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# Synthesis of Ag–Pt alloy nanoparticles in aqueous bovine serum albumin foam and their cytocompatibility against human gingival fibroblasts

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ABSTRACT

Foams of bovine serum albumin (BSA) have been utilized for synthesizing *in situ* protein capped Ag–Pt alloy nanoparticles. The *in vitro* cytotoxicity and the rate of proliferation of human gingival fibroblasts (HGFs) in presence of the above synthesized alloy nanoparticles is investigated. Expression profile of protein involved in detoxification, i.e. metallotheonein (MT) were assayed by ELISA and expression of mRNA transcripts by reverse transcription polymerase chain reaction (RT-PCR). Cytotoxicity results suggested that protein capped nano-alloys might be promising candidates for implants and prosthetic material. RT-PCR and ELISA confirmed the expression of MT, in cells treated with the alloy nanoparticles. Morphology variation studied by SEM also confirms that cells treated with alloy nanoparticles present an intact morphology.



## 1. Introduction

Designing new synthetic protocols for nanoscale biocompatible materials is currently receiving considerable attention. Interest in nanomaterials arises due to new properties and functions stemming from their dimension and modified electronic structure [1]. Among various nanomaterials, with respect to biomedical applications, much attention had been paid towards alloy and composite materials for their superior properties compared to the individual components constituting the alloy/composite [2–5]. With respect to applications related to dentistry some nanoparticulate systems have crossed the realm of researchers and have gained entry to the market also. Particular mention should be made of Ag–Cu being used in dental implants and toothpastes that claim to contain nanoparticles which over a period of time fill up the cavities [6]. In the recent past Ag–Pt alloy nanoparticles (NPs) aroused much interest of researchers because of their novel application in dentistry, bone and breast surgical implants on account of their corrosion resistance and improved mechanical properties [7–9]. A key challenge to material scientists is to synthesize biocompatible Ag–Pt alloy NPs for their application *in vivo* that imposes stringent conditions of compatibility, non-immunogenecity, etc. A strategy towards such biocompatibility is to conjugate biocompatible, non-immunogenic protein molecules to the alloy NPs. While post-synthetic conjugation is a possibility, a much better alternative is to synthesize these materials in a template made-up of the protein of interest.

Bimetallic colloids can be prepared by simultaneous co-reduction of two kinds of metal ions with or without the protective agent (usually a polymer or a surfactant) or by successive reduction of one metal over the nuclei of another involving physical or chemical route [10–12]. Alloy systems are usually realized by the simultaneous reduction of the metal ions [13]. For example, for the chosen system here, i.e. Ag–Pt alloy the precursors of silver and platinum ions are taken together and subjected reduction concurrently. Usually the salt precursors used for silver are $Ag^+$ ions while those for platinum are $PtCl_6^-$ ions. A stumbling block in this procedure is the resultant alloy NP's phase purity, i.e. to avoid the separate nucleation of the individual nanoparticles along with the alloy NPs. Traditional surfactants compound this problem, as these are normally either positively or negatively charged and preferentially bind to one of the ions and hence promote separate nucleation. A way to circumvent this problem is to achieve a thorough mixing of the metal ions that constitute the alloy at atomic scale and then subject them to reduction. Here we use the bovine serum albumin as a template and stabilizing agent, which is zwitterionic in nature at its pI. The zwitterionic nature is also favorable for its foaming and some of us have previously reported the advantages of using foams for obtaining phase pure alloy NPs [14]. In the case investi-

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gated here, the alloy formation is simply accomplished by carrying both Ag and Pt ions in BSA foam simultaneously and reduction of metal ions was achieved by passing vapors of hydrazine hydrate through the foam. It is observed that the silver ions get reduced with hydrazine hydrate, which reduces the platinum ions by transmetallation reaction due to their favorable redox potentials for a spontaneous reduction of $Pt^{4+}$ by $Ag^0$ [15]. At appropriate concentration of the two ions alloy formation occurs and since these are formed inside the foam matrix the resultant alloy NPs are *in situ* capped by the protein molecules.

Then, in order to probe the resultant alloy NPs biocompatibility, especially with dental applications in mind, we investigated the effect of the alloy NPs on proliferation and cytotoxicity on human gingival fibroblast (HGF) cells cultured *in vitro*. Our data indicate that formed alloy NPs are more tissue compatible with HGFs compared to the salt precursors ($Ag_2SO_4$ and $H_2PtCl_6$) [16]. After ascertaining the alloy NPs nontoxic nature, we studied the events at molecular level by ELISA and RT-PCR to assess the mechanism involved in detoxification. It is well known that the expression of the protein MT and its presence in culture conditions is indicative of cell survival in spite of presence of salt [17]. In our study with alloy and silver salt precursors, MT expression is seen suggesting prima facie that BSA capping provides compatibility of NPs with the cells *in vitro*. SEM reveals that with salt precursors HGFs present loose cell morphology but remain intact in alloy environment, supporting their utility for *in vivo* applications. Details of studies carried out are presented below.

## 2. Experimental

### 2.1. Chemicals

Silver sulphate ($Ag_2SO_4$), chloroplatinic acid ($H_2PtCl_6$), and hydrazine hydrate ($N_2H_4$), all were obtained from Aldrich chemicals and used as received. Bovine serum albumin (BSA 98%, 66 kDa mol. wt.) was obtained from SRL Chemicals, India Ltd. and used as received.

### 2.2. Synthetic procedure

In a typical experiment, a rectangular column of 50 cm in height and with a square base of 10 cm × 10 cm with sintered frit embedded at the bottom was used for foam generation. 25 mL of $Ag_2SO_4$ ($1 \times 10^{-3}$ M, that leads to a final concentration of $2 \times 10^{-3}$ M $Ag^+$ in the final solution) and 25 mL of $H_2PtCl_6$ ($3 \times 10^{-3}$ M) were mixed and added to 50 mL bovine serum albumin (BSA) solution (3 mg/mL). The pH of the solution was adjusted to 4.7 which is the pI of BSA. The solution was taken in the bottom of foam column. Nitrogen gas was allowed to pass through the frit at the bottom of the foam column to generate the foam. The foam was allowed to raise into the column up to a height of 45 cm and the gas flow was stopped. The excess liquid from foam was drained for 20 min so that a dry foam condition is created. A Petridish containing hydrazine hydrate was kept under the foam column such that the vapors pass through foam and the reduction occurs. After 25 min of reaction, the color of foam column changes to grayish. The reduced alloy NPs in foam are collected by spraying water from the top of the foam column. The collected solution was centrifuged at 10,000 rpm for 15 min for the separation of protein from the sample. The pellet sample was washed with distilled water and recentrifuged described as above for complete removal of uncapped protein from sample. The pellet sample was used for the further UV, XRD, TEM, XPS characterization, etc. We also tried to synthesize individual platinum and silver nanoparticles in BSA aqueous foam by same method as described above. In these cases a 50 mL solutions of $Ag_2SO_4$ or $H_2PtCl_6$ ($1 \times 10^{-3}$ M) were taken instead of the mixture. The reduction was observed only when silver ions alone were taken and with platinum alone no reduction took place.

### 2.3. UV–vis spectroscopy studies

The optical properties were monitored on a Jasco UV–vis spectrophotometer (V570 UV-VIS-NIR) operated at a resolution of 2 nm.

### 2.4. TEM measurements

TEM samples were prepared by placing a few drops of their aqueous dispersions on carbon coated copper grids and allowing them to dry. TEM measurements were performed on a JEOL model 1200EX instrument operated at an accelerating voltage at 80 kV.

### 2.5. X-ray diffraction measurements

Thick films on glass substrate prepared from the aqueous solutions were used for this purpose. The samples were characterized on the X'pert Pro model X-ray diffractometer from PanAlytical instruments operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation.

### 2.6. XPS measurements

XPS measurements of Ag–Pt alloy nanoparticles were carried on a film deposited onto copper substrate by the drop coating technique. VG Micro Tech ESCA 3000 instrument operating at a pressure better than $10^{-9}$ Torr was used. The general scan was performed and C 1s, N 1s, O 1s, S 1s, Ag 3d and Pt 4f level spectra were recorded with un-monochromatized Mg Kα radiation (photon energy = 1253.6 eV) at pass energy of 50 eV and electron take off angle (angle between electron emission direction and surface plane) of 60°. The overall resolution of measurement is 1 eV for the XPS measurements. The core level spectra were background corrected using the Shirley algorithm [18] and chemically distinct species were resolved using a nonlinear least square procedure. The core level binding energies (BEs) were aligned with respect to the C 1s binding energy of 285 eV.

### 2.7. Cytocompatibility test

Human gingival fibroblasts (HGFs) were isolated from three patients under local anesthesia from normal skin (two males and one female from 18 to 35 years of age) with their informed consent without any history of oral pathology. Proper Ethical Cleavances were taken from appropriate authorities. Before culture, the samples were rinsed with phosphate-buffered saline (PBS), and deep dermal layers and necrotic tissues were removed. Tissue were cut into small fragments by sterilized scalpel and then transferred to Petridishes containing 20 mL dispases (20 mg/mL). Tissue pieces were again rinsed with PBS and further minced with scalpel in order to loosen the tissue matrix and facilitate the migration of fibroblast into the medium. HGFs were next isolated from tissue pieces by overnight digestion with Type I collagenase (80 U/mL, Worthington Biochemical Corporation, Freehold, NJ) with Dulbecco modified Eagles media [DMEM] containing 20% fetal calf serum (FCS from Gibco BRL, Carlsbad, CA) at 37 °C and plated in 75 $cm^2$ plastic culture flask. Now fibroblast were further propagated in DMEM supplemented with 10% FCS, penicillin and streptomycin [100 μg/mL each, Gibco Invitrogen Corporation], and amphotericin B [2 ng/mL Gibco] under standard culture conditions (i.e. at 37 °C in a humidified atmosphere containing 5% $CO_2$) and cell culture medium were changed regularly at an interval of every 48 h. After attaining 80% confluence, cells were trypsinized and passaged into 1:3 split ratios. After first passage, cells were routinely maintained