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Synthesis of Size Monodisperse Water-Soluble Metal Nanoclusters for Protein Quantification by Elemental Mass Spectrometry †

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Abstract: The use of metal immunoprobes, defined as recognition molecules (e.g., antibodies) labeled with metal tags, constitutes an interesting strategy for the analysis of proteins in biological samples. Fluorescent and biocompatible metal nanoclusters (MNC) have been recently established as powerful tags for detection by spectrofluorimetry, but also by elemental mass spectrometry (MS). Detection of such immunoprobes by elemental MS allows not only the qualitative analysis of the proteins but also their absolute quantification. However, the deviation associated with the MNCs polydispersity will limit the analytical precision, particularly in those samples where the concentrations of the sought protein are very low (e.g., single cell analysis). In this work the synthesis of size monodisperse gold nanoclusters (AuNCs) is investigated by using different experimental conditions such as reaction time and temperature, solvent, reducing agent, and pH, among others. Characterization of AuNCs was performed by spectrofluorimetry, dynamic light scattering (DLS) and high resolution transmission electron microscopy (HR-TEM) measurements.

Keywords: gold nanoclusters; elemental mass spectrometry; ICP-MS; protein quantification; dynamic light scattering; Spectrofluorimetry; High resolution transmission electron microscopy

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1. Introduction

Strategies based on the use of metal immunoprobes to quantify biomolecules by inductively coupled plasma mass spectrometry (ICP-MS) have been widely investigated in recent years in the bioanalytical field [1]. This type of approaches is described as the specific interaction between the analyte (e.g., protein biomarker) and the recognition biomolecule (e.g., antibody) previously labeled with several metals or a metal nanostructure which are easily detected by elemental MS [2].

The metal nanostructures proposed up to date are diverse, from complexes with several atoms (e.g., Maxpar® and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid, DOTA) [3] to metallic nanoparticles (NP) with typical diameters above 10 nm [3]. In this context, metal nanoclusters (MNC) have been recently introduced as an interesting alternative for the analysis of biomolecules by elemental MS. MNCs exhibit a low size (<3 nm) and, additionally, they are formed of hundreds of atoms of the same metal, providing a

high amplification signal with a lower risk of blocking antibody recognition sites compared to larger labels (e.g., Maxpar® or typical NPs). Therefore, low concentrations of analytes can be detected through the signal's amplification and the high sensitivity offered by ICP-MS detection [4]. Furthermore, MNCs have unique properties compared to common metallic NPs due to their smaller size, such as molecular fluorescence. Thus, MNCs can be employed for bimodal detection by fluorescence and elemental MS.

MNCs of gold, silver or platinum (AuNCs, AgNCs, and PtNCs) have been recently reported as elemental tags for the determination of proteins in serum samples and biological tissues by using ICP-MS (i.e., serum) and laser ablation (LA) coupled to ICP-MS (i.e., tissues and cultured cells). For example, PtNCs were used as bimodal labels in a competitive immunoassay for immunoglobulin E determination in serum samples [5]. The huge amplification achieved by ICP-MS detection gave rise to a better limit of detection than the fluoroimmunoassay and the commercial ELISA kit. Concerning the analysis of tissues and cells, the use of LA solid sampling technique allows to determine the sough proteins concentration and their distribution along the micrometer structures of the samples (lateral resolution in the range of 1 to 5 μm has been reported). In this case, AuNCs, AgNCs, and PtNCs have been successfully investigated as elemental tags for proteins determination in human brain and ocular tissues [6–8]. However, the main limitation of using MNCs for quantification purposes is related to their size variability. The dispersion associated with the MNCs diameter generates a high variability in the number of metals per nanostructure and, therefore, limits the possibility to obtain accurate and precise quantification of the biomolecules (particularly in samples with low analyte concentrations, such as single cells).

In previous studies, we successfully synthesized water-soluble AuNCs following a bottom-up approach by chemical reduction of the Au (III) to Au (I, 0) in the presence of a thiolated ligand [7]. Nevertheless, the deviation associated with the AuNCs diameter must be decreased in order to apply them for quantification of biomolecules in single cells by elemental MS detection. Thus, in this work we introduce our latest efforts to achieve a proper synthesis method which allows to obtain AuNCs with a narrow size distribution.

2. Materials and Methods

2.1. Reagents and Materials

For the synthesis of AuNCs, $NaAuCl_4 \cdot H_2O$ (99% powder; Sigma–Aldrich, San Luis, CA, USA) was used as the metal precursor. Lipoic acid (>98% powder; Across Organics, Geel, Belgium), dihydrolipoic acid, and reduced L-glutathione (>98% powder; Sigma–Aldrich) were evaluated as the capping agents. $NaBH_4$ (>98% powder; Sigma–Aldrich) was employed as the reductor. Moreover, different solvents were used, such as propan-2-ol (Fisher Scientific, Hampton, VA, USA), acetone, and methanol (both from VWR chemicals, Radnor, PA, USA). In order to purify AuNCs, Amicon ultra centrifugal filter units (3 kDa pore size, Merck Millipore, Darmstadt, Germany) and syringe filter units (PVDF, 0.22 μm pore size, Olim Peak, Teknokroma Analitica S. A., Barcelona, Spain) were employed. The materials and instrumentation needed to carry out the synthesis were a cylindrical flask, a magnet, a stirring plate (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for the synthesis and a pH meter (Mettler Toledo, Barcelona, Spain) to control the pH.

2.2. Instrumentation

Fluorescence measurements in solution were performed with a spectrofluorimeter (Cary Eclipse, Agilent Technologies,CA, USA) using a suprasil quartz cuvette model 101-Qs from Hellma® (Sigma–Aldrich). A high-resolution transmission electron microscope (HR-TEM) (JEOL JEM-2100, Tokyo, Japan) with an energy dispersive X-ray (EDX) spectrometer for microanalysis allowed morphological characterization of the AuNCs. Measurements of hydrodynamic size, as well as the polydisperse index (PDI), were carried out with a Zetasizer Nano ZS instrument (Malvern analytical Ltd., Malvern, UK).

2.3. Experimental Procedures

In order to reduce the dispersion associated to the AuNCs diameter, different experimental parameters were evaluated, starting from the previously synthesized AuNCs [6]. Briefly, the synthesis protocol was as follow: a total of 20 mL of ultrapure water and 50 μL of NaOH (2 M) were collected in a cylindric flask, then 6.3 mg of lipoic acid were added and, subsequently, the solution was stirred until complete dissolution. Then, 400 μL of $NaBH_4$ solution (50 mM) were slowly dropwise. Next, several modifications of this protocol were tested to achieve higher control in forming the nanostructures.

First, the kinetics of the reduction reaction were studied. For such purpose different conditions were evaluated, such as the temperature of the synthesis (25 or 8 °C), the solvent of the synthesis (water or methanol), the solvent used to dissolve the reducing agent (acetone or methanol), the pH of the synthesis, and the reaction time, among others. In addition, 2 thiol-ligands (used for capping) were evaluated: reduced lipoic acid and glutathione. Besides, a post-treatment of the just synthesized AuNCs was also evaluated. Specifically, polydisperse AuNCs were subjected to an aging treatment to form monodisperse AuNCs by incubating the synthesis at 50 °C for 2 h.

All experiments assayed for the synthesis of AuNCs were characterized by measurements using dynamic light scattering (DLS) to know the hydrodynamic diameter, as well as the polydispersity index (PDI). On the other hand, the AuNCs fluorescence emission (λ_{em}, 720 nm) was always corroborated by spectrofluorimetry measurements. Finally, selected AuNCs synthesis (i.e., using the optimized conditions) was measured by transmission electron microscopy (HR-TEM). Studies about electron diffraction (i.e., crystalline structure) and X ray diffraction (i.e., composition) were also performed.

3. Results and Discussion

The first study carried out for the synthesis of monodisperse AuNCs was the evaluation of the reaction time. For such purpose, different reaction times in the range from 0 to 24 h were studied. Experimental results obtained by DLS measurements showed that longer reaction times increase the dispersion in size of AuNCs: by decreasing the time from 15 to 4 h, a significant improvement in polydispersity index (PDI) from 0.4 to 0.28 was observed. On the other hand, fluorescence emission was also measured, and the higher fluorescence emission was observed using 4 h as the reaction time for the AuNCs synthesis, confirming that high-quality AuNCs were formed.

The second attempt was based on the hypothesis that slower kinetics of the chemical reduction can allow a better control in the growth of the AuNCs, leading to more homogeneous sizes. Thus, basic media, organic solvents, or low temperatures were used to decrease the reduction power of the reducing agent (i.e., $NaBH_4$). In this case, experimental results showed that the use of a basic pH during the AuNCs synthesis (pH = 13) allowed to reduce the PDI up to 0.12 value (synthesis at pH = 11, the original, showed a PDI of 0.4).

Regarding to the use of different thiolate ligands, bidentate thiols (e.g., lipoic acid) were confirmed as those to provide better control during the growth of AuNCs. The use of lipoic acid, particularly at its reduced form (i.e., dihydrolipoic acid, DHLA), ensured that the sulfhydryl groups were available for the direct chemisorption into the Au surface. DLS measurements of AuNCs synthesized with DHLA showed a PDI of 0.18. In contrast, the use of monodentate ligands (e.g., glutathione) for the synthesis of AuNCs showed to turn out bad quality measures by DLS: different size populations were found as well as the worst PDI value (denoted as 1 value).

The last strategy evaluated for the synthesis of monodisperse AuNCs was based on the employment of a post-synthesis step (denoted as aging step), consisting in incubating the synthesized AuNCs at 50 °C for 2 h (just after the synthesis). This approach produced highly monodispersed AuNCs with a PDI lower than 0.1.

Fluorescence measurements were also carried out with the different synthesis, showing in all cases an intense emission. The intensity of fluorescence was slightly affected

(compared to the original synthesis) when low reaction times and basic pH (pH = 13) was used. However, fluorescence emission decreased significantly when other ligands were employed as capping agents (i.e., dihydrolipoic acid and glutathion). A decrease in fluorescence emission was also observed after submitting the synthesis to the size-focusing treatment.

Additionally, Figure 1 collects the HR-TEM image obtained for the AuNCs synthesized with the reduced lipoic acid. An average diameter for the AuNCs was found to be 1.99 ± 0.04 (n = 300). It should be noted that most of the nanoparticles collected in the Figure 1 have this measured diameter, however aggregations of more than one nanostructure can be found. It is probably due to the drying process of the solution containing the AuNCs on the HR-TEM grids.

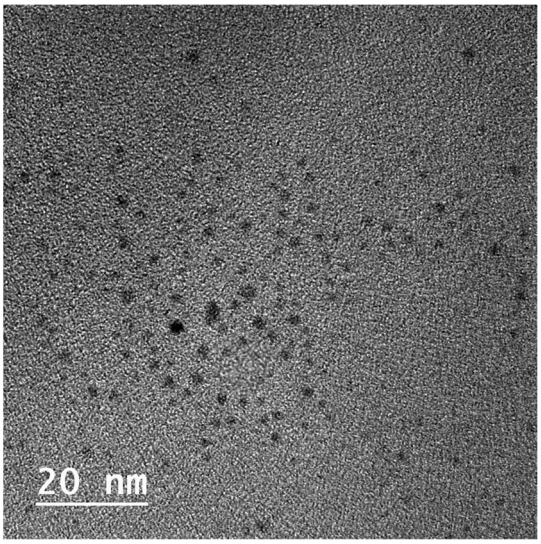


Figure 1. HR-TEM image of the AuNCs synthesis using reduced lipoic acid as ligand.

4. Conclusions

In this work, we assayed different strategies to achieve the synthesis of monodisperse AuNCs for their further application as elemental labels in the determination of proteins by elemental MS. Experimental results showed that low reaction times (4 h), the use of bidentate thiol ligands as capping agents, and basic pH media reduced the polydispersity of AuNCs considerably: PDI was reduced from 0.4 to 0.12. Moreover, the addition of a last step to the synthesis (an aging post-treatment) provided the best results, allowing to obtain AuNCs with a PDI of 0.09. Fluorescence measurements performed with the different AuNCs synthesis showed in all cases an intense fluorescence emission (λ_{ex}, 325 or 390, λ_{em} 720). Finally, HR-TEM images showed that it was possible to obtain monodisperse AuNCs.

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