Development of Non-ionic Surfactant and Protein-Coated Ultrasmall Silver Nanoparticles: Increased Viscoelasticity Enables Potency in Biological Applications

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ABSTRACT: To enhance the interactivity with biological cells, we developed ultrasmall (5 nm in diameter) Ag NPs coated with a mixture of Tween-20 (Tw-20) surfactant and human serum albumin (HSA) or hemoglobin (Hb) proteins. These were tested with cancerous and healthy cell lines to investigate the therapeutic applicability. Using the established concept of generation of reactive oxygen species (ROS) and the ROS-induced oxidative stress in carcinogenic cells by Ag NPs, we found that the presently synthesized Ag NPs selectively destroyed the cancerous cells. A mixture of Tw-20 with protein, where the surfactant was in large excess, created a coating over the Ag NPs resulting weaker protein–protein interactions and facilitating interfacial protein–surfactant interactions, which leads to an increase in the film viscoelasticity to enhance the stability of the Ag NPs and cell viability. Moreover, this concept has been applied to drug delivery using a model fluorophore (fluorescein) on Ag NPs to explore the prospects in photodynamic therapy. The results are encouraging and deserve further investigation.

1. INTRODUCTION

Silver nanoparticles (Ag NPs) are probably the most widely studied nanomaterials because of their multiple beneficial functions and applications. These have stemmed from the immensely useful anti-bacterial activities of the Ag NPs along with their catalysis capabilities and sensing properties.1–3 These ultrasmall particles provide a large surface area that enhances their properties even in biological substances including serum, mucus, etc.4 It is proven that the release of silver ions (Ag⁺) in the medium due to oxidation of the Ag NPs imposes cytotoxicity, genotoxicity, and apoptosis.5–7 In spite of the large number of studies on Ag NPs, much is left unexplored regarding cytotoxicity as well as their behavior on short- or long-term exposure to living beings.8,9 In vitro and in vivo studies indicate that the ionization of Ag triggers the generation of reactive oxygen species (ROS) that, in turn, plays a big part in the toxic effects of the Ag NPs.10,11

Because of the large varieties of applications of Ag NPs, these are produced in huge amounts using different techniques mostly yielding uncoated Ag NPs. The synthetic methods involve chemical reduction,1 laser ablation,12 γ-radiation,6 electrochemical and microemulsion techniques,7,13 microwave-assisted synthesis,14 and photoinduced reduction.15 As these methods involve intrinsically toxic substances, hence attempts to synthesize green Ag NPs gained importance, and in this context, microorganisms and plant extracts were used as reducing and stabilizing agents to Ag salts.8 In addition to this, the reducing and stabilizing properties of surfactants have been used to synthesize Ag NPs to prevent particle aggregation.16 For example, studies on protection of Ag NPs with surfactants showed that polyvinylpyrrolidone (PVP) protects more
compared to polyethylene glycol (PEG). PVP influences the preparation of Ag NPs by polyol method. Looking into the mechanism of action of the Ag NPs in releasing Ag⁺ ions or formation of large aggregates that determines toxicity, these were protected by lung surfactant, such as dipalmitoylphosphatidylcholine (DPPC), and investigated as a function of pH. The results helped understanding the stability of the Ag NPs in the deep lung. Prolonged research showed that the cytotoxicity of uncoated Ag NPs is greater than that of coated Ag NPs. Cytotoxicity is controlled by the coating agents by restricting the release of the Ag⁺ ions. In one report, Martin et al. used in situ ultrasmall-angle X-ray scattering (USAXS) results to quantify the dissolution, agglomeration, and stability limits of Ag NPs coated with bovine serum albumin (BSA) protein. They showed that the loss of Ag from the NPs matches with a second-order kinetic rate reaction on disruption of the BSA corona. It is important to understand the interaction of the NPs with the protein medium while investigating their biological activities since the protein corona is more or less the deciding factor. The protein corona formation around the NPs is dynamic in nature involving exchange between the surface bound and the outer proteins accompanied by a slow formation of hard corona that determines the biological identities of the NPs.

Variation in particle size largely determines the cytotoxicity of Ag NPs. The effect of the Ag NPs on many biological functions depends on their size. Carlson et al. found that hydrocarbon-coated 15 nm Ag NPs generate more ROS compared to the 55 nm ones. Liu et al. found that PVP-coated 5 nm Ag NPs were even more toxic compared to the 20 and 50 nm varieties, as also was found for citrate-protected Ag NPs by Kaba et al. However, there is controversy on the dependence of the toxicity threshold on the particle size. A study on commercially obtained Ag NPs of different sizes showed that the smallest (20 nm) in the lot are the most cytotoxic. In another study on size-dependent acute toxicity of Ag NPs, the authors found the smallest citrate-stabilized Ag NPs (10 nm) to be the most toxic on normal mice. Similar were the observations with Ag NPs derived from reverse micelles with a narrow size distribution (4.6–9.3 nm).

Choice of proteins as protecting agents was found to produce differently shaped Ag NPs, which revealed the absence of shape-induced toxicity.

The larger surface area of smaller NPs promotes intracellular penetration providing enhanced antimicrobial activity. Ivask et al. studied the action of Ag NPs of various sizes on biomolecules and concluded that release of Ag⁺ ion is the principal cause of cellular toxicity for particles in the 20–80 nm diameter range, whereas those with diameters of 10 nm and smaller were cytotoxic due to prominent cell–particle interaction. Herein, we have synthetically produced ultrasmall Ag NPs with a diameter of ~5 nm protected with a neutral surfactant-infested proteins (namely, human serum albumin (HSA) and hemoglobin (Hb)). The intention of the study is to look into the applicability, stability, and biological cell viability and estimate any differential cytotoxicity aiming toward selective destruction of cancerous cells by the ultrasmall Ag NPs. In this context, it is pertinent to mention that application of Ag NPs to unicellular organisms could be made with protein tagging.

It is known that the uncontrolled growth of tumor cells has close association with oxidative stress. Such a situation arises due to redox imbalance or irregular ROS-scavenging. Hence, understandably any ROS-promoting substance would exert additional oxidative stress to such cells and consequently lead to cell death. Such examples are known in works with MCF-7 human breast cancer cells where Zn-doped titanium oxide NPs were used. MDA-MB231 human breast cancer cells were treated with Ag NPs of 20 nm in diameter using the same mechanism. To increase interactivity with biological cells, we have developed ultrasmall (5 nm in diameter) Ag NPs coated with a mixture of Tween-20 (Tw-20) surfactant and HSA or Hb proteins, where the surfactant concentration is 1000 times higher than the proteins and exploited the concept of ROS-induced oxidative stress. Carcinogenic cells were treated with these NPs, and the results were compared with healthy cells. At such a high concentration, the surfactant molecules act as denaturant to the protein due to higher surfactant–protein interaction. This results into weaker protein–protein interactions and facilitates interfacial protein–surfactant interactions leading to an increase in the film viscoelasticity. Thus, our synthetic procedure might induce biocompatibility as well as stability to the Ag NPs on one hand and the benefits of the ultrasmall size on the other. Presumably, the same mechanism of oxidative stress to eliminate the cancer cells over the healthy cells is operative in this case. Furthermore, we have successfully used fluorescein (FL) as a model fluorophore to explore its binding dynamics with the newly developed delivery vehicles (surfactant–protein coated Ag NPs) where we got interesting results that produced new pathways to FL-aided photodynamic therapy (PDT).

2. RESULTS AND DISCUSSION

Ag NPs show toxicity under various circumstances depending on their environment and size. Controversies exist about the size-induced interactions of the Ag NPs with biological cells. Some reports mention that larger Ag NPs (ca. 100–150 nm) show less toxicity compared to the smaller ones (ca. 20–50 nm). On the other hand, larger surface area of the smaller NPs induces better cell penetration increasing their antimicrobial activity. The interaction of Ag NPs with biological substances depends on their surface coating. Ag NPs coated with bio-compatible proteins, lipids, and surfactants show better results in cell viability. Proteins form corona on the surface of the NPs, which, in turn, interacts with the cell membrane during cellular intake. Internalization of the Ag NPs triggers ROS generation due to oxidation of Ag. It has been reported that smaller Ag NPs generate more ROS compared to the larger ones. Hence, the principal concern of the present study laid on the construction and the stability of the surface coating of the NPs that will help in internalization and subsequent cellular environment dependent actions of the Ag NPs based on the ROS generation. To tackle the first problem, that is, stable coating of the Ag NPs, we adopted the principle of dilution of protein–surfactant mixtures. A much larger concentration of Tw-20 compared to that of the protein (HSA or Hb) enabled mixing of the two components to construct a stable form. The resulting weaker protein–protein interaction and enhanced interfacial protein–surfactant interaction increases the film viscoelasticity. Such a substance provides a stable coating to the Ag NPs. We intended, principally, to use these bio-friendly Ag NPs in treating the cancer cells and compare the effects with healthy cell lines.

Tumor development and metastasis are the characteristic results of uncontrolled cellular growth, which is commonly
known as cancer. The most popular treatment till date, chemotherapy, has the drawback of non-specific cellular interactions resulting into adverse effects on the healthy cells. To this end, Ag NPs have appeared as promising anticancer agents. The Ag NPs penetrate the cell membrane by endocytosis followed by degradation to release of Ag⁺ triggering the generation of ROS and reduction in glutathione (SGH) level. The transmembrane potential of mitochondria, thus, gets altered due to enhanced cellular superoxide radicals influencing the signal transduction pathways resulting in cell death. Following the proven hypothesis of association of oxidative stress with uncontrolled growth of tumor cells (cancer) resulting into irregular ROS-scavenging, we have designed our experiment to compare the effects of the ultrasmall Ag NPs on healthy and cancer cell lines. The 5 nm Ag NPs supposedly generate more ROS, and hence the absence of proper scavenging mechanism will result into cancer cell death. The obtained experimental results support this view.

Cytotoxicity values of the Tw-20-HSA and Tw-20-Hb coated Ag NPs were measured in triple-negative metastatic breast cancer cell line (MDA-MB231) and human embryonic kidney cell line (HEK293T). The comparison of IC50 (Table 1) values indicates that both the varieties of the Ag NPs have more potency to affect cell viability in MDA-MB231 cell line. The IC50 values determined for MDA-MB231 on treatment with the Tw-20-HSA and Tw-20-Hb coated Ag NPs were 45.53 ± 3.24 and 41.00 ± 0.54 μM, respectively, whereas no significant cell death was observed for HEK293T cells even at 200 μM concentration of Ag NPs. The projected IC50 value is more than 200 μM, which is around five times higher than what we observed for MDA-MB231 cells. This gives a clear idea that the Ag NPs specifically affect the viability of the MDA-MB231 cell lines even at micromolar level. The IC50 values obtained are well within the clinically acceptable concentration of 100 mg/L or 926 μM Ag.

The cell viability results are represented by Figure 1a,b for HEK293T and MDA-MB231, respectively. The absorbance decreases by 33% on applying about 200 μM Ag NP for HEK293T, while the change is 73% for MDA-MB231 on addition of about 60 μM Ag NP. IC50 values estimate the potency of the Ag NPs in inhibiting cell death. The IC50 values are remarkably different in the two cases. The healthy HEK293T cells are affected very little compared to the tumor cells (MDA-MB231). The results obtained are surprisingly better than those reported so far with different types and sizes of Ag NPs. In the present case, Ag NPs of 5 nm in diameter have been used, which are reported as potentially cytotoxic in several reports as mentioned earlier. However, using the modified bio-compatible coating and the concept of generation of a larger amount of ROS and along with the process of higher oxidative stress in tumor cells and lack of ROS scavengers, we could achieve the targeted results.

The Tw-20-HSA and Tw-20-Hb coated Ag NPs were further explored as a host for FL, which is a well-known dye with very high fluorescence quantum yield and useful in PDT. PDT is a well-known and vividly applied medical process where a photosensitive drug is infused into the site of treatment in animal tissue. The accumulated dye is retained at the treatment site in greater proportion than the healthy regions. The region of treatment is irradiated with a targeted non-thermal laser correlated to the absorption peak of the accumulated drug, which in turn treats the affected region. PDT has been successfully applied in healing various types of cancers by non-invasive techniques. In a recent report, Ag NPs were immobilized over silica NPs and decorated with transferrin for cancer cell targeting and treatment. The dye-based photosensitizers used in PDT are principally obtained from the tetrapyrroles, tricyclic dyes, and flurocoumarins.

Table 1. IC50 Values of Tw-20-HSA and Tw-20-Hb Coated Ag NPs on Interaction with the HEK293T and MDA-MB-231 Cell Lines

| Coatings of Ag NPs | HEK293T (μM) | MDA-MB231 (μM) |
|--------------------|-------------|----------------|
| Tw-20-HSA          | >200        | 45.53 ± 3.24   |
| Tw-20-Hb           | >200        | 41.00 ± 0.54   |

Figure 1. Cell viability experiments show that both the varieties of Ag NPs have high activity on the HEK293T cells and MDA-MB231 cell line. (a) The HEK293T cells and (b) the MDA-MB-231 cells were treated with various concentrations of the two types of Ag NPs, and the cell viability is determined from the absorbance values at 595 nm using MTT assay.
The dye, FL, lies among the tricyclic dyes and is effectively used in PDT.51 Considering the utility of FL as a photosensitizer and the PDT enabling properties of the synthesized Ag NPs, we attempted to cargo the dye on the NPs. Most astonishingly, we witnessed a remarkable increase in the absorbance of FL on addition of the Tw-20-protein protected Ag NPs in aqueous buffer (pH 7). The enhancement in absorbance was 28, 40, and 55 times the original optical density of FL on adding 26, 80, and 200 μM Ag NPs (Figure 2). We eliminated the absorption band due to the surface plasmon effects of the Ag NPs to monitor the changes on the FL signal. This result is remarkable since not only such an increase in light absorptivity will make FL a brilliant candidate for PDT but also the effect of the Ag NPs in this process remains to be a subject of further investigation.

The physical characteristics of FL interacting with the applied Ag NPs are shown in Figure 3. The intensity of FL fluorescence initially increased on addition of the Ag NPs up to 16 μM and subsequently quenched to some extent (Figure 3b,e). This indicates that FL is cooperatively interacting with the Ag NPs only after accumulation of a certain amount of the NPs. The portions of quenching of the fluorescence of FL was analyzed using the Stern–Volmer plots (Figure 3c,f), which fitted with straight lines indicating one particular type of quenching. The nature of the quenching of the FL fluorescence was examined from the time-resolved emission measurements exciting the fluorophore at 475 nm and monitoring the 510 nm emission. The decay data could be fitted with a single exponential decay routine, which provided the excited state lifetimes of FL to be 3.72 ns in the absence of the proteins and 3.73 and 3.6 in the presence of Hb and HSA, respectively. The practically unchanged fluorescence lifetime of FL indicates that the quenching is static in nature due to binding of FL to the surfactant–protein matrix.56 Fluorescence quenching is better for the Tw-20-Hb Ag NPs compared to the HSA variant indicating the Hb variant to act better in PDT among the two, although both of them look promising. The results show that a small concentration of FL adsorbed over the Ag NPs will be a good candidate to be retained at the treatment site. The strong absorption of light by FL at the target site on irradiation will suffice in treating the affected region.

3. CONCLUSIONS

A new variety of protected Ag NP was synthesized using the dilating property of protein−surfactant mixtures. The enhanced viscoelasticity of such a mixture with a larger share of surfactants than protein provides more stable and bio-compatible coating to the in situ formed Ag NPs. The results obtained herein on interacting the two varieties of ultrasmall (5 nm) Ag NPs (with TW-20-HSA and Tw-20-HB coatings) showed that such a small size facilitates cancer cell destruction. We used HEK293T and MDA-MB231 cell lines (healthy and cancerous, respectively) to apply the Ag NPs, which showed that the cancer cell lines are affected much more compared to the healthy ones, the degree of which is unprecedented. In addition to this discovery, we have also applied FL to bind to the Ag NPs so that further applications can be looked into in

Figure 2. Absorption spectra of FL (1 μM) in aqueous buffer added with (a) Tw-20-HSA and (b) Tw-20-Hb coated Ag NPs. The experiments were performed in a double-beam spectrophotometer keeping the blank Ag NP-buffer solutions in the reference compartment to eliminate the surface plasmon signal from the Ag NPs.

Figure 3. Fluorescence emission spectra of FL (1 μM) (a, d) with enhancement in the Ag NP concentrations (λex = 450 nm), (b, e) plots of relative change in the fluorescence intensity of FL at the maximum with increase in the Ag NP concentrations, and (c, f) relative quenching of fluorescence plots for FL with added Ag NPs in concentrations larger than 16 μM following the Stern–Volmer equation: \( F_0/F = 1 + K_{SV}[Q] \), where \( F_0 \) and \( F \) are the fluorescence intensities in the absence and presence of the Ag NPs, respectively, \( K_{SV} \) is the Stern–Volmer (or quenching) constant, and \([Q]\) is the concentration of the quencher.

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terms of PDT. Ag NP increases the absorptivity of light energy by FL manifold on binding to it, which is significant to enhance the photosensitizing ability of FL.

4. MATERIALS AND METHODS

All the chemicals and solvents (spectroscopy grade) were either procured from Sigma, USA or Merck, India. The purified proteins were bought in powder form from Sigma, USA. HPLC-grade water procured from Merck, India was used throughout the experimental processes. Phosphate buffer (pH 7) was used in the studies on biological cells. The HEK293T (healthy cells from kidney) and MDA-MB231 (breast cancer cells) cell lines were used in the studies of cell viability using MTT assay.

4.1. Synthesis of Ag NPs. The synthetic route of the Ag NPs was adopted from a reported study and done with minor modifications. Briefly, 1.5 mL of 0.1 mM Tw-20 was taken in a glass round-bottom flask, and 1.5 mL of 0.77 × 10^{-6} M HSA or Hb was added to it. The mixture was added with 30 μL of 0.1 mM silver nitrate solution. Finally, 200 μL of 0.01 M ice-cold NaBH4 was added dropwise to it with continuous stirring. The process produced Ag NPs coated either with Tw-20-HSA or Tw-20-Hb. The solution was stored in the refrigerator for future use. The shelf-life of the product was found to be fairly good.

4.2. Characterization of the Synthesized Surfactant–Protein Coated Ag NPs. The NPs were well dispersed in aqueous solution. High-resolution transmission electron microscopy (HR-TEM) images provided in Figure 4a,b show that the Tw-20-HSA and Tw-20-Hb coated Ag NPs were around 5 nm in diameter. The synthesized Ag NPs were further characterized by their respective absorption spectra in aqueous medium as shown in Figure 5a,b. The spectra show the presence of absorption signal from the proteins at around 280 nm as well as the surface plasmon peaks for the Ag NPs at around 410 nm in aqueous phosphate buffer (pH 7).

Non-ionic surfactants interact weakly with proteins because of the absence of electrostatic forces. Unlike with ionic surfactants, proteins do not undergo significant structural change on interacting with non-ionic surfactants, and their interaction with the proteins are more favorable compared to that among themselves.

Figure 4. HR-TEM micrographs of Ag NPs coated with (a) Tw-20-HSA and (b) Tw-20-Hb. The insets show the selected area electron diffraction (SAED) pattern of silver nanoparticles. The scale bars are 20 nm.

Figure 5. Absorption spectra of Ag NPs coated with (a) Tw-20-HSA and (b) Tw-20-Hb in aqueous medium. The insets show the corresponding spectra of HSA and Hb, respectively.
showing hydrogen bond formation between ethylene oxide chains of non-ionic surfactants and BSA molecules leading to alteration of the protein conformation.55,56 It has been established that proteins containing tryptophan residues and non-ionic surfactant molecules interact through the tryptophan residues and the vicinal amide groups.57 Hence, it can be considered that, in the present case, the large excess of Tw-20 induces denaturation of HSA and Hb form a coating over the Ag NPs produced due to the reduction of AgNO3 by NaBH4.

4.3. Methods. The steady-state absorption and fluorescence spectra of the samples were measured using a QM-40 spectrophotometer from PTI, Inc. and U2900 spectrophotometer from Hitachi, respectively. In the experiments with FL, the samples were excited at 470 nm. The HR-TEM images were obtained from a JEML, JEM-2100F microscope using a 200 keV electron source at the DST-FIST facility in IISER Kolkata.

4.4. Cytotoxicity Assay. Cytotoxicity values of the Tw-20-HSA and Tw-20-Hb coated Ag NPs were determined in HEK293T cells and MDA-MB-231 cell line following Pal et al.58 Two hundred microlitres of the cells (HEK293T and MDA-MB-231) was seeded in each well of a 96-well plate and kept at 37 °C for 48 h. The initial medium was replaced with fresh DMEM containing 1 mg/mL MTT (Thermo Fisher). The cells were incubated for 3 h, and then the medium was discarded followed by dissolution of the cells in dimethyl sulfoxide (DMSO; Molecular Biology grade). The inhibition of cell growth induced by the tested complexes was detected by measuring the absorbance of each well at 595 nm using a Spectra Max M2e plate reader. The data were plotted in dose-dependent inhibition using Prism, and the half maximum inhibitory concentration (IC50) was evaluated.

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