand generated in Marvin Sketch and Marvin Space.

functionalized with ligands containing carboxyl end groups as described previously. Apart from them, the AuNPs surface can be specifically tailored to a particular target by choosing a ligand that best suits the capture of the biomolecule of interest. Several groups recently produced AuNP-NTA, or other derivatives, with high, non-covalent affinity for proteins and peptides containing his-tag [72] (Figure 2a). Besides facilitating the selection of his-tag labeled peptides and proteins, these studies also aimed to create labeling nanoconjugates that successfully bind to proteins and find their location in complex assemblies [72] Translating these results to further applications, it could be possible to select labeled proteins after heterologous expression using similar nanoparticles. Other affinity-based conjugation methods occur through the binding of a metal ion belonging to the protein and selectively capture them from biological samples (Figure 2b).

It is also possible to capture rare proteins and proteins of low concentration in complex samples. Such sensitivity has been tested against a common method using trichloroacetic acid (TCA) for protein precipitation from crude samples.[73] The TCA method presents some drawbacks such as low protein concentration and deficiency in dissolving the protein precipitate without enzyme digestion. The aim of this study was to use AuNPs to capture proteins present in urine samples diluted in large volumes. The AuNP–protein conjugates were successfully separated by denaturant electrophoresis to observe the captured proteins and identified by mass spectrometry. The study showed that with the TCA method there was no enrichment effect in samples of more than 2 mL or low protein concentration such as 4 ppm. With the aid of AuNPs it was possible to achieve protein enrichment from a sample of 1.33 ppm of protein content, showing that AuNPs can selectively detect low protein concentration independent of sample volume. Other successful studies followed and such was the case where PEG functionalized gold nanorods conjugated with aptamers were used to effectively enrich and detect thrombin in blood plasma.[74]

Another strategy is to take advantage of the interactions between antibodies and antigens. In several experiments, AuNPs conjugated with antibodies are able to detect their specific counterpart in biological fluids in low concentration [75] (Figure 2c).

With the purpose of removing unbound protein from AuNP–protein conjugates in a sample, ultracentrifugation or ultrafiltration is necessary. To suppress the necessity of expensive machinery, Nash and coworkers [76] showed that it was possible to maximize the separation process combining iron oxide magnetic nanoparticles with AuNPs. In this case, Au/iron aggregates incorporated the intrinsic gold properties for bioconjugation leading to biomarker enrichment and magnetic properties that made separation from the complex sample easier just by using a magnet.

Another common method to obtain proteins of interest in the purest and most concentrated form is by using immobilization supports such as chromatography columns. A capillary column was obtained by using a monolithic polymer support functionalized with thiol ligands and AuNPs. These particles

were functionalized with thiolated ligands in order to selectively capture peptides containing cysteines from complex samples.[77] Chromatography columns with AuNPs can therefore be designed to selectively capture biomolecules from samples and achieve the purification desired for posterior analysis.

So far, AuNPs have shown to be efficient in protein enrichment in diluted and low protein concentration samples, and in detecting specific proteins, which makes them a viable option as a method for protein enrichment prior to mass spectrometry analysis.

Post-translational modifications

As previously discussed in this review, one of the interests in proteomics is the study of proteins and peptides with PTMs. These modifications allow proteins to regulate various biologic processes. The main challenge still resides in the low concentration of proteins with PTMs in biologic fluids and their detection being compromised by the high availability proteins that mask their signal. Once more, prior to mass spectrometry analysis, it is important to have an enrichment step in order to improve selectivity and sensitivity.

Glycosylation, the addition of a sugar moiety to proteins and peptides, is one of the most common PTMs. These modified proteins play essential roles in cell adhesion, receptor activation and signal transducing. Alterations in this process have been implicated in inflammatory and neurodegenerative disorders.[78] In this case, other than the boronic acid-functionalized AuNPs strategy,[79] an interesting approach might be to functionalize AuNPs with polysaccharides where enrichment of glycoproteins relies on sugar-sugar interactions as it has been done with other types of nanoparticles.[80]

Nitrosylation is another common PTMs where the S-nitrosylated sites occur in the presence of reactive species of nitrogen able to nitrosylate protein thiols. Nitrosylated proteins are of great interest because they are associated with regulation, subcellular compartmentalization, protein degradation, and signaling pathways with impact in health.[81] However, these sites are sensitive and hard to detect and isolate. Since AuNPs are able to bind to SNO-protein, they selectively capture and enrich peptides to identify S-nitrosylated (Figure 2d), S-glutathionylated and S-alkylated sites in proteins by mass spectrometry.[81] With great success, this strategy showed protein thiol affinity for AuNPs, even after modification, and with further optimization this method was able to differentiate between the different S-modified sites in study.

The addition of phosphate groups to proteins catalyzed by kinases, a process called phosphorylation, is perhaps the most important modification since it regulates the increase or decrease of enzymatic activity, protein degradation, protein trafficking and protein interactions.[82] Very recently, a novel method for the detection of protein kinase inhibitors was developed by Bhalla et al. [82] This method combined the localized surface plasmon resonance (LSPR) of AuNPs with electrolyte insulator semiconductor (ESI)-based proton detection with promising results for the rapid detection of thiophosphorylated proteins and their inhibitors.

Structure identification

The protein three-dimensional structure is one of the objectives in proteomics. An understanding of how the protein folds *in vivo* opens up the possibility to comprehend their function and interactions with other proteins, inhibitors or drugs. To date, X-ray Crystallography and Nuclear Magnetic Resonance (NMR) has achieved the resolution of more than 92 000 structures according to Protein Data Bank. Crystallography relies on protein precipitation and the formation of an organized arrangement of protein to form a suitable crystal for X-ray diffraction. Some methods include the use of metals, precipitants, nucleating agents and the variation of crystallization conditions.

AuNPs have been used for the crystallization of lysozyme, commonly used as a crystallization standard for its reliability. Citrate-stabilized AuNPs and AuNPs functionalized with thiolated ligands with carboxyl end groups were effective in increasing the nucleation of both lysozyme and ferritin.[83] The work conducted so far shows that AuNPs might be a valuable option to optimize existent crystallization conditions.

On the other hand, NMR is often used to characterize molecules bound to metal surfaces since the electronic environment of the examined nuclei interferes with the internal interactions (e.g. chemical shift).[84] Therefore, solid-state NMR has been used to study the three-dimensional structure and dynamics of proteins and peptides in AuNPs conjugates by measuring the isotropic chemical shifts.[85] With this technique, it is possible to discover which residues bind directly to the surface of the particle, as was the case of AuNP-ubiquitin adsorption site in solution.[86]

Laser desorption/ionization mass spectrometry matrices

AuNPs can also be used in other aspects of proteomics besides obtaining selected biomolecules for analysis. Laser desorption/ionization mass spectrometry (LDI-MS) is an identification technique often used to identify biomolecules, such as peptides, proteins, polymers or lipids with high sensitivity.[87] The matrixes used, capable of UV light absorption and formation of charged analyte and matrix ions, are often organic and encompass several drawbacks. For instances, problems can occur in signal reproducibility, chemical noise due to cluster ions leading to complex spectra in the small molecular range (<800 m/z) and matrix crystallization.[87] Nanoparticles provide advantages such as the elimination of matrix ion interference, improvement in sample homogeneity, higher detection sensitivity, high surface area-to-volume ratio which helps in efficient desorption/ionization of analytes, and flexibility in sample preparation and deposition.[88]

Since the early studies of Tanaka,[89] which used a matrix of combined cobalt nanoparticles and glycerol to ionize peptides, metal nanoparticles have been seen as a valid matrix option. Metal nanoparticles, due to intense light absorption, makes possible the use of visible laser pulses to ionize samples sensitive to photo degradation.[87]

McLean et al. [90] reported the use of size-selected AuNPs as low concentration and selective matrices for the ionization of biomolecules with LDI. In comparison with organic

compounds, metal compounds have some advantages regarding sample preparation and flexibility in sample deposition conditions. Spherical AuNPs below 10 nm can perform as enhanced LDI matrices, and selectively ionize several peptides and small proteins when co-deposited with them. In the following years, the use of AuNPs as matrices for LDI-MS improved significantly.

Glycoproteins have been detected with the help of AuNPs signal amplification. Glycoproteins covalently bound to the surface of carboxyl functionalized AuNPs were directly deposited for identification in LDI-MS with high sensitivity [91]

Recently, mass spectrometry for molecular tissue imaging field has also benefited from the utilization of AuNPs to study the morphological features of organs, correlate them with the pathological symptoms and find biomarkers which can help in the diagnosis. Huang et al. [92] were able to develop a conjugate between AuNPs, graphene oxide and a protein aptamer that was able to selectively bind to a biomarker associated with several malignant tumors. The results showed highly amplified signals during LDI-MS analysis of breast cancer cells.

Biosensors

The goal for a robust bionanoconjugate is not only to aid in research methods to discover and study biomarkers, but to also apply them in clinical performances. Biomarkers, which are associated with specific health conditions, are usually present in fluids such as blood, urine, saliva or amniotic fluid. Since these fluids are somewhat accessible, they are the primary source to be analyzed when searching for a disease, predicting the appearance of a disease or monitor the response to a treatment. Therefore, in order to detect and quantify these biomarkers there should be standardized methods with high sensitivity, specificity and easily manipulated. Nowadays there is a wide variety of proteomic based techniques to identify biomarkers, such as mass spectrometry, protein microarrays or immune-based methods. Nonetheless, the current methods do not meet all the necessary requirements. For example, some methods lack high sensitivity, which is an important factor for successful biomarker identification with more relevance in the early stages of a disease where concentrations are low. AuNPs emerged as a possibility to develop highly sensitive, quick sensors that do not require highly skilled personnel and uses minimal infrastructure to evaluate the results. It is possible to take advantage of detection methods like SPR peak shift to develop colorimetric assays [93] fluorescence, surface-enhanced Raman spectroscopy,[SERS] [30] or electrochemical detection strategies such as voltammetry [11] to detect a wide variety of biomarkers in biological samples (Table 2).

Colorimetric and SPR-based techniques are the most commonly used when it comes to develop AuNP-based biosensors for analyte detection. Some of these analytes are antigens that can specifically interact with their antibody counterpart. Understanding the interactions between antibodies and antigens is important in clinical diagnosis and pharmaceutical areas. Enzyme linked immunosorbent assay (ELISA) is a widely used analytical method with great interest in the diagnosis field due to its sensitivity and easy manipulation. Even so, common ELISA methods encounter the shortcoming of low surface-to-volume ratio inherent to the polystyrene plate which leads to lower sensitivity. Additionally, it is necessary to take into account the random orientations of antibodies or antigens unfavorable for antibody-antigen recognition,[110] AuNPs offered an immobilization platform with both high surface-to-volume ratio and the possibility of orienting the antibodies towards the medium for easy antigen detection. When using AuNPs in sandwich ELISA procedures, studies showed improved binding capacity, signal intensification and sensitivity towards a biomarker for carcinoembryonic antigen in human plasma.[110] AuNPs have not only been used in ELISA as immobilization supports but their formation has also been used to detect antibody-antigen with the aid of the plasmonic shifts at the naked eye. De la Rica et al. [111] developed a plasmonic-based ELISA where the catalytic activity of the enzyme label reduces gold ions to nanoparticles with color change depending on the success of analyte detection tested with a prostate specific antigen and a HIV-1 capsid antigen. In the absence of the analyte, the reduction of gold ions by hydrogen peroxide formed non-aggregated AuNPs of red color. On the other hand, in the presence of the analyte, hydrogen peroxide was consumed by catalase producing a blue colored solution of aggregated AuNPs. Nonetheless, some ELISA performances still require a considerable amount of time to obtain results and reagent consumption. Alternatively, a great deal of immunosensors have been developed with AuNPs to develop quick colorimetric immunoassays without the need for enzyme or substrate.[44] AuNPs aggregation induced by conjugated antibody-antigen complex formation AuNPs are one of the examples for the recognition of specific analytes.

Mirkin and colleagues [112] have devoted their research efforts to develop AuNP-based biosensors to detect polynucleotide sequences based on colorimetric assays. In one of their studies, AuNPs functionalized with alkanethiol oligonucleotides were able to detect a target sequence and differentiate between fully complementary targets and targets with deletions, insertions or mismatches.

Another emerging field in spectroscopy allied to AuNPs optical properties is the surface enhanced Raman spectroscopy (SERS). As described in the Gold Nanoparticles section, Raman spectroscopy highly benefited from the use of AuNPs, as a surface enhancer, to improve the weak scattering signal characteristic of molecules. SERS has been used to study biomolecule interactions with the help from AuNPs due to their intense SPR band and is currently an emerging field in spectroscopy. With SERS, and due to the metal-analyte interactions, it is possible to study the structure, conformation and charge transfer in biomolecules and understand how they interact.[113] Such was the studies of Shin et al. [114] that recently were able to develop a highly sensitive SERS-based immunoassay, which conjugates the antibody-antigen interactions with AuNPs signal enhancement, to detect proteins in low concentrations in a mixed protein solution.

Future perspectives in proteomics

In a five year perspective, the continuous growth of AuNPs potentialities is expected, as an advantage to enhance several

Table 2. List of techniques and applications of gold nanoparticles in biosensors.

Biosensor	Conjugate	Application	Reference
Colorimetric	Spherical AuNPs functionalized with CTAB	Detecting the hydrolysis of antibiotics by penicillin G acylase	[94]
	Spherical AuNPs used as the seeds for the growth of palladium nanostructures	Detection of prostate-specific antigen (PSA) coupling with an enzyme-cascade-amplification strategy	[95]
	Spherical AuNPs functionalized with 4-amino-3-hydrazino-5-mercapto- 1,2,4-triazol (AHMT)	Detection of adrenalin in human serum sample	[96]
	Spherical AuNPs functionalized with PEG and conjugated with Cys-PEG11-SNKTRIDEANQRATKNorL-biotin peptide and AuNPs functionalized with Neutravidin	Detection of Botulinum Neurotoxin	[97]
	Spherical AuNPs conjugated with 12- or 20-amino-acid peptide that contains the sequence of co-activator for activating nuclear receptor by an agonist ligand	Detection of agonists of estrogen receptors	[98]
	Spherical AuNPs conjugated with 6-mercapto-1-hexanol (MCH), gelatin and casein	Detection of matrix metalloproteinase (MMP) activity and screening of potential MMP inhibitors	[99]
SPR	AuNP conjugated with anti-IMA (Ischemia Modified Albumin)	Detection of IMA for the detection of myocardial ischemia prior to necrosis	[100]
SERS	AuNPs conjugated with 4-ATP-azo	Detection of thrombin	[101]
	AuNPs as cell surface receptor labels	Identification of markers of interest in chronic lymphocytic leukemia (CLL) and lung cancer	[102]
	AuNPs conjugated with anti-MMP-7 or anti-CA 19-9 and 5,50-Dithiobis(succinimidyl-2-nitrobenzoate) as reporter molecule	Detection of pancreatic adenocarcinoma biomarkers: serum carbohydrate antigen 19-9 (CA 19-9) and matrix metalloproteinase-7 (MMP-7) in human serum	[103]
Fluorescence	Spherical AuNPs functionalized with MUA and conjugated with heat shock protein 70 antibody	Detection of malaria anti-heat shock protein 70 from *Plasmodium falciparum*	[41]
	Spherical AuNPs conjugated with fluorescent proteins	Detection of multiple caspase activity in live cells	[104]
Electrochemical	Spherical AuNPs conjugated with Single domain V_HH antibodies (sdAbs)	*Clostridium difficile* toxin detection associated with several infections	[105]
	Spherical AuNP functionalized with PAMAM-dendrimer and conjugated with anti-PSA antibody	Detection of prostate-specific antigen (PSA)	[75]
	AuNP conjugated with 1,6-hexanedithiol and antibody-binding fragments (Fab') of anti-hemagglutinin H5 monoclonal antibodies Mab 6-9-1	Detection of peptides derived from avian *influenza hemagglutinin* H5	[106]
	Spherical AuNPs as electroactive labels conjugated with goat polyclonal antibodies anti-human IgG and magnetic nanoparticles	Detection of human IgG antibodies anti-hepatitis B surface antigen in human serum	[107]
	Spherical AuNP-Citrate with poly-(diallyldimethyl-ammonium chloride) (PDDA) and carbon nanotubes	Detection of cholesterol in human serum	[108]
Lateral flow	Spherical AuNPs conjugated with anti-troponin I antibody and AuNPs conjugated with anti-BSA antibody	Detection of troponin I	[109]

proteomic techniques already in use to study the proteome, its proteins and protein-protein interactions. The three greatest characteristics of AuNPs: size and morphology, surface functionalization according to the application, and unique optical and electrochemical features, make them increasingly attractive to be implemented in laboratory routines as tools for protein enrichment, structural characterization and analyte detection. In spite of the rapid advance of proteomic techniques, they still have limitations, but with the implementation of AuNPs it is expected that, in the future, some of the common techniques will exclusively adopt the use of these nanoparticles to enhance their performance. So far, AuNPs have been used in proteomics with great success and yet, the application possibilities remain endless.

It is important to note that even though many progresses have been made with the introduction of nanoparticles in proteomic techniques, there remain several difficulties when it comes to the difference between detecting and identifying proteins and small molecules. Proteins are complex structures that require the combination of several techniques and high amounts of data in order to be identified. On the other hand, small molecules have relatively uncomplicated spectra and the use of nanoparticles as matrixes helped greatly in aspects such as ion interference. With the constant use of MS techniques, the future should bring MS instrumentation to their full sensitive and selective potential.

Additionally, production costs are relatively low when taking into account the production yield and the versatility of modifications and applications that can be achieved. In the

following years we will witness the entrance at the market level of kits containing AuNPs for protein enrichment and protein quantity measurements which will be simple to use and surpass the sensitivity levels of the currently existent kits for the same purposes.

The structural and conjugational information obtained from proteomic analysis complemented with toxicity information is extremely relevant and useful in order to design new therapeutic models. The protein structure and their interactions with other proteins and analytes lead to the discovery of new potential targets and the development of more efficient therapies.

On the other hand, *in vivo* use of these nanoparticles in medical treatment and drug delivery is a challenge that might take more time to overcome. When the human body is concerned and the potential toxicity of AuNPs is in question, the scientific community is divided. Many drug-delivery and therapeutic strategies with AuNPs have been proposed over the last years with attested success,[115] but the information of long-term risks (or lack of them) for AuNPs application *in vivo* is still limited. The lack of conclusive studies regarding potential toxicity is why *in vivo* applications are slowly progressing and still lacking proper application in human patients.

Interestingly, as many AuNPs have improved proteomic techniques, very recently, the very same techniques have been used to study the effects of these nanoparticles in pathways and cellular mechanisms and how they might be modified upon cellular uptake.[116] In future years, proteomic tools will become essential to understand nanotoxicity and improve AuNPs safety for clinical applications. When that threshold is finally surpassed, AuNPs might become a powerful resource for cancer therapies or even a commonly used drug delivery method.

AuNPs also became an asset in the development of biosensors, due to the combination of protein enrichment techniques and structural affinity information with the colorimetric characteristics, with promising results. These characteristics will lead to the development of inexpensive, but highly sensitive screenings, which will not need trained personal or expensive equipment to be operated. A mass market production of such tests is expected in the near future and their distribution, for example, in third world countries, to rapidly diagnose the population for diseases such as malaria or tuberculosis and design early course of treatments to avoid massive transmission among the communities.

There is still much work ahead to be made in this specific area, especially in terms of studying AuNPs surface modifications and biocompatibility in order to adapt to each performance. Nevertheless, the work conducted so far supports the fact that AuNPs are reliable, versatile and with a high correlation between production costs and benefits.

Conclusions

The last decade has seen enhanced growth in nanotechnology, in particularly the use of gold nanoparticles and their application in several areas. From proteomics to medicine, AuNPs have proven time and time again to be an excellent material with enhanced physical, optical and electrical properties that was able to achieve substantial results to support their continued use. In the following years, AuNPs will achieve an even greater status in laboratory applications in terms of improving proteomic techniques as well as becoming essential tools for clinical applications.

Declaration of interest

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