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GOLD NANOPARTICLES FUNCTIONALIZED WITH ANTICANCER BIOCOMPOUNDS

Madalina Anca UJICA[a], Ionel MANG[a], Ossi HOROVITZ[a], Aurora MOCANU[a], Maria TOMOAIA-COTISEL[a,b,*]

ABSTRACT. The focus of this work is on the functionalization of gold nanoparticles, GNPs, with doxorubicin, D, an anticancer drug, both in the absence and in the presence of natural adjuvant biomolecules, like piperine, P, resveratrol, R, resveratrol-piperine, RP, complex, and icariin, I, which are therapeutic molecules with demonstrated anticancer and anti-inflammatory activity, to form highly stabilized colloidal dispersions. The green syntheses of GNPs, as cores, loading self-assemblies of various selected biomolecules, adsorbed on their surface, as shells, was confirmed by observing surface plasmon resonance at about 538 nm. Further, gold nanoparticles stabilized by resveratrol, GNP-R, are functionalized with various concentrations of selected biomolecules: D, P, R, RP, and I, resulting in different D/P/R/RP/I@GNPs-R composite nanoparticles for various compositions. Another series of stabilized colloidal dispersions is generated as GNP-R1, where the initial GNP-R is centrifuged and washed and then it is dispersed in aqueous solutions and further functionalized with said selected biomolecules. This study proves the functionalization of GNPs, as composite nanoparticles of high stability, in the presence of phosphate buffer saline, PBS, as confirmed by UV-Vis spectra of their colloidal aqueous dispersions.

Keywords: *gold nanoparticles, doxorubicin, adjuvant anticancer molecules, UV-Vis spectroscopy, surface plasmon resonance*

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INTRODUCTION

Gold nanoparticles, GNPs, are regularly used in cancer treatment due to their capability to carry drugs and therapeutic adjuvants to designated targeting tumors, while reducing the drug side effects [1, 2] and also improving the efficiency of pharmaceuticals with low water solubility [3--5]. The production and characterization of gold nanoparticles have generated a distinctive progress in the field of cancer treatment [6-8]. Firstly, the synthesis of spherical GNPs was initiated by Turkevich [9]; HAuCl4 is treated with citric acid in boiling alkaline aqueous solution, in which sodium citrate acts as both reducing agent and stabilizing agent. Later, Frens [10] revealed that different proportions of $HAuCl_4$ and citrate can be used to produce spherical GNPs of controllable size, which is still in use today. The GNPs provide a large surface area, easily to be functionalized and ability for translocation into the cells, achieving varied functions for many uses [11]. The GNPs allow controlled release of therapeutic constituents, while enhancing the efficiency of cancer treatment [1, 2, 11].

Therapeutic adjuvant substances, such as curcumin and flavonoids [12,13], as well as carotenoids [14,15] play a crucial role as antioxidants, anti-inflammatory and anticancer compounds. It is also demonstrated that carotenoids, such as astaxanthin [14] and canthaxanthin [15] are able to induce apoptosis in various tumor cells. This effect is due to their remarkable electronic and optical characteristics [16] and to their molecular orientation at biological interfaces [17], as well as to their interaction with various lipids from cell membrane as the Langmuir lipid layers [18-20].

Therapeutic medicine, such as doxorubicin, D, is considered as an effective anticancer drug used in chemotherapy of various types of cancer, e.g., leukemias, breast, ovarian, and lung cancer [21]. Its mechanism of action is based on doxorubicin intercalation with DNA, and finally blocking the DNA and RNA synthesis and leading to cell death. Accordingly, doxorubicin inhibits DNA and RNA production, by impeding an enzyme known as topoisomerase 2, leading to cell death. However, like other anticancer drugs, its administration is accompanied by an induced adverse effect which can be associated to neurotoxicity [21, 22], and/or cardiotoxicity [23] in a dose dependent manner, which limits the usefulness of doxorubicin.

Recently, an additional strategy is proposed namely to use natural adjuvant compounds as inducers of immunogenic cell death, which might activate immune cells to fight cancer cells and develop the cancer immunotherapy [24]. Further research is needed to investigate doxorubicin side effects at various doxorubicin doses and increase its activity on diverse cancer cells, including drug resistance cells, specifically MCF-7 cells [25] and ovarian cancer cells [26]. Novel core-shell nanoparticles (i.e., nanocomplexes or nanocomposites)

need to be designed to bind doxorubicin that might serve as efficient doxorubicin carriers to overcome the drug resistance mechanism in cancer cells, due to P-glycoprotein, PGP, which might be over expressed and can mediate export of doxorubicin from intracellular to cellular exterior [26]. Alternatively, some cancer cells might be expressing an array of genes that could confer intrinsic resistance.

For the first time, we developed a new strategy to effectively use a therapeutical agent, trans-resveratrol, R, to mediate the effects of doxorubicin in two human cervical tumor cell lines, specifically HeLa and CaSki [27]. Resveratrol is a polyphenol produced by plants and can form complexes with GNPs [27-29] whose biocompatibility has been demonstrated, making it a good candidate for drug delivery [30-33] and as an anti-oxidant for clinical implications [33].

Several studies have demonstrated the beneficial effects of resveratrol on the living organism, for instance, the anti-inflammatory action, modulation of lipid metabolism and inhibition of platelet aggregation [30, 34-36]. Resveratrol might have the therapeutic potential against emerging respiratory vital infections [36]. Even alone, resveratrol has a slight anticancer action [37].

Piperine, P, is an alkaloid obtained by extraction from *Piper* longum and *Piper nigrum*, which has been used for a long time [38, 39] for its antioxidant, anti-inflammatory and immunomodulatory effects, with a potential role in cancer prevention [40] and cancer treatment [41]. Piperine has the ability to modulate the bioactivity and bioavailability of resveratrol [42]. Piperine in combination with resveratrol, either as a physical mixture, R:P, or as a RP co-crystal, jointly enhance radiosensitivity of tumor cells, increasing cellular death through apoptosis [43].

Icariin is a natural flavonoid with pharmacological activities [44], anti-inflammatory and immunoregulatory effects [45], and a drug for anticancer treatment.[46] Icariin is presented as an active participant in the increased intracellular accumulation of doxorubicin in hepatocellular carcinoma [47], inducing immunogenic cell death. Moreover, icariin enhances the cytotoxicity of doxorubicin in human multidrug-resistant osteosarcoma cells [48]. Icariin has a broad inhibitory effect on breast cancer, colon cancer and hepatocellular carcinoma.

This study presents the green synthesis of gold nanoparticles [49, 50], under alkaline conditions [27], and the strategy of their functionalization with doxorubicin, in the absence and the presence of said natural biomolecules. Further, these new formulations containing functionalized GNPs are used in various cell cultures to evaluate *in vitro* conditions the anticancer activity of doxorubicin in the presence of the selected adjuvant biomolecules.

RESULTS AND DISCUSSION

UV-VIS characterization of GNPs colloidal solutions obtained by reduction with resveratrol

The procedure described by Mohanty at al. [29] was followed, but for the $HAuCl_4$ concentration indicated by the authors (4 mM) we did not obtain a colloidal gold solution. The color of the mixture turned rapidly to red, and after 30 min an absorption peak at 551 nm was observed; but then a yellowish-brown muddy suspension resulted (Fig. 1a). Therefore, a more diluted $HAuCl_4$ solution (10^{-3} M) was used [27]. The color of the reaction mixture changed rapidly to red, signifying the formation of gold nanoparticles, GNPs, with their surface coated by resveratrol molecules, denoted GNP-R or as GNP_R, and the maximum absorbance increases during the first hour. The absorption maximum is observed at about 538 nm (Fig. 1b). This GNP-R colloidal solution was stable for more than a year (Fig. 1c).

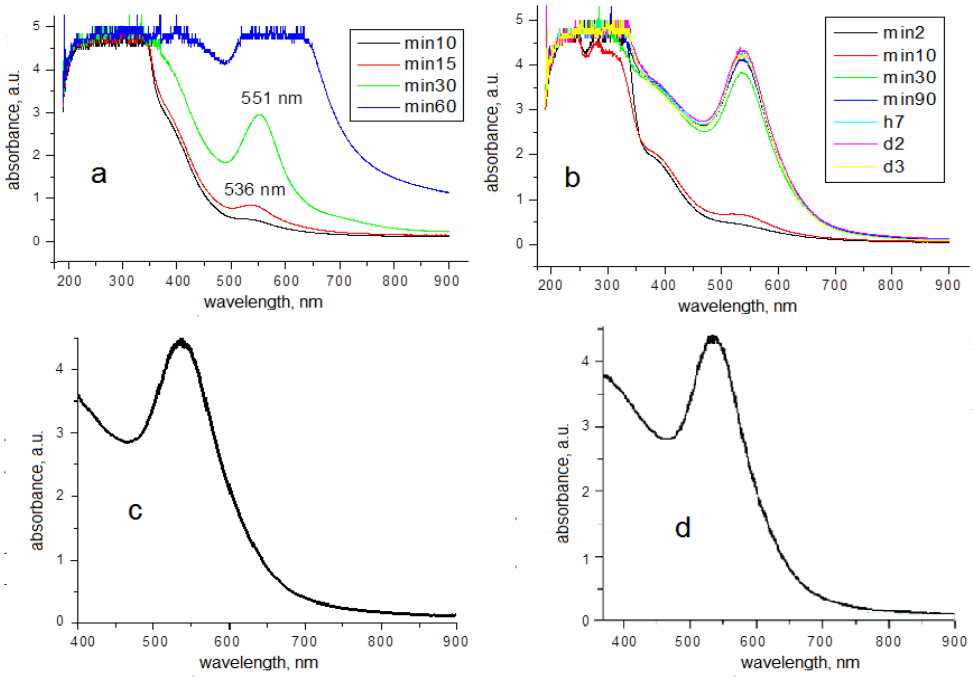

Fig. 1. Time evolution of the UV-Vis spectrum for a mixture of 4 mM $HAuCl_4$ and resveratrol, in NaOH solution (pH 12) at room temperature (a)**;** the 1 mM $HAuCl_4$ solution and resveratrol (b); UV-Vis spectrum of GNP-R solution after 1 year (c); UV-Vis spectrum of GNP-R1 solution at 1 year (d)

By centrifugation and washing with ultrapure water, a concentrated gold colloidal solution was obtained, without residual resveratrol or its oxidation products. After dilution for 70 times with ultrapure water, the maximum intensity of the absorption peak (at 535-538 nm) is similar to that of the initial GNPs solution (Fig. 1d).

Assuming a total reduction of Au(III) to Au(0), the GNPs concentration in the two solutions, namely GNP-R initially synthesized in aqueous alkaline solution, and GNP-R1 finally obtained, after its centrifugation, and dispersed in deionized water was 0.91 mM (179 mg/L), as presented in Table 1.

Interaction of GNP-R with PBS solution

Since *Dulbecco's phosphate buffered saline* (DPBS, noted also PBS) solution was later used for the preparation of all solutions, and further utilized in cell cultures, its interaction with the GNP-R solution was also investigated by UV-Vis as shown in Fig. 2.

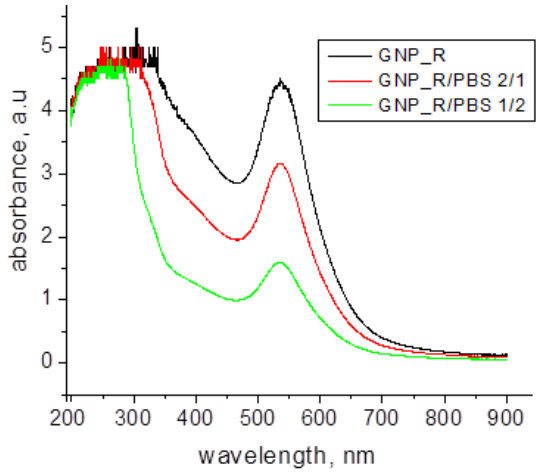

Fig. 2. UV-VIS spectrum of GNP-R (179 mg Au/L) mixed with *Dulbecco's* phosphate buffered saline (PBS) in different volume ratios (v/v) as given in the insert.

In the presence of PBS, as shown in Fig. 2, the surface plasmon resonance (SPR), namely the maximum of absorption band of the GNP-R, does not modify its position, only a decrease of absorbance with increasing dilution is observed.

Functionalization of GNP-R with doxorubicin hydrochloride, D

At physiological pH 7.4, the doxorubicin molecule is mainly protonated at the N atom (positively charged cationic form), while resveratrol molecule is almost non ionized, but at higher pH, resveratrol is negatively ionized (anionic forms).

Adding to the colloidal GNP-R solution (179 mg Au/L, which is equivalent to $0.91 \cdot 10^{-3}$ M) increasing amounts of doxorubicin ($7.2 \cdot 10^{-5}$ M (D) aqueous solution, containing 42 mg/L doxorubicin hydrochloride, as shown in Table 1, causes a slight shift of the absorption maximum from 538 to 542 nm, but for a higher D content in the GNP-R-D (i.e., D@GNP-R) mixture the nanoparticles aggregate in time and finally precipitate. For the GNP-R/D, volume/volume ratio 2, after 5 days the sedimentation is almost complete (Fig. 3a). For a lower D content (GNP-R/D = 6.25/1, v/v; or D@GNP-R = 1/6.25 v/v) the colloidal system is stable at least for 5 days (Fig. 3b).

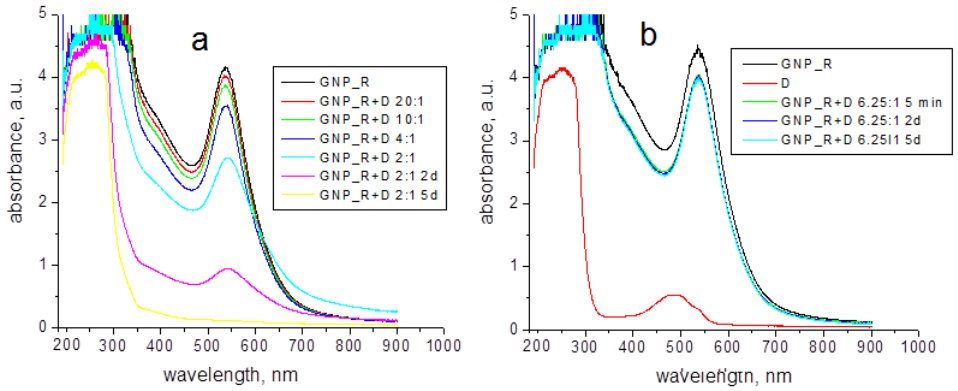

Fig. 3. UV-Vis spectra of GNP-R (179 mg Au/L) aqueous dispersion, and its mixtures with doxorubicin hydrochloride (D, 42 mg/L) aqueous solution, in deionized water, for different volume ratios as given in the insert, and the time evolution, immediately after preparation (at about 5 min), as well as at 2 and 5 days, as shown in the insert (a); UV-Vis spectra for GNP-R dispersion, D solution, and their GNP-R/D mixture for v/v, 6.25 mL: 1mL ratio, at different times after mixing (b), as shown in the insert

For the GNP-R/D volume/volume ratio 2, after 5 days, the GNP peak practically disappears (yellow curve is at the bottom in Fig. 3a), due to the almost complete sedimentation of constituent particles. For a lower D content, such as GNP-R/D = 6.25/1, v/v; or D@GNP-R = 1/6.25, v/v, the colloidal system is stable at least for 5 days (Fig. 3b).

The mixtures of the three solutions for, GNP-R with PBS and D, were unstable and deposited gold sediment was observed, for higher then 4 mg/L, of D content. Therefore, many different systems were tested to discover the optimum quantity of Au, requested for the high stability of the GNP-R functionalized with doxorubicin, also noted as D@GNP-R nanocomposite in aqueous dispersion.

Lastly, a mixture of GNP-R solution (12.5 mL), PBS (25 mL) and D solution (4 mL), with an Au content of 54 mg/L and D content of 4 mg/mL, was identified to be stable for several months (Fig. 4a).

Accordingly, the most stable colloidal solution was identified, as given in Fig. 4, and subsequently it was used *in vitro* research on various cancer cells (unpublished results).

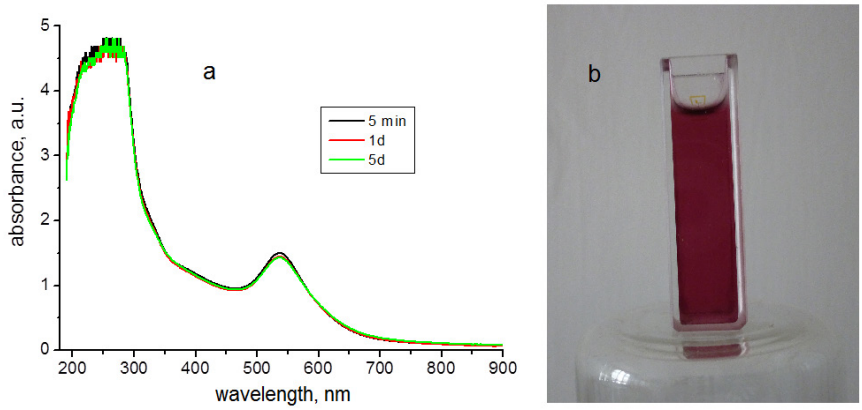

Fig. 4. UV-Vis spectrum of a dispersion, containing GNP-R functionalized with D, symbolized as D@GNP-R nanocomposite in PBS, obtained in a mixture of 12.5 mL of diluted GNP-R (Au 54 mg/L) and 4 mL of D (4 mg/L) solution and 25 mL PBS, measured in time (a), and a photo of this colloidal solution (b)

Thus, the surface functionalization of GNP-R with anticancer drugs, such as doxorubicin, is leading to one of the most favorable D@GNP-R nanocomposites with a high stability, as demonstrated in Fig. 4, having a strong shell formed of doxorubicin mixed with resveratrol, which is covering the core of GNPs, and is capable of resisting to the required washing steps and incubation conditions, especially in biological studies for biomedical applications.

This functionalization is realized by noncovalent interactions, particularly through electrostatic interactions, hydrogen bonds, and van der Walls forces. The advantage of this steady self-assembled layer of biomolecules covering the GNPs core of composite nanoparticles consists of the fact that these

biomolecules are not attached by various chemical modifications to the GNPs core and thus, are easier released in the interior of cancer cells. Certainly, the binding core-shell needs to be strong enough to produce stable shell surfaces capable of surviving during biological studies in cell cultures.

After functionalization, the new structure, spherical core (GNP)-shell (adsorbed as outer coating) of biomolecules, is realized particularly by electrostatic interactions, which leads to a non-covalent modification of the GNP surface and might have the great potential to be optimized and stabilized for various drug delivery systems [27].

On the other hand, the treatment with doxorubicin can increase reactive oxygen species and other adverse effects potentially leading to neurotoxicity and/or cardiotoxicity. In these situations, natural compounds with therapeutic activity are selected for use to reduce the cellular oxidative stress and inflammatory effects and simultaneously enhance the anticancer activity of doxorubicin.

In agreement with the therapeutic properties of resveratrol, piperine and resveratrol-piperine complex, and icariin, I, these biocompounds are used in achieving innovative composites in the presence of GNPs, obtained by reduction of $HAuCl_4$ with resveratrol, namely further resulting gold nanoparticles coated by a layer of resveratrol, GNP-R (also noted as GNP_R) and followed by centrifugation and redispersion in deionized water or in PBS, resulting in GNP-R1 (noted as well GNP_R1).

The surface of GNP-R and GNP-R1 nanoparticles is further functionalized with anti-cancer drug, e.g., doxorubicin, and various therapeutic adjuvant biomolecules, such as resveratrol, piperine, resveratrol-piperine complex, and icariin. The concentration of each component in solution is given in Table 1, as mg/L and mmol/L.

Functionalization of GNP-R with resveratrol-piperine, RP, complex

The UV-Vis spectra of resveratrol (R, saturated solution in PBS comprising 30 mg/L), piperine (P, saturated solution in PBS containing 40 mg/L) and of the resveratrol-piperine (RP) complex (50 mg/L) are compared in Fig. 5a. The concentration of these biocompounds in PBS solutions is shown in Table 1.

The addition of the resveratrol-piperine complex solution in PBS (50 mg/L) to the GNP-R colloidal solution (Au 179 mg/L), in the GNP-R/RP = 2/1 (v/v) ratio does not shift the absorption band of the GNP-R (Fig. 5b).

Also adding small amounts of doxorubicin solution, D (42 mg/L) in the volume ratios GNP-R/RP/D = 6/3/1 (v/v/v) did not modify the UV-Vis spectrum

(Fig. 5b). Thus, the mixtures of GNP-R with RP complex and with doxorubicin are leading to D/RP@GNP-R nanocomposite significantly stable, but color change and sedimentation occur after 8 days.

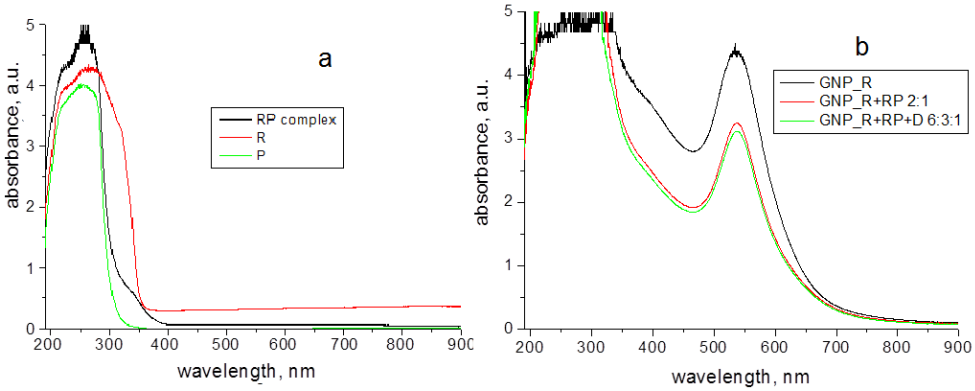

Fig. 5. (a) UV-Vis spectrum of the resveratrol-piperine, RP, complex (50 mg/L) in PBS, of a saturated piperine (40 mg/L) solution in PBS, and of a saturated resveratrol (30 mg/L) solution in PBS; (b) UV-Vis spectra of GNP-R dispersion (Au 179 mg/L), of RP@GNP-R nanocomposite in PBS dispersion (GNP-R: RP, 2:1 v/v ratio), and of GNP-R (Au 179 mg/L) mixed with resveratrol-piperine (RP) complex (50 mg/L), and with doxorubicin (D 42 mg/L) solutions at the volume v/v/v ratios of 6:3:1 as given in the insert of Figure 5b, resulting in stable D/RP@GNP-R nanocomposites in PBS dispersion.

This investigation aimed to determine the effect of a combination of doxorubicin and resveratrol-piperine complex, which contains piperine as a bioenhancer of resveratrol activity, and jointly adsorbed on the surface of GNP-R, and also improve required stability of D/RP@GNP-R nanocomposite in aqueous dispersions.

Functionalization of GNP-R with icariin, I

The UV-Vis spectra of DMSO, and of icariin (I, 100 mg/L) dissolved in DMSO and PBS (1mL/1mL, v/v ratio) mixture, recorded directly after mixing, and after 2 and 3 days are shown in Fig. 6a. The concentration of icariin in DMSO and PBS solution is shown in Table 1. After preparation, the resulted icariin (I, 100 mg/L) solution was stable and there were no UV-Vis spectra modifications.

Accordingly, the solution of *icariin* in DMSO and PBS (1:1, v/v ratio) mixture was stable the first day, after preparation and from day 2 a yellow sediment appeared.

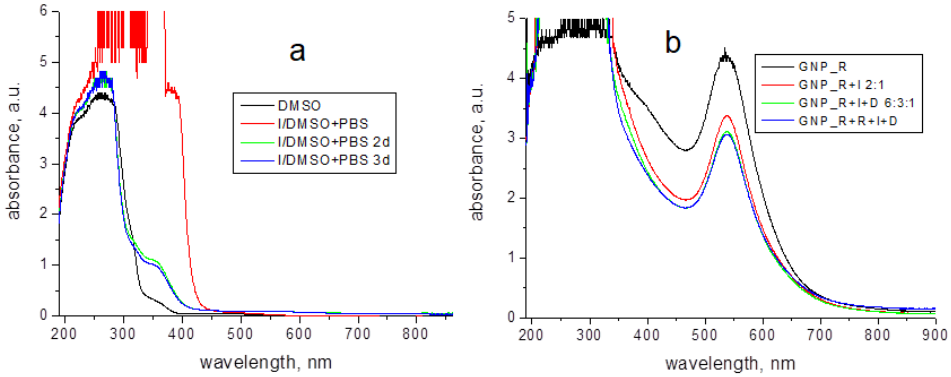

Fig. 6. UV-Vis spectra of DMSO, and of icariin (I, 100 mg/L) solution in the DMSO and PBS (1mL/1mL, v/v ratio) mixture, recorded directly after mixing, and after 2 and 3 days (a); UV-Vis spectra of GNP-R dispersion (Au 179 mg/L), of I@GNP-R nanocomposite in PBS dispersion (GNP-R: I for 2:1 v/v ratio), and of GNP-R (Au 179 mg/L) mixed with I (100 mg/L) solution and D (42 mg/L) solution, at the volume v/v/v ratios of 6:3:1 as given in the insert of Figure 6b, resulting in D/I@GNP-R nanocomposites in PBS dispersion as well as for GNP-R (1.8 mL) + R (0.4 mL) + I (0.5 mL) + D (0.3 mL) resulting in D/I/R@GNP-R nanocomposite in PBS dispersion.

The addition of this icariin (I, 100 mg/L) fresh solution in the GNP-R (179 mg Au/L) colloidal solution for GNP-R:I = 2:1 (v/v) ratio, does not change the absorption band of the GNP-R (Fig. 6b). The addition of doxorubicin solution, D (42 mg/L) in the volume ratios GNP-R/I/D = 6/3/1 (v/v/v) did not initially affect the UV-Vis spectra; neither did the addition of resveratrol (30 mg/L) in PBS solution to the mixture of GNP-R, I and D resulting in GNP-R (1.8 mL) + R (0.4 mL) + I (0.5 mL) + D (0.3 mL) change the spectrum just after mixing (Fig. 6b). Additionally, the evolution in time, after the first day of the D/I/R@GNP-R composite preparation, shows that the functionalized gold nanoparticles are rather stable for a week, when particles started to aggregate and precipitate.

This investigation is the first to assess the combinatorial effect of icariin, resveratrol and doxorubicin, together in functionalization of GNP-R, resulting in D/I/R@GNP-R nanocomposite stable in PBS dispersions. So, the self-assembled layer of these biomolecules is adsorbed on the surface of gold nanoparticles, GNP-R1, exemplified through SPR in UV-Vis spectroscopy, reaching a good stability of this nanocomposite, designed as a potential drug delivery system (Fig. 6b).

Functionalization of GNP-R1 with various biomolecules

The GNP-R was centrifugated and then diluted with bidistilled deionized water resulting in GNP-R1 (also noted GNP_R1) colloidal solution (of the same concentration in gold as in GNP-R initially synthesized in aqueous dispersion, as revealed in Fig. 1b. Both colloidal solutions of GNP-R and GNP-R1 have a very high stability as shown in Fig. 1c and Fig. 1d, respectively.

Moreover, the GNP-R1 colloidal solution displays a behaviour similar to that of GNP-R colloidal solution, in its interaction with solutions of various drugs, but the resulting mixtures with GNP-R1 are more stable in time. Therefore, several examples regarding the functionalization of GNP-R1 with resveratrol, piperine, resveratrol-piperine complex and doxorubicin are presented

The strongest effect on the absorption band is manifest for piperine (P) solution, and for the complex resveratrol-piperine (RP), both initially in PBS solutions. The UV-Vis spectra of mixtures formed of GNP-R1 colloidal solution and various solutions of resveratrol (R), doxorubicin (D), piperine (P) and/or resveratrol-piperine (RP) complex, at different volume ratios, are given in Figure 7. The concentration of these biocompounds in used solutions, is shown in Table 1.

The present study aimed to determine the effect of a combinative effect of doxorubicin and resveratrol as well as doxorubicin and resveratrol-piperine complex versus resveratrol, piperine, resveratrol-piperine complex, or doxorubicin effect on the stability of functionalized GNP-R1 in PBS dispersions.

The results confirmed a good stability for almost all studied systems, but the P@GNP-R1 nanocomposite appears to show a large maximum (of low absorbance) which probably denotes a stronger interaction among components.

The current investigation was hence proposed to study the combined effect of doxorubicin and several selected adjuvant biomolecules, adsorbed on the surface of GNPs, on the stability of functionalized GNPs designed for future drug delivery systems to be employed *in vitro* and *in vivo*. The most stable aqueous solutions (dispersions) will be employed later on as advanced models for their anticancer examination on different cancer cells, and thus, for establishing their potential therapeutic effect on various human cancers.

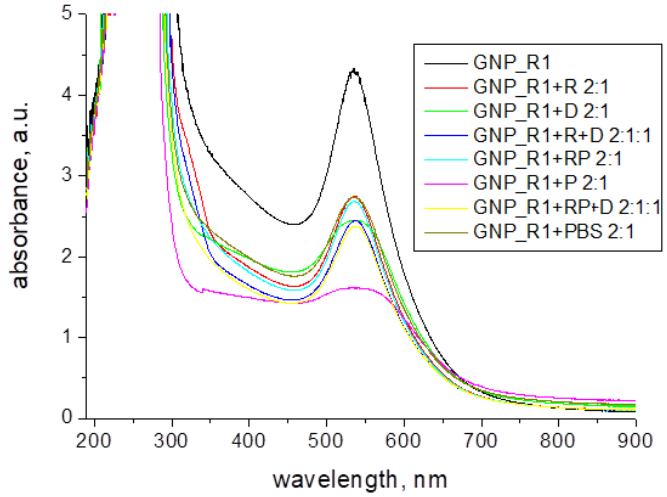

Fig. 7. UV-Vis spectra of GNP-R1 (Au 179 mg/L) aqueous dispersion; of R@GNP-R1 nanocomposite obtained from GNP-R1**:** R, with R (30 mg/L) in PBS solution, at 2:1 v/v ratio; of D@GNP-R1 nanocomposite for GNP-R1**:** D, with D (42 mg/L) aqueous solution, at 2:1 v/v ratio; of D/R@GNP-R1 nanocomposite for GNP-R1**:** R, with R (30 mg/L) in PBS solution, and D, with D (42 mg/L) aqueous solution, at 2:1:1 v/v/v ratio; of RP@GNP-R1 dispersion for GNP-R1**:** RP, with RP (50 mg/L) in PBS solution, at 2:1 v/v ratio; of P@GNP-R1 nanocomposite for GNP-R1**:** P, with P (40 mg/L) in PBS solution at the volume v/v ratio of 2:1; of D/RP@GNP-R1 nanocomposite for GNP-R1 mixed with RP (50 mg/L) in PBS solution and D (42 mg/L) aqueous solution at the volume v/v/v ratio of 2**:**1**:**1**;** and GNP-R1 (Au 179 mg/L) aqueous dispersion and PBS solution for volume v/v ratio, 2**:**1; altogether in final dispersions as given in the insert of Figure 7.

CONCLUSIONS

The GNP-R and GNP-R1 colloidal solutions obtained by the above green synthesis by reduction with resveratrol, were used together with doxorubicin, piperine, resveratrol, the resveratrol-piperine complex, and icariin, in the synthesis of multifunctional GNPs-anticancer drug composites, namely, D/P/R/RP/I@GNPs-R composites. These advanced composites, also named complexes, were made by self-assembly of biomolecules on the surface of gold nanoparticles, already stabilized with a coating layer of resveratrol. The UV-Vis study highlighted the interactions between these components and it is obviously to be further completed with other physico-chemical methods of characterization and with biological investigation on various cancer cells.

One major goal of this study was to optimize the functionalization of GNPs to be beneficial for achieving the union of multifunctional array and multi-therapeutic components, within the adsorbed shell on the surface of GNPs aimed at reaching a high stability in various aqueous dispersions, including cell cultures. The formation of self-assemblies of biomolecules might take advantage of electrostatic interaction as well as molecular changes by hydrogen bonds and intermolecular van der Walls forces among molecules.

As highlighted in this study, GNPs have a large ability to be easily functionalized with biomolecules as determined by surface plasmon resonance, SPR. The constructed multifunctional GNPs provide the colloidal solutions of high stability in various PBS conditions making them useful for biological investigation in various cell cultures, as tools for pharmaceutical and medical applications.

In the future research, the multifunctional GNPs will be used and further developed from their interaction with cancer cells and certainly new formulations will be tailored to obtain optimized therapeutic combinations, and thus, making them to support medical applications.

Moreover, the interaction among molecules adsorbed on GNPs might achieve highly ordered self-assemblies, which can also be preserved within the cells modifying the mechanism of doxorubicin action in cancer cells with resistance to anti-cancer drugs. Surely, the gold nanostructures should be evaluated *in vitro* and *in vivo* research for their efficiency.

EXPERIMENTAL SECTION

Materials and methods

Gold nanoparticles (GNPs) were obtained by the reduction of tetrachloroauric(III) acid, $HAuCl_4$, with trans-resveratrol (R). The tetrachloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$) 99.5% (Merck - Darmstadt, Germany), trans-resveratrol ≥ 99% (HPLC assay, from Sigma-Aldrich, Buchs, Switzerland) and NaOH reagent grade ≥ 98% (Merck KGaA, Darmstadt, Germany) were used in aqueous solutions prepared with bidistilled deionized water. Doxorubicin hydrochloride (about 98%) was purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). Piperine ≥98% (HPLC assay), was obtained from AlfaAesar (Karlsruhe, Germany) and icariin, analytical standard (≥94%) was obtained from Sigma-Aldrich (Steinheim, Germany). Resveratrol-Piperine complex was prepared in our Research Center of Physical Chemistry (*unpublished results*). Dimethyl sulfoxide (DMSO) for cell culture was purchased from Sigma-

Aldrich (Schnelldorf, Germany). Dulbecco's phosphate buffered saline (DPBS, noted also PBS), without $CaCl_2$ and $MgCl_2$, (pH 7.4), was purchased from AlfaAesar (Karlshure, Germany).

Synthesis of GNPs

For the preparation of GNPs using trans resveratrol as reducing and stabilizing agent, by a modified method [29], we started with 100 mL 10^{-3} M aqueous $HAuCl_4$ solution; a freshly prepared solution obtained from 25 mg resveratrol dissolved in 10 mL 0.02 M NaOH solution was added under continuous stirring at 500 RPM, for 10 min at room temperature (about 22 ^oC). The initial resveratrol concentration was therefore 10^{-3} M and the pH about 12.

This GNP-R colloidal solution was concentrated by centrifugation (at about 14 000, RPM) for about 20 min, and the supernatant comprising the excess of resveratrol was removed. The obtained colloid gold particles, covered with a protective layer of resveratrol, from their initial colloidal solution, were washed with ultrapure water and dispersed in deionized water for further studies. The final solution was diluted for 70 times with ultrapure deionized water to attain the same gold concentration as in the initial colloidal solution (GNP-R), representing the GNP-R1 sample.

The GNP-R and GNP-R1 colloidal dispersions obtained by the above green synthesis were mixed in different proportions with anti-cancer drug solutions of doxorubicin hydrochloride, icariin, piperine, resveratrol and piperine-resveratrol complex to explore the functionalization of gold nanoparticles.

All used solutions in this study are given in Table 1. Since icariin is not soluble in water, it was solubilized in dimethyl sulfoxide (DMSO, 11 mg/ 10 mL) and then 100 mL PBS were added to the solution.

Table 1. Solutions used in this study

Solution	Solvent	Chemical formula	Molar mass (g/mol)	Concentration	
				mg/L	mmol/L
GNP-R, GNP-R1	Water	Au	197	179	0.91
Doxorubicin.HCl, D	water	$C_{27}H_{29}NO_{11} \cdot HCl$	580.0	42	0.072
Resveratrol, R	PBS	$C_{14}H_{12}O_3$	228.2	30	0.131
Piperine, P	PBS	$C_{17}H_{19}NO_3$	285.3	40	0.140
Resveratrol-Piperine, RP	PBS	$C_{14}H_{12}O_3 \cdot C_{17}H_{19}NO_3$	513.5	50	0.097
Icariin, I	DMSO, PBS	$C_{33}H_{40}O_{15}$	676.7	100	0.148

UV-Vis spectroscopy

The *UV-VIS absorption spectra* were measured with a Jasco UV/Vis V650 spectrophotometer, from 900 to 200 nm wavelength. The stability of the GNPs-R and GNPs-R1 and their further functionalization with various biomolecules, used as therapeutic adjuvants, was studied as function of PBS content, composition and time.

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