Arabian Journal of Chemistry (2015) xxx, xxx–xxx

King Saud University

Arabian Journal of Chemistry

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ORIGINAL ARTICLE

Sapota fruit latex mediated synthesis of Ag, Cu mono and bimetallic nanoparticles and their *in vitro* toxicity studies

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Received 16 May 2014; accepted 16 December 2014

KEYWORDS

Alloy nanoparticles;
Cytotoxicity;
Latex

Abstract Ag, Cu metallic and bimetallic nanoparticles (NPs) with diverse compositions were efficiently synthesized using the fruit latex of *Achras sapota Linn*. Spectroscopic and cyclic voltammetry results suggested that reduction of Ag was assisted by ascorbic acid, reducing sugars and other phenolic compounds present in the latex. However, the reduction of Cu and alloy NPs required additional ascorbic acid. Comparative *in vitro* toxicity of as synthesized nanoparticle solution was assessed in 3T3L1 cells using MTT assay and fluorescent microscopy. A minimal impact was observed on cell viability and morphology during 72 h. This demonstrates great potential for use in biomedical applications such as cellular imaging or photothermal therapy.

1. Introduction

Noble metal nanoparticles (NPs) offer extensive potential for use in several biological applications (Mallikarjuna and Verma, 2008; Sperling et al., 2008). Bimetallic NPs can be synthesized by the simultaneous reduction of two kinds of metal ions (De Barros et al., 2005) or by the successive reduction of one metal over the nuclei of another (Nadagouda and Varma, 2006; Dahl et al., 2007). Depending on the preparation methods used, either the alloy or the core–shell (layered) NPs are produced. Alloy NPs have received special attention due to the possibility of tuning their properties over a broad range by simply varying the alloy composition.

Various wet chemical synthesis methods have been reported for the formation of metal NPs (Du et al., 2007; Wiley et al., 2004). However, for delivery of NPs at cellular levels and *in vivo* stability it is necessary to cap NPs with biocompatible coatings. Thus there is a current drive to integrate all the "green chemistry" approaches to design environmentally

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Peer review under responsibility of King Saud University.

ELSEVIER Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.arabjc.2014.12.042

benign materials and processes (Nadagouda and Varma, 2006; Kanipandian et al., 2014; Valodkar et al., 2011a; Castillo-López and Pal, 2014). Such an approach will also be of advantage for the integration of metal and alloy NPs into biologically relevant systems. A vast array of biological resources available in nature including plants and plant products, vitamins, algae, fungi, yeast, bacteria, and viruses could all be employed for synthesis of NPs (Das and Velusamy, 2013; Zhang et al., 2013; Mohanpuria et al., 2008). A number of reviews have been published recently which list the natural resources employed for the synthesis of noble metal NPs (Mittal et al., 2013).

Completely green synthesis of NPs and their biological applications has been subject of interest of our group (Valodkar et al., 2010, 2011b,c; Thakore et al., 2014). We have observed that reduction of silver ions can be induced by components of various natural extracts while reduction of Cu ions requires additional reducing agent. One such green reducing agent is ascorbic acid which was observed to be a major component of latex of *Achras sapota Linn.* commonly known as sapota. The latter belongs to sapotaceae family known to possess medicinal properties. In this work, we report an easy and rapid synthesis of Ag, Cu and their bimetallic NPs induced by sapota latex. The advantage of such non-toxic aqueous synthesis is that the NPs can be used as such for biological applications without isolation or purification (Valodkar et al., 2010, 2011b,c). We have also observed that the capping by different plant components affects the cytotoxic potential of metal NPs. Since the toxicity of bimetallic NPs has not been extensively studied, we decided to assess their role in biological systems.

2. Experimental

2.1. Materials

Copper nitrate ($Cu(NO_3)_2{\cdot}3H_2O$), Silver nitrate ($AgNO_3$) and Ascorbic acid were all purchased from Merck Mumbai, India. All the solutions were prepared using double-distilled deionized water. Crude milky white latex obtained from the fruit of *Achras sapota Linn.* was stored under refrigeration at −20 °C.

2.2. Cell culture

Mouse embryonic fibroblast (3T3L1) cells (obtained from National Centre for Cell Sciences, Pune, India) were seeded (1×10^5 cells/25 mm T Flask) and cultured in Dulbecco's minimum Eagle's medium (DMEM, Himedia, Ahmedabad, India) containing 10% Fetal Bovine Serum (FBS, Biosera, Mumbai, India) and 1% at 37 °C with 5% CO_2 (Thermo scientific, forma II water jacketed CO_2 incubator). Cells were subsequently sub-cultured every third day by trypsinization with 0.25% Trypsin–EDTA solution. All the reagents were filtered through 0.22 μ filter (Laxbro Bio-Medical Aids Pvt. Ltd.) prior to use for the experiment.

2.3. Synthesis of metallic and bimetallic nanoparticles (NPs)

$AgNO_3$ and ($Cu(NO_3)_2{\cdot}3H_2O$) solutions were prepared using double distilled de-ionized water.

Synthesis of NPs was carried out using 1% (v/v) solution of the extract. In a typical process, 10 ml of the diluted extract was mixed with 0.6 ml of 0.01 M $AgNO_3$ solution. It was heated at 60 °C for 30 min whereby the formation of silver NPs was marked by characteristic coloration of the solution. Similar reactions were performed with 0.1 and 0.5 M $AgNO_3$ solution to assess the potential of the latex at higher metal concentrations.

When synthesis was carried out with $Cu(NO_3)_2$, the formation of cuprous oxide (Cu_2O) NPs was observed at 60 °C. Since the extract acts as a mild reduction agent the reduction of copper was difficult. Hence additional ascorbic acid 10% (w/v) was used as reducing agent. For the formation of bimetallic NPs equal volume of metal precursor was added and was reduced simultaneously at room temperature using ascorbic acid.

2.4. Characterization of NPs

Characteristic optical properties of the NPs solutions were recorded using PerkinElmer Lambda 35 UV–vis spectrophotometer. FT-IR spectra of latex and vacuum dried NPs-latex composites were recorded as KBr pellet on Perkin Elmer RX1 model in the range of 4000–400 cm^{-1}. Size and shape of the NPs were determined by using TEM on a Philips, Holland Technai 20 model operating at 200 kV. The sample for TEM was prepared by putting one drop of the suspension onto standard carbon-coated copper grids and then drying under an IR lamp for 30 min. The particle size distribution and zeta potential were measured using a 90 Plus DLS unit from Brookhaven (Holtsville, USA). XRD was determined by using PANalytical 'X'PERT-PRO XRPD of Cu Kα radiation (λ = 0.15406 nm) with scanning rate of 2°/min and 2θ ranging from 30° to 80°. Cyclic voltammograms were carried out on CH 600 C INSTRUMENT, using a Pt electrode as the working electrode, Pt rod as the counter electrode and Ag/AgCl as the reference electrode.

2.5. Cell viability by MTT assay

Mitochondrial MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction assay, as an index of cell viability was performed as reported elsewhere (Valodkar et al., 2011b). Briefly, 3T3L1 cells (5.0×10^3 cells/well) were maintained in 96 well plates (Tarson India Pvt. Ltd.) for a period of 72 h, in presence of Ag/Cu NPs (50–1000 ng/ml) or control. Absorbance was measured at 540 nm in ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc., Winooski, VT). At the end of 72 h, cells were fixed in 4% paraformaldehyde for 10 min, mounted in glycerin and examined under Leica DMIL inverted microscope (200×). Photographs were taken with Canon power shot S 72 digital camera (Valodkar et al., 2011b). Acridine orange (AO) and ethidium bromide (EB) AO/EB staining and statistical analysis were carried out as per reported procedure (Valodkar et al., 2011c).

3. Results and discussion

The digital photographs in Fig. 1 indicate that with increase in concentration of precursor solution from 0.01 M to 0.5 M, the

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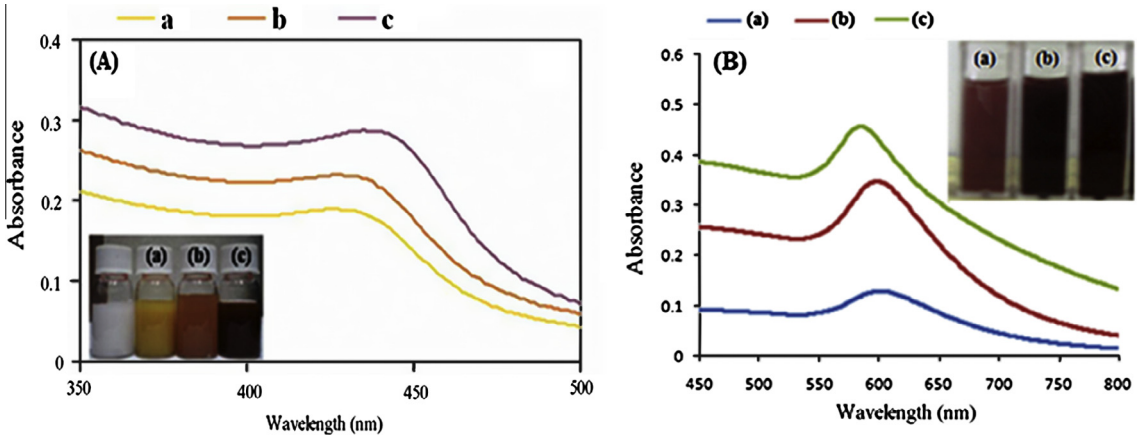

Figure 1 UV–vis absorption spectra of latex induced NPs at different molar concentrations of the metal precursor. (A) Ag (B) Cu (a) 0.01 (b) 0.1 (c) 0.5 M. Inset shows the digital photographs.

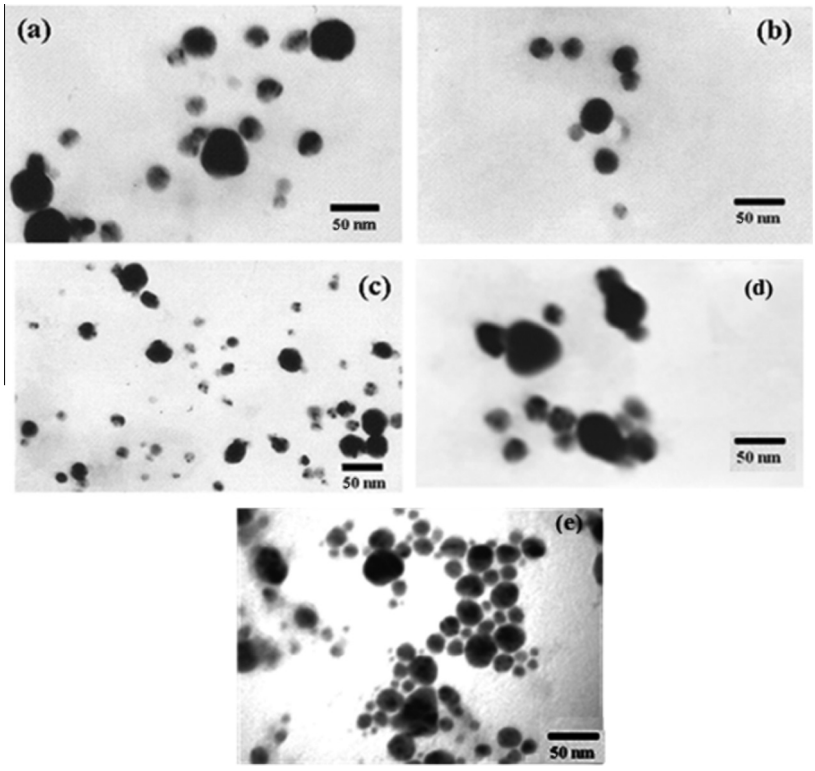

Figure 2 TEM image of the latex stabilized NPs of (a) Ag, (b) Cu, (c) AgCu (25:75), (d) AgCu (50:50), and (e) AgCu (75:25) synthesized using 0.01 M precursor solution.

color of solution changes from yellow to brown for Ag and light red to dark red for Cu. The surface plasmon absorption band shows red shift with increasing metal content. The SPR values 433 and 603 (Supporting information) are characteristic of AgNPs and CuNPs respectively. For the alloy nanoparticles, the plasmon band appeared in between the two. With an increase in Cu content the absorbance wavelength increases (Valodkar et al., 2011d).

The typical TEM images (Fig. 2) of the monometallic and alloy NPs show that the particles are spherical in shape and more or less of similar size (~20–40 nm) although some aggregates are seen. XRD data revealed that the crystallite size of alloy NPs and CuNPs is smaller than AgNPs (Table 1). This is also evident from TEM images and DLS data of these nanoparticles. This may be because of additional capping of CuNPs due to ascorbic acid used as reducing agent.

The formation of alloy nanoparticles was further confirmed by XRD analysis. Fig. 3A shows the XRD patterns of monometallic and bimetallic NPs. The peaks due to Bragg's reflection along with lattice parameter of metallic and bimetallic NPs are shown in Table 1. It is observed that the Ag (1 1 1) peak appears prominently in the spectrum of the AgNPs. On

Table 1 Peaks due to Bragg's reflection observed at 2 theta value, lattice parameter and crystallite size of metallic and bimetallic NPs.

Nanoparticles	2 theta (°) corresponding to				Lattice parameter (Å)	Crystallite size (nm)
	(111)	(200)	(220)	(311)		
AgNPs	38.55	44.71	65.09	78.07	4.089	47
AgCu NPs (75:25)	38.79	45.01	65.91	78.05	4.071	31
AgCu NPs (50:50)	38.55	44.79	65.07	78.09	4.058	32
AgCu NPs (25:75)	36.73	43.37	50.61	74.57	3.973	30
CuNPs	43.43	50.65	74.35	–	3.615	33

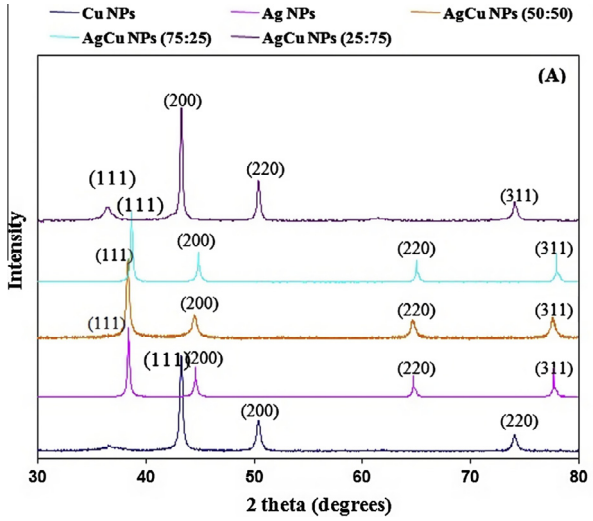

Figure 3A X-ray diffraction patterns of latex stabilized metallic and bimetallic NPs.

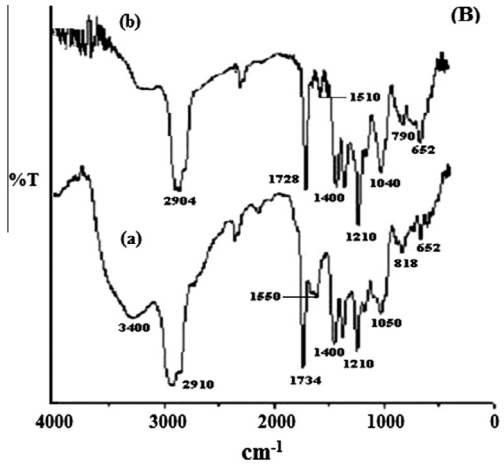

Figure 3B FT-IR spectra (a) sapota latex and (b) sapota latex stabilized AgNPs.

the other hand, peaks due to face-centered cubic (fcc) copper metal (200) are prominently in the spectrum of the Cu rich samples. The peaks corresponding to bimetallic NPs fall in between the peaks of Ag and Cu indicating that the new phases formed are homogenous Ag—Cu alloy phases rather than completely separated phases of Ag and Cu. The difference in lattice parameter also indicates the formation of alloy NPs (Valodkar et al., 2011d).

The chemical composition of sapota latex revealed that it is one of the rich sources of reducing sugars, proteins, ascorbic acid, phenolics, and carotenoids (Kulkarni et al., 2007), as clearly visible in the IR spectra (Fig. 3B). The spectra of latex as well as latex stabilized AgNPs exhibited a band at 1050, 1100 and 1210 cm^{-1} which is a depiction of 1°, 2° alcoholic groups and phenolic derivatives. The bands visible at 652–818 cm^{-1} signify the presence of R—CH group and aromatic C—H bending respectively. The intense bands at ~1350 and ~1400 to 1450 cm^{-1} are due to C—O stretching and aromatic C=C stretching which is abundantly found in sapota latex. A major difference in both spectra is that the intensity of the band at 3400 cm^{-1}, due to the —OH group present in ascorbic acid and other phenolic compounds decreases in the spectra recorded after bioreduction of Ag ions. A significant band at 1734 cm^{-1} due to carbonyl groups of reducing sugars shifts to 1728 cm^{-1} and that at 2910 cm^{-1} shifted at 2904 cm^{-1}. All these changes signify the role of —OH groups and participation of —C=C group in bio-reduction suggesting conversion of ascorbic acid into dehydroascorbic acid (Supporting information Scheme S1). Other bands which are visible in both the spectra (before and after reduction) in bunch are at, 652, 818, 1029, 1315 and 1400–1450 cm^{-1}. In order to investigate the mechanism of the reduction further, the electrochemical behavior of the latex was examined in the presence and absence of metal ions.

Cyclic Voltammetric studies also supported the reducing nature of the extract. Fig. 4 shows the typical cyclic voltammogram (CV) of the extract and extract stabilized Ag and Cu NPs recorded at a scan rate of 0.03 V s^{-1} in the potential window between −1.30 and 1.20 V. The direction of the scan is shown by an arrow. It can be seen from the figure that there appeared to be an oxidation peak at the potential of about −0.65 V in the latex which may be due to the redox activity of the components of latex. This suggests that the components of the latex might contribute to an effective process for obtaining electrons on the Pt electrode. The reductive materials in the latex which are responsible for causing the reduction, gain electrons and gets oxidized in the surrounding of an electric field (Thounaojam et al., 2011). In case of AgNPs ions latex induces complete reduction and therefore no reduction peak is observed in latex treated silver solution. CuNPs show the reduction peak at −0.3 V indicating that latex being the mild reducing agent was unable to reduce Cu. The reduction peaks at 0.125 V and −0.235 V correspond to the reduction of $CuO \rightarrow Cu_2O$ and $Cu_2O \rightarrow Cu$, respectively. The above peaks were also observed in case of alloy NPs indicating that latex was unable to reduce alloy NPs (data not shown). The cyclic

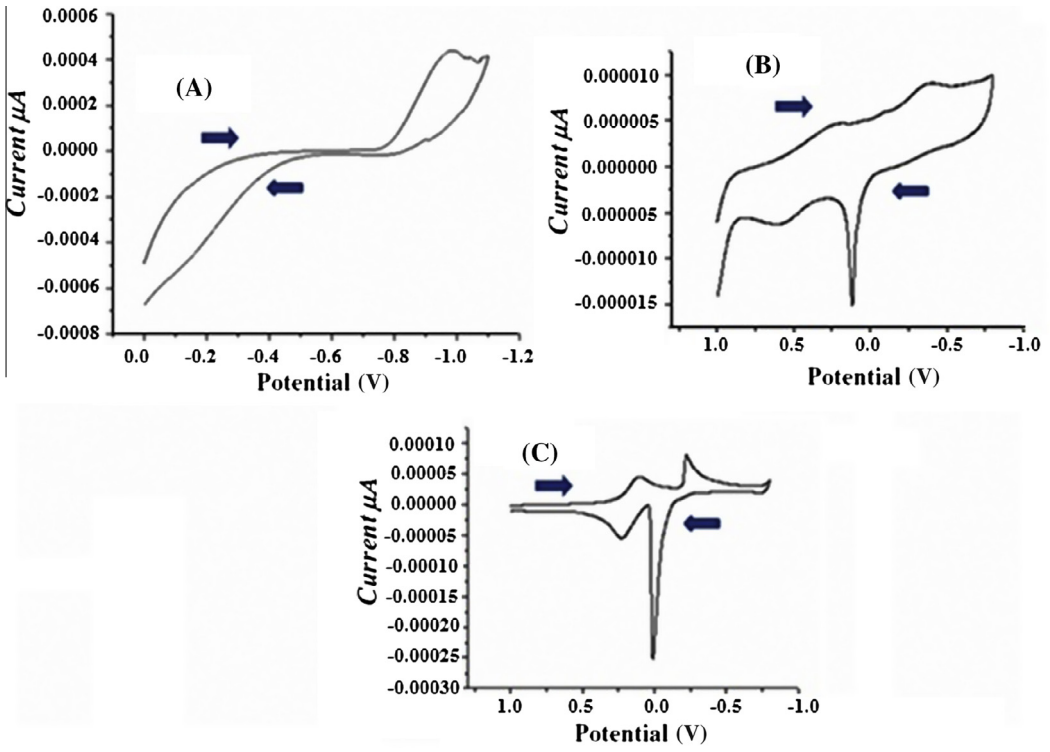

Figure 4 Cyclic voltammogram spectra of (A) latex stabilized AgNPs, (B) Sapota latex and (C) latex stabilized CuNPs.

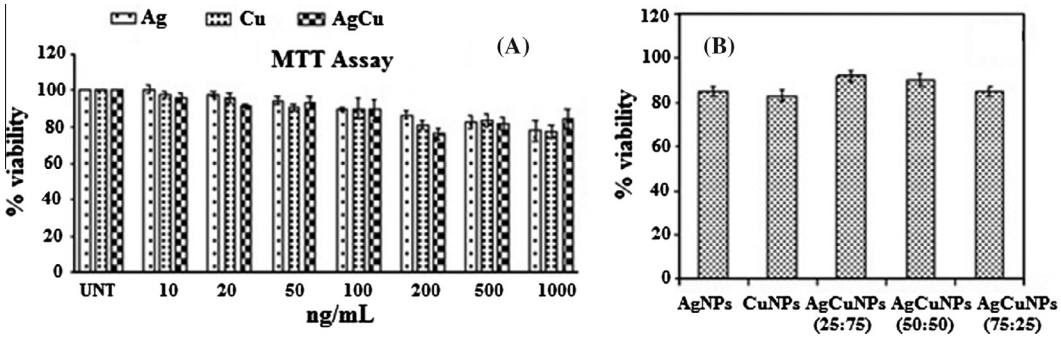

Figure 5 Effect of sapota latex capped metal and alloy NPs on 3T3L1 cell viability at (A) various concentrations and (B) at 1000 ng/ml. Results are expressed as mean ± S.E.M. for $n = 3$ (replicates). The changes were not significant.

voltammetry data supported the fact that the reduction of silver was feasible with latex while that of copper was not feasible (Fig. 4).

In vitro cytotoxicity of metallic and bimetallic NPs was evaluated in 3T3L1 cells using MTT cell viability assay. MTT is a tetrazolium dye that undergoes reduction by the mitochondrial enzymes to form a blue colored formazan. Cells with non-functional mitochondria cannot carry out this reaction and hence, color intensity of soluble formazan crystals is directly proportional to cell viability. Thus, it is a useful tool to detect cytotoxicity. Exposure of 3T3L1 cells for 72 h to the latex control and latex capped nanoparticles in the dose range of 10–1000 ng/ml did not record significant cytotoxicity (Fig. 5A) except, 50:50 alloy which recorded moderate degree of cytotoxicity with doses 500 and 1000 ng/ml. A comparison of viability assay with uncapped forms of Ag and Cu NPs revealed that capping with latex biomolecules significantly minimized its cytotoxicity (Supporting information Fig. S1) (Valodkar et al., 2012) while, comparison of alloys at maximum dosage also revealed non-cytotoxic nature at all compositions (Fig. 5B). However the cell viability at these concentrations was about 85% which suggests that higher dosage would prove to be cytotoxic. Further, acridine orange/ethidium bromide florescence assay was used for qualitative cytotoxicity evaluation. Acridine orange is a nucleic acid selective fluorescent cationic dye that is cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions respectively. When bound to DNA, it is very similar spectrally to fluorescein, with an excitation maximum at 502 nm and an emission maximum at 525 nm (green). Whereas, ethidium bromide is impermeable to the viable cells and hence, taken up only by non-viable cells and emits red

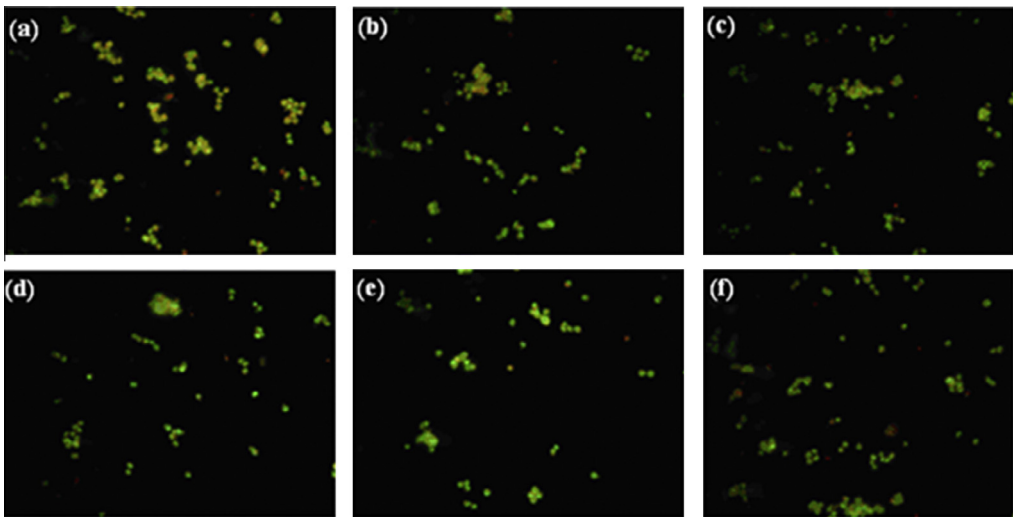

Figure 6 AO/EB photomicrographs of stained 3T3L1 cells exposed to (a) Sapota latex and 1000 μg/ml of sapota latex stabilized NPs, (b) Ag, (c) Cu, (d) AgCu (25:75), (e) AgCu (75:25) and (f) AgCu (50:50).

fluorescence by intercalation into DNA, presently recorded observations as seen in Fig. 6 clearly indicated majority of the cells with green colored florescence up to a dosage of 1000 ng/ml. This suggests non-toxic nature of NPs.

The NPs, especially if made in aqueous solution, have a surface charge to stabilize them against aggregation via electrostatic repulsion. Measurement of zeta potential reveals that the latex as well as latex capped NPs showed negative zeta potential values (Supporting information Table S2). Interaction of cationic particles with negatively charged cancer cells has been well reported in the literature (Alkilany and Murphy, 2010). Dasgupta et al., have also observed that neither size nor zeta potential alone determine the optimal cellular response induced by NPs (Patra and Dasgupta, 2011). The components of cellular growth media used *in vitro* studies could interact with NPs and change their physiochemical properties. Thus size, aggregation state, surface charge and surface chemistry would be significantly modified via electrostatic screening which in turn could influence their ability to interact with or enter cells (Nikolov and Wasan, 2007). It has also been proposed that, in regions where the columbic repulsion of similar charges is not too pronounced, the presence of a high electric field may cause local electroporation. Such high electrical fields may succeed even with negative zeta potential owing to small size of NPs. This phenomenon is known to facilitate permeation of various nanoscale objects through biological membranes.

4. Conclusions

Silver and copper alloy nanoparticles were synthesized using latex of sapota fruit. *In vitro* studies revealed that the aqueous latex capped NPs were significantly less toxic than uncapped NPs or ions. Formation of alloy did not have significant effect on cytotoxicity. They were also successfully able to bind with 3T3L1 cells despite negative zeta potential. The biological interactions of the NPs may be attributed to their nanosize and aggregation state in the latex. A therapeutical window might exist for the application of the biocompatible latex capped metallic and alloy NPs in medical products.

Acknowledgments

One of the authors (MV) is grateful to CSIR, New Delhi, for senior research fellowship. Special thanks to Dr. Bhavna Trivedi and Mr. Shardul Bhatt Department of Chemistry, for cyclic voltammetry studies.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arabjc.2014.12.042.

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