Evaluation of Gold, Silver and Silver–Gold (Bimetallic) Nanoparticles as Radiosensitizers for Radiation Therapy in Cancer Treatment

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Abstract One of the most recognized and widely used treatment modality in cancer is radiation therapy which depends on the radiosensitivity of tumour tissue. Over the past few years there has been lot of interest in the use of formulations to enhance radiotherapeutic effects, especially using metallic (mainly gold) based nanoparticles. Our goal here is to fabricate nanoparticles (NPs) that can be delivered to tumor tissue to increase its radio-sensitivity. This would increase efficiency of radiation absorption by the tumor tissue and reduce radiation doses delivered during radiotherapy. This could potentially decrease radiation exposure related side effects to patients. We have achieved this by synthesizing nanoparticles of high Z elements such as gold, silver and a more efficient bimetallic silver-gold size ranging from 3nm to 72nm using chemical reduction and hydrothermal method. The synthesized metallic nanoparticles were characterised using Ultraviolet (UV)-Visible Spectroscopy, Fluorescence spectroscopy and Dynamic Light Scattering. The metallic nanoparticles showed radiosensitizing activity in colloidal form by absorbing radiations when irradiated by 60Co source which emits two gamma rays of energy 1173keV and 1332keV. Based on our results, we are of the opinion that such radiosensitizing agent if injected into the tumour tissue would increase radiation absorption and enhance treatment effect with lower therapeutic radiation dosage.

Keywords Radiosensitizers, Silver-Gold (Bimetallic) Nanoparticles, Gamma Radiation, Gold Nanoparticles, High-Z Metal Nanoparticles, Enhanced Radiation Therapy

1. Introduction

Every year 10.9 million people worldwide are diagnosed with cancer, and there are 6.7 million reported deaths from the disease [1]. There are continuous efforts across the globe to come up with more efficient and safe treatment modalities for cancer. Often radiation therapy is used in cancer treatment [2]. The success of radiation therapy depends on the radiosensitivity or radioresistance of tumour tissue in consideration with surrounding normal tissue. Radiation resistance of tumour tissue is a key challenge in oncology [3]. Radiosensitizers are agents that enhance the positive effects of radiation therapy. Over the past few years there has been lot of interest in the use of formulations to enhance radiotherapeutic effects, especially using metallic (mainly gold) based nanoparticles [4]. Nanoparticles can be combined with radiation therapy where metallic particles can selectively absorb and/or scatter the high energy gamma radiations. This allows for better targeting of cellular components within the tumour tissues allowing for more localized and enhanced damage. NPs also provide enriched interaction cross-section with the photons from X-rays or high energy radiations and gamma irradiations [5,6].

Delivering a therapeutic dose of radiation to tumour tissues while preserving normal surrounding tissues is still a great task in radiation therapy. Acquired radiation resistance during treatment is one of the major causes of radiation therapy failure and subsequent tumour relapse. Three major approaches for the improvement of efficacy of radiation therapy and minimize associated risks/complications have been put forward (I) enhancing radiosensitization of tumour tissue (II) reversing of radiation resistance in tumour tissue and (III) enhancing radioresistance of the healthy tissue [7].

Radiosensitization is done by strategically using high-Z NPs. These NPs can intensify the production of secondary electrons or Auger electrons and reactive oxygen species (ROS) that in turn enhance radiation therapy effects [8]. The most studied NPs are gold-based NPs (GNPs) that were
widely described in particular by Hainfeld et al. [9]. Literature shows that of late titanium oxide nanotubes, lanthanide-based NPs or cadmium selenide quantum dots are being used [10-13]. Other studies have shown use of silver-based NPs which take advantage of its excellent surface enhanced Raman scattering and broad-spectrum antimicrobial activities [14]. However, silver NPs cause dose dependent toxicity and induce oxidative stress and DNA damage in triple negative breast cancer [15]. Radiosensitization by gold NPs (GNPs) are dependent upon six factors that include intracellular localization, size, concentration, radiation dose, cell type and modified surface of these nanoparticles [16].

Chitrani et al demonstrated that GNPs around 50 ± 5 nm diameter had the highest radio enhancement factor (REF) (1.43 at 220 kVp) compared with GNPs of 14 and 74 nm (1.20 and 1.26, respectively) [17]. The highest cellular uptake of GNPs was found to be at 50nm particle size [18]. Hence, we have considered the size of NPs (50±5nm) to be one of important factors in our work while synthesizing bimetallic silver-gold, silver and gold NPs.

Our hypothesis is that bimetallic silver-gold NPs will be the best radiosensitizing agent as they may have better absorption coefficient and non-toxic. Gamma radiation transmission experiment was conducted to evaluate the radio sensitizations of NPs. For this purpose, a narrow beam transmission experiment was carried out using 60Co source with silver, gold and silver-gold nanoparticle solutions of various size distribution as targets by employing a high resolution hyperpure germanium detector. The variation of photon mass attenuation coefficient for each sample so obtained was correlated with the radiosensitizing capacity of NPs. Possible conclusions are drawn based on the present study.

2. Materials and Methods

2.1. Reagents

Chloroauric acid (HAuCl₄, Aldrich chemicals, Silver Nitrate (AgNO₃, Sigma chemical company), Tri-Sodium Citrate Dihydrate (HiMedia), ultrapure water (purity up to 18.2 MΩcm⁻¹ Elga Option Q7).

2.1.1. Synthesis of Nanoparticles.

Colloidal NPs such as gold, silver and silver-gold (bimetallic) were synthesized by chemical reduction and hydrothermal method. Precursors, reducing agent and concentration for synthesis of various nanoparticles are given in table 1.

Gold nanopolyhedra were grown using the seeded growth method. Au NPs are most commonly synthesized through the reduction of chloroauric acid (HAuCl₄) by sodium citrate resulting in an aqueous Au NPs colloid [19, 20]. Au NPs size (often measured in terms of diameter) can be modified by adjusting the chloroauric acid to sodium citrate ratio. Similarly Ag NPs are synthesized by reduction of Silver Nitrate (AgNO₃) by sodium citrate resulting in an aqueous Silver nanoparticles colloid [21].

2.1.2. Chemical reduction

The nanoparticles were synthesized in different batches. This was done by placing G1 sample i.e., 25ml of 1 mM HAuCl₄ solution in a round bottom flask. Round bottom flask was kept in a bowl of glycerine and heated indirectly. A motorized stir bar was placed in the solution and set to a spin rate of 400 rpm and temperature was set 300°C. The gold solution was heated until solution was boiling. To achieve a homogenous size of the gold nanoparticles solution, 1ml of 40 mM sodium citrate solution was quickly added to the batch during continuous stirring. A colour shift from yellow to red was seen as the gold solution was reduced and the nanoparticles were synthesized. The solution was left to boil for another 10 minutes before the heating mantle was turned off. The size of gold nanoparticles generated depends on the concentration of HAuCl₄, the amount of sodium citrate added and the duration of heat supplied. Also G3 was prepared in same way.

Similarly for silver nanoparticles precursor 25ml of 1mM AgNO₃ was used. By using chemical reduction, samples S1, S2, SG1 and SG3 were synthesized by altering the precursors and maintaining amount of the reducing agent to be 1ml of trisodium citrate at same concentration 40mM, except in S2 sample where 5ml of reducing agent was added in order to alter the size of nanoparticles. S3 was prepared just by mixing ingredients in a vial and kept undisturbed for 1 day.

| Sample                | Precursor               | Reducing agent               |
|-----------------------|-------------------------|------------------------------|
| G1 gold NPs           | 25ml of 1mM HAuCl₄     | 1ml of 40mM trisodium citrate|
| G2 gold NPs           | 25ml of 1mM HAuCl₄     | 1ml of 40mM trisodium citrate|
| G3 gold NPs           | 25ml of 1mM HAuCl₄     | 1ml of 40mM trisodium citrate|
| S1 silver NPs         | 25ml of 1mM AgNO₃      | 1ml of 40mM trisodium citrate|
| S2 silver NPs         | 25ml of 1mM AgNO₃      | 5ml of 40mM trisodium citrate|
| S3 silver NPs         | 25ml of 1mM AgNO₃      | 1ml of 40mM trisodium citrate|
| SG1 silver –gold NPs  | 12ml of AgNO₃, 12ml of HAuCl₄ Wt % 1:1 | 1ml of 40mM trisodium citrate|
| SG2 silver –gold NPs  | 12ml of 1mM AgNO₃, 12ml of 1mM HAuCl₄ | 1ml of 40mM trisodium citrate|
| SG3 silver –gold NPs  | 12ml of 1mM AgNO₃, 12ml of 1mM HAuCl₄ | 1ml of 40mM trisodium citrate|
2.1.3. Hydrothermal Synthesis

For the hydrothermal synthesis of gold nanoparticles, 25ml 1mM HAuCl₄, and 1ml of 40 mM trisodium citrate were added to a glass vial. Final mixture was then transferred to the Teflon liner (Vₗ₁₁ = 26 ml), which was later placed inside a general-purpose autoclave. The autoclaves were provided with Teflon liners of 50 ml capacity. Then each assembled autoclave was kept in an oven with a temperature programmer-controller. The temperature was programmed and kept at 180°C for 1 hr and autogenous pressure maintained. After the experimental run, autoclaves were cooled to room temperature. The resultant product in the Teflon liner was then transferred to clean glass vials. The sample SG2 was prepared at same temperature and methodology. The sample G2 was prepared at 180°C for 2 hours in the oven.

0.9% Saline was used as an analogue of biological medium in a previous experiment by V. Apanasevich et al (2014) [22]. In our experiment we avoided using 0.9% of saline as an analogue because every material contributes to absorption of gamma rays during transmission experiment or total attenuation of gamma radiation experiment. Also addition of electrolyte or salts causes agglomeration and change in dynamics of nanoparticles in the colloidal solution which has been shown in previous experiments [23, 24]. The biological activity of nanoparticles depends on many physico-chemical factors and is regulated by virtue of stability. A reduction in stability leads to aggregation and consequently total or partial loss of nanoscale properties [25].

Synthesised particles demonstrated colours varying from red for smaller particles to purple for larger particles (Figure 1). The gold NP solution with particle size less than 30nm was red whereas the larger size NP solutions were blue. The silver nanoparticles showed yellowish brown and silver–gold bimetallic nanoparticles developed a dark-violet brownish colour.

2.2. Characterization of Nanoparticles

2.2.1. Dynamic Light Scattering (DLS)

Nanotrac analyzers from Microtrac were used to determine the size of nanoparticles in the prepared colloidal solution. The average mean diameter of the nanoparticles in colloidal solution as determined by dynamic light scattering measurements (Figure 2, 3 and 4) showed different size distributions of particles in the solution from S1 to SG3 with peaks of 4 nm to 73 nm. Since particles are of ellipsoid in nature, they are characterised by two parameters namely major axis (diameter) and minor axis (width). Along with the average mean diameter and width of nanoparticles other important data have emerged out during DLS characterisation such as Zeta potential, Mobility, Charge and Polarity as shown in table 2. All the particles are less than 100 nm range and above 3 nm. Zeta potential of nanoparticles showed up to upper limit of device capacity -200mV. The samples will be termed according to the constituents of nanoparticles formed and size associated with it.
Figure 2(b). Dynamic light scattering distribution of 14.3nm gold nanoparticles.

Figure 2(c). Dynamic light scattering distribution of 45.5nm gold nanoparticles.

Figure 3(a). Dynamic light scattering distribution of 3.36nm silver nanoparticles.

Figure 3(b). Dynamic light scattering distribution of 41.7nm silver nanoparticles.

Figure 3(c). Dynamic light scattering distribution of 50nm silver nanoparticles.

Figure 4(a). Dynamic light scattering distribution of 17.4nm silver-gold (bimetallic) nanoparticles.
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**Figure 4(b).** Dynamic light scattering distribution of 52.9 nm silver-gold (bimetallic) nanoparticles.

**Figure 4(c).** Dynamic light scattering distribution of 72.4nm silver-gold (bimetallic) nanoparticles.

| Sample | Size Diameter (nm) | Width | Zeta Potential | Mobility | Charge | Polarity |
|--------|--------------------|-------|----------------|----------|--------|----------|
| G1     | 4.21               | 1.39  | -16.8 mV       | -1.31 u/ s / V / cm | -0.00054 fC | Negative |
| G2     | 14.35              | 7.01  | -32.2 mV       | -2.51 u/ s / V / cm | -0.00549 fC | Negative |
| G3     | 45.5               | 27.14 | -200.0 mV      | -15.63 u/ s / V / cm | -0.20923 fC | Negative |
| S1     | 3.36               | 0.79  | -156.0 mV      | -12.19 u/ s / V / cm | -0.0367 fC | Negative |
| S2     | 41.7               | 24.96 | -200.0 mV      | -15.63 u/ s / V / cm | -0.07315 fC | Negative |
| S3     | 50                 | 33.9  | -200.0 mV      | -15.63 u/ s / V / cm | -0.08898 fC | Negative |
| SG1    | 17.4               | 10.2  | -0.1 mV        | -0.01 u/ s / V / cm | -0.00002 fC | Negative |
| SG2    | 52.9               | 33.7  | -200.0 mV      | -15.63 u/ s / V / cm | -1.42024 fC | Negative |
| SG3    | 72.4               | 39.4  | 200.0 mV       | 15.63 u/ s / V / cm | 2.890 fC | Positive |

2.2.2. Ultraviolet–visible (UV–VIS) spectroscopy

Thermo Scientific Varioskan Flash Spectral Scanning Multimode Reader was used to carry out UV-Visible spectroscopy. It had plate types 6 – 384 well plates reading capacity, light source being Xenon flash lamp and wavelength range 200 – 1000 nm and bandwidth of 5nm.

The absorbance maxima for gold, silver and gold-silver NPs were determined by UV-Vis spectrophotometer. The spectra are as shown in figure 5 (a–c). The absorbance maxima for gold NPs were found to be at 515, 527 and 517nm which indicates that the gold nanoparticles are present based on the surface plasmon resonance. The absorbance maximum peaks of silver NPs were found to be at 418, 415 and 456 nm which indicates that the silver nanoparticles were present based on the surface plasmon resonance. Absence of secondary peak in these spectra indicated that the nanoparticles were spherical in shape. Also surface plasmon absorption bandwidth is a maximum of 418 nm indicating the presence of spherical or roughly spherical.
2.2.3. Fluorescence Spectroscopy

Thermo Scientific Varioskan Flash Spectral Scanning Multimode Reader was used for Fluorescence spectroscopy.
In this study, all nine samples were shown to possess fluorescence properties though at different degrees of intensity in addition to emission peak variance. The excitation of the samples at 325 nm resulted in the emission spectra as shown in Figure 6 with the emission peak maxima identified. The nanoparticles of gold, silver and silver gold bimetallic exhibited a single high intensity peak at 450 nm while there was little variation with regard to intensity comparison shown in figure 7(a-c).

Liu et al reported similar results with very small gold nanoparticles (5.1 nm) stabilized with Bovine serum albumin (BSA) excited at 320 nm exhibiting an emission peak at 404 nm [26]. The presence of a single high intensity fluorescence peak for the samples after irradiation at 325 nm indicates that it is useful for bio-labelling applications as potential alternatives to organic fluorophores.

2.3. Experimental Determination of Mass Attenuation Coefficient of Nanoparticles

The photon mass attenuation coefficient was determined by a transmission experiment. The transmission experiment was performed in a setup similar to the one used in an earlier study [27]. The $^{60}$Co source used in this study had a source strength of 132 kBq and was procured from the Bhabha Atomic Research Centre, Mumbai, India. Gamma rays of energy 1173 keV and 1332 keV emitted by the $^{60}$Co source were made to fall on the target. The transmitted intensities were detected using an EG & G ORTEC model 23210 (USA) Gamma-x high purity germanium detector. The signal from the detector was suitably amplified and the spectrum was recorded in a personal computer based multichannel analyzer supplied by M/S Nucleonix Corporation Ltd, Hyderabad, India.

The nanoparticles under investigation were confined in cylindrical plastic containers. The samples were weighed in an electrical balance correct to the third decimal place. The weighings were repeated to obtain concordant values of the mass. The mean of this set of concordant values was taken to be the mass of the sample. The inner diameter of each container was determined separately with the help of a travelling microscope by the usual method. Based on the mean value of mass and inner diameter, the mass per unit area (pt) of each sample was determined.

In the transmission experiment, spectra were recorded by placing the container with and without sample alternately in the path of the beam. The attenuation of the photon beam by the material of the empty container was negligible. The counting time was so chosen that at least $10^4$ – $10^5$ counts were recorded under the photo peak.

3. Results and Discussion

Gold NPs of size 4.21, 14.35 and 45.5 nm were synthesized and width being 1.39, 7.01, 27.14 nm respectively. Silver NPs of size 3.36, 41.7 and 50nm were synthesized and width being 0.79, 24.96, 33.9 nm respectively. Silver–gold NPs of size 17.4, 52.9 and 72.4 nm were synthesized and width being 10.2, 33.7, 39.4 nm respectively.

The highest zeta potential and highest mobility in each group was found for 45.5nm Au NPs, 41.7 and 50nm Ag NPs as well as 52.9 and 72.4 nm Au-Ag bimetallic NPs suggestive of a colloidal formation which could remain stable for a longer time than the NPs of other sizes respectively in each group.

UV-visible spectroscopy indicating a shift from 527 nm to 437 nm in synthesis of Ag-Au NPs, as well as colour appeared, indicates a lower percentage of gold outside compared to silver, may be attributed to the Au core and Ag shell [28]. Alloy formation is evidenced by the appearance of a single peak depending strongly on composition. The Plasmon band is blue-shifted with increasing amount of silver at surface. The absorbance maxima were found to be at 440 nm region which indicates that the silver NPs formed the outer shell with gold as core.

From fluorescence spectroscopy it was found that Au 45.5nm, Ag 50nm and bimetallic Ag-Au 52.9nm NPs have the peak intensity maximum, which is more desirable as a fluorescent agent compared to other size nanoparticles of respective group. Lower wavelength in UV region corresponds to higher frequency and hence, higher Force Constant (molecules are executing simple harmonic motion (SHM) and associated with this we have a force constant) which is essentially means higher capacity to absorb incident radiation. Potential energy diagram of energy can be visualized for such process as in Figure 6. It essentially indicates that nanoparticles of gold, silver and silver–gold (bimetallic) have transition from higher energy to lower energy state in the excited region without emission of radiation and then there is emission of radiation when they make transition back to ground state with emission of radiation less compared to absorption frequency.

A typical energy spectrum of silver-gold nanoparticle
sample with particle size 52.9nm is as shown in figure 8. Similar spectra which differed only in terms of their counts/channel were obtained for all other samples of interest. A suitable Lorentzian was fit to the photopeaks in each sample spectrum and the areas under the two photopeaks within a common region of interest were taken to be the intensity I. By using these intensities the mass attenuation coefficient $\mu/\rho$ was calculated from the well-known Beer’s law, using the equation

$$\mu/\rho = \ln(I/I_0)/\rho t$$

(1)

This was done for all samples of present interest (nanoparticles – colloidal solutions) for 1173 keV and 1332 keV respectively.

The main source of error in the transmission experiment was due to counting statistics. This error was less than 0.3% as the area under the peak was $10^4 - 10^5$ counts. The sample size was so chosen that the error due to multiple scattering was negligible.

There were several other possible sources of error in the present method. These were due to

a) Small-angle scattering contributions: In this experimental setup, a distance of 15cm was maintained between the source and the detector. According to the theoretical estimates, the contribution of coherent as well as incoherent scattering at such small angle to the measured cross section at the energy of interest is negligibly small.

b) Photon build-up and pulse pile up effects: The photon dose buildup effects and pulse pile up effects were kept to a minimum by choosing an optimal count rate and counting time. The photon dose buildup is a function of the sample thickness, its atomic number, and the incident energy. It is also a consequence of multiple scattering occurring inside the sample. Since the multiple scattering effects are corrected, and an optimal count rate and counting time as well as a detector of good resolution were employed, it is expected that the effect of photon dose build up and pulse pileup was a minimum in the present study.

c) Dead time of the counting instrument: There was built-in provision for dead-time correction in the multichannel analyzer.

Thus the overall error on the measured mass attenuation coefficient was below 4% - 5%.
3) At 1173 keV, the \(\mu/\rho\) of Ag-Au NPs exhibits a maximum at a particle size of 50nm.

4) At 1332 keV the \(\mu/\rho\) of Au NPs exhibits a maximum at 15nm.

Radiosensitizing mechanism of Ag NPs according to Su and coworkers is due to release of Ag\(^+\) cation from the Ag nanostructures inside cell. Ag\(^+\) cation has the ability to capture electron and thus functions as an oxidative agent, which could further reduce the ATP content of the cell and increase production of ROS. ROS formation when radiation interacts with gold NPs may be one of the mechanisms that mediate gold NP radiosensitization [29].

4. Conclusions

This is the first in vitro study shows radio sensitisation capacity of silver, gold and silver-gold (bimetallic) nanoparticles as per the absorption studies. All the three types of nanoparticles were prepared using trisodium citrate as reducing agent via Chemical reduction and Hydrothermal synthesis resulting in fabrication of spherical type of nanoparticles in colloidal solution ranging from 4nm to 70nm as desired.

According to DLS data size of all nanoparticles was determined and also nanoparticles had negative charge on surface at around -200mV suggestive of a stable colloidal solution of nanoparticles.

From UV-Visible spectroscopy we can infer the presence of silver, gold and bimetallic silver-gold NPs with their maximum absorption peaks at 400-450nm, 525nm and 400-450nm respectively. The bimetalic silver-gold NPs maximum peak at 400-450nm suggests that this consists of gold core with silver shell.

Fluorescence emission spectra for all 3 varieties of nanoparticles showed a peak at 450nm which suggested that these were good fluorescent agents as well as bio-labelling materials in cancer cells. Particularly NPs of gold, silver and silver-gold around 40-50nm showed higher intensity of emission. Hence, 50 nm particles are desirable as fluorescent agent.

From the gamma ray transmission experiment we observe that silver nanoparticles have the lowest mass attenuation coefficient, which is a measure of absorption. Gold nanoparticles had relatively uniform and higher mass attenuation coefficient for particle size up to 50nm. The maximum mass absorption coefficient was observed for particle size in the range 40-50nm for all the three types of samples. Silver-gold had the maximum absorption coefficient corresponding to 52.9 nm at 1173 keV. Ag-Au nanoparticles were more desirable due to size and having enhanced gamma ray absorption capacity. Among the 3 types of NPs silver-gold NPs showed an increased radio sensitisation effect which is due to alloy of high Z elements attributing to increase in the electron cloud and also by synergistic combination of Ag and Au NPs radiosensitization mechanism.

On the whole, based on the present study it is felt that Ag-Au nanoparticles and Au nanoparticles may prove to be versatile radiosensitizing agent for treating cancer tissues with relatively less radiation dosage up to 50 times. It is noteworthy that the non-toxicity and biocompatibility of these NPs has already been proven by studies reported earlier.

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