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Novel Nanocomposites based on a Strawberry-like Gold-coated Magnetite (Fe@Au) for Protein Separation in Multiple Myeloma Serum Samples

José E. Araújo¹,², Carlos Lodeiro¹,², José L. Capelo¹,², Benito Rodríguez-González³, Alcindo A. dos Santos⁴, Hugo M. Santos¹,², (✉) Javier Fernandez-Lodeiro¹,²,⁴ (✉)

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**Novel nanocomposites based on a strawberry-like gold-coated magnetite (Fe@Au) for protein separation in multiple myeloma serum samples**

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¹ BIOSCOPE Research Group, REQUIMTE, Department of Chemistry, Faculty of Science and Technology, University NOVA of Lisbon, 2829-516 Caparica, Portugal. ² PROTEOMASS Scientific Society, Madan Parque, Rua dos Inventores, 2825-182 Caparica, Portugal. ³ Scientific and Technological Research Assistance Centre (CACTI), University of Vigo, Vigo, Spain. ⁴ Instituto de Química, Universidade de Sao Paulo, Av. Prof. Lineu Prestes, 748, CxP. 26077, Sao Paulo 05508-000, Brazil.
Synthesis and characterization of a new strawberry-like gold-coated magnetite (Fe@Au) nanocomposites.

Fe@Au nanocomposites are used as a proteome fractionation tool to identify potential biomarkers of multiple myeloma.

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Novel Nanocomposites based on a Strawberry-like Gold-coated Magnetite (Fe@Au) for Protein Separation in Multiple Myeloma Serum Samples

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¹ BIOSCOPE Research Group, REQUIMTE, Department of Chemistry, Faculty of Science and Technology, University NOVA of Lisbon, 2829-516 Caparica, Portugal.
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ABSTRACT
A new process to produce magnetite partially coated strawberry-like gold nanoparticles in aqueous medium is reported. Fast response to magnetic fields and optical properties of gold nanoparticles-based colloidal systems are the two main advantages of this new Fe@Au nanomaterial. Both advantages allow for the use of this new colloidal nanomaterial for various purposes in proteomics and biomedicine as proteins can bind the surface, and the surface can be functionalized as well. As proof-of-concept, the new Fe@Au nanoparticles have been assessed in biomarker discovery as a tool for pre-concentration and separation of proteins from complex proteomes. To this end, sera from healthy people were compared with sera from patients diagnosed with multiple myeloma. The application of this new Fe@Au nanomaterial combined with mass spectrometry has allowed for the identification of 53 proteins, and it has also revealed the heat shock protein HSP75 and the plasma protease C1 inhibitor as potential biomarkers for diagnostics and control of multiple myeloma progression.

1 Introduction
Multifunctional Hybrid Nanoparticles (MHNPs) has attracted much attention recently since they are nanomaterials with unique advantages with respect to mono-functional nanoparticles [1, 2]. Iron oxide nanoparticles have been explored extensively for medical applications being fascinating new tools used in diagnostics, therapeutics and for theranostic [3, 4]. The most studied systems are based in pure iron oxide nanomaterials as maghemite, magnetite and alloys, due to their relatively low toxicity and biological tolerance [5]. However, despite its relatively low toxicity, these nanomaterials may be

Address correspondence to First A. Firstauthor, email1; Third C. Thirdauthor, email2
involved in the formation of oxygen reactive species (ROS) causing cell damage [6]. As a mechanism to prevent this toxicity, gold-coated magnetic nanocomposites can be explored as MHNPs, showing unique physical and chemical properties for biomedical studies [7]. The absorption of biological molecules (proteins, peptides, amino acids, etc.) to the nanoparticle surface confers to these nanomaterials a new perspective in the biological and medical arena [8, 9]. These materials have been recently subjected to extensive research since the gold shells could improve biocompatibility, functionality and stability in biological media. Different approaches have been addressed for the synthesis of gold-coated magnetic nanocomposites, including microemulsion, sonochemical synthesis, self-assembly, seed-mediated growth method, and laser irradiation [10].

Proteomics has gained momentum in biomarker discovery. The term biomarker(s) in proteomics refers to a protein(s) that can be used to trace a biological state or condition. In medicine protein biomarkers are used for the early detection of diseases, in prognosis and to monitor medical treatment [11–13]. Citrate gold nanoparticles, used to reduce the proteome complexity of serum samples, in combination with classic proteomics workflows has recently allowed for the classification of three groups of people as follows: patients with myeloma, patients with lymphoma and healthy people [14].

Heat shock proteins (HSP) are a family of stress proteins classified in groups as a function of their molecular weight as follows, HSP110, HSP90, HSP70, HSP60 and the smaller HSP27. The main function of these proteins is to address the correct folding for misfolding proteins, preventing this way its aggregation [15]. HSP70 family is formed by at least 13 different proteins, which have been linked with cancer. It is well known that at the initial stages of tumorigenesis, the HSP70 can protect cells undergoing transformations due to oncogenic stress induced by overexpression of oncogenes [16,17].

In this paper, a new simple synthetic methodology to obtain strawberry-like coated gold magnetic nanoparticles was developed. As proof-of-concept, the new nanoparticles were used to pre-concentrate and separate proteins from sera samples of patients with multiple myeloma as well as from healthy people. As a result, 53 proteins were identified, and it was also revealed that the heat shock protein HSP75 as a potential biomarker for diagnosis and monitoring of multiple myeloma progression.

2 Experimental

2.1 Reagents and characterization methods

Reagents and solvents were purchased from Alfa-Aesar and Sigma-Aldrich and were used without further purifications. A Power Pac Basic power supply from Bio-Rad (CA, USA) was used for SDS-PAGE protein separation. Protein quantification was accomplished by measuring the absorbance at 280 nm with the use of a NanoDrop 1000 Spectrophotometer from Thermo Scientific. Gold nanoparticles were characterized by UV-vis spectroscopy using a spectrophotometer from JASCO V-650 UV-vis (Easton, MD, UK) and Transmission Electron Microscopy, Philips CM20. High-resolution transmission electron microscopy (HRTEM) and STEM images of the strawberry-like MHNPs were obtained with a JEOL JEM 2010 FEG-TEM operating at an acceleration voltage of 200 kV. X-Ray energy dispersive spectra (EDS) were obtained using an Inca Energy 200 TEM system from Oxford Instruments. Elemental maps were acquired coupling the X-ray spectrometer to a STEM unit, equipped with a dark field High Angle Annular Dark Field Detector (HAADF).

2.2 Synthesis of Gold Coated Magnetite MHNPs (Fe@Au)

The synthesis of the new Strawberry-like partial coated gold magnetic nanoparticles was done in three steps:

2.2.1 Gold Nanoparticles AuNP (1)

First, Au@citrate nanoparticles were prepared in aqueous solution following the Turkevich methodology [18–20]. An aqueous solution (125 mL) of hydrogen tetrachloroaurate (0.125 mmol, 49.5 mg) was added rapidly to a solution of 1% (w/v) sodium citrate (12.5 mL) that was heated under reflux. The heating under reflux was continued for an additional 5 min. During this time, the colour changed to deep red.

2.2.2 Fe2O3 Nanoparticles. Fe@SO42-(2)

In a second step, precipitation and aging of magnetite nanoparticles were carried out following modifying a method previously published by M. A. Vergés et al. [21]. Two different solutions were prepared. A solution (A) containing 90 mL of distilled water, and 90 mL of absolute ethanol with NaOH 7×10-2 M and KNO3 0.1 M was placed in a three-necked round bottom flask. A second solution (B) was prepared using 10 mL of distilled water and 10 mL of absolute ethanol containing H2SO4 1×10-2
M and Fe(SO₄)₂ 5×10⁻² M. Both solutions were kept under inert atmosphere separately for one hour. Maintaining the nitrogen atmosphere, solution (B) was drop-wise to solution (A). When ending the precipitation, the nitrogen bubbling was continued for ten minutes. All the reaction mixture was heated at 90 °C for 24 h.

When the reaction end, the round bottom flask was cooled in an ice / water bath. The solid magnetic nanoparticles were separated by magnetic decantation and washed five times with distilled water. The final magnetic solid was re-suspended in 80 mL of Milli-Q water. Strong magnetite nanoparticles with near cube-shaped was obtained.

2.2.3 Strawberry-like Gold Coated Magnetite nanoparticles: Gold-Shell Formation (3)

In the third step, following a previous procedure described by Caruso et al. [22-25] poly-diallyldimethylammonium chloride (PDADMAC) and poly-sodium 4-styrenesulfonate (PSS) were alternately adsorbed onto the negative surface of the magnetic nanoparticles Fe@SO₄²⁻. A solution of 5 mL of the previous Fe@SO₄²⁻ synthesized NPs (2) was resuspended in 100 mL of Milli-Q water during 2 minutes assisted with ultrasonic energy. 100 mL of a water solution containing 1 mg/mL of PDADMAC was added. The solution was maintained in an ultrasonic bath during a minute, and under magnetic stirring during two hours. The NPs obtained were separated from the supernatant by magnetic separation and washed 10 times with Milli-Q water. This process was repeated for the adsorption of the PSS layer, and for a second monolayer of PDADMAC.

The three-layer polyelectrolyte magnetite nanoparticles washed previously, were re-suspended in 100 mL of Milli-Q water and mixed with 5 mL of NaCl 0.2 M solution. This solution was keeping it during one minute in an ultrasonic bath. After this time, 20 mL of previously synthesized gold nanoparticles (1) was added, and the entire solution was maintained with magnetic stirring during 2 hours. The final MHNP's were separated from the supernatant by magnetic decantation, and washed ten times with Milli-Q water being finally re-suspended in 100 mL of Milli-Q water.

The formation and growth of the partial gold shell were performed by adding to the solution an aliquot of 100 μL of Au³⁺ (5×10⁻⁴ M) followed by the addition of 100 μL of ascorbic acid (0.34×10⁻³ M) under ultrasonic stimulation to help nanoparticle separation during the growth process. The strawberry-like nanostructures Fe@Au NPs were separated by magnetic decantation and washed ten times with Milli-Q water. Subsequently re-suspended in 20 mL of Milli-Q water.

Samples for TEM, HRTEM, STEM and EDS were prepared by pipetting a drop of the colloidal dispersion onto an ultrathin carbon coated copper grid and allowing the solvent to evaporate.

2.3 Human serum samples

Human serum samples were purchased from Patricell Ltd (BioCity Nottingham, UK). In this study, serum samples from five patients diagnosed with multiple myeloma were used. For control purposes, a pool of two individuals (mixed gender) was also employed (Table 1).

<table>
<thead>
<tr>
<th>Diagnostic</th>
<th>Age</th>
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2.4 Depletion of multiple high abundant proteins

Protein depletion with DTT was performed according to the protocol described by Warder et al. [26, 27]. Fresh DTT 500 mM (2.2 μL) was mixed with 20 μL of human serum and vortexed briefly. Samples were then incubated until a viscous white precipitate persisted (60 min), followed by centrifugation at 14000×g for 20 min. Supernatants were transferred to a clean Lo-Bind tube prior to protein alkylation and Fe@AuNPs fractionation.

2.5 Fe@Au protein fractionation

After protein depletion, the reduced SH-groups were alkylated with IAA for 45 min at room temperature and protected from light, and then the sample’s pH were adjusted to 5.8 with citrate/ citric acid buffer as described by Lópex-Cortés et al. [14]. Finally, 100 μL of Fe@Au GNP's and the resulting solution were added and then the samples were incubated for 2 h at room temperature. The Fe@Au GNP's were harvested by magnet separation and the pellet containing proteins bound to Fe@Au nanoparticles were washed twice with 100 μL of
citrate/ citric acid buffer to remove unbound proteins. Both supernatant and Fe@Au GNP s were mixed with Laemmli samples buffer and loaded onto a 12.5% SDS-PAGE. After electrophoresis, each gel was fixed for 30 minutes with 40% (v/v) ethanol and 10% (v/v) acetic acid and then stained overnight with colloidal coomassie blue [28]. Gels were rinsed with distilled water and a 0.5 M sodium chloride solution until a clear background was observed. Gel imaging was carried out with a ProPicII-robot (Digilab-Genomic Solutions, USA) using 16 ms of exposure time and resolution of 70 μm [29].

2.6 In-gel protein digestion

Protein bands were excised manually and transferred to 0.5-mL Lo-Bind tubes, and then washed twice with water and then with 50% (v/v) acetonitrile/ 25 mM Ambic until the blue colour disappears. Prior to trypsin digestion, gel spots were washed with 25 mM Ambic and dehydrated with acetonitrile. Then, 15 μL of trypsin (20 ng/μL in Ambic 12.5 mM / 2% (v/v) acetonitrile) was added to the gel spots and incubated for 60 min on ice. After this time, gel spots were inspected, and all the trypsin solution not absorbed into the gel were removed, and the gels were covered with 50 μL of 12.5 mM Ambic. Samples were incubated 12h at 37 ºC. Then 25 μL formic acid 5% (v/v) was added and the supernatant was transferred to new Lo-Bind tube and the peptides were further extracted from the gel with 50% (v/v) acetonitrile / 0.1% (v/v) trifluoroacetic acid. Samples were dried-down and stored at -20 ºC until MS analysis [29].

2.7 MALDI-TOF/TOF MS analysis

Prior to analysis, samples were resuspended in 10 μL of formic acid 0.3%, and 1 μL of sample was hand-spotted onto a MALDI target plate (384-spot ground steel plate) then 1 μL of a 7 mg/mL solution of a-cyano-4-hydroxycinnamic acid matrix in 0.1% (v/v) TFA and 50% (v/v) ACN was added and allowed to air dry.

Mass spectrometry data were acquired using an Ultraflex II matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF-TOF) instrument (Bruker-Daltonics, Bremen, Germany) equipped with a LIFT cell and 50 Hz nitrogen laser. The mass spectrometer was operated in positive ion mode using a reflectron, and thus, spectra were acquired in the m/z range of 600-3500. A total of 500 spectra were acquired for each sample at a laser frequency of 50 Hz. External calibration was preformed with the [M+H]+ monoisotopic peaks of bradykinin 1–7 (m/z 757.3992), angiotensin II (m/z 1046.5418), angiotensin I (m/z 1296.6848) substance P (m/z 1758.9326), ACTH clip 1–17 (m/z 2093.0862), ACTH18–39 (m/z 2465.1983) and somatostatin 28 (m/z 3147.4710). Peptide mass fingerprints (PMF) were searched with MASCOT search engine with the following parameters: (i) SwissProt Database2012_04 (535698 sequences; 190107059 residues); (ii) molecular weight of protein: all; (iii) one missed cleavage; (iv) fixed modifications: carbamidomethyl (C); (v) variable modifications: oxidation of methionine and (vi) peptide tolerance up to 50 ppm after close-external calibration. The significance threshold was set to a minimum of 95% (p≤0.05). A match was considered successful when protein identification score is located out of the random region, and the protein analysed scores first [29].

3 Results and discussion

Coating colloidal gold solutions with magnetite via layer-by-layer polymer deposition leads to the formation of the so-called strawberry-like partial gold cover magnetite surface nanoparticles. Such nanocomposites maintain the magnetic properties and present the red colour of the colloidal gold nanoparticles. Each polyelectrolyte coating film over the surface of the magnetic nanoparticles was fixed onto by electrostatic interactions. The first adsorption of the polyelectrolyte film of PDADMAC has positive charge, being the opposite of the negatively charged magnetic Fe@SO42-nanoparticles. At the end of the adsorption of the three-polyelectrolyte layers (PDADMAC/PSS/PDADMAC), the synthesized nanomaterial presents a positive net charge and a spongier-like topology. Nano-particle size is critical, because as the number of polymeric layers is increased, the particles tend to precipitate. In addition, the highest is the size; the worst is the magnetic behaviour. Different numbers of polymeric layers were tested. According to the aforementioned tips, the best number of layers to avoid precipitation maintaining magnetic properties was three. With this strategy we designed a system with substantial potential for allowing the pre-concentration of proteins present in complex proteomes by anchoring them to the Fe@Au surface, and then their separation by the straightforward application of an external magnetic field.

The UV-visible absorption spectrum of Fe@Au nanoparticle colloidal solution immediately after
agitation (see Fig. 1a) and after standing, demonstrating the aggregation evidenced by the attenuation of the plasmon band (Fig. 1b); the nanoparticle solution before and after magnetic separation is depicted in Fig. 1c; a pictorial composition of the magnetic nanoparticles before and after decoration with gold nanoparticles, as well as the TEM images of the magnetic strawberry-like Fe@Au nanoparticles, is shown in Fig. 1d.

This new system clearly shows the characteristic absorption band of colloidal systems gold nanoparticles centred at 567 nm. The magnetic aggregation process in the absence of external magnetic field or mechanical agitation cause decantation of the colloidal system after a few minutes, evidencing the high magnetism inherent to the Fe@Au colloidal nanocomposite. Two bright field TEM images and two dark field STEM images of the magnetite@Au nanoparticles are shown in Fig 2. In these images is noted how magnetite octahedral like nanoparticles displays a very different contrast than the nearly spherical gold nanoparticles. These images also show two interesting facts: (i) Magnetite nanoparticles were aggregated forming a chain like core and (ii) Almost all gold nanoparticles were attached to the surface of the magnetite nanoparticles that formed the chain. We hypothesised that gold nanoparticles were attached to the magnetite chain after the one was formed. The quality of the linking between gold and magnetite nanoparticles was demonstrated by TEM images, by the fact that there are no isolated gold nanoparticles in the surrounding of the strawberry like structures. This means that the electrostatic attraction was very effective to the magnetite core. Once the gold shell was not complete leading to the strawberry-like aspect, the resulting system keeps the strong magnetic property of the pure Fe_3O_4 nanoparticles.
moreover maintaining the plasmonic resonance property of the gold-NP shell. The observed aggregation property confers this new material perfect application as protein scavenger substrates.

Fig. 3a and 3b show a dark field STEM image together with the corresponding X-ray EDS elemental mapping displaying both the distribution of Fe in red and Au in green. As may be seen, the gold cubic-octahedral nanoparticles are mainly distributed in the outer part of the magnetite aggregates that form the core of the chain-like aggregates. The EDS spectrum, corresponding to the same area as the elemental mapping confirms the principal composition of the composite aggregates. The EDS spectrum showed Fe Kα and Au Mα as the most intense peaks, but also smaller O, C and Cu peaks. From these images, and from Fig. 2, it is clear that the almost spherical gold nanoparticles (15 nm as the average diameter) were mainly situated in the outer part of the magnetite aggregates that form the core of the chain-like aggregates. Magnetite aggregates are in the core and are formed by octahedral Fe2O3 nanoparticles with 38 nm as the average length of the edges of the octahedron.

Figure 2. TEM images of the magnetite@Au nanoparticles showing darker spherical Au nanoparticles together with octahedral like magnetite nanoparticles (a, c). Dark field STEM images showing Z contrast, gold nanoparticles display brighter contrast due to higher Z number (b, d). Note that in all the images all the gold nanoparticles are always attached to the group of magnetite nanoparticles in the core.

3.1 Proof-of-concept: protein fractionation using Fe@Au nanoparticles

As proof-of-concept, a simple and fast method for protein identification in human serum samples that entails major protein depletion with DTT and subsequent protein fractionation with Fe@Au nanoparticles was developed. In addition, to demonstrate further applicability, the method was applied to two different groups of people, healthy versus non-healthy. Thus, samples from five patients diagnosed of Multiple Myeloma were used. For comparative purposes, two-serum samples from healthy individuals were pooled and used as one sample. In brief, proteins were first depleted from major proteins using DTT as previously reported [26, 27] and further processed as described in 2.5. Afterwards, the Fe@Au nanoparticles were quickly separated using a magnet. Two-protein fractions were thus obtained, one in the supernatant and the second one attached to the Fe@Au nanoparticles. Then the supernatants and Fe@Au nanoparticles were separately loaded onto a 1D-SDS-PAGE. Proteins were separated and, after staining, gel bands

Figure 3. Bright field STEM images of a magnetite@Au Strawberry-like nanoparticles group (a). X-ray EDS elemental mapping of the same area as image b showing the distribution of Fe in red and Au in green (b). EDS elemental mapping of the area displayed in (a, b and c). Main peaks are due to Fe and Au, O is due to magnetite nanoparticles, C and Cu are due to the carbon coated copper grid used to TEM sample preparation.
were excised and submitted to the sample treatment described in 2.6. The resulting pools of peptides were then analyzed by MALDI-TOF/TOF MS for protein identification.

Fig. 4 shows the 1D gels for the two-protein fractions obtained as described above. As may be seen, the gel profiles for the healthy sample (HS) and the patients (from A to E) are similar. The same is observed for the gel profiles obtained with the protein fractions obtained from Fe@Au nanoparticles. When the gel profiles of both fractions are compared, it is easily noted that there is a difference in the intensity of the bands. However, no conclusion can be drawn unless the proteins are identified. Thus, for the healthy control, 31 proteins in the supernatant and 22 proteins in the Fe@Au NPs pellet were identified (redundancy not excluded). On the other hand, for the five patients used in this study, a total of (redundancy not included) 35, 33, 36, 29 and 34 proteins were identified in the supernatants, and 22, 20, 18, 22 and 17 proteins were identified in the Fe@Au NPs pellets (see Fig. 4c, 4d and 4e).

Remarkably, fractionation of the proteome using Fe@Au NPs allows for the identification of the plasma protease C1 inhibitor and the protein HSP75 in the supernatant of all patient samples. Furthermore, these proteins were not identified in the healthy control neither in the samples without fractionation. A complete list of the proteins identified is given in supplementary material. The plasma protease C1-inhibitor is an acute-phase protein that may play a crucial role in regulating important physiological pathways including complement activation, blood coagulation, fibrinolysis, the generation of kinins and inflammation. Survival and proliferation of multiple myeloma cancer cells is critically dependent of signals coming from the microenvironment [30]. These include inflammatory processes. Inflammation is an essential innate immune response to perturbed tissue homeostasis and it is known that inflammatory processes affect all stages of tumour development as well as therapy [31]. It is known that HSPs contribute to myeloma survival and chemoresistance via their roles in multiple pathways known to be important in the progress of myeloma Cancer [32].

**Figure 4.** 1D-SDS-PAGE of human serum fractionated with Fe@Au NPs, (a) supernatants and (b) Fe@Au NPs pellet. (c) Number of identified protein without protein fractionation and Venn diagram showing the common proteins found in the healthy control (HS) and in Multiple Myeloma patients. (d) Number of identified proteins found in the supernatant after fractionation with Fe@Au NPs and Venn diagram showing the common protein found in the healthy control (HS) and in Multiple Myeloma patients. (e) Number of identified proteins found in the Fe@Au NPs pellet after fractionation with Fe@Au NPs and Venn diagram showing the common protein found in the healthy control (HS) and in Multiple Myeloma patients. (f) Venn diagrams showing the common protein found without NPs fractionation, supernatant after fractionation with Fe@Au NPs and Fe@Au NPs pellet for the healthy control (HS) and Multiple Myeloma patients.

The HSP70 family still participates in general folding of unfolded or misfolded proteins exposing hydrophobic regions and preventing their aggregation. Furthermore, it has been claimed that HSPs proteins may be involved in binding protein fragments from dead malignant cells helping this way the immune system to recognize them. Moreover, intracellular heat shock proteins are overexpressed in cancerous cells, seeming to play an important role in their
development [33]. In addition, high expression of HSP70 is correlated with poor prognosis in a wide range of cancers [32, 34]. As HSP75 can be easily isolated through Fe@Au NPs fractionation, as demonstrated above, this method holds great potential in diagnostic, prognostic, and prediction over multiple myeloma cancer [32].

4 Conclusions

A new simple synthetic methodology based on layer-by-layer polymer deposition to obtain strawberry-like coated magnetic gold-coated nanoparticles, Fe@Au NPs, was developed. As proof-of-concept, the new Fe@Au nanoparticles were assessed in biomarker discovery as a tool for pre-concentration and separation of proteins from complex proteomes. To this end, sera from healthy people were compared with sera from patients diagnosed with multiple myeloma. The application of this new Fe@Au nanomaterial combined with mass spectrometry has allowed for the identification of 53 proteins, and it has also revealed the plasma protease C1 inhibitor and the heat shock protein HSP75 as potential biomarkers for diagnosis and control of multiple myeloma progression.

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Electronic Supplementary Material: Proteins identified in 1D-SDS-PAGE of Multiple Myeloma serum samples: Mascot Score, protein sequence coverage for the identified proteins.

Electronic Supplementary Material is available in the online version of this article at http://dx.doi.org/10.1007/s12274-***.***.* (automatically inserted by the publisher).

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[10] Jiang, H.; Zeng, X.; He, N.; Deng, Y.; Lu, G.; Li, K. Preparation and Biomedical Applications of Gold-coated...
Electronic Supplementary Material

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¹ BIOSCOPE Research Group, REQUIMTE, Department of Chemistry, Faculty of Science and Technology, University NOVA of Lisbon, 2829-516 Caparica, Portugal.
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Address correspondence to First A. Firstauthor, email1; Third C. Thirdauthor, email2