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ORIGINAL ARTICLE

L-Leucine for gold nanoparticles synthesis and their cytotoxic effects evaluation

Camelia Berghian-Grosan · Liliana Olenic · Gabriel Katona · Maria Perde-Schrepler · Adriana Vulcu

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Abstract This work reports the preparation of water-soluble leucine capped gold nanoparticles by two single-step synthesis methods. The first procedure involves a citrate reduction approach where the citrate is used as reducing agent and leucine as capping/stabilizing agent. Different sizes of gold nanoparticles, citrate reduced and stabilized by leucine, Leu-AuNPs-C, with the mean diameters in the range of 21–56 nm, were obtained by varying the macroscopic parameters such as: concentration of the gold precursor solution, Au (III):citrate molar ratio and leucine pH. In the second procedure, leucine acts both as reducing and stabilizing agent, allowing us to obtain spherical gold nanoparticles, Leu-AuNPs, with a majority of 80 % (with the mean diameter of 63 nm). This proves that leucine is an appropriate reductant for the formation of water-soluble and stable gold nanoparticles colloids. The characterization of the leucine coated gold nanoparticles was carried out by TEM, UV–Vis and FT-IR analysis. The cytotoxic effect of Leu-AuNPs-C and Leu-AuNPs was also evaluated.

Keywords Leucine capped gold nanoparticles · Citrate reduction method · Leucine reduction method · Cytotoxic effect · HaCaT cells · A431 cells

Introduction

In the last years, gold nanoparticles (AuNPs) have received special attention owing to their unusual chemical (surface reactivity, catalytic), electronic, magnetic, optical properties and their wide-ranging applications (Daniel and Astruc 2004). In this context, the amino acid capped gold nanoparticles were synthetized and used for different studies (Zhong et al. 2004; Joshi et al. 2004; You et al. 2005; Lim et al. 2007; Ghosh et al. 2008; Majzik et al. 2010; Stobiecka and Hepel 2011).

Functionalization of gold nanoparticles with amino acids can be realized by two methods: (1) the ligand exchange reaction and (2) the amino acid reduction method. On the first case, the gold nanoparticles were obtained by the reduction of Au^{3+} with sodium tetrahydridoborate ($NaBH_4$) or trisodium citrate (Na_3Cit). The most common method used for the synthesis of the monodisperse gold nanoparticles is the reduction of tetrachloroauric acid ($HAuCl_4$) by citrate in aqueous media and at high temperature (Turkevich et al. 1951; Frens 1973). During this reaction, the sodium citrate acts both as a reducing agent and as a capping/stabilizing agent. Then, the amino acid capped gold nanoparticles were obtained by aging amino acid and gold hydrosol aqueous solutions (Zhong

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C. Berghian-Grosan (✉) · L. Olenic · A. Vulcu (✉)
National Institute for Research and Development of Isotopic and Molecular Technologies, 67-103 Donat Street, 400293 Cluj-Napoca, Romania
e-mail: camelia.grosan@itim-cj.ro

A. Vulcu
e-mail: adriana.vulcu@itim-cj.ro

G. Katona
Faculty of Chemistry and Chemical Engineering, Babes-Bolyai University, 11 Arany Janos Street, 400028 Cluj-Napoca, Romania

M. Perde-Schrepler
Department of Radiotherapy, Tumor and Radiobiology, Ion Chiricuta Oncologic Institute, 34-36 Republicii Street, 400015 Cluj-Napoca, Romania

et al. 2004; Aryal et al. 2006a). However, some limitations of this method should be noticed: the poor monodispersity of the nanoparticles greater than 30 nm and the necessity to get an exchange reaction of the citrate for another capping ligand, if some functional groups are needed to the gold surface. The second method is a "green" synthesis of AuNPs. The amino acid behaves as reducing and capping agent and thus, it is fixed directly on AuNPs during the synthesis. Tryptophan, lysine, cysteine, histidine, aspartic and glutamic acids are known to reduce gold salts and to yield stable gold nanoparticles (Selvakannan et al. 2004; Mandal et al. 2005; Ma and Han 2008; Liu et al. 2010; Mandal et al. 2002; Wangoo et al. 2008). Tan et al. (2010) studied the Au (III) reduction capability of the 20 natural α-amino acids under the same reaction conditions and theirs Au^0-binding affinity. They found out that leucine exhibits a weak reducing power for $AuCl_4^-$ and a weak binding interaction with gold.

L-Leucine is one of the three proteinogenic BCAAs (Branched-Chain Amino Acids) and one of the nine essential amino acids. BCAAs are used for different studies (Choudry et al. 2006; Takeshita et al. 2012), including their effect on the immune system (Calder 2006). The complex nanoparticles of bismuth and leucine (with the average diameter of 80 nm) are prepared at room temperature by solid–solid reaction (Jia et al. 2005), while the salbutamol sulphate nanoparticles coated with L-Leucine are prepared in the gas phase (Lähde et al. 2008). A series of amino acids including leucine were bound to nano-sized magnetite by two-step transformation process (Tie et al. 2006; Lee et al. 2008). Regarding the leucine-capped gold nanoparticles, obtained by a two-step procedure, there is a study about the nanoparticles aggregation in the presence of various concentrations of electrolyte (Aryal et al. 2006b).

For biological and biomedical applications, it is important for colloidal solutions to be biocompatible and to have non-cytotoxic effects. Because gold is chemically stable and less cytotoxic, the gold nanoparticles are widely employed in biomedical applications (Dykman and Khlebtsov 2012). However, there are scientific reports that present gold nanoparticles as being nontoxic (Connor et al. 2005), toxic (Goodman et al. 2004; Pernodet et al. 2006) or size-dependent cytotoxic (Pan et al. 2007). Alkilany and Murphy (2010) discussed the recent results obtained in the field of gold nanoparticles toxicity, both in vitro and in vivo, and highlighted the controversial conclusions regarding this subject. Chithrani et al. (2006) proved that the spherical shaped NPs are better internalized than the rod shaped ones. The uptake of NP can alter cellular function, but there is the possibility to have no negative effects. The NP interaction with the cellular surface can also have toxic effect on the cells, even if they are not internalized (Johnston et al. 2010).

In this work, the synthesis of leucine capped gold nanoparticles by two different methods was investigated. The first method implied the reduction of $HAuCl_4$ by a reductant, trisodium citrate, and the displacement of citrate ions by amino acid, while the second one used the amino acid both as reducing and stabilizing agent. The size-control of gold nanoparticles (citrate reduced and stabilized by leucine, Leu-AuNPs-C) by varying parameters such as gold precursor solution concentration, Au:citrate molar ratio and leucine pH was realized. The gold nanoparticles reduced and stabilized by leucine (Leu-AuNPs) were obtained. Although leucine is known as amino acid with a weak reducing power for gold salts, we have shown that its use as reductant leads to the formation of stable, water soluble gold nanoparticles. The TEM, UV–Vis and FT-IR analysis were used to characterize the gold colloidal solutions or gold nanoparticles surfaces. For the assessment of cytotoxicity of the leucine coated gold nanoparticles on skin cells, two cell lines were used: HaCaT, a normal, spontaneously immortalized keratinocytes cell line and A431, an epidermoid carcinoma cell line.

Experimental

Materials and instruments

Tetrachloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$, 99.5 %), and trisodium citrate ($Na_3Cit \cdot 2H_2O$) were purchased from Merck and L-Leucine 99 % was bought from Biosynth. The 99 atom % ^{15}N-L-Leucine was obtained by our research group. All chemicals were used as received and all solutions were prepared using double-distilled water. For in vitro cytotoxicity studies, normal keratinocytes (HaCaT) were purchased from the cell line service of the German Cancer Research Center (Heidelberg, Germany).

A shimadzu spectrophotometer was used for characterization of the new colloidal solutions. The morphology and size distribution of synthesized gold nanoparticles were realized using a transmission electron microscope (H-7650 120 kV Automatic TEM, Hitachi, Japan). Fourier transform infrared (FT-IR) studies were performed to establish the structure of the ligand that covers the gold nanoparticles. The dried gold nanoparticles were analyzed by a FT-IR spectrometer (JASCO 6100); the infrared spectra were recorded with a resolution of 4 cm^{-1} from 4000 to 500 cm^{-1} and using KBr pellet technique.

Procedure for gold nanoparticles synthesis

Leucine-functionalized gold nanoparticles were synthesized, in aqueous media and at high temperature, using two chemical reduction methods: (1) the trisodium citrate reduction (Turkevich et al. 1951) and (2) the amino acid (L-Leucine) reduction. On the first case, the citrate serves as reducing agent and the citrate ions are exchanged in situ by amino acid, while for the second one, the amino acid acts also as reducing and stabilizing agent.

Synthesis of citrate reduced gold nanoparticles coated with L-Leucine

Citrate reduced gold nanoparticles coated with L-Leucine were prepared according to Turkevich-Frens method with small variation. Thus, to 100 mL heated solution of $HAuCl_4$ 0.254 mM (Au 5 mg, 0.0254 mmol), 10 mL solution of L-Leucine 10^{-2} M (13.12 mg, 0.1 mmol) was added. When the mixture started to bubble, 3.6 or 0.75 mL of trisodium citrate 38.8 mM (see Online Resource) was added under vigorous stirring. This solution was boiled for 5 min, under constant stirring, to get a wine-red colour and then, it was let to reach the ambient temperature.

Synthesis of L-Leucine reduced gold nanoparticles (Leu-AuNPs)

During this reaction the amino acid acts both as reducing agent and as capping/stabilizing agent; the Au:leu molar ratio is 1:4. In a typical experiment, when the 100 mL solution of $HAuCl_4$ 0.254 mM (5 mg Au, 0.0254 mmol) reached the boiling point, 10 mL solution of L-Leucine 10^{-2} M (13.12 mg, 0.1 mmol) was added under vigorous stirring. The mixture has boiled for another 5 min and then let to achieve the room temperature. During the reaction, the solution colour changed from yellow pale (the first 2–3 min after the loading of amino acid) to a mauve pale and mauve with a brown suspension (to the final of the 5 min of reaction).

Cytotoxicity assay

The cell lines were maintained in specific media: HaCaT in high glucose (4.5 g/L) DMEM, with 10 % foetal calf serum, glutamine and antibiotics; for A431, the media was MEM, supplemented with 1 % NEA, 10 % foetal calf serum, glutamine and antibiotics. Cells were plated in 96-well plates, 20,000 cells/well. The cells were kept at 37 °C in a humid incubator with 5 % CO_2. Three types of colloidal solutions were investigated: AuNPs-C, Leu-AuNPs-C and Leu-AuNPs. The series of dilution (dilution factor 200×, 80×, 40×, 20×, 10×, 4×, 2×, 1.33×) of gold nanoparticles in the medium were added to the plates. For each experiment, three wells were used for each concentration and at least three wells were left untreated (control). The toxicity effect was assessed by microscopic observations of cell viability. After 24 h of the treatments, cell viability was assessed by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide]. The tetrazolium salt (MTT) is reduced to a colored compound (formazan) only by metabolically active cells, by activation of mithocondrial hydrogenase. Absorbance was read on a Biotek Synergy 2 microplate reader, at 492 nm. Each individual experiment was repeated at least three times.

Results and discussion

Leucine coated gold nanoparticles synthesis (Leu-AuNPs-C and Leu-AuNPs)

The preparation, in one-step, of the gold nanoparticles functionalized with the amino acid L-Leucine, was investigated by two procedures: the first method implied the reduction of $HAuCl_4$ by a reducing agent (trisodium citrate) and the displacement of citrate ions by amino acid, while the second one used the amino acid both as reducing and stabilizing agent. The characteristic features of leucine capped gold nanoparticles synthetized by the two methods are depicted in Figs. 1 and 2.

The UV–Vis spectroscopy was first used for physical characterization of gold nanoparticles, and the size of the gold nanoparticles was confirmed by the TEM measurements. We observed that a molar ratio Au:leu of 1:4 leads to the obtaining of gold nanoparticles functionalized with leucine, no matter which reducing method was used (Fig. 1). The diversity of shapes must be noticed: spheres, triangles, rodes, pentagons, obtained using the reducing method with leucine. In this case, the spheres have a majority of only 80 %, as opposed to the case with the citrate reducing method, when there were obtained only monodisperse spherical gold nanoparticles.

In the UV–Vis spectrum, the sharp absorbance peak located at 519 nm, for Leu-AuNPs-C, indicates the formation of spherical small nanoparticles with good monodispersity. The TEM image and the particles size distribution realized over 200 nanoparticles confirm the UV–Vis analysis: the majority of leucine capped gold nanoparticles obtained by citrate reduction method (Leu-AuNPs-C) have a diameter of 21 nm and a good monodispersity. For leucine reduced/capping gold nanoparticles (Leu-AuNPs), we can see two absorbance peaks in the visible region, a sharp absorbance at 553 nm and a broad shoulder at a higher wavelength (around 757 nm). The red shift of 34 nm observed for Leu-AuNPs indicates an increase in the size of

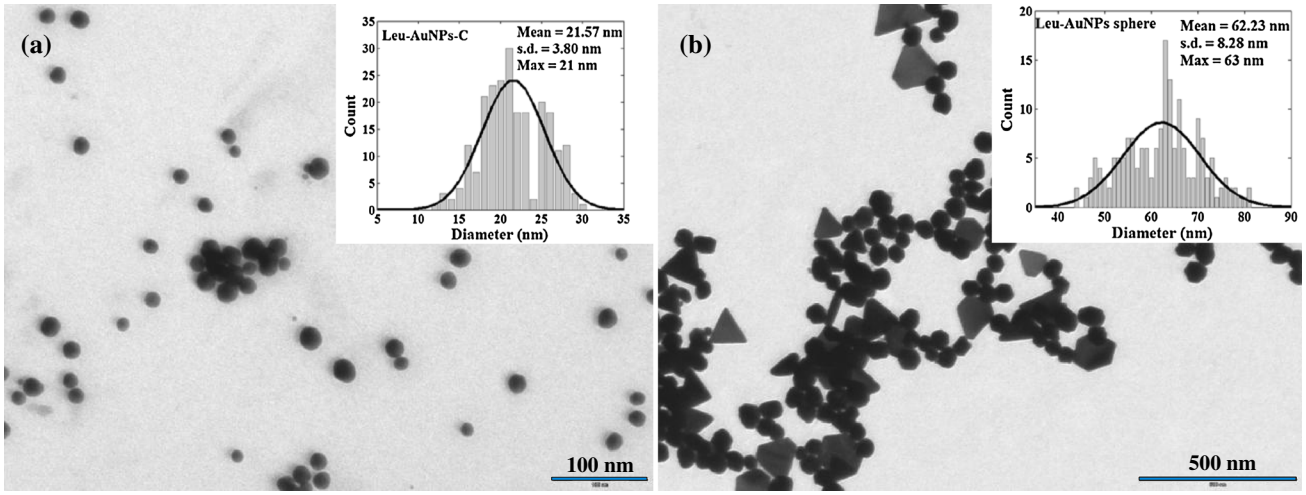

Fig. 1 **a** Representative TEM image of leucine capped gold nanoparticles obtained by citrate reduction method (Leu-AuNPs-C), *inset*: particle size distribution of Leu-AuNPs-C, **b** representative TEM image of leucine capped gold nanoparticles obtained by leucine reduction method (Leu-AuNPs), *inset*: particle size distribution of spherical Leu-AuNPs

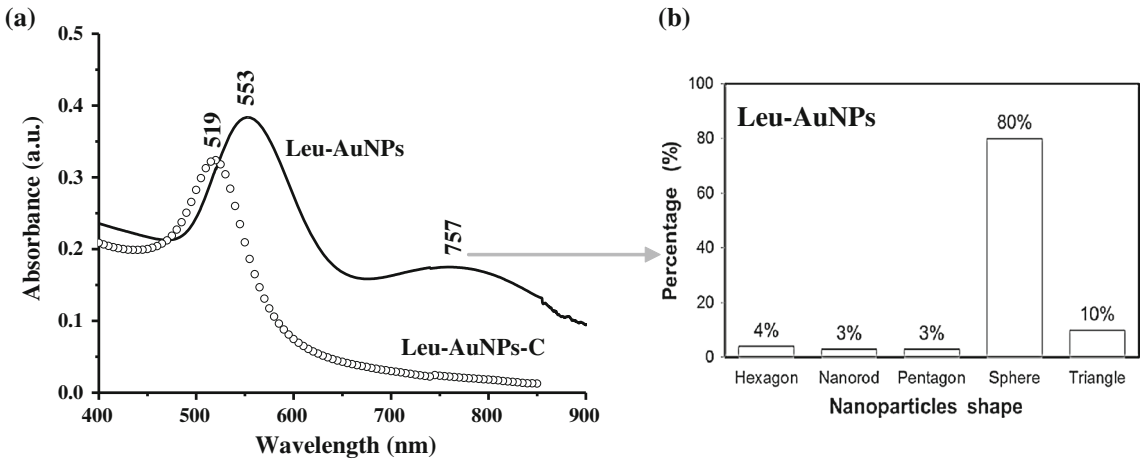

Fig. 2 **a** UV–Vis spectra of Leu-AuNPs-C and Leu-AuNPs, **b** abundance of different nanoparticles shapes for Leu-AuNPs

nanoparticles, from 21 nm, for Leu-AuNPs-C to 63 nm for Leu-AuNPs (Fig. 2a) while the broad shoulder from 757 nm can be assigned both to the aggregated and anisotropic structures from the colloidal solution.

In conclusion, using our one-step protocol, we obtained with good monodispersity, leucine-coated gold nanoparticles with a maximum of 21 nm diameter. On the other hand, the usage of leucine as reducing and capping agent allowed us to obtain larger spherical gold nanoparticles (max 62 nm) but only with 80 % percentage (Fig. 2b).

Leucine coated gold nanoparticles synthesis at a low citrate level (Leu-AuNPs-C_{min})

Furthermore, we are interested to employ a low quantity of reducing agent to obtain larger spherical gold nanoparticles. Thus, we have used a 1:4:1.15 Au:leu:citrate molar ratio; at this low level of citrate, the leucine ability to reduce could be seen, and also its reduction and capping capacities would be influence of its pH level. Taking account of its p*K*a and p*I* levels, we decided to test three types of leucine solutions with a pH of 9.2, 5.7 and 2.1. The characteristics of the colloidal solutions obtained from leucine with different pH values are presented in Figs. 3 and 4.

The colloidal solution obtained from a leucine with pH 5.7 (common pH of aqueous leucine solution) has a relatively good monodisperisity and contains spherical gold nanoparticles with a maximum diameter of 56 nm (Fig. 3a); the Vis absorbance peak for this solution is located at 539 nm (Fig. 4). Starting from a leucine solution with a pH of 9.2, the gold nanoparticles obtained revealed a

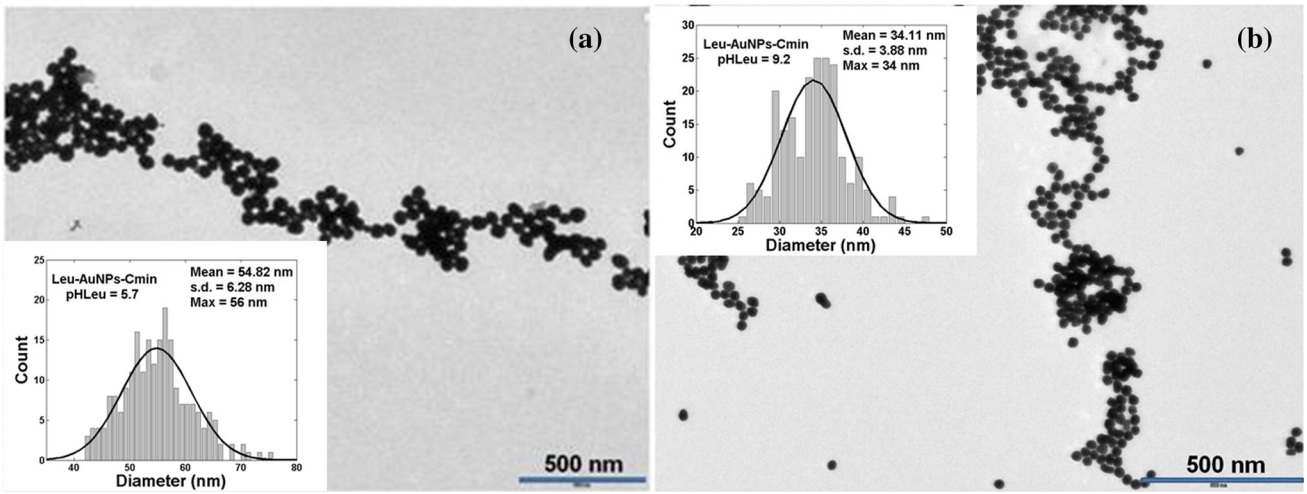

Fig. 3 **a** Representative TEM image of leucine capped gold nanoparticles obtained with a low level of trisodium citrate (Leu-AuNPs-C_{min}) and a leucine solution with a pH 5.7, *inset*: particle size distribution of Leu-AuNPs-C_{min}, pH-Leu 5.7, **b** representative TEM image of leucine capped gold nanoparticles obtained using a low quantity of citrate reduction agent (Leu-AuNPs-C_{min}) and a Leucine solution with a pH 9.2, *inset*: particle size distribution of Leu-AuNPs-C_{min}, pH-Leu 9.2

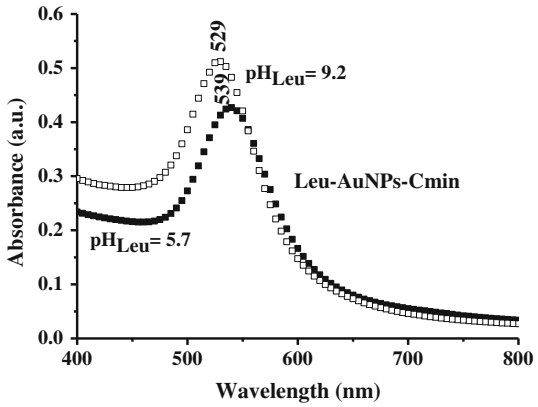

Fig. 4 UV–Vis spectra of Leu-AuNPs-C_{min} obtained using different pH Leucine solutions

visible peak at 529 nm, a spherical shape and a maximum diameter of 34 nm (Figs. 3b and 4). It must be noticed that, using a leucine solution with a pH of 2.1, the preparation of gold nanoparticles wouldnot be possible.

Gold nanoparticles surface characterization

To further characterize the structure of the ligand that covers the gold nanoparticles, the FT-IR analysis were conducted, see online resource for AuNPs purification procedure. To identify unambiguously the observed absorption bands, the effect of ^{15}N-labelling on the absorption frequency was first measured and compared with the corresponding ^{14}N-L-Leucine vibrations. The FT-IR spectra of L-Leucine and ^{15}N-L-Leucine are presented in online resource, Fig. S1.

It is well known that in solid state, the amino acids exist in the zwitterionic form. This situation is also encountered in our case. The spectrum of L-Leucine was found identical to that of ^{15}N-L-Leucine, except in what concerns the bands related to the isotope ^{15}N, Fig. S1. Thus, in the 3500–400 cm^{-1} spectrum of 99 atom % ^{15}N-L-Leucine, only several significant ^{15}N-sensitive peaks ($\Delta\nu > 3$ cm^{-1}) are observed at 3039 (NH_3^+ stretching, ν NH_3^+), 1509 (symmetric NH_3^+ deformation, δ_s NH_3^+), 530 cm^{-1} (C-C-N deformation, δ CCN), as compared with the corresponding ^{14}N-L-Leucine bands at 3055, 1515 and 535 cm^{-1} (Colthup et al. 1990; Wolpert and Hellwig 2006). Additionally, the band at 2128 cm^{-1} is due to a combination band of asymmetric NH_3^+ deformation (δ_{as} NH_3^+) and NH_3^+ hindered rotation, whereas the peak at 1607 cm^{-1} is assigned to the asymmetric NH_3^+ deformation, δ_{as} NH_3^+. These vibrations are shifted by 2 cm^{-1} to the high frequency in the ^{14}N-L-Leucine spectrum. The peaks at 1582 and 1407 cm^{-1} are unshifted and consequently, the two bands could be attributed to the asymmetric and symmetric COO^- stretching vibrations respectively (ν_{as} and ν_s COO^-).

In Fig. 5 are plotted for comparison the spectrum of L-Leucine and L-Leucine coated gold nanoparticles obtained by citrate reduction method (low level of reducing agent). In the spectrum of Leu-AuNPs-C_{min}, we observed the disappearance of the bands located at 3055, 1609 and 1515 cm^{-1} related to NH_3^+ vibrations and the appearance of a peak at about 1626 cm^{-1} than can be assigned to the bending mode of NH_2 group, δ NH. In addition, the broad band in the region 1260–975 cm^{-1} with a maximum at about 1086 cm^{-1} could be due to the intermixed NH_2

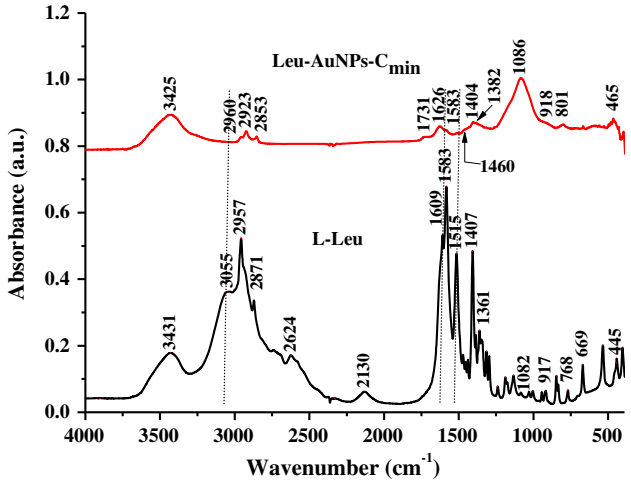

Fig. 5 FT-IR spectra of L-Leucine and L-Leucine capped gold nanoparticles Leu-AuNPs-C_{min} (obtained using citrate reduction), in the 4000–400 cm^{-1} spectral domain

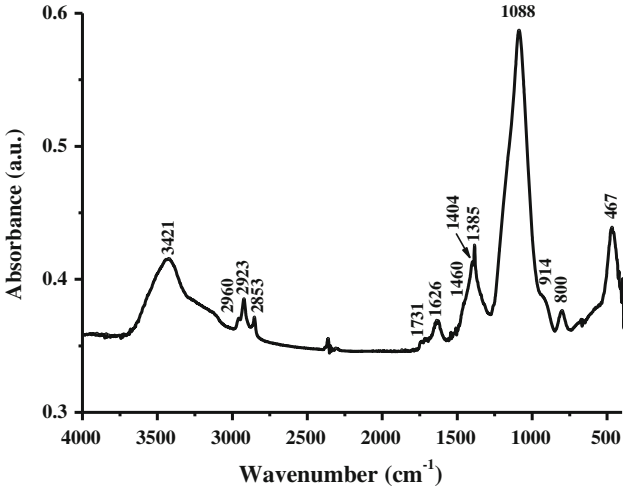

Fig. 6 FT-IR spectra of L-Leucine capped gold nanoparticles, Leu-AuNPs in the 4000–400 cm^{-1} spectral domain

wagging (ρ NH_2) and C–N stretching (ν CN) vibrations. The intensity of this peak reveals that the amino group is bounded to the gold surface. The other two vibrations located at 1460 and 1382 cm^{-1} could be associated with the CH_2 scissors and the CH_3 asymmetrical deformations, δ CH_2 and δ CH_3. The presence of the asymmetric and symmetric carboxylate stretches, ν_{as} COO^- and ν_s COO^-, at about 1583 and 1404 cm^{-1} (in L-Leucine these band appears at 1583 and 1407 cm^{-1}) together with the ν C=O vibrations of the COOH groups at 1731 cm^{-1} also reveals that the two groups (COO^- and COOH) are present at the surface of gold nanoparticles.

In addition, the existence of the asymmetric and symmetric vibrations of carboxylate COO^- shows that this group is adsorbed at the surface of gold nanoparticles in an asymmetric mode (with the oxygen atoms at different distances from the surface). Combining all these data, we suppose that the amino acid is preponderantly adsorbed to the gold surface in its anionic form (NH_2RCOO^-).

Figure 6 shows the FT-IR spectra of the gold nanoparticles reduced/stabilized with leucine (Leu-AuNPs). The band related to ν_{as} COO^- is not clearly seen in the spectrum of Leu-AuNPs. This situation might occur when the COO^- from amino acid is bound symmetrically to the gold surface, but further investigations need to be made to determine exactly the modes in which the functional groups are adsorbed to the gold surface. The bands related to the CH_2 scissors and CH_3 asymmetrical deformations, δ CH_2 and δ CH_3, are located at 1460 and 1385 cm^{-1}. The intensity of the band related to NH and C–N vibrations with a maximum at about 1088 cm^{-1} proves that the amino acid bonds to gold nanoparticles through the amino group. Similar to Leu-AuNPs-C_{min}, the amino acid is adsorbed to the gold surface in its anionic form (NH_2RCOO^-).

Effect of the gold nanoparticles on HaCaT and A431 cells viability using MTT assay

For the assessment of gold nanoparticles cytotoxicity on skin cells, two cell lines were used: HaCaT, a normal, spontaneously immortalized keratinocytes cell line and A431, an epidermoid carcinoma cell line. The in vitro effect of the three types of colloidal solutions, citrate capped gold nanoparticles (AuNPs-C), leucine coated gold nanoparticles, obtained by citrate reduction method (Leu-AuNPs-C) and gold nanoparticles functionalized with leucine, prepared by amino acid reduction protocol (Leu-AuNPs) was investigated. We are interested to test the obtained colloidal solutions of citrate coated gold nanoparticles (AuNPs-C) and leucine capped gold nanoparticles (Leu-AuNPs-C and Leu-AuNPs) without significant modifications (see online resource for AuNPs-C synthesis and AuNPs purification procedure). In this regard, the treatment implies the purification and concentration procedure to obtain threefold higher concentrated solutions. The results reveal a more important decrease in cell viability on A431 cells compared to HaCaT at all doses (Fig. S2).

In addition, in Fig. 7, we present the regression curves (second order polynomial) for the differences between the survival rates of HaCaT and A431 cells when the gold concentration is variable.

On one hand, we observed that for gold concentrations higher than 150 μM, the highest difference is obtained for Leu-AuNPs-C while the lowest is for AuNPs-C. This situation could be due to the presence of leucine to the surface of gold nanoparticles, while the diameters of the two types of gold nanoparticles, Leu-AuNPs-C and AuNPs-C, have close values. However, the usage of larger leucine-capped gold nanoparticles leads to a lower survival

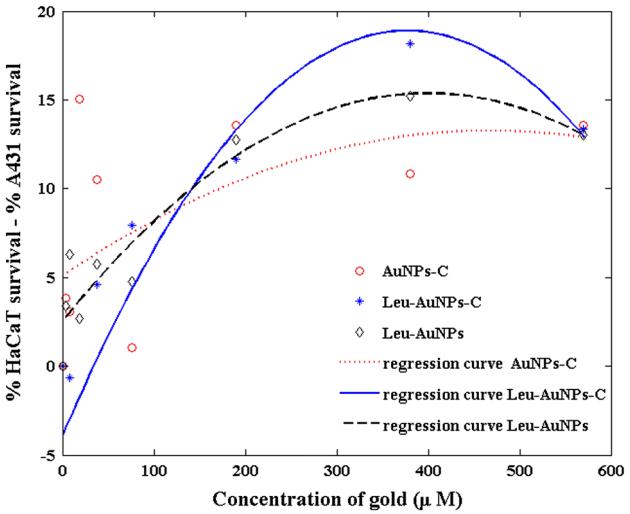

Fig. 7 Difference of survival rate of keratinocytes (HaCaT) and epidermoid carcinoma (A431) cells for gold nanoparticles AuNPs-C, Leu-AuNPs-C and Leu-AuNPs

difference. We suppose this happens because of the dimension of Leu-AuNPs nanospheres (63 nm) which is much higher than the dimension of Leu-AuNPs-C (21 nm). The agglomeration of larger nanoparticles can also greatly influence the interaction between cells and gold nanoparticles. On the other hand, the presence of different non-spherical shapes (20 %) in the Leu-AuNPs colloidal solution could also induce a cytotoxic effect.

Conclusions

We demonstrated the capability of leucine to bind to the gold nanoparticles surface and also its reducing capability for gold ions.

Using citrate reduction procedure and a relatively low Au precursor concentration solution, we obtained well-dispersed or long-branched chains gold nanoparticles; the control of nanoparticles size was realized by adjusting the Au:citrate ratio or leucine pH.

When leucine was used as reducing and capping agent, we observed a tendency to anisotropic growth (formation of nanoplates 20 %), but the spherical gold nanoparticles were preponderated (80 %). The colloidal solutions and gold nanoparticles surface characterization was realized by TEM, UV–Vis and FT-IR analysis. The results showed the presence of leucine at the surface of gold nanoparticles.

Regarding the cytotoxic effect, all nanoparticles decreased cell viability in a dose-dependent manner. This reduction in cell viability was more important on A431 cells compared to HaCaT at all doses. Further investigations need to be made to evaluate the role of non-spherical shapes towards the induced toxicity and also to elucidate the mechanism of cell death.

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Conflict of interest The authors declare that they have no conflict of interest.

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