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"Theranostic" role of bile salt-capped silver nanoparticles - gall stone/ pigment stone disruption and anticancer activity

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ABSTRACT

Silver nanoparticles (AgNPs) have been synthesized in situ in micelles formed by the bile salt sodium deoxycholate (NaDC). The AgNPs exhibit "green" fluorescence. It has been shown in the present study that they can disrupt the components of gall stones/pigment stones. This unique ability of the AgNPs has been observed upon detailed study of the interaction between the endobiotic pigment bilirubin (BR) and bile salt (NaDC). In addition, these AgNPs show significant cytotoxicity towards the breast cancer cells (MCF-7). Thus the AgNPs synthesized in this work show important physiological activity and can serve as prospective "*Theranostic Materials*" in future. Their green fluorescence bears relevance to future *diagnostic* applications while their anticancer activity and disruptive action upon BR aggregates in bile salt micelles is extremely important for *therapeutic* purpose. This is the first report of the use of metal nanoparticles in disruption of components of gall stones/pigment stones and thus the present work has very important physiological significance. The detailed spectral studies indicate that bile salts increase the dimerization of BR which could be linked to increased solubilisation of BR in bile salt media and consequent bile stone/pigment stone formation. Importantly, an increase in red fluorescence was observed (upon dimerization of BR), which is important for cancer detection and studying the metabolism of biological tissues.

1. Introduction

Noble metal nanoparticles (NPs) are a hot topic of research due to their high photostability, good photoluminescence and low toxicity [1–5]. Among them, silver nanoparticles (AgNPs) are promising [2,5]. There are reports of photochemical, radiolytic, sonochemical and chemical synthesis of AgNPs using templates like thiols, peptides, proteins among several others [6–11]. In this work, we report on a bile salt-mediated green methodology for synthesis of fluorescent AgNPs. Bile salts are "biological surfactants" that play an important role in various stages of solubilisation of cholesterol, fat and dietary lipids in the gastrointestinal tract. The main aim of the work was to study the effect of these synthesized bile salt-capped AgNPs on biologically relevant systems. Two such biological systems have been selected for study - (i) the biological pigment bilirubin and (ii) an in vivo system i.e. the breast cancer cell line MCF-7.

In contemporary times, breast cancer has claimed a significant percentage of human life [12]. Over the past decade, cancer treatment has become very challenging due to development of multiple drug resistance of the malignant cells [13]. In many cases, nanoparticles have been invoked as a promising alternative for the treatment of cancer. Due to their small size and affinity for binding to the surface of biomolecules, nanoparticles offer potential advantages over drugs for diagnostic and therapeutic uses [14–17]. In this work we have studied the effect of the bile-salt capped AgNPs on the breast cancer cell line MCF-7. Other workers have recently reported on the antioxidant and anticancer activity of AgNPs [18,19].

Bilirubin (BR) is the yellow-orange endobiotic substance constantly produced in the human body via breakdown of heme. BR has drawn much attention due to its biomedical importance in abnormal bile-pigment metabolism and also its cytotoxic role in a person suffering from jaundice. It is a substituted tetrapyrrole consisting of two dipyrrole halves joined by a $-CH_2$ group [20]. Unconjugated BR adopts a folded, biplanar ridge-tile or folded book conformation stabilized by intramolecular hydrogen bonds [21]. BR has anti-oxidant properties [22]. It can also play a protective role in cerebral and cardiovascular systems [23]. But its low solubility in water leads to many physiological problems. Increase in concentration of BR or decrease in concentration of bile salts in the gastrointestinal tract leads to formation of gall stones [24]. Pigment stones, a variety of gall stones form mainly due to

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increase in concentration of unconjugated BR and particularly BR aggregates [25]. These BR aggregates combine with bile salts to form pigment stones. Consequently, it is interesting to study the interactions of BR with bile salts and how AgNPs synthesized in bile salts affect these interactions.

In this work, AgNPs were synthesized in micelles formed by the bile salt NaDC. These AgNPs were completely characterized by various techniques. Further the biological applications of these AgNPs were studied using the breast cancer cell line MCF-7 and also their effect on bilirubin aggregates (implicated in gall stone formation) was studied.

2. Materials and Methods

2.1. Materials

The bile salt sodium deoxycholate (NaDC) was purchased from Sigma-Aldrich (USA). Silver nitrate ($AgNO_3$) was from Merck (India). Bilirubin IX was purchased from TCI Chemicals (Japan). 8-Anilinonaphthalene-1-sulfonic acid (ANS) was from Fluka (Switzerland) and double distilled water was used to prepare all the solutions. Ascorbic acid was purchased from Merck (India).

2.2. Methods

2.2.1. Synthesis of Ag Nanoparticles

AgNPs were synthesized in bile salt assemblies at different concentrations of NaDC using ultraviolet (UV) radiation. AgNPs were synthesized both in absence and in presence of the reducing agent ascorbic acid (AA), the samples being named AgNP1 and AgNP2 respectively. The concentration of NaDC was varied from 5 to 120 mM and the absorption spectra were recorded at regular time intervals. The optimized concentrations of NaDC, the precursor $AgNO_3$ and ascorbic acid are 30 mM, 0.1 mM and 0.5 mM respectively. The pH values of the respective solutions with and without ascorbic acid were 7.1 and 7.8.

2.2.2. Cell Line and Culture

The breast cancer cell line MCF-7 was purchased from the National Centre for Cell Science (NCCS, Pune). Cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen) medium, supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Invitrogen) and 100 U/mL penicillin and streptomycin antibiotics (Gibco, Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2. All the studies were performed with exponentially growing cells. The cytotoxicity studies of AgNPs were carried out for the incubation period of 24 h.

2.2.3. Cell Viability Assay

Cell viability was determined using the Trypan Blue Exclusion principle. Briefly, 5×10^4 cells were inoculated into each well of a 6-well plate and then treated with solutions containing AgNP1 and AgNP2 in a dose dependent manner for 24 h. After completion of the incubation period, the cells were trypsinized and then counted using a hemocytometer under a light microscope (Dewinter) after Trypan Blue staining.

2.2.4. Cell Proliferation Assay

The effect of AgNP1 and AgNP2 on the proliferation of MCF-7 breast cancer cells was determined using the MTT assay. The proliferation of cultured cells was determined by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan crystals. 8×10^3 cells were inoculated per well into a 96-well plate, treated with AgNP1 and AgNP2 at various concentrations (1 μM and 2 μM). After incubating the cells for 24 h, they were washed twice with phosphate-buffer saline (PBS) and then MTT (100 μg/0.1 mL PBS) was added to each well. Cells were incubated at 37 °C to dissolve the formazan crystals. The plates were read using a Thermo Scientific Multiscan Go Nanodrop micro plate reader at 570 nm.

2.2.5. Phase Contrast Microscopy

1×10^5 MCF-7 cells were seeded in per well of 6 well plates. After 24 h of incubation, cells were treated with AgNP1 and AgNP2. The control cells received no treatment. Cells were then incubated for 24 h and observed under a phase contrast microscope (Dewinter) at 20 × objective. Snaps were taken using the Biowizard software.

2.2.6. Cell Cycle Distribution – Flow Cytometry

MCF-7 cells were seeded in 6 well culture plates at a density of 5×10^4 cells per well and incubated in DMEM medium containing 10% FBS for 24 h. After the cells have seeded, they were incubated with both AgNP1 and AgNP2 (1 μM and 2 μM concentration treatments) for 24 h. Next, the cells were collected by trypsinization and centrifuged at 2000 rpm for 5 min. The cell pellet was fixed with 70% ethanol and then permeabilized with 1 × PBS solution containing 0.1% Triton X-100 with RNase (40 μg/mL) for 45 min. The cells were then stained with Propidium Iodide (PI) solution (50 μg/mL) on ice for 30 min. The PI fluorescence was measured through a FL-2 filter (585 nm) using a FACS Verse (BD Accuri™ Flow Cytometer) and 10,000 events were acquired. Flow cytometry data was analyzed using BD Accuri C6 Software. The histogram display of DNA content (X-axis, PI-fluorescence) versus counts (Y-axis) was displayed.

2.2.7. Instrumentation

A Philips 16 W lamp was used for UV irradiation. The UV–vis spectra were recorded in a Shimadzu UV-2401PC spectrophotometer. The fluorescence emission spectra were recorded in a Perkin-Elmer spectrofluorimeter, Model No. LS-55. Time resolved fluorescence studies of the solutions were conducted using a Horiba Jobin Yvon Fluorocube-01-NL time correlated single photon counting (TCSPC) apparatus employing two separate picosecond laser diodes as excitation sources, operating at λ_{ex} = 375 nm and 440 nm. Dynamic Light Scattering Studies (DLS) were carried out in a Malvern Nano-ZS instrument. Transmission electron microscopy (TEM) studies of the nanoparticles were carried out at a resolution of 1.9 Å with a JEOL JEM-2100 electron microscope. TEM specimens were prepared by placing micro-drops of solution on a carbon film supported by a 300 mesh copper grid. X-ray diffraction (XRD) patterns were recorded using Philips (PAN analytical) instrument (Cu Kα radiation). X-ray photoelectron spectroscopic analysis was performed on a Kratos Axis Ultra DLD X-ray photoelectron spectrometer with monochromatic Al Kα (1486.708 eV) radiation. Spectra were resolved into components using Specwin software. The circular dichroism measurements were done with a JASCO J – 815 CD spectrometer (model No. J-815-150 S).

2.2.8. Statistical Analysis

Flow cytometry experiments were done by analyses of variance and the results are represented by the error limits in figure. Data points were obtained from the mean of at least three replicates.

3. Results and Discussion

3.1. Bile Salt Assemblies - Dynamic Light Scattering (DLS) Results

The hydrodynamic diameters (d_h) of the NaDC assemblies at various concentrations were determined by DLS. DLS results are shown in Fig. 1. NaDC concentrations were varied from 5 to 120 mM. If we compare the number plots in the 5–30 mM window we observe a sharp decrease in d_h from 90 nm (at 5 mM) to 40 nm (at 10 mM) to 0.7 nm (at 30 mM) NaDC, indicating the formation of micelles. Bile salts are known to exhibit stage-wise micellization behaviour [26–30]. The onset of micellization has been reported to be ~5 mM and the formation of secondary micelles is ~30 mM. The d_h of 0.7 nm corresponds to individual small aggregates constituting the secondary micelles. The sizes of the secondary micelles as reported by previous workers are ~0.8 nm. Thus our results are in good agreement with previous work [31,32].