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Influence of a blend of guar gum and poly (vinyl alcohol) on long term stability, antibacterial and antioxidant efficacies of silver nanoparticles

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This paper describes detailed procedure for the synthesis of highly stable (almost for 60 days) and monodispersed silver nanoparticles (average particle size of 16 nm which changed to 42 nm only on 60th day of standing under refrigeration) in presence of blend of a polysaccharide (guar gum) and a hydrophilic synthetic polymer (poly (vinyl alcohol)) where both the polymers provided synergistic effect in reduction and stabilization of incipient nanoparticles. Time dependant stability of aqueous dispersions of silver nanoparticles in various compositions of polymeric matrices was studied from UV-Vis spectroscopy, DLS and zeta potential characterization data. FTIR spectrum and HRTEM image along with EDX result of the best optimized compositions in respect of particle size and particle size distribution of silver nanoparticles were also obtained. A study displayed how viscosity of the polymeric matrices played a vital role in the long term stabilization of aqueous dispersions of silver nanoparticles. This is for the first time we are reporting that silver nanoparticles synthesized by green technique, using blend of two highly viscous polymers, exhibited excellent stability. The aqueous dispersions of silver nanoparticles also displayed strong antibacterial and antioxidant properties. Hence our synthesized silver nanoparticles may be suitable for commercialization in future.

Introduction

Silver nanoparticles (AgNPs) are prevalently used in various consumer products,¹⁻⁴ food additives and packaging,⁵ optoelectronic devices,^{6, 7} biomedical devices,^{8, 9} health care products¹⁰ and waste water treatment units¹¹ as one of the essential components. Presence of large proportions of surface particles with high energy due to higher surface area (surface effect) with respect to the bulk of the material and squeezing of electrons into a small area (quantum confinement effect) enable AgNPs to easily adsorb over variety of molecules or macromolecules even under harsh environmental conditions.¹²⁻¹⁴ AgNPs exhibit highly distinguishing size and shape tunable properties (optical,¹⁵ surface plasmon,¹⁶ chemical,¹⁷ catalytic activities,¹⁷ electrical,¹⁸ and thermal¹⁹) which make them potential candidates in wide spectrum of products, many of which have already been commercialized.²⁰ The good antimicrobial efficacy of AgNPs even at very low dosage owing to oligo dynamic action²¹ and reduced problem

of development of microbial resistance in any environment, promotes design of many consumer and biomedical products where antimicrobial property in nano-dimension is essential.^{22, 23} AgNPs also exhibit antifungal activities and hence find application in filter beds.²⁴ Interestingly, AgNPs effectively inhibit replication of HIV-1 viruses.²⁵ Other useful properties of AgNPs that provoke more and more research in this field include anti-inflammatory activities,²⁶ antiviral effects,²⁵ metal enhanced fluorescence properties²⁷ and plasmonic heating properties.²⁸ However, exploration of antioxidant properties of AgNPs is quite an overlooked field. Abdel-Aziz *et al* in a recent study highlighted antioxidant properties of AgNPs.²⁹ Oxidation is desirable in all living organisms including microbes, for production of energy. However, during oxidative stress in human body, highly reactive radicals (e.g.; hydroxyl radicals from splitting of water molecules on exposure to harmful gamma/ UV rays or superoxide radicals from biochemical reactions between oxygen and bio-macromolecules like adrenaline, dopamine or some components of mitochondria etc) are generated which hinders normal functioning of DNA, RNA, proteins and lipids.³⁰ As a consequence of the generation of free radicals in human cells, often cells are injured and cause necrosis or apoptosis leading to various diseases like cancer, cardiovascular problems, diabetes, ageing etc.³¹ Inclusion of AgNPs in medicinal compositions and various anti-ageing formulations may prove to be beneficial in scavenging any undesirable free radicals. Often packaged foods are spoiled by growth of microorganisms and by free radicals

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Electronic Supplementary Information (ESI) available: Zeta potential graphs of AgNPs of different compositions at different time and HRTEM image of 60 days old best optimized AgNPs are provided. See DOI: 10.1039/x0xx00000x

generated from their metabolism processes. However, commercial antioxidants are highly toxic. Thus to deal with the issue of spoilage of food, Kanatt *et al* used non toxic extracts of aqueous mint and pomegranate peels as highly efficient antioxidants and antimicrobials in a biodegradable film of chitosan blended PVA for active food packaging.³² Again, isolation of plant extracts always proves to be highly tedious and expensive processes for which most of the greener antimicrobials and antioxidants are deliberately avoided in commercial grounds. On the contrary, commercially viable aqueous dispersions of AgNPs if incorporated in biodegradable and water soluble film formulations may produce cost effective active packaging systems. Often AgNPs tend to leach out from polymeric compositions.³³ Although AgNPs are toxic yet when they leach out of the polymer compositions they tend to agglomerate very fast and hence reduce toxicity. Also, AgNPs that leach out of any highly viscous polymer stabilized compositions, are generally in very small concentration and thus are not lethal to human. Hence here we tried to prepare a composition by *in situ* generation of AgNPs in a biocompatible polymer blend which may be used safely in commercial products. Among the various techniques followed in the *in situ* synthesis of AgNPs (chemical reduction method using hazardous chemicals,^{34, 35} electrochemical method,³⁶ photochemical method,^{37, 38} radiochemical method,³⁹ sonochemical method,^{40, 41} laser ablation technique⁴² etc), green synthesis technology is the most favored one owing to the reduced environmental hazards associated with the synthesis procedure and economical viability of the green technology for large scale productions. Natural polymers especially polysaccharides owing to the presence of reactive functional groups (eg.; hydroxyls, aldehydes, amines etc) are widely used to reduce silver precursors and to stabilize AgNPs. Monodispersed AgNPs were successfully prepared using starch,⁴³ chitosan,⁴⁴ dextran,⁴⁵ gum arabic⁴⁶ etc in a one pot process. An abundantly available edible, non ionic, branched and pH responsive polysaccharide, guar gum (GG) is water soluble owing to the presence of extensive galactose units (i.e.; larger number of hydroxyl groups is present as compared to other polysaccharides). Hence GG may be used as a reducing cum protective agent for synthesis of AgNPs in an aqueous solution. In fact GG was successfully utilized by Pandey *et al* to develop AgNPs for ammonia sensing.⁴⁷ However, till date many safer green synthesis methods of AgNPs have not been commercialized owing to poor stability of stored aqueous dispersions of AgNPs. A research group of Pinto claimed borohydride reduced and citrate stabilized AgNPs were morphologically stable for an extended period of one year.⁴⁸ Similarly, Popa's group produced AgNPs which were stable for a month in presence of short chains of polyethylene glycol acting as solvent, reducer and stabilizer.⁴⁹ However, none of the gum or plant extract stabilized aqueous dispersions of AgNPs were stable for an extended period even under refrigeration. In our work, we tried to develop a greener method which could produce long term stable aqueous dispersions of AgNPs. We thought reducing and stabilizing power of GG during *in situ* preparation of AgNPs could be

further enhanced by using another hydrophilic polymer rich in hydroxyl groups and highly compatible with GG. Poly (vinyl alcohol) (PVA) is highly compatible with GG owing to the formation of intra molecular hydrogen bonds. PVA being of synthetic origin would not only increase reducing and stabilizing power of GG by providing more hydroxyl groups to the system but would also impart biocompatibility, biodegradability, good mechanical properties combined with film forming ability to the AgNPs-(GG + PVA) conjugate. Here we prepared highly stable aqueous dispersions of AgNPs (stable nearly for 2 months) by a one pot synthesis approach using an optimized blend of GG and PVA. We then precisely enumerated antibacterial and antioxidant properties of the polymer compositions impregnated with AgNPs.

Experimental details

Materials

GG with an average molecular weight of 1.06×10^6 gm mol⁻¹ was obtained as a gift from Hindustan Gum and Chemicals Ltd, Haryana, India. PVA (number average molecular weight 1.15×10^5 gm mol⁻¹, degree of hydrolysis ~98% and purity 99%), AgNO₃ (99.9% pure), NaOH pellets, KOH pellets, KH₂PO₄, concentrated HCl, H₂O₂, acetone and KBr (spectroscopic grade) were purchased from Merck, India. *Bacillus subtilis* [MTCC 736] bacterial strains and agar powder were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Butylated hydroxyl toluene (BHT), ethylenediaminetetraacetic acid (EDTA), ferrous chloride, ferric chloride, ascorbic acid, nitroblue tetrazolium (NBT), trichloroacetic acid (TCA), thiobarbituric acid (TBA), 2-deoxyribose, sodium phosphate, methionine, riboflavin and ferrozine disodium salt were purchased from Sigma chemicals Co. (St. Louis, MO, USA). All reagents and solvents were of analytical grade and were used without further purification. For preparation of any aqueous solution, milli Q water was used. For synthesis of AgNPs, all glass wares that were used were thoroughly cleaned with chromic acid and double distilled water.

In situ green synthesis of AgNPs using GG, PVA and a combination pack of GG and PVA

In a typical process, an aqueous solution of GG of maximum permissible concentration (1.0 w/v) was prepared under continuous stirring at a temperature of 50°C. We ensured no lumps of gum were present in the resultant solution of GG and a completely soluble form was obtained only after 48 hours of continuous stirring. We were unable to prepare completely soluble higher concentrated aqueous solution of GG above 1.0 w/v and hence we discarded any higher concentrated solution of GG for synthesis of AgNPs. The aqueous solution of GG was then cooled to room temperature. Three different sets of GG solution in milli Q (1.0 w/v) were prepared by adjusting pH to ~ 4.0, ~ 6.0 and ~ 8.0 respectively. Finally AgNO₃ solution (1 ml, 0.1 (N)) was added to the pH adjusted solution of GG and stirred vigorously for 1 hr. The reactions were carried out at 25°C, 50°C, 70°C and 100°C respectively. At the end of the

reactions, the final dispersions containing AgNPs were diluted five times with milli Q water and centrifuged at 10,000 r.p.m for 30 minutes. The supernatant solutions were carefully isolated and allowed to undergo sonication for further 15 minutes. Finally the aqueous dispersions were stored under refrigeration. Similarly, an aqueous solution of PVA (5.0 w/v) and an aqueous solution of a mixture of GG of strength 1.0 w/v (30 wt %, 50 wt %, 70 wt % w.r.t PVA) and PVA of strength 5.0 w/v (70 wt %, 50 wt % and 30 wt % w.r.t 30 wt %, 50 wt % and 70 wt % GG respectively) were used to synthesize AgNPs dispersions at different pH (~ 4.0, ~ 6.0 and ~ 8.0) and different temperatures (25°C, 50°C, 70°C and 100°C) by the procedure as described above. All the experiments were also repeated thrice to check reproducibility of the synthesis processes.

Characterization of aqueous dispersion of AgNPs

UV-Vis spectra of aqueous dispersions of polymer stabilized AgNPs (concentration 150 µg/ ml, adjusted after dilution) were recorded against air at 25°C in a UV-Vis spectrophotometer (CECIL CE7200, 7000 series, UK) from 200 nm to 600 nm using a quartz cuvette of 1 cm optical path length. Milli Q water was used as the blank. Formation of miscible blend of GG with PVA and alteration in structure of the blend on development of AgNPs was studied from FTIR spectra in transmission mode on Shimadzu IR affinity-18000 FTIR spectrometer. Thoroughly dried KBr pellets from the respective samples were prepared for FTIR spectroscopic studies. Hydrodynamic diameter and zeta potential of various compositions of polymer stabilized aqueous dispersions of AgNPs (concentration 150 µg/ ml) in milli Q was studied on Zeta Sizer Nano Series, Malvern, USA. Particle size and particle size distribution of polymer stabilized AgNPs in the dry state was studied by HRTEM (model JEM, 2010 EM, fitted with a CCD camera) at an accelerating voltage of 50kV. For HRTEM analysis, an aqueous dispersion of AgNPs_{70/30/6.1} (concentration 50 µg/ ml) was drop casted on a carbon coated copper grid of 300 mesh size and vacuum dried for 2 days prior to capturing of images. Finally EDX mapping of the same sample was also done using the EDX attachment to HRTEM in order to analyze the exact elemental composition of the dried colloids.

Assay of antibacterial efficacy of GG + PVA stabilized AgNPs

Agar diffusion method using *Bacillus subtilis*, a gram positive bacteria strain [MTCC 736] was used to analyze the antibacterial properties of AgNPs stabilized by GG and PVA. In a typical process, MTCC 736 bacterial strains were grown on liquid nutrient agar medium for 24 hours just prior to the experiment being seeded by pour plate technique. Four different cups like cavities were made using a cork borer (10 mm) at more or less equal distances. The cavities were chronologically filled with an aqueous solution (1 ml) of I) AgNO₃ (0.1 N) + GG (0.2 mg/ ml), II) AgNO₃ (0.1 N) + PVA (0.2 mg/ ml), III) AgNO₃ (0.1 N) + GG (0.14 mg/ ml) + PVA (0.06 mg/ ml) where 70 wt% GG: 30 wt% PVA ratio was maintained and IV) an aqueous dispersion of AgNPs_{70/30/6.1} (0.2 mg/ ml). Finally the plate was incubated at 37°C for 24 hours.

Assay of superoxide anion radicals scavenging activity of GG + PVA stabilized AgNPs

A method developed by Martinez *et al*⁵⁰ for determination of the superoxide dismutase with modification in the riboflavin-light-nitrobluetetrazolium (NBT) system⁵¹ was followed. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM EDTA, 75 µM NBT and 500 µl test sample solution at a concentration of 2 mg/ ml (containing aqueous solution of Gum_{100/0/6.2}, Gum_{0/100/6.1}, AgNPs_{70/30/6.1} or AgNPs_{50/50/6.1}). Reaction was initiated by illuminating sample by a fluorescent lamp. Finally the production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination. Identical tubes with the reaction mixture were kept in the dark and served as blank. Percentage scavenging of superoxide anion radicals was studied by comparing absorbance at 590 nm with the solution of control (50 mM sodium phosphate buffer) and the test samples (with antioxidants). A blank test was also performed using test samples without containing any antioxidants.

Assay of hydroxyl radicals scavenging activity of GG + PVA stabilized AgNPs

Here we adopted the work of Halliwell *et al* for determination of hydroxyl radicals scavenging efficiency of test samples.⁵² In a typical process, a freshly prepared reaction mixture (1 ml) contained 2- deoxyribose (2.8 mM), KH₂PO₄- KOH buffer (20 mM; pH 7.4), FeCl₃ (100 mM), EDTA (104 µM), H₂O₂ (1 mM), ascorbate (100 µM) and 500 µl test sample at a concentration of 2 mg/ ml (containing aqueous solution of Gum_{100/0/6.2}, Gum_{0/100/6.1}, AgNPs_{70/30/6.1} or AgNPs_{50/50/6.1}). Reaction was started by addition of H₂O₂ and then incubated at 37° C for 1 h. Finally approximately 2 ml TBA- TCA solution (acidified solution of TBA and TCA: 100 ml containing 380 mg TBA, 20 mg TCA) were added in order to inhibit the reaction initiated by H₂O₂ and then incubated at 100°C for 15 min. On sufficient cooling of the test sample, absorbance was measured at 535 nm against the sample where TBA-TCA was added prior to reaction mixture. BHT was used as the positive control. Percent inhibition of 2-deoxyribose degradation by the test sample at a concentration of 1mg/ml over the control was measured.

Chelating ability test of ferrous ions GG + PVA stabilized AgNPs

Chelating ability of various samples was determined according to the method of Dinis *et al*. (1994).⁵³ Aqueous solution of test sample (containing aqueous solution of Gum_{100/0/6.2}, Gum_{0/100/6.1}, AgNPs_{70/30/6.1} or AgNPs_{50/50/6.1}) was mixed with water (3.7 ml) so that final concentration of the samples became 1mg/ml. Finally FeCl₂ (0.1 ml, 2 mM) was added to the resultant mixture. The reaction was initiated by the addition of ferrozine (0.2 ml, 5 mM). After 10 min at room temperature, the absorbance of the mixture was determined at 562 nm against a blank (without any test samples but containing another chelating agent, EDTA).

Results and discussions

Preparation and characterization of AgNPs in aqueous solutions of GG, PVA and a blend of GG with PVA

An environment friendly bottom up method was used to prepare highly stable and monodispersed AgNPs. All the compositions of polymer matrices that were varied to synthesize AgNPs are tabulated in Table 1.

Table 1. Sample designation, reaction conditions and various properties of AgNPs of different compositions*

Sample designation	GG (wt%)	PVA (wt%)	Exact pH maintained during synthesis of AgNPs	Conc. Of AgNO ₃ (N)	Time of reaction (hr)	Time of recording data counted from the end of reaction	Average hydrodynamic diameter of AgNPs in dispersions (nm)	Zeta potential values of dispersion of AgNPs, ξ (mV)
AgNPs _{100/0/6.2}	100	0	6.2	0.1	1	2 hrs	950	-0.48
AgNPs _{0/100/6.1}	0	100	6.1	0.1	1	2 hrs	866	-2.41
AgNPs _{30/70/6.0}	30	70	6.0	0.1	1	2 hrs	72	-7.88
AgNPs _{50/50/6.1}	50	50	6.1	0.1	1	2 hrs	37	-9.00
AgNPs _{70/30/6.1}	70	30	6.1	0.1	1	2 hrs	20	-11.00
AgNPs _{100/0/8.0}	100	0	8.0	0.1	1	2 hrs	700	-4.93
AgNPs _{0/100/8.3}	0	100	8.3	0.1	1	2 hrs	1425	-3.43
AgNPs _{70/30/8.1}	70	30	8.1	0.1	1	2 hrs	230	-6.45
AgNPs _{70/30/6.1}	70	30	6.1	0.1	1	24 hrs	22	-10.70
AgNPs _{70/30/6.1}	70	30	6.1	0.1	1	7 days	35	-9.96
AgNPs _{70/30/6.1}	70	30	6.1	0.1	1	30 days	48	-9.78
AgNPs _{70/30/6.1}	70	30	6.1	0.1	1	60 days	52	-1.96
AgNPs _{50/50/6.1}	50	50	6.1	0.1	1	30 days	140	-4.40
AgNPs _{30/70/6.0}	30	70	6.0	0.1	1	30 days	153	-2.18

*All the reactions were carried out in water medium at 70°C. In our work, AgNPs is an abbreviated form of silver nanoparticles in aqueous dispersions.

GG being a reducing sugar with high hydroxyl content favors reduction of mono cationic silver precursor molecules and stabilizes the growing AgNPs in aqueous medium. Four different sets of temperature (25°C, 50°C, 70°C and 100°C) and three sets of pH (~ 4.0, ~ 6.0 and ~ 8.0) of the reaction medium were maintained in order to optimize the composition leading to most stable and finest of AgNPs. Similarly PVA was also used to prepare AgNPs in aqueous medium at different temperatures and pH. In the presence of GG and PVA separately, at 70°C, color of the aqueous solution containing AgNO₃ (at pH ~ 6 and ~ 8) turned yellow or reddish brown at the end of 1 hr which indicated the formation of AgNPs in solution. It was found that when the reaction was carried out at very high temperature (100°C) either in the presence of GG

or PVA, resultant solutions turned black indicating formation of bulk silver with no control over the reduction of silver precursor to silver particles. Interestingly, there was no visible change in the reaction medium when the reduction of AgNO₃ was carried out either in the presence of GG or PVA at 25°C or 50°C, even at the end of 6 hrs. This might be due to insufficient activation energy provided to the reaction mixture, which is needed for reduction of Ag⁺ to Ag⁰. Hence temperature of the resultant aqueous solution mixtures of all compositions was maintained only at 70°C during reaction. Again on varying pH of the reaction medium, we observed that at pH ~ 4.0, reaction mixture either containing GG or PVA and AgNO₃ at a temperature of 70°C remained colorless even after 6 hrs of addition of all the reaction components. When the pH of the reaction medium was at ~ 6.0 and ~ 8.0, reaction mixtures turned colored (from yellow to reddish brown depending upon various compositions of reducing and stabilizing agents used in the synthesis of AgNPs (Figure 1 (III)), indicating formation of smaller sized silver particles. Thus we carried out all the experiments by maintaining the pH of the reaction mixture either at pH ~ 6.0 or at ~ 8.0 and at a temperature of 70°C for 1 hr. AgNPs_{100/0/6.2} and AgNPs_{100/0/8.0} exhibited very low intense peaks at 411 nm (Figure 1 (I)) and 410 nm (Figure 1 (II)) respectively. Again AgNPs_{0/100/6.1} and AgNPs_{50/100/8.3} showed a broad low intense spectrum around 420 nm (Figure 1 (I)) and a sharp peak at 418 nm (Figure 1 (II)) respectively.

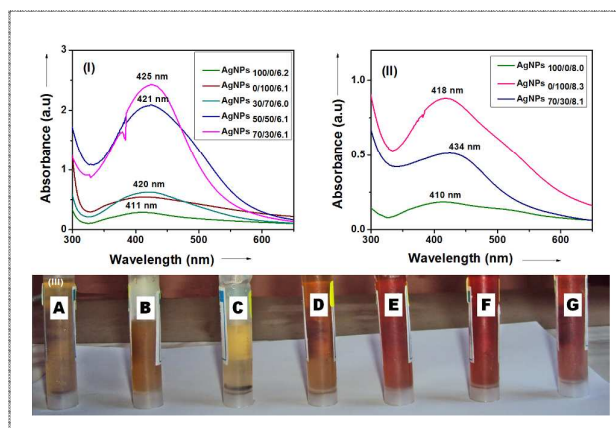
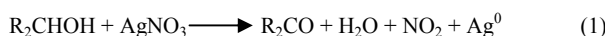


Figure 1. UV-Vis spectra and digital photograph of aqueous dispersions of AgNPs stabilized by GG, PVA and various blends of GG with PVA I) & II) UV-Vis spectra for aqueous dispersion of AgNPs_{100/0/6.2}, AgNPs_{50/100/6.1}, AgNPs_{30/70/6.0}, AgNPs_{50/50/6.1}, AgNPs_{70/30/6.1}, AgNPs_{100/0/8.0}, AgNPs_{50/100/8.3} and AgNPs_{70/30/8.1} respectively synthesized at 70°C. III) Photograph of aqueous dispersions of A) AgNPs_{100/0/6.2}, B) AgNPs_{50/100/6.1}, C) AgNPs_{100/0/8.0}, D) AgNPs_{50/100/8.3}, E) AgNPs_{30/70/6.0}, F) AgNPs_{50/50/6.1}, G) AgNPs_{70/30/6.1}. All data were recorded on the same day of the formation of AgNPs. Each test samples were diluted five times with milli Q water (concentration 150 µg/ml), sonicated for 1 hr, filtered and then analyzed.

Here we believe that both GG and PVA being rich in hydroxyl content, successfully reduced Ag⁺ to Ag⁰ through coordination chemistry and stabilized growing AgNPs. Both in neutral and in alkaline aqueous medium, aldehyde groups of the reducing sugar units get oxidized to carboxylic acid and carboxylate groups respectively, by nucleophilic addition of free hydroxyl groups at the carbonyl centers. During the nucleophilic addition reaction, one electron is delivered from one reducing

sugar which subsequently reduces Ag^+ to Ag^0 . On the other hand, secondary hydroxyl groups of mannoses, galactoses and PVA (R_2CHOH) in neutral as well as in highly basic medium get negatively charged whose number density varies with pH of the medium. Porel *et al* showed that negatively charged hydroxyl groups form complexes with positively charged silver ions which progressively dissociate to nucleating particles of silver atoms (reduced form) and carbonyl groups (oxidized form) as depicted through a “polyol route” in the Equation 1.⁵⁴



However, in an acidic medium the reducing groups of GG and PVA remained protonated for which their reducing efficiency dropped and were unable to generate any AgNPs in medium. Now on standing AgNPs, stabilized either by GG or PVA alone in a neutral or alkaline medium, for 7 days, the dispersions turned black indicating rapid agglomeration of AgNPs. In fact, stability of AgNPs for a considerable period of time, prepared by various techniques is one of the gravest problems. Hence in an attempt to prepare highly stabilized AgNPs with better control over particle size and particle size distribution, aqueous solution of a mixture of GG and PVA (pH ~ 6.0 and ~ 8.0) was used as the reducing cum capping agent for synthesis of AgNPs. Strong absorption spectra (Figure 1 (I)) were recorded at 425 nm, 421 nm and 420 nm for freshly prepared, filtered and well agitated AgNPs_{70/30/6.1}, AgNPs_{50/50/6.1} and AgNPs_{30/70/6.0} respectively. Here we propose that GG effectively reduced Ag^+ to Ag^0 and stabilized the nucleating particles of silver. The presence of PVA enhanced the reducing power of GG and thus helped in generating uniformly distributed AgNPs. From the UV-Vis results, we believe that probably PVA is a poor reducing agent as compared to GG in a medium of pH around 6.0 and hence produced lesser number of AgNPs in medium at the end of 1 hr, when 30 wt% GG + 70 wt% PVA blend was used as the polymeric matrix. Blend of 70 wt% GG with 30 wt% PVA and 50 wt% GG with 50 wt% PVA produced significant amount of AgNPs at the end of 1 hr in an aqueous medium of pH around 6.0. However blend of 70 wt% GG with 30 wt% PVA produced rapidly agglomerating AgNPs in solution at pH ~ 8.1 which might be due to enhanced reducing power of GG as well as of PVA in an alkaline medium. In an alkaline medium, compositions including 30 wt% GG + 70 wt% PVA and 50 wt% GG + 50 wt% PVA reduced Ag^+ to Ag^0 so fast that the growing silver particles could not be stabilized and thus agglomerated to bulk form of silver at the time of reaction. The hydrodynamic diameter of AgNPs in various stabilizing matrices were also found to be in correlation to the results obtained from UV-Vis spectra of respective samples as depicted in Figure 2 and tabulated in Table 1.

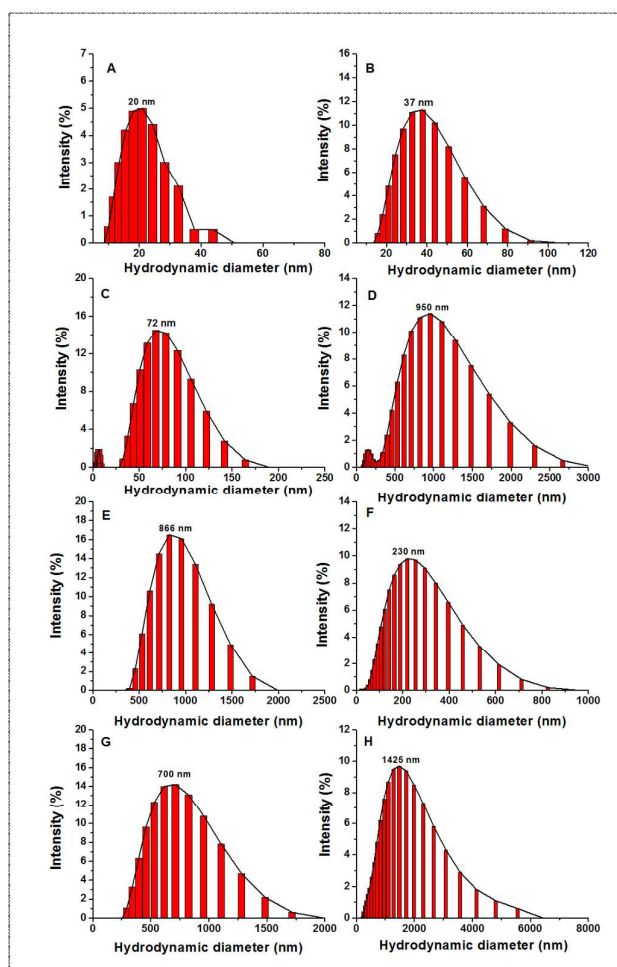


Figure 2. Particle size distribution of A) AgNPs_{70/30/6.1}, B) AgNPs_{50/50/6.1}, C) AgNPs_{30/70/6.0}, D) AgNPs_{100/0/6.2}, E) AgNPs_{50/100/6.1}, F) AgNPs_{70/30/8.1}, G) AgNPs_{100/0/8.0}, H) AgNPs_{50/100/8.3}. All data were recorded by DLS technique on the same day of the formation of AgNPs. Each test samples were diluted five times with milli Q water (concentration 150 $\mu\text{g}/\text{ml}$), sonicated for 1 hr, filtered and then analyzed.

The hydrodynamic diameter of AgNPs_{70/30/6.1} was around 20 nm with a polydispersity index of 0.098 which indicated highly monodispersed AgNPs in aqueous medium. Even the hydrodynamic diameter of AgNPs_{50/50/6.1} and AgNPs_{30/70/6.0} were recorded as 37 nm and 72 nm with a narrow polydispersity index of 0.13 and 0.198 respectively. However, AgNPs_{100/0/6.2}, AgNPs_{100/0/8.0}, AgNPs_{50/100/6.1}, AgNPs_{50/100/8.3}, AgNPs_{70/30/8.1} were highly polydispersed and quite larger in size. Thus particle size and size distribution of AgNPs_{70/30/6.1} was best controlled than those in other compositions. Indeed HRTEM image of dried AgNPs_{70/30/6.1} displayed highly monodispersed particles with an average particle diameter of 16 nm (Figure 3). However, average particle diameter acquired by HRTEM varied slightly from those obtained by DLS technique. This might be due to solvent-polymer interaction in aqueous medium owing to presence of large fraction of hydroxyl groups in capping polymers which resulted in greater hydrodynamic diameter of AgNPs. EDX spectrum (Figure 4A and B) confirmed presence of silver and other elements like

carbon (from carbon grid and polymer), oxygen (from polymer coatings) and gold (from instrument) in a typical dried sample of GG and PVA capped AgNPs.

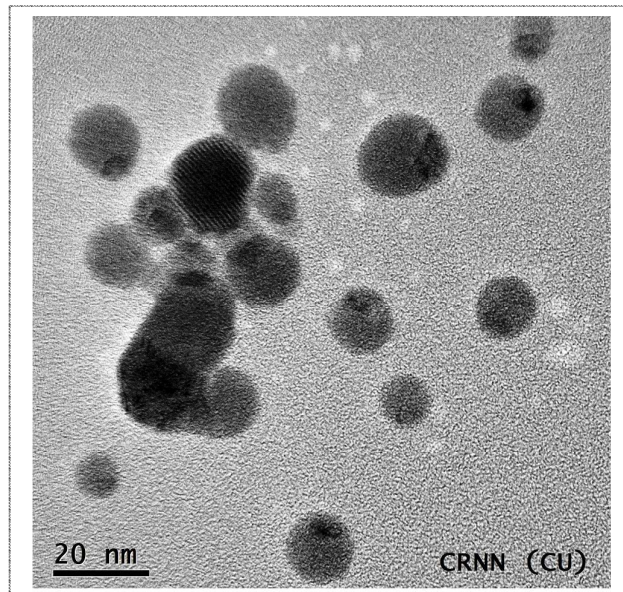


Figure 3. HRTEM image of dried AgNPs_{70/30/6.1}. All data were recorded within 72 hours of the formation of AgNPs.

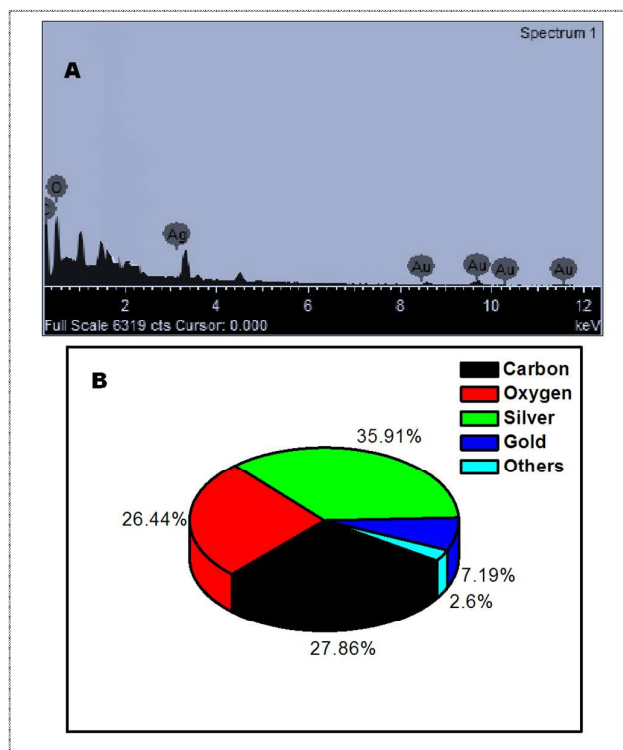


Figure 4. A) EDX spectrum and B) Percent distribution of various elements present in dried AgNPs_{70/30/6.1}. All data were recorded within 72 hours of the formation of AgNPs.

Stability of AgNPs, stabilized by various compositions of GG, PVA or both GG and PVA were studied from their respective zeta potential values. As recorded in Table 1 and Figure S1 (in the Supporting Information), zeta potential values of AgNPs_{70/30/6.1}, AgNPs_{50/50/6.1} and AgNPs_{30/70/6.0} were found to be -11.0 mV, -9.0 mV and -7.88 mV respectively. However, AgNPs_{100/0/6.2}, AgNPs_{100/0/8.0}, AgNPs_{50/100/6.1}, AgNPs_{50/100/8.3}, AgNPs_{70/30/8.1} exhibited low zeta potential values of -0.48 mV, -4.98 mV, -2.41 mV, -3.43 mV and -6.45 mV respectively. Undoubtedly, blend of GG and PVA provided better stabilization to the growing AgNPs in a neutral aqueous medium. Zeta potential value of AgNPs with least hydrodynamic particle size i.e.; AgNPs_{70/30/6.1} being around -11.0 mV implied that AgNPs were electrically neutral and stable. Here we believe that in a neutral aqueous medium (pH ~ 6.0), both GG and PVA in a blend exhibited low but enough negative potential owing to the absence of free negatively charged hydroxyl and carboxyl groups, which induced very little positive charge on the surface of the growing AgNPs. Hence the induced charged AgNPs repelled each other and prevented any agglomeration (electrostatic stabilization). For this reason when GG or PVA was used alone as reducer cum stabilizer at pH ~ 6.0, they did not provide sufficient free negatively charged hydroxyl and carboxyl groups to stabilize any growing AgNPs by induction effect. However in an alkaline medium, although AgNPs could be better stabilized yet due to very fast reduction of Ag⁺, particle size and particle size distribution of AgNPs could not be controlled precisely. We would like to add that in our case electrostatic stabilization was not solely responsible for long term stability and narrow particle size distribution of AgNPs. Blend miscibility and viscosity of various mixtures of GG and PVA might also have played a pivotal role in the generation of smaller sized AgNPs and their subsequent stabilization for a prolonged time. In the FTIR spectrum (Figure 5A), the sharp peaks observed at 3854 cm⁻¹, 3747 cm⁻¹ and 3616 cm⁻¹ corresponds to -OH bond stretching of macromolecular associations (secondary alcoholic groups of GG, primary alcoholic groups of GG and PVA respectively). The band observed at 2926 cm⁻¹ and 2892 cm⁻¹ are assigned to asymmetric and symmetric stretching vibrations of methylene groups of PVA. Other characteristic peaks which appeared at 1643 cm⁻¹, 1516 cm⁻¹, 1445 cm⁻¹, 924 cm⁻¹ and 762 cm⁻¹ are due to C=O stretching mode (typical of saccharide absorption due to -COOH group), glycoside bond stretching, C-H bend of methylene, 1-4 linkage of galactose and 1-6 linkage of mannose respectively. Thus FTIR spectrum confirmed that GG formed a highly miscible blend with PVA as there were characteristic peaks of both GG and PVA. Interestingly, FTIR spectrum of dried AgNPs_{70/30/6.1} (Figure 5B) exhibited few observable changes. The shift in spectral band from 1643 cm⁻¹ to 1736 cm⁻¹ definitely suggests that COO⁻ groups present in GG bound with Ag⁺ during process of reduction. Thus carboxylate groups of GG and free hydroxyl groups of GG and PVA were responsible for reduction of Ag⁺ to Ag⁰ and subsequent stabilization of AgNPs.

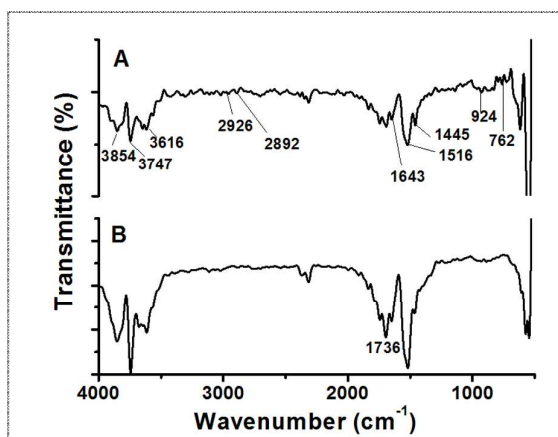


Figure 5. FTIR spectrum of A) blend of 70 wt% GG and 30 wt% PVA B) dried AgNPs_{70/30/6.1}

We further studied role of viscosity of the solutions of GG, PVA and GG + PVA on the particle size distribution and stability of AgNPs in aqueous medium. In Table 2, we compared reduced viscosities of various solutions of freshly prepared GG, PVA and GG + PVA at a temperature of 25°C and found that undoubtedly blend of GG and PVA exhibited higher viscosity than individual solutions of GG or PVA, both at pH around 6.0 and 8.0. Hence any blend of GG and PVA arrested fast movements of growing AgNPs in aqueous medium and prevented their agglomeration for a longer time. However other important factors as discussed earlier in this section were also responsible for stabilization of AgNPs. For this reason, although Gum_{30/70/6.0} exhibited maximum reduced viscosity yet they did not produce most stable AgNPs.

We also studied reproducibility of the above described synthesis process for generation of AgNPs. Indeed AgNPs_{70/30/6.2} and AgNPs_{70/30/6.0} exhibited hydrodynamic diameter in the range 20-30 nm. Thus we may claim that an optimized blend of GG and PVA produces almost monodispersed AgNPs with similar particle size distribution every time we repeated synthesis of AgNPs.

Table 2. Comparison of reduced viscosities of aqueous solutions of neat GG, neat PVA and various blends of GG and PVA at different pH and at a temperature of 25°C

Sample designation*	GG (wt%)	PVA (wt%)	pH of aqueous solution	Reduced viscosity, η_{sp}/c (dl/g)
Gum _{100/0/6.2}	100	0	6.2	45.25
Gum _{0/100/6.1}	0	100	6.1	61.08
Gum _{30/70/6.0}	30	70	6.0	70.77
Gum _{50/50/6.1}	50	50	6.1	69.43
Gum _{70/30/6.1}	70	30	6.1	69.22
Gum _{100/0/8.0}	100	0	8.0	42.44
Gum _{0/100/8.3}	0	100	8.3	59.00
Gum _{70/30/8.1}	70	30	8.1	66.55

*All the samples were devoid of silver nanoparticles

Time variant study on stability of AgNPs stored under refrigeration

Use of two highly viscous polymeric components not only served the purpose of a good reducing agent for Ag⁺ as comparable to NaBH₄ but also stabilized growing AgNPs for 60 days when stored under refrigeration. This is for the first time we are showing such a highly stable aqueous dispersions of AgNPs prepared by an environmental benign technique. We provided a chronological change in morphology of AgNPs_{70/30/6.1} with time through images, DLS results (Figure 6 A-E) and UV-Vis spectra (Figure 6F). Interestingly, AgNPs_{70/30/6.1} showed very gradual change in average particle size from 20 nm to 22 nm, 35 nm, 48 nm and 52 nm at the end of 2 hrs, 24 hrs, 7 days, 30 days and 60 days respectively from the moment reduction of Ag⁺ was stopped. The HRTEM image (Figure S2 in the Supporting Information) which was acquired on the 60th day of the formation of AgNPs revealed quasi spherical and slightly aggregated AgNPs with an average particle diameter of 42 nm. Zeta potential values of the respective samples also dropped accordingly from -11.0 mV to -10.7 mV, -9.96 mV, -9.78 mV and -1.96 mV respectively. Drop in zeta potential values of AgNPs_{70/30/6.1} indicate that indeed there was certain fraction of agglomeration of AgNPs in the aqueous medium. Nevertheless, AgNPs_{70/30/6.1} are more stable than any other dispersions of AgNPs, prepared by other methods worldwide so far. Even the UV-Vis spectra of AgNPs_{70/30/6.1} shifted from 425 nm to 427 nm, 431 nm, 430 nm and 453 nm at the end of 2 hrs, 24 hrs, 7 days, 30 days and 60 days respectively from the end of reaction. According to conventional Mie theory, red shifting in UV-Vis spectra of AgNPs_{70/30/6.1} with time envisage that there were slight distortion in spherical shapes of AgNPs along with aggregation of AgNPs.⁵⁵ In our system, AgNPs might have grown larger in size by coalescent growth mechanism. AgNPs in the sub 20 nm range are expected to be highly unstable owing to high energy surface area of AgNPs. Hence, in an attempt to reduce energy of the system, smaller sized AgNPs tend to coalesce at the surface to form larger particles with time.⁵⁶ However, as the viscosity of the medium was very high, the process of coalescence of smaller sized AgNPs was slow. For this reason, slightly modified morphology of AgNPs

were obtained even at the end of 60 days. On the other hand, AgNPs_{50/50/6.1} and AgNPs_{30/70/6.0} were stable only up to 30 days (results of DLS and zeta potential are tabulated in Table 1). Hence in our case, aqueous solution of 70 wt% GG + 30 wt% PVA produced smallest and monodispersed AgNPs, which were stable nearly for 60 days.

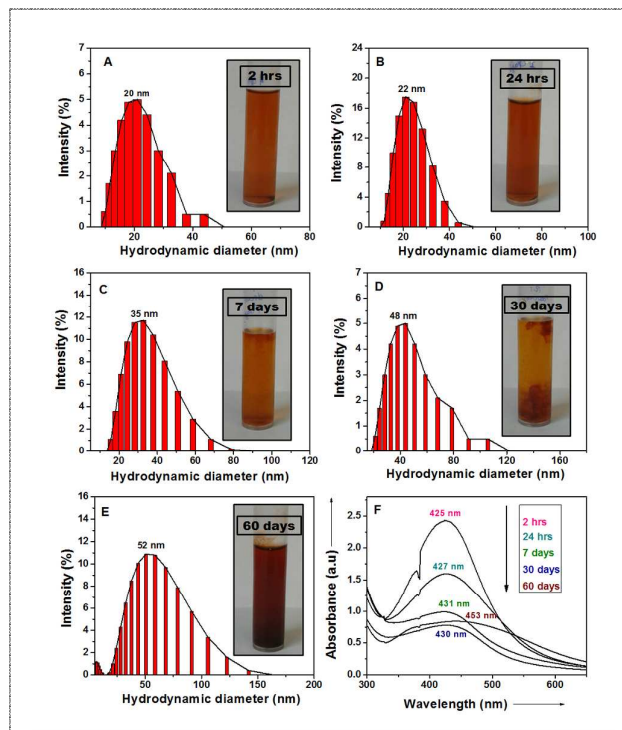


Figure 6. A-E) Change in particle size distribution of AgNPs_{70/30/6.1} as recorded by DLS technique at the end of 2 hrs, 24 hrs, 7 days, 30 days and 60 days respectively. Inset displays visual change in aqueous medium containing AgNPs_{70/30/6.1} with respective to time. Images were acquired immediately after taking out of refrigerator. F) Change in UV-Vis spectrum of AgNPs_{70/30/6.1} with time. Prior to recording of DLS data and UV spectra, all samples were ultra sonicated for 1 hr and filtered.

Antibacterial study of AgNPs stabilized by a blend of GG and PVA

Antibacterial activity of the *in situ* generated AgNPs by synergistic assistance of GG and PVA was explored by the conventional cup plate method. AgNPs_{70/30/6.1} indeed exhibited strong antibacterial activity against *B. subtilis* strains as compared to the behavior of controls (GG + AgNO₃, PVA + AgNO₃, GG + PVA + AgNO₃) used in the experiment against the same bacterial strains. We observed a radial diameter of inhibitory zone of 36 mm, 30 mm, 21 mm and 18 mm respectively around the zone filled with AgNPs_{70/30/6.1} (0.2 mg/ml)- nano, PVA + AgNO₃ (1 ml)- C2, GG + PVA + AgNO₃ (1 ml)- C1 and GG + AgNO₃ (1 ml)- C3 as depicted in the Figure 7. All the results of radial diameter of inhibitory zones around the respective cavities were mean of three different experiments. An inhibitory zone in the agar plate was observed in all the control cavities because of antibacterial power of AgNO₃ alone. However the antibacterial efficiency of AgNO₃ was suppressed in the presence of GG which is highly susceptible to bacterial attack (C3). Even in the blend of GG and PVA,

AgNO₃ could not clear the surrounding zone (C1). PVA is less compatible with microbes and hence created a good clearing zone around the cavity in the presence of AgNO₃ (C2). Antibacterial effect of AgNPs_{70/30/6.1} was very high due to high toxicity of metal nanoparticles to any cellular organisms.²² We also believe that there would be hardly any change in antibacterial activity of AgNPs_{70/30/6.1} with time as we have already reported that morphology and other properties of AgNPs_{70/30/6.1} almost remained similar even after 60 days.



Figure 7. Antibacterial study on different test samples by agar diffusion method in a petri dish. C-1 contained GG + PVA + AgNO₃, C-2 contained PVA + AgNO₃, C-3 contained GG + AgNO₃, nano contained AgNPs_{70/30/6.1} at a concentration of 1mg/ml. Image clearly showed an inhibitory zone of various diameters around each cavity. Antibacterial study was initiated using 3 days old AgNPs_{70/30/6.1}.

Antioxidant study of AgNPs stabilized by a blend of GG and PVA

Antioxidant activities of best optimized AgNPs (with respect to stability) were assayed by three different methods (superoxide scavenging assay, hydroxyl radical scavenging assay and chelating ability of ferrous ions). Superoxide anion radicals (SO or O₂⁻) were deliberately generated by photochemical reduction of riboflavin. Subsequently O₂⁻ reduced NBT and formed blue formazan (NBT²⁺). Presence of O₂⁻ in the medium and gradual scavenging of O₂⁻ by test samples were monitored by recording UV-Vis absorbance at 590 nm which decreased due to reduction in formation of NBT²⁺ in the absence of free O₂⁻ in the medium. Percentage inhibition in O₂⁻ generation in the medium by four different test samples was deduced by using the Expression (1):

$$\% \text{ Inhibition in generation of } O_2^- = [1 - A/A_0] \times 100 \quad (1)$$

where A is the absorbance by test samples containing antioxidants and A₀ is the absorbance by control.

In the present study, we found that AgNPs_{70/30/6.1} and AgNPs_{50/50/6.1} exhibited enhanced scavenging of O₂⁻ generated in riboflavin-NBT light system as compared to that of

Gum_{100/0/6.2} and Gum_{0/100/6.1} (Figure 8I). Both GG and PVA have very low antioxidant property. Presence of AgNPs significantly affected inhibition in the generation of any undesirable $O_2^{\cdot-}$. $O_2^{\cdot-}$ are highly detrimental as they produce large number of secondary highly active radicals like hydroxyl radicals, hydrogen peroxide, singlet oxygen etc. Hence we further tried to assay antioxidant activity of AgNPs against various reactive oxygen species (ROS). Hydroxyl radicals (HR of OH^{\cdot}) were purposely generated from Fe^{2+} -ascorbate-EDTA- H_2O_2 system (by Fenton's reaction) which attacked 2-deoxyribose and set off a series of reactions that eventually resulted in the formation of malondialdehyde (MDA), measured as a pink MDA-TBA chromogen at 535 nm. When ferric-EDTA was incubated with H_2O_2 and ascorbic acid at a pH of 7.4, free OH^{\cdot} were formed in the solution and were detected spectrophotometrically by their ability to degrade 2-deoxyribose into fragments that formed a pink chromogen upon heating with TBA at low pH. When the test samples were added to the reaction mixture, they removed OH^{\cdot} from the medium and showed decreased absorbance owing to inhibition in the formation of pink chromogen in absence of free OH^{\cdot} . Finally OH^{\cdot} scavenging activity of the test samples were calculated from the general Expression (2):

$$\% OH^{\cdot} \text{ scavenging effect} = [1 - A_1 / A_0] \times 100 \quad (2)$$

where A_1 is the absorbance by the test samples containing antioxidants and A_0 is the absorbance by the control without any antioxidants

Here we observed that AgNPs_{70/30/6.1} and AgNPs_{50/50/6.1} could effectively remove even the highly reactive OH^{\cdot} from the system as compared to Gum_{100/0/6.2} (Figure 8II). Our studies clearly revealed that Gum_{0/100/6.1} could not scavenge OH^{\cdot} . OH^{\cdot} are highly active species and thus lead to premature ageing of cells and deterioration of packaged food. Hence highly active antioxidants are often added in many formulations to scavenge OH^{\cdot} in order to prevent any further generation of other harmful radicals. Very often OH^{\cdot} are generated by reaction of H_2O_2 with Fe^{2+} (abundantly present in food and biological systems). Hence undesirable OH^{\cdot} generation can be circumvented through formation of complexes with Fe^{2+} by using suitable chelating agents. Ferrozine quantitatively forms complexes with Fe^{2+} . In the presence of external chelating agent, the complex formation of Fe^{2+} with ferrozine gets disrupted owing to a competitive reaction between ferrozine and chelating agent for association with Fe^{2+} and thus there is a reduction in UV-Vis absorbance (in the red zone), which subsequently allows estimation of the chelating ability of the coexisting chelator. The percentage of inhibition of ferrozine- Fe^{2+} complex formation was calculated from the Expression 3:

$$\% Fe^{2+} \text{ chelating ability} = [1 - A_2 / A_0] \times 100 \quad (3)$$

where, A_2 is the absorbance of the solution of stabilized AgNPs and A_0 is the absorbance of the control.

Here we observed that AgNPs_{50/50/6.1} and AgNPs_{70/30/6.1} demonstrated a marked capacity for iron binding ability of 69.61% and 46.24% at a concentration of 1 mg/ml respectively (Figure 8III). We believe that AgNPs in presence of both GG and PVA were able to chelate Fe^{2+} might be due to presence of carboxyl groups of GG and hydroxyl groups of GG and PVA. Thus presence of AgNPs indeed helped in formation of stronger complexes with ferrous ions adsorption mechanism as compared to neat GG or PVA. From the antioxidant studies we thus demonstrated that GG and PVA capped AgNPs exhibited good antioxidant property. In the present case, two factors might have governed the antioxidant property of the blend of GG and PVA capped AgNPs, one is the inherent antioxidant feature of GG and other is the presence of AgNPs with high surface activity. GG being a derivative of alcohol, is weakly acidic in nature. Hence release of a proton in aqueous medium forms a free radical on the backbone of GG which subsequently combines with a generated free radical and neutralize its effect.⁵⁷ Moreover the electron withdrawing carboxyl and aldehyde groups present in the GG activate the hydrogen atom of sugar residues, release more free hydrogen atoms to the surrounding medium which in turn combine with the free radicals to form more stable radicals. Also, GG contains many repeated heterocyclic pyrane moieties. The hetero oxygen atoms present in the structure coordinate with Fe^{2+} ions and form stable five membered chelating rings.⁵⁸ Thus the capping agent for AgNPs definitely plays a crucial role in developing antioxidant properties in the composition. However, PVA scavenged only less reactive $O_2^{\cdot-}$ (< 10%) and formed poor complexes with Fe^{2+} owing to presence of few hydroxyl groups. Silver is an electropositive metal. Thus if highly reactive radicals are present in the vicinity of silver, silver readily share its free electrons with the unpaired electrons and thereby scavenges the harmful radicals. Again due to very small size of AgNPs, their surface area is very high and can potentially entrap large number of free radicals on their surface. This enhances the antioxidant efficiency of matrix material (GG) in which AgNPs is stabilized. Also, AgNPs being electropositive in nature with very high surface energy can collect more electronegative metal ions like Fe^{2+} . This explains why the chelating ability of blend of GG and PVA rich in AgNPs exhibited such a high chelating ability for Fe^{2+} . It is expected that as morphological stability of AgNPs_{70/30/6.1} remained unchanged for a prolonged time, then antioxidant property of AgNPs_{70/30/6.1} would also remain unchanged with time. Hence here we did not perform any time dependant antioxidant studies of AgNPs.

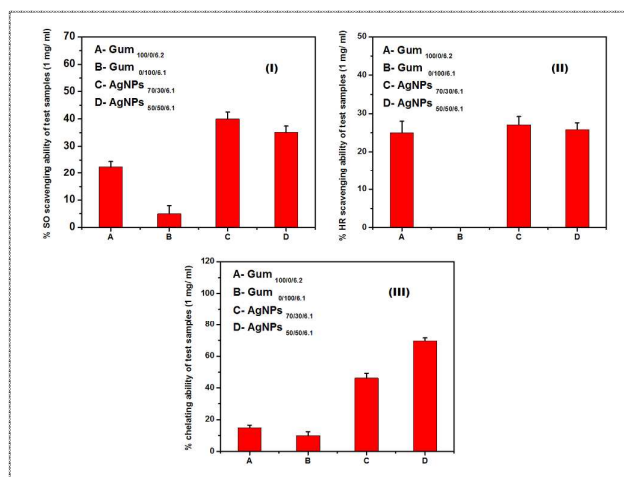


Figure 8. I) Superoxide radical scavenging activity II) Hydroxyl radical scavenging activity and III) Ferrous ion chelating ability of A) AgNPs_{100/0/6.2}, B) AgNPs_{50/100/6.1}, C) AgNPs_{70/30/6.1} and D) AgNPs_{50/50/6.1}. Results are the mean \pm SD of three separate experiments, each in triplicate. All antioxidant studies were initiated using 3 days old AgNPs_{70/30/6.1} and AgNPs_{50/50/6.1}.

Conclusion

In the present work, we have successfully prepared highly monodispersed AgNPs which were stable for a considerable period of time (almost 60 days). Use of blends of GG and PVA in various proportions as reducer cum stabilizer in an aqueous medium at pH around 6.0, not only encouraged reduction of Ag^+ to Ag^0 but also stabilized the growing AgNPs for a longer time as compared to AgNPs in either GG or PVA alone. Blend compatibility, high medium viscosity and presence of various functional groups of both GG and PVA highly encouraged generation of AgNPs, arrested the incipient particles to a size until AgNPs acquired sufficient system stability and kept AgNPs in dispersions for a long time. In our study, AgNPs_{70/30/6.1} in aqueous medium, were smallest in size (16 nm as obtained from HRTEM result), highly monodispersed and stable for 60 days. Thus the current green methodology may be used as an efficient route to develop long term stable and monodispersed AgNPs. AgNPs_{70/30/6.1} also exhibited stronger antibacterial and antioxidant activities than neat GG (as GG exhibit intrinsic antioxidant properties). Thus here we conclude that *in situ* prepared AgNPs, reduced and stabilized by two highly viscous polymers may be used in various commercial products.

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