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Characterization and Hemolysis Evaluation of Carboxy Methyl Cellulose Stabilized Serum Capped Silver Nano Particles.

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Abstract

Objective: Silver nanoparticles are increasingly being employed as antimicrobial agents especially as a new approach for combating infectious diseases caused by multidrug resistant microorganisms. In this study, we will determine in vitro hemolytic potential of Carboxy Methyl Cellulose (CMC) stabilized serum capped silver nano particles (AgNPs) in hu man blood characterizing physical, antimicrobial and hemolytic properties of AgNPs that contribute to Red Blood Cell (RBC) morphology defining proper safety margins.

Methods: The colloidal silver nano-particles had been prepared from silver nitrate by carboxy methyl cellulose (CMC) as stabilization agents, serum as capping agents, glucose as reducing agents & characterized for physical and antimicrobial properties. The hemolytic properties of car boxy methyl cellulose stabilized serum capped silver nano particles are determined by observation of morphological changes by prepared silver nanoparticles on human erythrocyte by electron, light microscope & in vitro hemolytic potential analysis of prepared silver nano particles in human blood by measurement of the total hemoglobin concentration of heparinized human whole blood using the cyanmethemoglobin method based on a hemoglobin concentration standard curve at an absorbance wavelength of 540 nm.

Results: Prepared serum capped CMC stabilized silver nanoparticles showed average size of 10 nm, with triangular shape. Their UV-absorption spectrum was 410 nm, zeta potential values of prepared silver nano particles were -28 mV.

The antimicrobial efficacy of the AgNPs were between 8192 to 32,768 folds.

The prepared silver nano particles did not cause any hemolysis even at the highest concentration which indicated that the test material did not cause any damage to RBCs and was safe for systemic use.

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Conclusion: As the prepared silver nanoparticles inter act with blood components, for application of these silver nanoparticles in the field of nanomedicines from a hemocompatibility point of view, the in-vitro hemolytic potential analysis may be useful for determining proper safety margins.

Introduction

Heavy metal based nano particles are increasingly being employed for clinical applications, especially as a new approach for combating infectious diseases. Infections caused by multidrug-resistant micro-organisms are emerging as causes of morbidity and mortality worldwide. So, Infections caused by multidrug-resistant micro- organisms is a big challenge in modern world.

As there is increase in the trend of microbial infection with the superbugs where no antimicrobial agents can be employed as potential antimicrobial agents, suitable nonconventional antimicrobial agents are being urgently needed [1,2]. Since the beginning of penicillin era, microbes have showed developing resistance against all newly developed antimicrobials within a few decades. The search for non-conventional antimicrobial agents is gaining much importance in view of dwindling of antimicrobial development and increasing irrational antimicrobial therapy in recent times [3,4,5].

Silver and other metal ions have been used as wound healing agent, antimicrobial agents since ancient times. Metallic silver has been used to combat microbial infections associated with medical devices such as wound dressings, catheters, orthopedic and cardiovascular implants with various degrees of clinical efficacy [6,7]. Silver was used for making water portable as early as 1000 B.C. The German obstetrician Carl S. F. Crede first treated ophthalmia neonatorum with 1% aqueous solution of silver nitrate (AgNO3) in 1881. The 1-2% solution of AgNO3 was also employed as topical germicide to treat mouth ulcer, root canal and for dressing burn wounds [8]. Silver nano particles shows a new ray of hope in biomedical applications due to greater surface area to volume ratio resulting enhanced reactivity. When employed on cardiovascular, neurosurgical catheters, wound &burn dressing, drug delivery and bioimaging, silver nano particles can be potential antimicrobial agents [9,10].

Among all heavy metals based nano particles, silver nanoparticles damages microbial cells mostly by protein coagulation and blocking sulfhydryl enzymes. To develop new selective anti replicant drug to prevent superbug infection, a new class of multi-targeted, potent, wide range of cyto-toxic agents can solve superbug infection problem by protecting host cells during systemic use. Among all heavy metal based antimicrobial nanoparticles, silver nano particles are potent antimicrobial agents with least toxicity and stronger antimicrobial action after physical transformation of native silver into a unique materialistic state containing plenty of unstable zero valent reduced silver atoms in robust core of stabilized silver nano particles due to higher enhanced antimicrobial action than equivalent concentrations of native silver [3,4,5,11,12,13].

A new approach has been made to synthesis a systemic compatible silver nano-particles (AgNPs) containing a robust core of aggregated reduced silver atoms (AgO) stabilized by Carboxy Methyl Cellulose (CMC) and homologous acellular components (serum) of human blood suitable for systemic use with appropriate margin of safety for drugs for man or animals. By targeting different biomolecules, the prepared CMC stabilized serum capped AgNPs produce reactive oxygen species (ROS) in systems. This colloidal AgNPs due to smaller size, triangular shape, higher negative values of zeta potential measurement have higher surface-volume ratio than native silver state and higher mobilization towards oppositely charged microbial cell membrane, leading to significant damage with higher drug influx and osmotic disbalance. The host's serum makes these CMC stabilized serum capped silver nano particles further stable state in blood circulation and before internalization into target cells it does not permit release of toxic core materials into the circulation like targeted nano missile with a pay load cluster of atoms. Owing to high zeta potential values of CMC stabilized serum capped AgNPs, these nanoparticles will rapidly interact with constantly multiplying negatively charged microbes and will be internalized by splitting of the microbial lipid membranes. Unlike non serum protein capped silver nanoparticles, these prepared serum capped CMC stabilized silver nanoparticles are unable to be endocytosed into normal host cells due to receptor ligand recognition inadequacy by self-protein capped particles. By employing blood group specific serum or pooled serum as capping agent, prepared silver nanoparticles will be system compatible antimicrobial agents. As the plasma components is outer capping structure of the prepared CMC stabilized serum capped AgNPs, these silver nano particles may spare host cells due to less receptor ligand attachment during affinity-based endocytosis. Such AgNPs for properties of non-toxicity of biomimicking de-capping products andleast chance of releasing further protein into the circulation, these AgNPs can be used as potential antimicrobial agents.

For the clinical application of CMC stabilized serum capped AgNPs, the safety of these AgNPs in the bloodstream should be determined. The basic tests in assessing the safety of a blood-contacting medical material are to evaluateits hemolytic potential in blood circulation in vitro. When the red blood cell (RBC) membrane is damaged and hemoglobin is released, leading to hemolysis, this can produce adverse health effects.

In this study, we determine the physical, antimicrobial and hemolytic characteristics of CMC stabilized serum capped silver nano particles and evaluation of the in-vitro hemolytic potential in human blood using modification of the test protocol from ASTM standard E2524-08 (ASTM International, West Conshohocken, PA, 2000) [6].

Methodology

Preparation of serum capped carboxy methyl cellulose stabilized silver nanoparticles (AgNPs)

Silver nanoparticles were prepared by chemical reduction of silver nitrate (AgNO3) solution (Sigma Aldrich, USA). To prepare systemic usable AgNPs by chemical reduction method, we used aqueous solution of silver nitrate (AgNO3) (Sigma Aldrich, USA) with pooled sterile human serum (from samples of serological testing laboratory) as capping agent.

Thus, sodium carboxy methyl cellulose (CMC, Amit Chemicals, India) was used as stabilization agents using dextrose (Sigma Aldrich, USA) as reducing agent.

In a beaker with magnetic stirrer, 100 ml sodium carboxy methyl cellulose (4000 Centipoises) solution at 9.64 mg/L concentration was taken. To this 50 ml 1mM AgNO3 solution (effective concentration in 200 ml AgNPs at 10.68 mg/ L silver from 17 mg AgNO3 / L) in HPLC grade water was added force fully with continuous stirring by magnetic stirrer under refluxing condition at room temperature. To this mixture 50 ml of sterile serum

was added. Then 100 ml 0.2 mM dextrose (effective con centration in 200 ml AgNPs at 36 mg/ L) was added forcibly into the solution and stirring was continued for one hour at 45°C [5]. The nano state transformation was indicated by change of color to golden yellow. Resultant mixture was stored in amber colored bottle, one at room temperature, another at 4°C refrigerator and another at oxygen depleted environment inside two step combustion modified candle jar [6] with loosely tight screw cap.

Physical Characterization of carboxy methyl cellulose stabilized serum capped silver nano particles (AgNPs)

Physical characterization of prepared CMC stabilized serum capped silver nano particles for phase identification were deter mined by UV-VIS absorption spectrophotometer (Jasco V 650 UV VIS Spectrophotometer, Japan). The documentation of size and size distribution of the AgNPs was performed by the log normal size distribution curve obtained from DLS (Malvern Zen 3600 Zetasizer, USA). Zeta potential (Malvern Zen 3600 Zetasize, USA)measurement were performed to ensure stability of such AgNPs in aqueous dispersion, while actual size and shape were determined by transmission electron microscope images (JEOL JEM 2100 HR with EELS, USA).FTIR analysis (Jasco FTIR 6300, Japan) were carried out to identify biomolecules responsible for capping and efficient stabilization of the prepared silver nanoparticles and enabled the in-situ analysis of interfaces to investigate the surface adsorption of functional groups on sil ver nanoparticles. XRD analysis (Bruker D8 Discover instrument, German) confirmed silver nano status.

Study on anti-microbial properties

The enhanced microbicidal action of CMC stabilized serum capped silver nano particles after nano conversion varied with size, shape, zeta potential, duration and method of preservation and test environment. So, such “bio- parameters” could be simpler and reliable indicators for all practical purposes. Related antimicrobial testing with CMC stabilized serum capped silver nanoparticles were carried out in accordance with institutional ethics for handling bio-hazardous materials.

Methicillin resistant (MRSA) reference strain *Staphylococcus aureus* ATCC 43300, susceptible strain Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853 and resistant strain biofilm positive Candida albicans ATCC 10231 (Microbiologics-Inc, USA) along with multidrug resistant clinical isolates of S. aureus, E. coli, P. aeruginosa, C. albicans were included as test organisms. Before performing test, organisms were isolated from stock culture, confirmed identification and antimicrobial susceptibility using VITEK-2 compact (Version08.01; BioMerieux, France).

Minimum Inhibitory Concentrations (MIC) for native silver nitrate solution (Ag+) and equivalent concentration of silver nano-particles (Ag^0) for the prepared AgNPs were deter mined by two-fold serial broth microdilution method. For each microorganism, three to five colonies from nutritive agar medium (incubated at 37 ^{O}C) for [18hours to 24 hours (for bacteria), and 48 hours (for yeast)] were touched with a loop and the growth were transferred to sterile cation adjusted Mueller hint on broth. The bacterial and yeast suspension were adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard and 1 McFarland standard respectively for the broth microdilution method. The prepared adjusted microbial suspension contained approximately 1 x 10^8 CFU/ml for microbes. The adjusted microbial suspension prepared as above was diluted in broth to give a final cell number concentration of 5 x 10^5 CFU/ml (range 2 x 10^5 CFU/ml to 8 x 10^5 CFU/ml). The 0.1 ml of standardized microorganism suspension were transferred to a tube containing 9.9 ml (1:100 dilution) of broth resulting in a suspension of 1 x 10^6CFU/ml. Then 50 µl of microbial suspension were added to an equal volume (50 µl) of aliquots of serially diluted AgNPs in HPLC grade water in a row of 96 wells of microtiter plates, resulting in a final inoculum of 5 x 10^5 CFU/ml.

Plates were observed for turbidity to note the end points as MICs after incubation at 37°C for 24 hours (for bacteria)/ (48hours for yeast). The subculturing method were performed to crosscheck the end points. The enhanced antimicrobial activity of AgNPs in comparison to equivalent ionic silver for different multidrug resistant and reference strains of microbes were calculated as increase of dilution factors of respective MIC values in two sets of micro broth dilution study. Validation of results were performed by triplicate study.

Synergism study of carboxy methyl cellulose stabilized serum capped silver nanoparticles with several antimicrobials against different microbial strains

Synergism study were performed by combination of silver nano particles with different antimicrobials manually by checkerboard method.

Morphological changes by carboxy methyl cellulose stabilized serum capped silver nanoparticles observed on human erythrocyte by light, electron microscope

Whole blood was centrifuged at 3000 rpm for 12 min, and the plasma, buffy coat, and top layer of cells was decanted. The remaining packed RBCs was washed two times with Phosphate Buffered Saline (PBS). After washing, 500 ul of packed RBCs was diluted to 1.5 ml with PBS (25% hematocrit). The diluted RBC suspension (0.3 ml) then was mixed with carboxy methyl cellulose stabilized serum capped AgNPs suspensions in PBS (1.2ml) at various concentrations (final hematocrit, 5%). RBC suspension in PBS (1.2 ml) without AgNPs was used as a control. The final combined suspensions were gently mixed and incubated at room temperature. To study the effect of incubation time on erythrocyte with AgNPs, the incubated samples was observed at 1-hour intervals upto 4 hours.

***In-vitro* study of hemolytic potential of carboxy methyl cellulose stabilized serum capped silver nano particles in human blood**

The total hemoglobin concentration of heparinized human whole blood was measured using the cyanmethemoglobin method based on a hemoglobin concentration standard curve at an absorbance wavelength of 540 nm.

The blood was then diluted to a hemoglobin concentration of 10 mg/ml with Ca^2+/Mg^2+-free DPBS. At various concentrations, the prepared serum capped CMC stabilized silver nano particles and positive & negative controls were analyzed in triplicate using blood from a different donor on each test day. Aliquots (100 µl) of prepared carboxy methyl cellulose stabilized serum capped silver nanoparticles (AgNPs) suspension in HPLC grade water were added to microcentrifuge tubes, followed by the addition of 700 µl of Ca^2+ /Mg^2+-free DPBS. Within 3 mins, diluted blood(l00µl) was added to each of the tubes. The incu bation of tubes was performed in a 37 °C water bath for 3 hours 5 minutes with gentle inversion of the sample tubes for 30 mins. After the incubation, the centrifugation was performed at 800x3 g for 15 mins at room temperature.

The resulting supernatants were mixed with CMH reagent in a 1:1 ratio and analyzed by O.D measurement at 540 nm. The measured absorbance was rectified for background interference (i.e., particles in DPBS without blood). The assessment of concentration of cell-free hemoglobin in each sample was performed from the hemoglobin standard curve and by calculating the 16-fold dilution factor for the samples and positive & negative controls. The percent hemolysis was calculated by dividing each sample's cell-free hemoglobin concentration by the total hemoglobin concentration (10 mg/ml).

PEG [final concentration (¼) 4.4%] was used as a negative control and TritonX-100 [final concentration (¼)1%)] was used as a positive control.

Results

Physical Characterization of AgNP-Serum-CMC-10

CMC stabilized serum capped silver nanoparticles showed average size of 10 nm with triangular shape. Plasmon absorption peaks were located at wavelengths of 410 nm and indicated the presence of **(Figure lA)** 10 nanometer-sized AgNPs while actual size and shape were determined by transmission electron microscope images **(Figure 1B)**. The size of 10 nm and a narrow size distribution of the AgNPs was documented by the lognormal size distribution curve were obtained from DLS results **(Figure lC)**. Zeta potential values of CMC stabilized serum capped silver nano particle ranging from -28 mV ensured high aggregation stability of such mixture of AgNPs in aqueous dispersion **(Figure 1D)**.

In **Figure 1E**, X-ray diffraction pattern recorded by preparing drop-coated film of serum capped CMC stabilized silver nanoparticles further validated the crystalline nature of the prepared CMC stabilized serum capped silver nanoparticles. In Figure 1 E, the well-defined peaks at 2θ values of 38.1°, 44.3°, 64.5°, and 77.4° corresponded to (111), (200), (220) and (311) planes of silver, respectively of the serum capped CMC stabi lized AgNPs. Those above values could be correlated with the face centered cubic (fee) lattice structure of crystalline silver.

In FTIR analysis **(Figure 1F)**, the presence of bands at 1422, 1608, 2931, and 3420 cm^{-1} corresponded to the stretching vibration of COO- (symmetric), COO- (asymmetric), C H (aliphatic), and O H groups of serum capped CMC stabilized AgNPs, respectively. In **Figure 1F**, FTIR spectrum of serum capped carboxy methyl cellulose stabilized silver nanoparticles produced characteristic bands of amide at 1608 and 1422 $cm^{-1}$and also exhibited C-N stretching vibration band of aliphatic amine at 1064 cm^{-1}. The bands present at 1608 and 1422 cm^{-1} in the spectrum of serum capped CMC stabilized AgNPs indicated that the carboxyl groups of Carboxy methyl cellulose were present in the colloidal silver suspension after crosslinking. From the spectrum of serum capped Carboxy methyl cellulose stabilized AgNPs, some typical peaks appeared at 1327, 1262, and 1064 cm^{-1}. The peaks at 1327 and 1262 cm^{-1} belonged to stretching vibration of CO C and C C stretching vibration respectively. The band observed at 1064 cm^{-1} indicated bending vibration of OH group.

Antimicrobial characterization of prepared AgNP-Serum-CMC-10

The bonus effects of CMC stabilized serum capped silver nanoparticles were between 8192 to 32,768 folds.

Nano transformation was confirmed by physical parameters of CMC stabilized serum capped silver nanoparticles. By antimicrobial characterization, it showed 8192 to 32768 folds higher antimicrobial action than that of equivalent ionic silver solution, termed as "Enhanced antimicrobial action (Bonus effect)" and considered as the microbiological marker of nano-antimicrobials **(Table 1)**. The MICs of the AgNPs for different test microbes were about 0.000625 mg/L for bacterial strains and 0.0003125 mg/L for candida strains.

The cut-off MIC values for sensitive drugs in automated system remained unchanged to same values, not excluding possibility of lower synergistic concentrations. For resistant antimicrobials, break-point synergism was under evaluated by limiting actual values between ≥ and ≤ values. The quantitative indica tion of enhancement of antimicrobial action could be possible by conventional microdilution method. Thus, automated system could only give a qualitative indication of synergism for resistant drugs only.

Synergism study of carboxy methyl cellulose stabilized serum capped silver nanoparticles with several antimicrobials against different microbial strains manually (By checkerboard method)

In checkerboard method (manually performed) of two drugs combination study, low or no additive effect was reflected for sensitive drug while for resistant drug greater lowering of MIC points towards degree of synergism, though exact combined effect might be even higher.

For rationale use of silver nano particles with combination of conventional antibiotics/antifungal agents at sub toxic dose of the prepared serum capped CMC stabilized silver nano particles, we were trying to develop regimen of their synergistic combina tion with sufficient margin of safety in a resistant-proof manner. **(Table 2)**.

Morphological changes by serum capped carboxy methyl cellulose stabilized silver nanoparticles observed on human erythrocyte by electron, light microscope

From observation that it was seen there were no morpho logical changes in human erythrocyte examination by light, electron microscope at 4 hours of incubation with different concentrations (1 ppm, 10 ppm, 100 ppm) of prepared serum capped carboxy methyl cellulose stabilized silver nanoparticles. For untreated control RBCs, there were no changes in shape of RBC, and no surface abnormalities were observed, i.e., the intact red blood cells morphology **(Figure 2)**. After 1 hour, treatment of RBCs with serum capped carboxy methyl cellulose stabilized silver nanoparticles produce no distinct changes in RBC shape **(Figure 3)**. After 2-hours, treatment of RBCs with serum capped carboxy methyl cellulose stabilized silver nano particles produce no distinct changes in RBC shape **(Figure 4)**. After 3 hours, treatment of RBCs with serum capped carboxy methyl cellulose stabilized silver nano particles produce no distinct changes in RBC shape **(Figure 5)**. After 4 hours, treatment of RBCs with carboxy methyl cellulose stabilized serum capped silver nanoparticles produce no distinct changes in RBC shape. i.e., the intact red blood cells morphology **(Figure 6)**. From microscopic observation, there was no hemolytic activity of carboxy methyl cellulose stabilized serum capped silver nanoparticles at all concentrations on in RBCs.

In Vitro Hemolytic Property of prepared serum capped carboxy methyl cellulose stabilized silver nano particles

We performed absorption spectrum of CMC stabilized serum

capped silver nanoparticles with human blood with PEG [final concentration¼ (4.4%)] as a negative control and Triton X-100 [final concentration(¼) 1%] as a positive control. The validation of the hemolysis assay was verified by the results. The determi nation of hemolysis assay was based on measurement of absorbance at 550 nm, with subtraction of the interference (par ticles in DPBS without blood) of Ag NPs. By the subtraction of the 0D550 nm of the AgNPs suspended in the solution from the measured 0D550 nm at the same concentration, this interfer ence was avoided. Triton X-l00(positive control) PEG (negative control) produced 100% and 0% of lysis, respectively.

The percent hemolysis was obtained by dividing each sam ple's cell-free hemoglobin concentration by the total hemo globin concentration (10 mg/ml). The percent hemolysis was increased as a function of increasing mass concentration of par ticles in the DPBS/blood mixture **(Supplementary material -1)**.

Determination of the hemolysis percentage from the formula

H (%) [%hemolysis] = (abs. of sample - abs of negative control)/ abs of positive control) x 100 =(O.D550nm sample – O.D 550nm tyrode)/ (O.D 550nm Triton X-100 1% - O.D 550nm tyrode) x 1OO.

The AgNPs at 1 ppm, 10 ppm and 100 ppm induced no he molysis in whole blood after 1 hour, 4 hours or 24hours.

With whole blood or washed RBC, no hemolysis was observed, in all the concentration of Serum capped carboxy methyl cellulose stabilized silver nanoparticles after 24 hours of incubation.

The prepared CMC stabilized serum capped silver nanoparticles did not cause any hemolysis even at the highest concentration which confirms that these nano particles do not produce any damage to RBCs and safe for systemic use. A negligible difference was observed in washed RBC with prepared serum capped CMC stabilized AgNPs at 100 ppm after 24 h of incubation in comparison with controls. No hemolytic effect was observed in whole blood than in washed RBC. This is probably because, in whole blood, an adsorption of plasma proteins on the AgNPs might occur and impact upon the hemolytic properties. Spectrophotometric analysis of the supernatants indicated that the silver nano particles did not shift the absorbance peaks of the hemoglobin released from the RBCs **(Supplementary material -1)**.

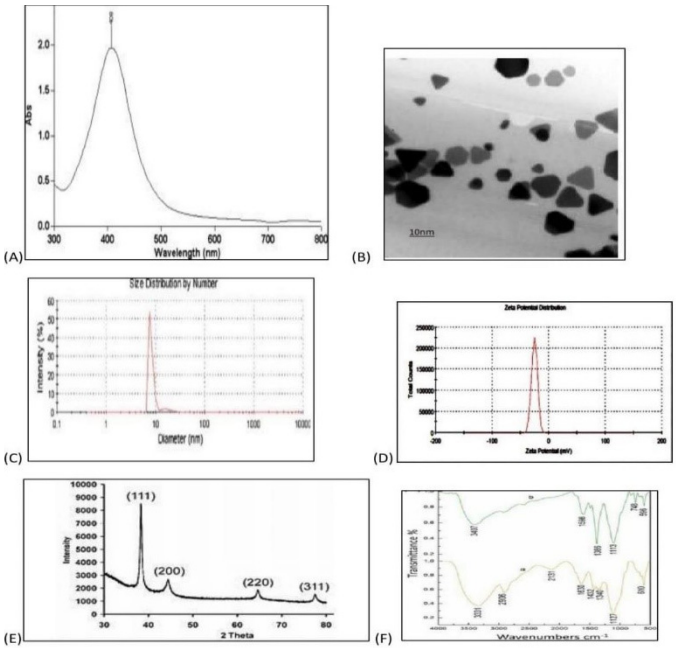

Figure 1: (A) UV-Vis absorption spectra of serum capped carboxy methyl cellulose stabilized silver nanoparticles at 410 nm and narrow distribution. **(B)** TEM image of triangular shaped serum capped carboxy methyl cellulose stabilized silver nanoparticles. **(c)** Size distribution obtained from DLS measurements of serum capped carboxy methyl cellulose stabilized silver nano particles: 10 nm). **(D)** Zeta potential of serum capped carboxy methyl callulos stabilized silver nanoparticles. **(F)(a)** FTIR spectrum of Carboxy methyl cellulose; **(b)** FTIR spectrum of serum capped carboxy methyl cellulose capped silver nanoparticle.

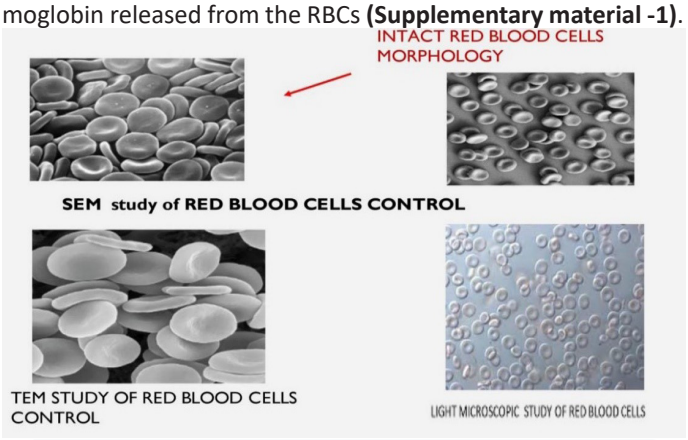

Figure 2: Microscopic study of red blood cells (before addition of Serum capped Carboxy methyl cellulose stabilized silver nanoparticles).

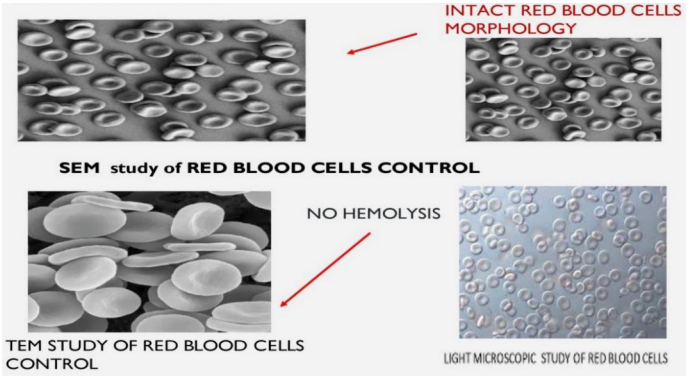

Figure 3: Microscopic study of red blood cells (After 1 hour of addition of Serum capped Carboxy methyl cellulose stabilized silver nanoparticles).

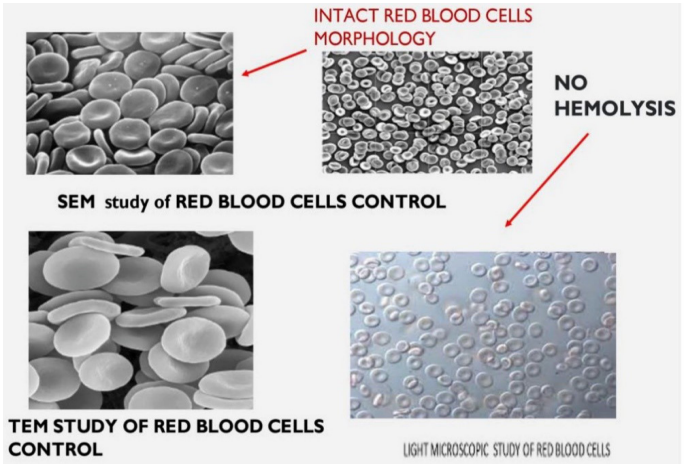

Figure 4: Microscopic study of red blood cells (After 2 hours of addition of Serum capped Carboxy methyl cellulose stabilized silver nanoparticles).

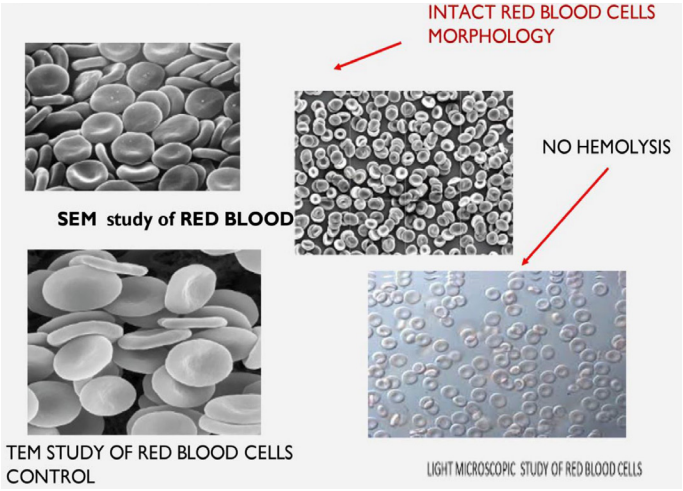

Figure 5: Microscopic study of red blood cells (After 3 hour of addition of Serum capped Carboxy methyl cellulose stabilized silver nanoparticles).

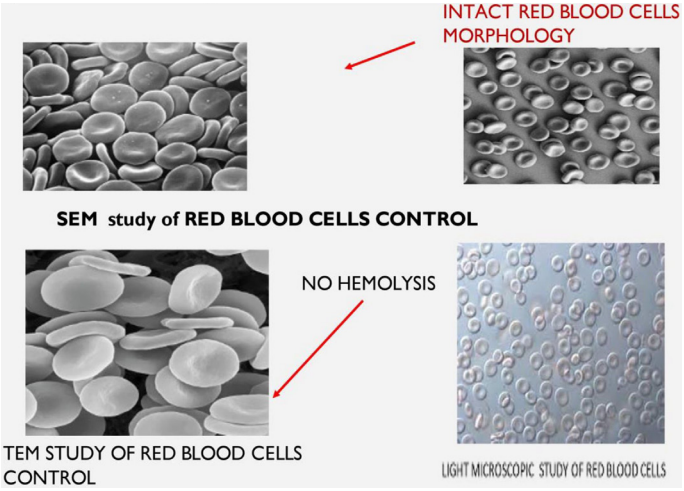

Figure 6: Microscopic study of red blood cells (After 4 hour of addition of Serum capped Carboxy methyl cellulose stabilized silver nanoparticles).

Supp Material 1: Hemolysis of human erythrocytes (%) after their incubation with the Serum capped carboxy methyl cellulose stabilized silver nanoparticles at given concentrations.

Serum capped carboxy methyl cellulose stabilized silver nanoparticles concentration	**Hemolysis of erythrocytes, %**					
	Serum capped carboxy methyl cellulose stabilized silver nanoparticles concentration (1 ppm)	Serum capped carboxy methyl cellulose stabilized silver nanoparticles concentration (10 ppm)	Serum capped carboxy methyl cellulose stabilized silver nanoparticles concentration (100 ppm)	Serum capped carboxy methyl cellulose stabilized silver nanoparticles concentration (1 ppm) after centrifugation	Serum capped carboxy methyl cellulose stabilized silver nanoparticles concentration (10 ppm) after centrifugation	Serum capped carboxy methyl cellulose stabilized silver nanoparticles concentration (100 ppm) after centrifugation
Minimum inhibiting concentration	0.2 + 0.025	0.6 + 0.6	0.9 + 0.89	0.1 + 012	0.5 + 0.5	0.8 + 0.79
Minimum inhibiting concentration X 2	0.3 + 0.3	0.4 + 0.4	0.8 + 0.79	0.4 + 0.40	1.0 + 1	0.8 + 0.8
Minimum inhibiting concentration X 4	0.3 + 0.29	1.1 + 1	1.5 + 1.51	0.9 + 0.90	1.0 + 1.2	0.9 + 0.9

Discussion

Among the heavy metal-based nanoparticles, silver nano particles have been considered as very stable, least toxic, wide range, potent antimicrobial agents. In this study, AgNP-Serum-CMC-10 are proved to be very potent anti-microbial against wide range of multidrug resistant strains of microbes. In the process of chemical reduction, it transfers electrons to silver ions (Ag+) by the reducing agent (dextrose employed) resulting in unstable silver atoms (Ag0). This in-turn lead to the formation of clusters of uncapped silver nanoparticles (as this cluster is uncapped, this cluster is very unstable). Polymeric stabilization of colloids involving polymeric molecules prevent uncontrolled amount of particle formation by electrostatic stabilization and the aggregation of the colloidal particles, by steric repulsion. This leads to the formation of stabilized molecule capped nano-sized (1-100 nm) colloidal particles containing a core of aggregated silver atoms with adsorption of ions to the surface which leads to the formation of an electrical double layer impairing columbic repulsion force between individual silver nano particles.

In this study, AgNP-Serum-CMC-10 are synthesized with carboxy methyl cellulose as stabilization agent, dextrose as reducing agent. In this chemical reduction procedure, serum contain various components of many capping and dextrose is reducing agents, leading to all physical and bio-functional characteristics of anti-microbial silver nanoparticles.

The silver nano particles can bind to the bacterial surface mainly by electrostatic forces (this force contribute to zeta potential of colloid AgNPs & helps AgNPs to interact with other particles with great differences of electrostatic surface charges) [14]. This AgNPs split lipid membrane which interact rapidly with negatively charged microbes [15,16] resulting higher cellular permeability, osmotic disbalance and release of cytoplasmic contents. Silver atoms invade the respiratory chains at mesosome level of the microbial cell membrane leading to cell death. This antimicrobial activities are mainly led by Reactive Oxygen Species (ROS) which is seen to be accumulated in the presence of oxygen and depleted in the presence of anti-oxidants [17]. This type of oxidative stress by production of large amount of ROS after interaction with Ag0 can occur inside the mammalian cell at mitochondria level, through the interaction of silver with the thiol group of the enzymes [18].This ROS can result in dephosphorylation of peptides on tyrosine residues and leads programmed cell death by affecting microbial signal transduction [12,19]. In comparison to the cytotoxic action of heavy metals, this type of ROS mediated “programmed cell death” may require longer time for completion of chain reactions, internalization of nanoparticles into mammalian cells mainly take place by endocytosis through receptor ligand interactions. The toxicity of silver nanoparticles to mammalian cells is mainly monitored by structure of surface capping molecules (20,21]. Small sized triangular shaped silver nanoparticles can deliver large Ag0 pay loads due to large surface area to volume ratio, presence of ac-

tive facets [22]. Their size and shape depend upon the method of nano preparation and choice of stabilization agents for the purpose. For all these reasons, AgNP-Serum-CMC-10 has shown strong antimicrobial action but appreciably no hemolytic action on mammalian cells.

Physicochemical Characterization: Particle Diameter Measurements

The physicochemical characteristics of silver nanoparticles are determined to perform the correlation of results in various scientific measurements affecting their unique size and composition. The prepared serum capped CMC stabilized silver nanoparticles are well dispersed in HPLC grade water and does not display any morphological alteration in TEM image analysis [6]. The size & shape measurements by TEM analysis may also be affected by the solvent used to disperse the silver nano particles prior to drying for TEM image analysis.

The size measurement by DLS analysis of uniformly dispersed colloidal solution of silver nano particles are dependent on the three-analysis parameter i.e., intensity, volume, number. Among the intensity, number, volume based DLS analysis, we perform the number based DLS analysis to estimate most abundant silver nanoparticles enabling us to correlate between different silver nano particles. In this analysis, the effect of solvent on nanoparticle size, agglomeration, and polydispersity are significant. The dilute plasma proteins in the solvent stabilize the silver nano particles, increase their dispersion into the solvent. This protein coating effect are employed to regulate the formation of stable agglomerates of silver nanoparticles in biological solvent so that they remain well dispersed for days [6].

Antimicrobial characteristics

In this study, CMC stabilized serum capped AgNPs shows nonspecific synergism [23,24,25] with conventional antimicrobial agents [26]. This property can be advantageously employed in therapeutic treatment of combination therapy for subtoxic dose use of both drugs. Alteration of cell membrane permeability in silver nano particles treated microbial cells maybe the primary reason for non-specific synergism for other drugs. Unlike conventional antimicrobial agents, the basic mechanism of antimicrobial action of silver nanoparticles are independent of cell replication. This prepared silver nano particles can kill dormant, non-replicating microbes within biofilms displaying bio film properties.

The single-dose infusion of 100 mL AgNP-Serum-10 is sufficiently enough for managing infections in terms of higher MIC achievable concentration in blood for most in-vitro tested MDR strains enabling the wide margin of safety for the host. By using 1/32768 th of AgNPs (10 mL), synergistically with one appar ently resistant antimicrobials, the elimination of the infection may be possible. This is supported by an in-vitro study adding 1/4th MIC of serum capped carboxy-methyl cellulose stabilized AgNPs at ultra-dilution in emulsion fluid of automated susceptibility testing device that reverts all resistant drugs into sensitive range. The quantity of silver delivered to the system with 100 mL AgNP- CMC-Serum-10 is only 10.8 mg (approximate achievable plasma concentration >2 mg/L adequate for antimicrobial action, while 0.1 mg silver/ kg body weight is bio-compatible with the host). After oxidation within cells, this type of silver nano particles are removed from body through kidney as ionic silver will not show toxicity to environment.

For preparation of this 10 mL AgNPs, only 2.5 mL patient's serum will be required, which can be easily collected from any adult patient **(Table 1 -2).**

Table 1: Anti-microbial enhancement of CMC Stabilized serum capped CMC Stabilized silver nanoparticles by shifting MIC following nano conversion.

AgNPs	Organisms	MIC of colloidal AgNP	MIC of AgN03 solution with equivalent silver	Antimicrobial efficacy
CMC Capped AgNPs. (10.6mg/L Silver from 17 mg AgNO3/L)	S.aureus ATCC 43300	1/32768 dil	1/2 dil	16384- fold
	S. aureus MDR	1/32768dil	1/2 dil	16384-fold
	E. coli ATCC 25922	1/32768 dil	1/2 dil	16384-fold
	E. coli MDR	1/32768 dil	1/2 dil	16384-f fold
	P. aeruginosa ATCC 27853	1/32768 dil	1/4 dil	8192- fold
	P. aeruginosa MDR	1/32768 dil	1/4dil	8192-fold
	Calbicans ATCC 10231	1/65536 dil	1/2 dil	32768-fold
	C. albicans MDR	1/65536dil	1/2 dil	32768-fold

Table 2: Synergism study of serum capped carboxy methyl cellulose stabilized silver nano-particles with different antimicrobials.

Organisms	MIC of Antimicrobials alone	MIC of AgNPs alone.	MIC of antimicrobials in combination withAgNPs	MIC of AgNPs In combination
MDR Candida albicans	1/2 dil=A	1/65536 dil=N	A/4	N/64
MDR Staphylococcus aureus	1/4 dil=A	1/32768 dil=N	A/4	N/16
MDR E.coli	1/4 dil= A	1/32768 dil= N	A/4	N/8
MDR Pseudomonas aeruginosa	1/2 dil=A	1/32768 dil= N	A/4	N/8

Hemolysis assay

The human serum constituents containing minor protein varies between different individuals due to presence of blood group iso-antibodies, acquired immune-bodies after vaccination or infection and presence of auto-antibody in persons with auto immune disease. If blood group specific human serum is included as capping agents in AgNP- Serum-CMC-10, the products of these newly prepared silver nano particles may have adverse effects by targeting selective host cells when mismatched. If the prepared silver nanoparticles interact with non-nucleated human RBCs [27] and release free Ag0 or ROS into circulation before attachment with target microbes, it may also produce adverse effects to other cells of the host. Therefore, it is better to employ matched serum especially with antibody of specific infection [28,29]. Due to instability or, mismatched viscous colloidal state of the prepared colloidal silver nano particles, adsorption of various protein components from circulation, the adverse effect to various component in human system, non-serum capped silver nano particles cannot be used for systemic use. Instead, it is advisable to use serum capped CMC stabilized silver nano particles [30,31].

Therefore, serum or plasma are considered as most suitable capping agent for systemic use not only to protect against hemolysis but also for the selective attack to microbes against which person carrying antibodies. Silver nanoparticles prepared from blood group matched serum of convalescence patients may be considered as life-saving infection controlling agent. During intravenous use of silver nanoparticles, nucleus and mitochondria free human RBCs which is a bag of oxygen carrying molecules are first to be interact with AgNPs. So, non-serum capped AgNPs release ROS into circulation even after depletion of 0.1% cells, can produce toxic effects to the host by endothelial damage, platelet aggregation, phagocytic oxidative burst, and genotoxicity. After achieving plasma derived corona coats, se rum capped CMC stabilized silver nano particles are no longer hemolytic.

Now a days, silver nanoparticles are being increasingly em ployed in blood-contacting biomedical applications and devices [6]. The increasing use of Ag NPs leads to the toxicological evaluation to ensure patient safety. The hemolytic potential evaluation comprises a two-step process:(1) physicochemical characterization of the nanomaterial (i.e., the prepared serum capped CMC stabilized silver nanoparticles in this case) and (2) biocompatibility assessment[6].

Nanoparticle surface chemistry

Serum proteins have been shown to have a significant effect on particle toxicity, possibly due to changes in agglomeration or surface chemistry [6]. In our study, the blood plasma proteins provide a stabilizing effect against the prepared silver nanoparticle agglomeration, [6] and increasing the protein concentration decreases the hemolytic activity [32-35].

Relevance of the Current Study and the ASTM E2524-08 Standard

For the assessment of biocompatibility evaluation of blood contacting materials, we have carried out the modified protocol of ASTM E2524-08 standard [6] By employing this protocol, various dimension of methodology can influence test results and interpretation. In our study, the prepared CMC stabilized serum capped silver nano particles should be well stabilized in a physiologic solution before undergoing the in -vitro hemolysis testing [36].

A lower level of hemolysis is seen in whole blood than in washed RBC due to that, an adsorption of plasma proteins of whole blood on the non-serum capped AgNPs may take place and influence the hemolytic properties. These can lead to ad verse side effects such as release of hemoglobin, anemia, pulmonary hypertension, renal toxicity. In contact with blood, metallic silver ionizes and releases ions in the bloodstream, which can interact with transmembrane proteins, whereas our serum capped CMC stabilized silver nano particles do not produce any hemolysis. In current study, we emphasize on the importance of the effect of serum proteins on particle size, dispersion, and cellular toxicity [6].

Silver nano Particle size, silver nano particle interference with the assay, coagulation of blood around silver nano particles can affect hemolysis. Therefore, it is advisable to employ nanoparticle-based positive and negative controls in the assay which can be easily evaluated through an interlaboratory study.

Our in vitro hemolysis results suggest that blood component contacting with silver nanoparticles, either in free or bound form, should be carefully tested for their hemolytic potential properties.

Prepared silver nanoparticles are highly antimicrobial and there is no hemolytic activity over the entire range of concentrations. For the development of nanomedicines from a hemocompatibility perspective, the hemolysis analysis may be useful for first-in-human exposure and for identifying appropriate safety margins. The prepared AgNP-Serum-CMC-10 can be a promising nonspecific microbiocidal nanomedicine safe for intravenous use.

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Author Contributions

A.G & P.K.M. conceived the idea of determining for physical, antimicrobial, hemolytic characterization of serum capped carboxy methyl cellulose stabilized silver nanoparticles. AG carried out experiments dealing with synthesis, characterizations, and microbiological experiments with AgNPs. Interpretation of physical characterization of prepared silver nanoparticles are done by MCG. Authors were involved with interpretation of obtained results, preparation and editing the manuscript.

Conflict of interest: None.

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