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Green synthesis of Ag nanoparticles using plant metabolites

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Abstract. Nano-biotechnology is one of the most promising areas in modern nanoscience and technology. In this emerging area of research, nanoparticles (NPs) play an important role since the large-scale production and huge numbers of utilization. Gold and silver nanoparticles are among the most extensively studied nanomaterials, since they show high stability and low chemical reactivity in comparison to other metals. They are commonly synthesized using toxic chemical reducing agents able to reduce metal ions into uncharged NPs and/or high energy supplied procedures. The most commonly used method for the synthesis of NPs requires toxic chemicals like N,N-dimethyl formamide (DMF) or trisodium citrate, but recently a green technique, based on natural reducing agents, has been suggested to substitute the nature-unfriendly chemical methods. Many scientific works put in evidence the efficacy of plant extracts to reduce metal salts into the respective NPs, but this process lacks a clear control of NPs shapes and dimensions, since many different metabolites present into the extracts could participate to the process. This paper aims to clarify the reducing action of single pure natural compounds usually present in plant tissues and to obtain a stable and reproducible protocol for NPs synthesis.

INTRODUCTION

Nanoscience is the study of matter with at least one dimension between 1 and 100 nm that displays properties different from its bulk counterpart and it represents one of the most rapidly growing and cross-disciplinary field in science. The relevance of nanoscale researches is underlined by USA federal funding through the National Nanotechnology Initiative, which has grown from $464 million in 2004 to $1.85 billion in 2011. In addition, the number of consumer goods that self-report the inclusion of nanomaterials has risen from 54 in 2005 to 1317 in 2010, as an indicator of the economic relevance of nanoscience through commercialization [1].

In particular, during the last decade, nanotechnology has become a cutting edge application in interdisciplinary research area of strong interest, including basic / environmental / material and medical sciences. Nanomaterials present unique physical (wider surface) and structural (shape and dimensions) characteristics that give them peculiar mechanical, chemical, electrical, magnetic, optical, and biological properties [2].

For these reasons, the use of nanomaterials and in particular nanoparticles (NPs) is getting an even more relevant and promising area in biological science.

Metal NPs have attracted interests of a broad scientific community for decades due to their remarkable physicochemical properties that are often substantially different from those of bulk material. In recent years, the size and shape-controlled synthesis of metal NPs becomes an important area in advanced materials, as almost every property within the nanometer regime is size and shape dependent. At the same time, synthesis approach will determine the surface properties and cost of as-prepared metal NPs, so controlled synthesis of metal particles is highly desirable and many efforts have been devoted to this aspect. [3].

Among the metals used for NPs formation, gold (AuNPs) and in particular silver nanoparticles (AgNPs) represent the most interesting and used metals, exhibiting intrinsic peculiar characteristics [4].

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AgNPs are known to be potent antimicrobial agents with a broad spectrum and a high efficacy against bacteria [5], widely used in consumer products thanks also to their optical properties (yellowish color in water with a characteristic absorbance peak around at 420 nm) [3]..

Thanks to the possibility to coat them, engineered nanoparticles have been extensively used in the agriculture industry as "nano carriers", containing herbicides, chemicals and other bio-molecules [6].

Different organic and inorganic agents, such as sodium borohydride ($NaBH_4$), sodium citrate, ascorbate, elemental hydrogen, N,N-dimethyl formamide (DMF) and poly-ethylene glycol (PEG) block copolymers are used to reduce metal ions into zero charged molecules, forming the NPs. Capping agents are also used for size stabilization of the nanoparticles. The large use of these toxic chemicals has been recently questioned, particularly considering their effects on human health / safety and the environmental problems concerning their disposal. A new nature friendly methodology using natural reducing agents has recently attracted large interest thanks to their low cost and easy scaling up for large scale synthesis of NPs. A lot of data has been reported in literature on green synthesis of NPs using natural molecules present into bacteria, fungi and particularly in plants [7].

The ability of plant extracts to reduce metal ions has attracted considerable attention within the last 30-years, although the role of the involved single reducing agents has not well understood. Plant extracts may act as both reducing and stabilizing agents in the synthesis of NPs and the different metabolites present at various concentrations in several plant tissues enhance even more the capacity of the extract to reduce and stabilize the NPs. Typically, a plant extract-mediated bio-reduction occurs at room temperature and is generally complete within a few minutes. In view of the number of different molecules involved, the bio-reduction process is relatively complex and not well controlled: shape and dimensions of the NPs can vary widely from one extract to the other and different plants can produce different types of the same metal NPs. In addition, dimensional distribution is rather wide and this is a generally undesired feature in terms of biotechnological applications [8].

Many different metabolites seem to participate to the reduction power of plant extracts, but only a specific group of them possess all the appropriate characteristic: sufficient molar concentration, high antioxidant/reducing power, direct involvement in heavy metal plant detoxification mechanism. This group is the polyphenol family. The most abundant polyphenols are the condensed tannins, belonging to the flavonoids sub-class and found in several families of plants. In plants flavonoids show different biological functions, including protection against ultraviolet (UV) radiation and phytopathogens, flower and fruit coloration, as well as a strong antioxidant activity [9].

Quercetin (QC) represents the most abundant flavonoid present in plant kingdom. Flavonoids share a common aromatic chemical structure that allows them to perform many different chemical reactions that involve sharing and exchanging of electrons.

Since the physical characteristics of the NPs seem to be the most desired features, the aim of this work is to characterize the role played by a flavonoid such as QC in AgNPs formation and clarify the characteristics of the synthesized NPs by comparing the effect of flavonoid concentration and different pHs of incubation buffer.

MATERIALS AND METHODS

Materials

All the reagents (buffer salts, quercetin and $AgNO_3$) were purchased by Sigma. Three different 10 mM buffer solutions were used based on MOPS and Borax salts.

Green NPs synthesis

The reduction step of $AgNO_3$ to the respective NPs has been conducted mixing a fixed concentration of $AgNO_3$ (25 μM) with two different concentrations of QC as reducing agent (0.1 mM and 0.01 mM). The experiments were performed in 10 mM MOPS/KOH solution at pH 7.5 and 8.5 or 10 mM Borax solution at pH 9.2. The reaction took place at room temperature for 30 minutes, in dark round bottom flasks, in constant shaking. After the synthesis the samples were kept at 4°C. The following data were the results of 3 independent replicates. Preliminary evidence of AgNPs formation was appreciated observing the color of QC 0.1 mM solution, which turned from light yellow to brown color (Fig. 1).

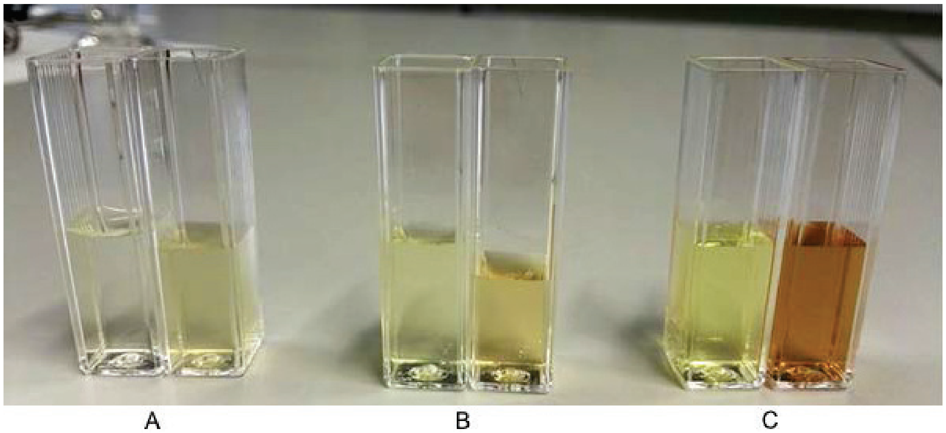

FIGURE 1. Preliminary evidences of AgNPs formation in response to pH variations (A=pH 7.5, B=pH 8.5, C=pH 9.2) are demonstrated by color change of the solution. The left-side cuvettes contained 0.1 mM QC and the buffer solution at different pH, while in the right-side cuvettes 25 µM $AgNO_3$ was further added.

Spectrophotometric and dynamic light scattering (DLS) characterization

The different solutions obtained in the previous paragraph were centrifuged at 100.000 *g* for 2 hours and the pellet resuspended in few mL of the same buffer solution. This resuspension was sonicated for 5 minutes and analyzed with Agilent 8453 UV-visible Spectroscopy System for the spectrophotometric analysis, and with Zeta Potential/Particle Sizer NICOMPTM 380ZLS for DLS analysis.

Single Particle Inductively Coupled Plasma Mass Spectrometry (spICP-MS)

The solutions obtained from the previous step were analyzed by spICP-MS (NexIon 350X, Perkin Elmer, USA). The solutions were sonicated for 5 minutes and diluted in order to obtain a final concentration of NPs in the range of 100.000 to 200.000 particles per mL. The spICP-MS parameters were set as follow: Dwell Time 100 µs, Sample Time 100 s, the Sample Flow Rate and the Transport Efficiency were daily calculated. The sample flow rate was obtained by aspirating MilliQ water for 5 minutes and checking the weight difference, while the transport efficiency was determined by using gold nanoparticles standards (AuNPs) of 30 and 60 nm size and gold dissolved solutions at 1, 2, 4 and 10 ppb, respectively.

Transmission electron microscopy (TEM)

The TEM samples were prepared by deposition of a single drop, almost 10 µl, of the solutions as it is on TEM Formvar/Carbon coated copper grid with 200 mesh and they were left to dry covered overnight.

RESULTS

Images obtained by TEM and DLS analysis

A first validation of the presence of silver NPs obtained using QC as reducing agent was obtained by TEM analysis (Fig. 2). A blank sample with a representative concentration of reducing agent (QC 0.1 mM) was used to validate the presence of silver NPs at the three pHs. Irrespective of the used pH, NPs were observed, although both in monodisperse and poly-disperse aggregates and with different dimensional classes. They were also almost characterized by a spherical conformation.

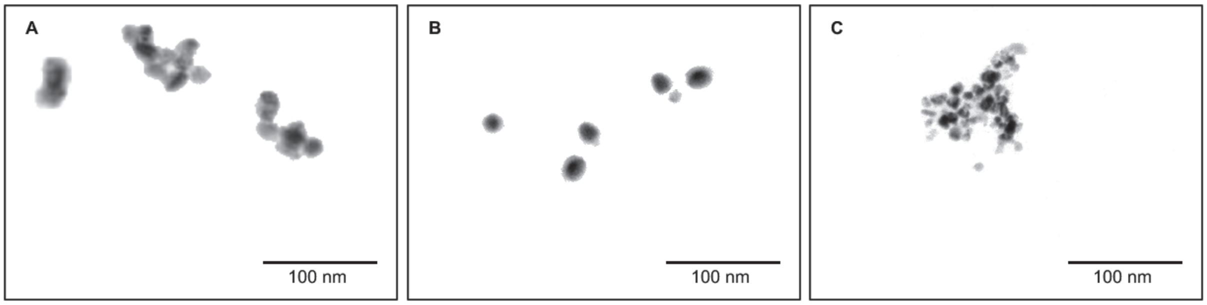

FIGURE 2. Representative TEM images of silver NPs obtained with QC (0.1 mM) at pH 7.5 (A), 8.5 (B) and 9.2 (C).

With regard to DLS analysis, at each pH two or three distinct groups of nanostructures were observed (Fig. 3). The information about each group of nanostructures is reported on Table 1.

The data show the presence of nanostructures with huge diameter, in particular 1180 nm, 191 nm and 106.1 nm at pH 7.5, 8.5 and 9.2, respectively. These nanostructures might be the consequence of QC polymerization and not of AgNPs formation (as later explained).

The DLS analysis puts on evidence the presence of one/two smaller groups of NPs, in particular 89.4 nm, 12/54 nm and 11.9 nm at pH 7.5, 8.5 and 9.2, respectively, exhibiting lower percentage of frequency than that one of the bigger aggregates. From these data, we observed that the smallest NPs increased from 3% to 14.8% when the pH value changed from 8.5 to 9.2.

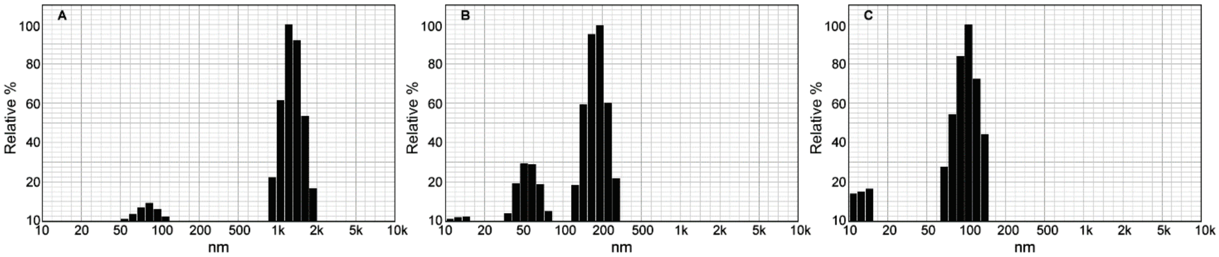

FIGURE 3. DLS analysis of silver NPs obtained with QC (0.1 mM) at pH 7.5 (A), 8.5 (B) and 9.2 (C).

TABLE 1. DLS analysis data for each group of nanostructure observed. Nanoparticles percentage values in respect to the bulk amount are reported between parentheses

	Parameter	1° group	2° group	3° group
pH 7.5	Diameter Av.	89.4 nm	1180 nm	
	St. Dev.	15.4nm (17.3%)	239.9nm (20.3%)	
	% of total	8.5%	91.5%	
pH 8.5	Diameter Av.	12 nm	54 nm	191 nm
	St. Dev.	1.6 nm (13%)	9.2nm (17.1%)	33.5nm (17.5%)
	% of total	3%	22%	75%
pH 9.2	Diameter Av.	11.9 nm	106.1 nm	
	St. Dev.	1.6 nm (13.4%)	23.8 nm (22.5%)	
	% of total	14.8%	85.2%	

Spectrophotometer analysis of silver NPs

After the AgNPs synthesis, 2 mL of silver NPs suspension were analyzed by a spectrophotometer to obtain plasmonic curves as a proof of the AgNP formation (Fig. 4). A previous spectrophotometric screening was performed to detect the best range of pH and $AgNO_3$/QC concentration able to induce high NPs synthesis. Figure 4 shows that the high concentration of QC (0.1 mM) was not able to induce a different amount of AgNPs if compared to the low concentration of QC (0.01 mM), at all tested pH, except for 9.2. Conversely, at this pH, NPs formation was strongly stimulated by 0.1 mM QC. The synthesis reaction was associated to the development of a yellow/brown coloration, whose intensity depended on QC concentration, as already shown in Fig.1.

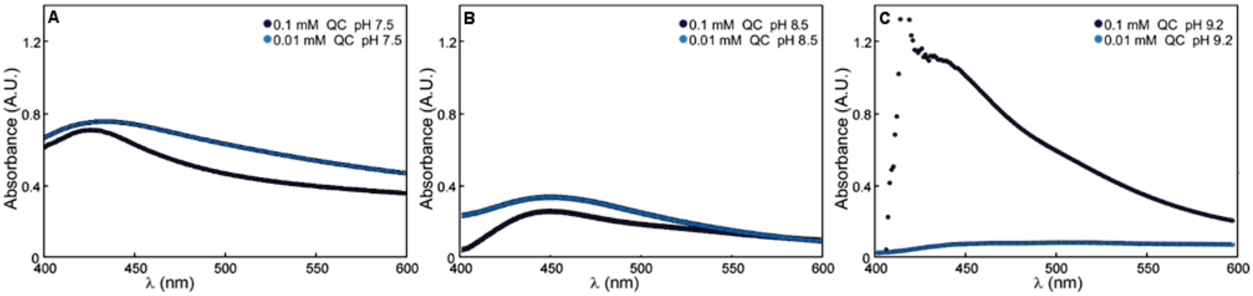

FIGURE 4. Absorption spectra of AgNPs suspension obtained with 0.1 (black) and 0.01 mM QC (blue), respectively, at pH 7.5 (A), 8.5 (B) and 9.2 (C). The synthesis of the AgNPs in buffer solution was monitored by recording the absorption spectra at a wavelength range of 400-600 nm.

spICP-MS

An aliquot of AgNPs suspension was analyzed using spICP-MS to obtain the distribution of AgNP dimensional classes present into each sample (Fig. 5). NPs formation was observed at three different pHs, although with minor differences in the medium size dimension. The solution with 0.1 mM QC appears to be the best reducing agent concentration, since the medium size of NPs was around 25 nm already at pH 8.5, whereas in the solution with 0.01 mM QC comparable NPs were present only at strongly basic conditions (pH 9.2).

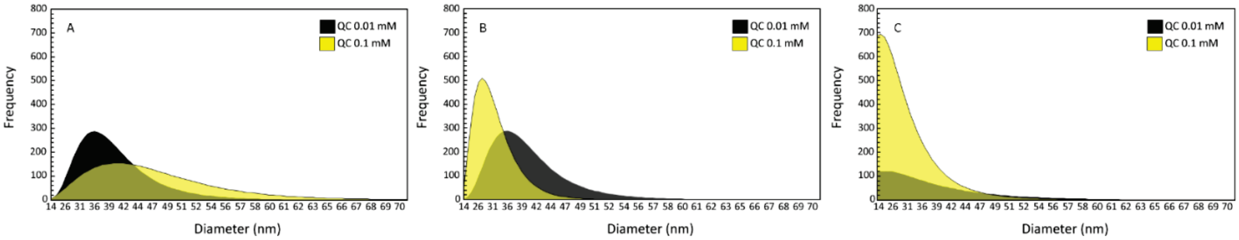

FIGURE 5. Frequency and diameters of AgNPs produced by 0.1 and 0.01 mM QC at respectively pH 7.5 (A), 8.5 (B) and 9.2 (C) and observed at spICP-MS.

DISCUSSION

Physico-chemical properties of NPs confer them a huge potential value in engineering (inks and microelectronics), medicine (bactericidal and fungicidal activity), industry (plastics, soaps, pastes, food and textiles) and for the environment (air disinfection, water purification, soil detoxification). According to the Project on Emerging Nanotechnologies (PEN) among 1300 nanotechnology-enabled products within the global market, there are 313 nanosilver products (24% of products listed), making it the largest and fastest growing class of NPs in consumer product applications. Because of their widespread applications, the scientific community and industry has paid special attention to AgNPs and in particular on green eco-friendly NPs synthesis. When AgNPs are produced by chemical synthesis, three main components are needed: a silver salt, a reducing agent to reduce ions to 0-charged molecules and a stabilizer or capping agent to control the growth of the NPs and prevent them from aggregation. In case of the green synthesis of AgNPs, molecules produced by living organisms replace the reducing agents and the stabilizers. These reducing and/or stabilizing compounds can be obtained from bacteria, fungi, yeasts, algae or plants [10].

Green chemistry principles maximize safety and efficiency and minimize the environmental and social impact of toxic raw materials. Green synthesis of NPs focuses on three important aspects i.e., (i) use of green solvents, (ii) use of an eco-friendly reducing agent, and (iii) use of a non-toxic material as a stabilizer [11].

Plant extracts contains enzymes (hydrogenases, reductases) and phytochemicals, such as terpenoids, flavonoids, phenols, substituted phenols and so on, acting both as reductants and capping agents in the presence of metal salt for NPs synthesis [12].

Although in literature is present a huge number of papers regarding hundreds of different plant extracts and plenty of information is available about the NPs characteristics (dimension, shape and quantity) for each extract, currently the knowledge on which molecules contribute to the process - and how - is quite poor [8, 13. 14].

With the aim to clarify this problem, we focused the attention on a common class of secondary metabolites (flavonoids), known to be an essential part of the reducing/oxidizing system of plants. Among this class of strong antioxidant molecules, we consider quercetin (QC) as a good candidate, as it is one of the most naturally abundant and easy to extract flavonoid.

Since dimensions and quantity are two of the most important parameters to be considered during industrial synthesis, an initial screening using spectrophotometric analysis (data not shown) was performed to set the better conditions and the most suitable parameters to obtain the largest amount of smaller NPs.

According to these preliminary assays, a fixed $AgNO_3$ concentration (25 μM), two QC concentrations (0.1 and 0.01 mM) and three different pH values (7.5-8.5-9.2) were used, performing the synthesis in round brown bottles, at room temperature for 30 minutes, with shaking. Once set the parameters, the highest concentration of reducing agent (QC 0.1 mM) was used as reference standard for the TEM and DLS analysis (Fig. 2-3), with the aim to characterize the formation of AgNPs. Considering the increasing solubility and color intensity of QC solution (without silver nitrate) at higher pHs, the NPs synthesis could be followed by observing a clear color change in all the three treatments (Fig. 1). This color change is typically due to AgNPs formation and the results are in agreement with the literature, which identifies the basic pH as the best condition for NPs synthesis [5]. In fact, in our experiment the most pronounced color change occurred at pH 9.2 (Fig. 1, panel C).

In TEM images, at each pH solution NPs formation could be observed, although characterized by different average (mean) sizes: the smallest ones at pH 9.2 and the largest ones at 7.5 and 8.5 (Fig. 2). Similar results are obtained by DLS analysis, even though presenting different distributions of dimensional sizes. Although the DLS technique is widely used for particle characterization, there are some limits in the case of measurement of samples with large-size distribution or multimodal distribution (as it is our case). In the case of polydisperse colloids, DLS analysis could not discriminate small objects or particles screened by the largest ones. In addition, a hydration/capping layer must be considered to cause a general increase in final measured particle diameter [15]. For all these reasons, only general considerations can be formulated:

(i) at increasing pH, there is a general increase in the small NPs;

(ii) at increasing pH, there is a general decrease in total particle sizes;

(iii) although actual quantity of each single NPs could not be deduced, the smallest NPs are less frequent with respect to the other dimensional classes;

In agreement with the results found at TEM and DLS analysis, the spectrophotometric assay evidences a highest broad peak at 420 nm and the strongest difference was observed when the treatments by the two QC concentrations at pH 9.2 were compared (Fig. 4). At 7.5 and 8.5 pH values, spectrophotometer analysis exhibits weaker peak intensity around 420 nm, without a large difference between the two concentrations. This effect is probably due to the poor solubility of QC at pH close to neutral [16] and to the positive effect exerted by basic environments on the synthesis of small sized NPs [17].

Among the techniques for NPs characterization, the most commonly and easy to use are DLS and UV-Vis spectroscopy, although none of them are actually able to carefully define the metal composition of the particle under analysis, giving only general information on the aggregate dimension of the solution. For this reason, spICP-MS was used to obtain more accurate information regarding specifically Ag nanomaterial. The Figure 5 shows that all the assayed pHs induce the AgNPs formation, although with a negligible difference in their average size, in accordance with the previous discussed results. Taking in account that the sensitivity and the accuracy of spICP-MS have a NPs size distribution detection limit (denoted as Dmin) of approx. 20 nm for AgNPs [18], the small NPs observed in DLS analysis and TEM could not be detected by the spICP-MS output. Considering the 0.1 mM concentration, the spICP-MS analysis can however evidence that the aggregates bigger than 100 nm, observed with DLS, seemed not to be ascribed to silver metal, because the spICP-MS that measures only the presence of silver element did not reveal any NP whose dimensional size exceeded 90 nm. From this evidence, we concluded that the larger aggregates detected in the DLS are not composed by silver element, but consist only in aggregates of the reducing agent QC.

The frequency labelled in the figure axis expresses the counts per second (CPS) and shows the distribution of the most frequent dimensional class of NPs contained into the sample volume, though not providing a true direct quantification of the NPs present into the sample.

These preliminary results need to be further corroborated with experiments to confirm, with a more accurate analysis by means of TEM images, the actual dimensional classes of AgNPs and their quantitative yield as an effect of different QC concentration use.

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