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In situ formation of silver nanoparticles in thermosensitive glycogels and evaluation of its antibacterial activity

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Copolymeric thermosensitive hydrogel, *N*-isopropylacrylamide and glycomonomer based on D-galactose have been synthesized by free radical polymerization using 2,2′-azobis-isobutyronitrile as initiator and ethylene glycol dimethacrylate as crosslinker. This hydrogel has been further used as template to synthesize AgNPs. The composite glycogel containing AgNPs has been characterized by different techniques and tested for antibacterial activity against *E. coli* and *P. aeruginosa.*

Keywords: Nanomaterials, Silver nanoparticles, Hydrogels, Thermosensitive materials, Antibacterial activity, Free radical polymerization

In recent years, great interest has been developed in metal nanoparticles due to their fascinating physicochemical properties and small size.[1] Precious metal nanoparticles have been used for various applications like biosensors, catalyst and biomedical applications etc.[2-5] Silver in any form such as ions, nanoparticles, colloids exhibits all of these properties and finds applications in various areas. Silver nanoparticles (AgNPs) show effective antibacterial activity against various bacterial strain as they penetrate into the bacterial cell wall, effectively killing the bacteria.[6,7]

These nanoparticles have a tendency to form aggregates as they have large surface energy. To avoid this, polymeric supports like hydrogels, dendrimers and microgels have been used which stabilize the formed nanoparticles.[8-10] In the case of *in situ* reduction of metal nanoparticles, hydrogels are found to be a better template as compared to the other polymeric supports since the size of the nanoparticle formed is mostly uniform and the formed particles are well stabilized in the hydrogel matrix.[11]

In situ reduction of metal ions often involves use of chemical agents such as sodium borohydrate ($NaBH_4$),[12] alkali amines[13] and hydrazine hydrate.[14] These reducing agents are hazardous and environmentally toxic. To overcome the problems associated with these chemical agents, natural polysaccharides have been used as reducing agents to achieve monodispersed colloidal AgNPs.[15] Methyl cellulose and dextran in combination with PVA have been reported.[16] Li & coworkers[17] have synthesized hybrid hydrogels based on tapioca dialdehyde starch (DAS)-chitosan for *in situ* reduction of Ag ions. These polyhydroxylated polymers also stabilize AgNPs, thus avoiding the aggregation of nanoparticles. Donati *et al.*[18] reported alditol bearing chitosan that showed the ability to reduce silver ions without any external reducing agent in mild conditions. Twu *et al.*[19] prepared silver/chitosan nanocomposites using basic chitosan suspension as a stabilizer and as a reductant in the absence of other chemicals agents and at higher temperatures.

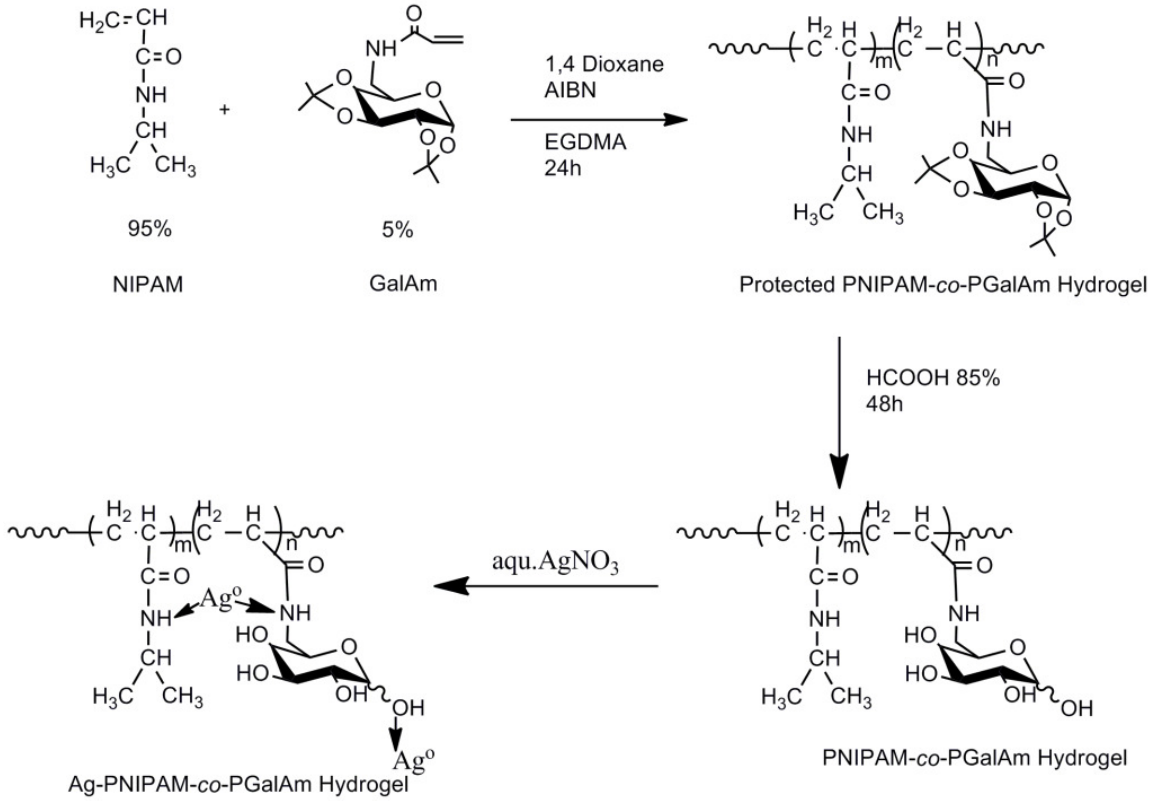
Furthermore, stimuli responsive hydrogel due to extensive swelling and deswelling behavior depending on the external conditions have been used in synthesis of nanoparticles.[20,25] Poly(*N*-isopropylacrylamide) is a classic example of thermosensitive polymers that exhibit thermodynamic lower critical solution temperature (LCST) in the range of 31-33 °C in water and show an inverse solubility behavior with an increase in temperature. Crosslinked hydrogels obtained from this polymer undergo a first order volume transition at the LCST.[21] Due to the unique property of sharp swelling to shrinking transition at 32 C, this temperature sensitive polymer yields uniformly distributed nanoparticles.[20] Combination of such stimuli responsive polymeric material with nanoparticles finds wide application in the field of biomedicine. Bajpai *et al.*[20] have reported *in situ* reduced AgNps in poly(*N*-isopropylacrylamide) [PNIPAm] based hydrogel. This thermoresponsive hybrid hydrogel was effective against *E. coli* bacteria. Other temperature sensitive polymers like Pluronic[22,23] have also been reported. PNIPAm along with the biopolymer, chitosan, has improved the antibacterial property of AgNps embedded in the hydrogel matrix.[24]

As a part of our continuing efforts in the area of glycopolymers,[25-27] herein we aim to synthesize a new hydrogel by free radical polymerization of NIPAm and galactose based monomer (GalAm), which we have further used to prepare AgNPs utilizing the reducing ability of the anomeric hydroxyl of sugars. Incorporation of glycomonomer in the hydrogel excluded the use of any external reducing agents for formation of AgNPs. The composite hydrogel was characterized with various techniques such as UV-visible spectroscopy, FTIR, FE-SEM and XRD. Subsequently, this hydrogel was tested for its antibacterial activity against *E. coli* and *P. aeruginosa.*

Experimental

Silver nitrate ($AgNO_3$) (Merck), D-galactose (Acros chemicals), acryloyl chloride (Fluka), ethyleneglycol dimethacrylate (EGDMA) (Fluka) were used as received. *N*-isopropylacrylamide (NIPAm) and 2,2′-azobis-isobutyronitrile (AIBN) were procured from Sigma Aldrich and recrystallized from methanol before use. All other chemicals were analytical-grade and were used as received.

The glycomonomer, 6-acrylamido-6-deoxy-1,2:3,4-di-*O*-isopropylidine-α-D-galactopyranoside (GalAm) was synthesized from D-galactose as shown in Scheme S1 (supplementary data) by modifying the reported procedure.[26,28] Details of synthesis, ^{1}H and ^{13}C-NMR spectra of GalAm are given as supplementary data. As shown in Scheme 1, the PNIPAm-*co*-PGalAm hydrogel was prepared by free radical polymerization technique. Briefly, NIPAm (0.872 g, 7.72 mmol) and GalAm (0.127 g, 0.404 mmol) were dissolved in 1,4-dioxane (6.0 mL). To this mixture, AIBN (40 mg, 0.24 mmol) was added as radical initiator and EGDMA (14 μL, 0.081 mmol) as the crosslinker. Nitrogen gas was purged for 30 min, and then the tube was sealed and kept in oven at 65 ^{o}C for 24 h under nitrogen atmosphere. The gel obtained was diced into small discs and stirred in 20 mL formic acid for 72 h at room temperature in order to deprotect the acetonide functionality of the sugar unit. Subsequently, the deprotected hydrophilic gel was washed with deionized water for 3 days followed by washing with acetone and dried in the oven at 65 °C.



Scheme 1

For the preparation of silver nanocomposite hydrogel, to a solution of $AgNO_3$ (20 g/L), dry hydrogel weighing (1.4 g/L) was added, and the solution was kept for stirring in dark. After 5 h, the gel and the external solution turned yellow, indicating the formation of colloidal AgNPs. These AgNPs loaded hydrogels were characterized by different techniques.

^1H and ^{13}C-NMR spectra of GalAm were recorded on Bruker AC 200MHz instrument. FT-IR was recorded on Bruker TENSOR- 37 spectrometer. The samples were dried completely, grounded to fine powder and scanned at the wavelength range of 400-4000 cm^{-1} at room temperature. UV-visible absorption spectra were recorded using a UV-2500 spectrophotometer (Shimadzu, Japan) in the scan range of 200-800 nm. HR-TEM images were recorded on Technai-20 transmission electron microscope with an accelerating voltage of 200 KeV. For HR-TEM measurements, finely grounded hydrogel samples were dispersed in 1 mL distilled water. A drop of this particle solution was placed on a copper grid and dried at room temperature. Powder X-ray diffraction patterns of the gels were recorded on Rigaku Ultima diffractometer IV with Cu K radiation (= 0.1546 nm) running at 40 kV and 200 mA. The gel disc was crushed to fine powder before analysis. The average crystallite size (D) of the AgNPs formed was calculated using Debye-Scherrer relationship, $D \alpha k = \beta \cos\theta$, here k is a constant ($k \alpha = 0.9$), is the wavelength of the incident X-ray, β is the full width at half maxima, and θ the Bragg's diffraction angle. The morphological and elemental studies of the synthesized AgNPs were investigated by FESEM (FEI, Nova NanoSEM 450) equipped with energy dispersive spectrometer (Bruker, XFlash®). The gels were cooled to –78 °C, crushed into fine powder and loaded on silicon wafer. It was sputter-coated with a thin layer of conduction chromium metal.

The swelling measurements were carried out by immersing the dry gel samples in excess water, which was maintained at a controlled temperature within ±0.5°C. The gels were allowed to equilibrate for 72 h during which constant weight was reached, after which they were quickly removed and weighed. The surface water was carefully wiped off before weighing. Swelling capacity was calculated as follows:

$$q = W_s\text{-}W_d/W_d$$

where W_s and W_d are the weight of swollen and dry samples, respectively.

Laboratory strains of ***P. aeruginosa*** PAO1 and ***E. coli*** were grown overnight on a shaker at 120 rpm in Luria Bertanni-(LB) broth (0.5 g yeast extract, 1.0 g caesine enzyme tryptone type I, 1.0 g sodium chloride in 100 mL distilled water and 7.2 pH). This ensured proper aeration of the culture. Stock solution of the Ag composite was prepared using LB broth (1 mg/mL). Then, 100 L of the culture was spread on each microtitre plate and a 2 mm bore was made. Varying concentrations of the test compound were added to each well. The plates were inverted and incubated at 30 °C for 48 h to allow for bacterial growth. Inhibition zone diameters (IZD) were measured in mm. All isolates were run in triplicate and reported as mean. Minimum inhibitory concentration (MIC) and sub-inhibitory concentration (SIC) were performed as previously described using the broth microdilution method. Briefly, the test compounds were diluted with LB medium by serial dilutions. The final concentration of bacterial cultures was about 10^5 cfu/mL. After incubation at 37 °C for 24 h, the absorbance at 600 nm was measured using a microplate reader (Thermo Electron Corporation) to assess the cell growth. The blank readings of each compound were subtracted from the test readings and a graph of dilution versus absorbance was plotted. The MIC value for the compound was determined from the plotted graph. The value preceding the MIC was the SIC.

Result and discussion

Glycopolymer hydrogel was synthesized by free radical technique using NIPAm and GalAm monomers in the ratio 95:05 as shown in Scheme 1. The acetonide protection of sugar moiety was removed under mild acidic conditions. The incorporation of glycomonomer in the hydrogel was confirmed by FT-IR spectroscopy. Figure 1 shows FT-IR spectra of PNIPAm, protected and deprotected hydrogel. The protected hydrogel showed two carbonyl frequencies at 1554 $cm^{-1}$and 1650 cm^{-1} due to presence of two amide groups, confirming the incorporation of sugar units. The deprotected gel showed broad peak around 3300 cm^{-1} due to free hydroxyl group of sugar, while the absence of peak at 990 cm^{-1} and 1200 cm^{-1} confirms the deprotection of isopropylidine group. After acetonide deprotection, the gel was washed with deionized water and used for

formation of AgNPs by *in situ* reduction of silver nitrate solution. The masked aldehyde group in the form of hemiacetal plays role as a reducing agent for conversion of Ag ions to AgNPs. The AgNPs were formed in hydrogel due to the carbohydrate at room temperature and neutral pH. The completion of reduction process required 5 h, which was verified by UV at regular intervals. The AgNPs were well dispersed and stabilized in the hydrogel matrix due to the thermoresposive PNIPAm and also the free hydroxyl of the sugar.

As PNIPAm is a temperature sensitive polymer and shows LCST at around 32 ^{o}C, we carried out the swelling analysis at 25 ^{o}C. Equilibrium swelling ratios of the protected PNIPAm-*co*-PGalAm gel, deprotected PNIPAm-*co*-PGalAm gel and Ag-PNIPAm-*co*-PGalAm gel were determined as the gels attained equilibrium after 48 h. The deprotected PNIPAm-*co*-PGalAm gel showed enhanced swelling as compared to the protected gel as the free hydroxyl group of the sugar moiety increases hydrophilicity of the gel. The Ag-PNIPAm-*co*-PGalAm gel showed highest equilibrium swelling capacity due to increase in porosity of the gel (Fig. 2).

The formation of silver nanoparticles in the hydrogel was confirmed by the absorption spectra. The absorption peak around 440 nm was observed for the silver nanoparticles in the gel nanocomposites as compared while the plain PNIPAm-*co*-PGalAm hydrogel which did not show any such peak.

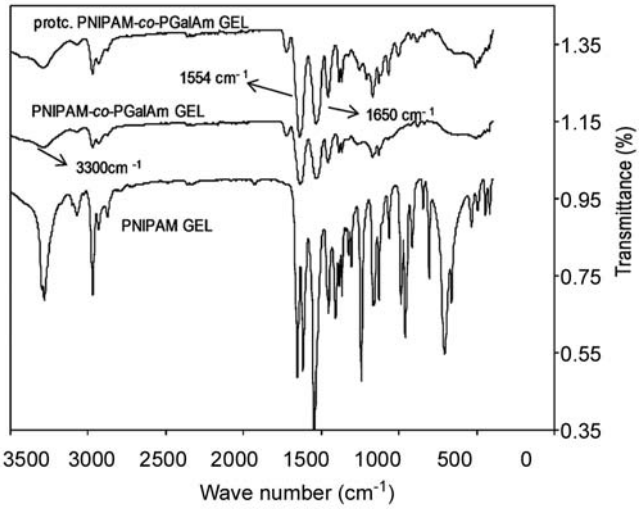


Fig. 1 – FTIR spectra of protected, deprotected and PNIPAm-co-PGalAm gels.

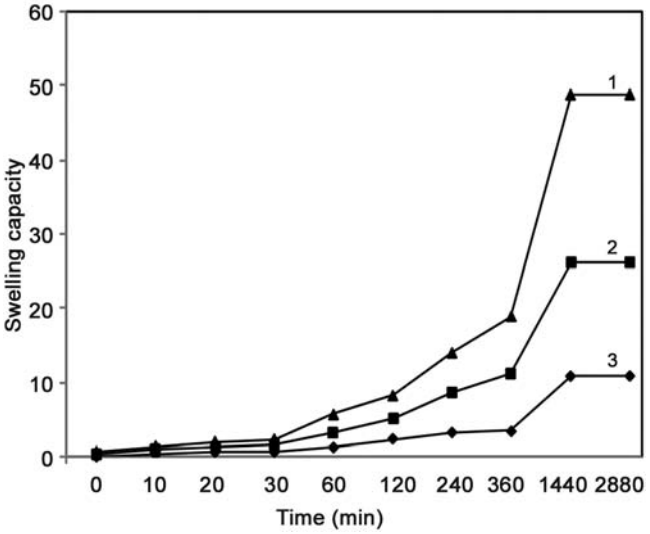
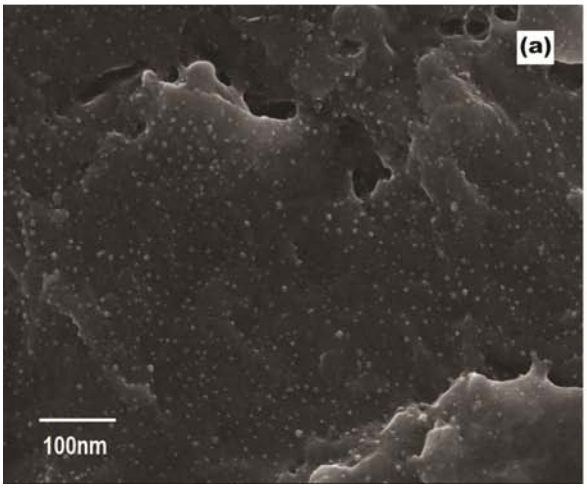


Fig. 2 – Swelling capacities versus time for Ag-PNIPAm-*co*-PGalAm gel (1), PNIPAm-*co*-PGalAm (2), and, Protected PNIPAm-*co*-PGalAm at 25 ^{o}C (3).



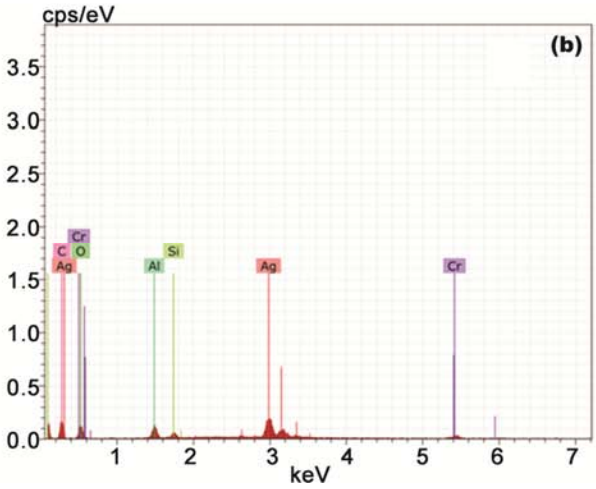


Fig. 3 – (a) FE-SEM image of Ag-PNIPAm-*co*-PGalAm gel [100X magnification], and, (b) EDX profile of Ag-PNIPAm-*co*-PGalAm gel].

The FE-SEM images of the Ag-PNIPAm-*co*-PGalAm gel showed the porous nature of the hydrogel. The AgNPs were uniformly distributed in the hydrogel matrix as seen in the Fig. 3a. These nanoparticles were not only seen on the surface of the hydrogel, but are also uniformly distributed inside the hydrogel as free anomeric sugar part is randomly distributed in the polymeric strands of the hydrogel. EDS showed a high concentration of AgNPs (~33%) as seen in Fig. 3b.

The HR-TEM of the hydrogel samples was recorded to confirm the presence of AgNPs. Figure 4 shows formation of spherical AgNPs of the size of around 30 nm, which is in good agreement with surface plasmon resonance peak at 440 nm. The crystal lattice arrangement of silver nanoparticles can be seen clearly under high magnification.

The formation of AgNPs was further confirmed by X-ray diffraction technique. The diffraction pattern of the plain and AgNPs containing hydrogel is depicted in Fig. 5. The XRD pattern of the PNIPAm-co-PGalAm hydrogel shows peak at 48.4° due to crystalline nature of some part in the hydrogel. The Ag-PNIPAm-*co*-PGalAm hydrogel exhibits diffraction peaks at 38.18°, 44.2°, 64.4° and at 77.28° corresponding to (111), (200), (220), and (311) planes respectively of the face-centered cubic of AgNPs. Such peaks were absent in the XRD pattern of PNIPAm-*co*-PGalAm hydrogels. The average size of the AgNPs formed in Ag-PNIPAm-*co*-PGalAm hydrogel are around 30 nm as calculated using the Debye-Scherrer equation,[29] which is agreement with the UV absorbance value.

Antibacterial activity of the gel was studied by calculating the zone of inhibition employing the disc diffusion method (Fig. 6). The Ag-PNIPAm-*co*-PGalAm hydrogel did not show any antibacterial

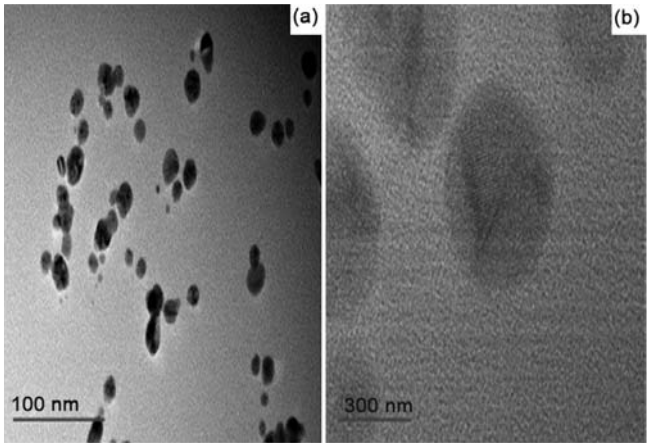


Fig. 4 – HR-TEM of Ag- PNIPAm-*co*-PGalAm gel. [(a) 100 nm scale and (b) 30 nm scale].

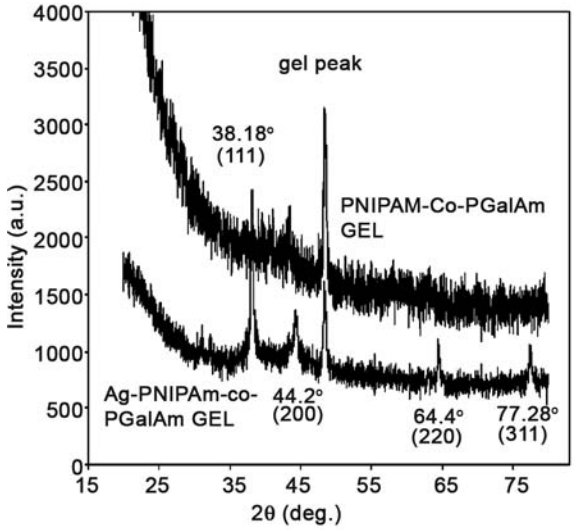


Fig. 5 – X-ray diffraction patterns of PNIPAm-*co*-PGalAm gel and Ag-PNIPAm-*co*-PGalAm gel.

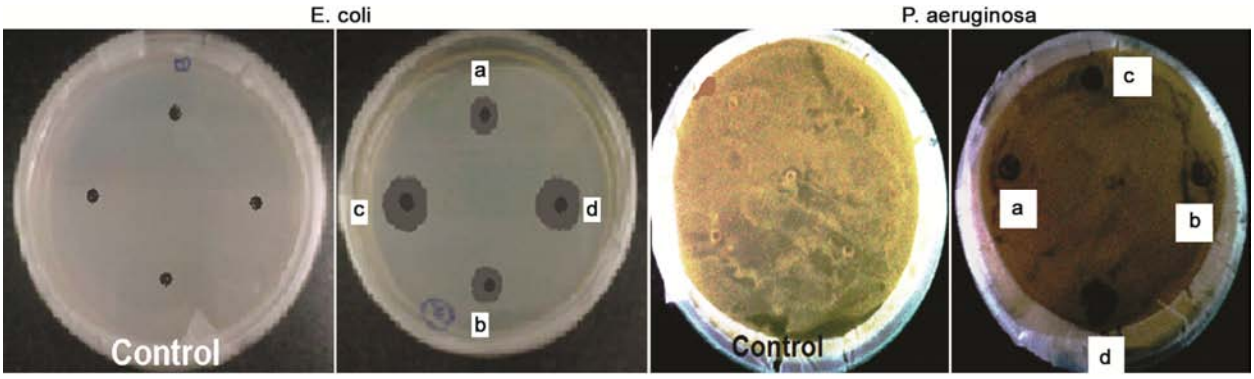


Fig. 6 – Zone of inhibitions of *P. aeruginosa* and *E. coli* with Ag-PNIPAm-*co*-PGalAm gels [control: 2 µg/mL, Concentrations of hydrogel: 0.5, 1, 2 and 3 µg/mL].

activity. The Ag-PNIPAm-*co*-PGalAm hydrogel at different concentrations: 0.5, 1, 2, 3 g/mL showed zone of inhibition 6, 7, 10, 12 mm for *P aeruginosa* and 4, 4, 5, 7 mm for *E. coli* respectively. It was found to be more effective against *P. aeruginosa* compared to *E. coli*. The suppression of bacterial growth may be due to the porous nature of hydrogel, which facilitates the diffusion of the liquid media frequently. This further releases the AgNPs and thus enhances the antibacterial activity against the bacterial strain. The MIC values were evaluated by dispersing the control as well as hydrogel sample in LB media. The MIC value of the Ag-PNIPAm-*co*-PGalAm hydrogel was 0.13 μg/mL and 0.25 μg/mL for *P. aeruginosa* and *E. coli* respectively. However, SIC value was found to be 0.25 μg/mL and 0.5 μg/mL for *P. aeruginosa* and *E. coli* respectively. The values indicate that these gels are effective at very low concentration against these bacterial strains, and have potential for various biomedical applications.

In summary, colloidal AgNPs were synthesized using PNIPAm-*co*-PGalAm hydrogel where anomeric masked aldehyde of sugar moiety played the role of reducing agent. The PNIPAm proved to be excellent stabilizer, and anchored the AgNPs in the hydrogel. PGalAm as a reducing agent proved to be beneficial as the AgNPs formed were well distributed on the surface of hydrogel. Also, the use of any harsh conditions/chemicals could be avoided. This gel composite showed enhanced antibacterial activity. The good MIC and SIC values indicate Ag-PNIPAm-*co*-PGalAm hydrogel to be a promising candidate for biomedical applications.

Supplementary data

Supplementary data associated with this article, viz., the detailed synthesis procedure and ^{1}H, ^{13}C NMR spectra of the monomer 6-acrylamido-6-deoxy-1,2:3,4-di-*O*-isopropylidine-α-D-galactopyranoside, GalAm are available in the electronic form at http://www.niscair.res.in/jinfo/ijca/IJCA_55A(04)441-446_SupplData.pdf.

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