Development of microbial resistant thermosensitive Ag nano composite (gelatin) hydrogels via green process
2.1 Introduction

The preparation and study of inorganic particles incorporated biodegradable hydrogels have been explored for wide range of biomedical applications [1, 2] over the last few decades, especially in wound dressing applications [3]. Of late, various biodegradable hydrogels were prepared from natural biopolymers which are protein based (e.g., elastin, collagen, gelatine, fibrin and globular proteins), due to their greater abundance in nature with low cost and having good biodegradability in nature [4]. Due to their specific properties, they are used as recombinants in DNA technology and for biomedical applications [5]. Moreover, gelatine is one of the natural protein polymer [6], having great abundance in nature and has inherent biodegradability in physiological environments. Therefore, gelatine is widely used in biomedical applications like additive in pharmaceutical formulations, wound dressing, drug delivery, cell cultures and tissue engineering [7] etc. Recently, Dash et al [8] have reported gelatine hydrogels cross-linked by cellulose nanowhiskers. In their study, they have reported that they which are useful for wound dressing, tissue engineering and sustained release applications. Chena et al [9] have prepared gelatine based hydrogels for non-healing wound treatment.

Similarly, biodegradable thermoresponsive hydrogels have shown great attraction in various biomedical applications, owing to their lower critical solution temperature (LCST) [10, 11]. Further, the embedding of inorganic particles inside the hydrogel network system significantly improves their usefulness in biomedical biomedicine. They are mainly prepared through chemical, photoinduced and microwave-assisted reduction methods, however, the chemical reduction methods
are the most common [12]. The production of inorganic nanoparticles procedures involves toxic chemicals [13, 14]. To overcome this problem, researchers have developed the green process in which toxic free reagents have been used to obtain inorganic nanoparticles [15]. In this green process, respective investigators have used plant leaf extracts as reducing agents for the production of metal nanoparticles, which are significantly cost-effective and principally employ ambient conditions for the reduction process [16]. Therefore, the development of metal nanoparticles basing on natural extracts is considered as the most appropriate clean method for obvious environmental reasons.

In view of the above discussion, the present research work is focused on the development of biodegradable temperature sensitive silver nanocomposite hydrogels by reducing the silver nitrate, using neem leaf's extracts, within the hydrogel networks. The resulted hydrogels were thoroughly examined for their structure, morphology, thermal properties, swelling behaviour, degradation and antibacterial properties. The effect of silver nanoparticles on the antibacterial activity of the P(GT-NIPAM) hydrogels was studied. Herein, a study about the design of P(GT-NIPAM) silver nano composites hydrogels for significant antibacterial applications is presented.

2.2 Materials

N-Isopropylacrylamide (NIPAM) was procured from Aldrich Chemicals, USA. Gelatin (GT) (Product No: 54045), N,N'-methylenebisacrylamide (MBA), ammonium persulphate (APS) and N,N,N',N'-teramethylene diamine (TMEDA) and silver nitrate (AgNO3) were obtained from S.D Fine Chemicals, India. Double distilled
water was used throughout the investigations for the preparation of all solutions and reactions.

2.3 Structure of Gelatin and its properties

Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides, each with extended left-handed proline helix conformations and containing between 50 - 1000 amino acids. The triple helix of type I collagen extracted from skin and bones, as a source for gelatin, is composed of two \( \alpha_1(1) \) and one \( \alpha_2(1) \) chains, each with molecular mass \( \sim 95 \) kD, width \( \sim 1.5 \) nm and length \( \sim 0.3 \) \( \mu \)m. Gelatin consists of mixtures of these strands together with their oligomers and breakdown (and other) polypeptides. Solutions undergo coil-helix transition followed by aggregation of the helices by the formation of collagen-like right-handed triple-helical proline/hydroxyproline rich junction zones. Gelatin is primarily used as a gelling agent forming transparent elastic thermoreversible gels on cooling below about 35 °C, which dissolve at low temperature to give 'melt in the mouth' products with useful flavor-release. In addition, the amphiphilic nature of the molecules endows them with useful emulsification (for example, whipped cream) and foam-stabilizing properties (for
example, mallow foam). On dehydration, irreversible conformational changes take place [397] that may be used in the formation of surface films. Such films are strongest when they contain greater triple-helix content. Although gelatin is by far the major hydrocolloid used for gelling and Gelatin is nutritionally lacking as a protein being deficient in isoleucine, methionine, threonine and tryptophan.

2.4 Preparation of the leaves extract

Leaves extract was prepared by green process technique, using the standard procedure described by Ravindra et al [17]. Neem leaves (Azadirachta Indica)(AI) were collected from the neem leaves tree and thoroughly washed with distilled water. Neem leaves broth was prepared by taking 25g of thoroughly washed and finely cut leaves in a 1000ml Erlenmeyer flask with 500ml of sterile distilled water. The mixture was heated at 100°C for 2min in order to extract the contents of the leaves and filtered through 0.45μm PVDF Millex Filter using 50ml syringe. The extracted neem leaves solution was stored at 4°C.

Structure of neem extract

The neem contains c=o as a major constituent which containing reducible groups.
2.5 Preparation of temperature sensitive silver nano composite hydrogels (TSSNH)

The preparation of TSSNH was achieved in three steps as mentioned below:

**Step 1: Preparation of temperature sensitive poly (Gelatin-N-Isopropylacrylamide) [P(GT-NIPAM)] hydrogel**

Synthesis of P(GT-NIPAM) hydrogel was achieved by free-radical polymerization technique as described in the earlier investigation [18]. Accordingly, 8.837mM of NIPAM was dispersed in 5 ml of aqueous solution containing different amounts of GT (0.05-0.15g), MBA (0.648mM) and APS (2.919mM) / TMEDA (0.172mM) as shown in Table 1. Each mixture was stirred separately for 30 min over a magnetic stirrer at 100 rpm. The gel matrix formed was safely transferred into 1 liter beaker containing 500 ml of distilled water, and distilled water was repeatedly changed for (every 5 hours) for 2 days to remove unreacted products such as, monomer, cross-linker, initiator and soluble polymers and allowed to dry in an oven (GUNA, Chennai, India) at 60°C.

**Step 2: Loading of silver salt into hydrogel**

100 mg of dry P(GT-NIPAM) hydrogel disks were equilibrated in distilled water for 3 days and these disks were transferred in to a beaker containing 50 ml of AgNO₃ (8.493 g/500 ml) aqueous solution and then allowed to equilibrate for 1 day. During this stage, the silver ions were exchanged from solution to the P(GT-NIPAM) hydrogel networks.
Step 3: Silver nano particle formation in the hydrogel network.

The silver salts loaded P(GT-NIPAM) hydrogels were surface water was wiped off using a tissue paper and transferred in to a beaker containing 50 ml of cold leaves extract of *Azadirachta Indica* (AI) solution. This beaker was kept in a refrigerator (4°C) for 8h in order to reduce the Ag⁺ into Ag⁰ nano particles. The obtained Ag⁰ nano particles inside the hydrogels are often termed as temperature sensitive silver nano composite hydrogels. Table 1 is illustrates the various components used in the preparation of P(GT-NIPAM) hydrogels.

<table>
<thead>
<tr>
<th>Hydrogel Code</th>
<th>NIPAM (mM)</th>
<th>GT (g)</th>
<th>MBA (mM)</th>
<th>APS (mM)</th>
<th>TMEDA (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(GT-NIPAM) 0</td>
<td>8.837</td>
<td>0.0</td>
<td>0.648</td>
<td>2.191</td>
<td>0.172</td>
</tr>
<tr>
<td>P(GT-NIPAM) 1</td>
<td>8.837</td>
<td>0.05</td>
<td>0.648</td>
<td>2.191</td>
<td>0.172</td>
</tr>
<tr>
<td>P(GT-NIPAM) 2</td>
<td>8.837</td>
<td>0.10</td>
<td>0.648</td>
<td>2.191</td>
<td>0.172</td>
</tr>
<tr>
<td>P(GT-NIPAM) 3</td>
<td>8.837</td>
<td>0.15</td>
<td>0.648</td>
<td>2.191</td>
<td>0.172</td>
</tr>
</tbody>
</table>

2.6 Swelling studies

The swelling characteristic of the physical hydrogels provides the information about the hydrogel network integrity after loading silver salt as well as formation of embedded AgNPs inside the gel networks [19]. To study this phenomenon, for all the types of hydrogels the same weights of dried hydrogels were equilibrated in distilled water at 25°C for 2 days. Swollen hydrogels were treated first with AgNO₃...
and then with leaves extract as mentioned in the previous sections “preparation of temperature sensitive silver nanocomposite hydrogels (TSSNH)”. The swelling ratio (Q) of the gels was calculated from the equation:

$$Q = \frac{W_e}{W_d},$$

where, $W_e$ is the weight of the swollen hydrogel and $W_d$ is the dry weight of the pure hydrogel.

2.7 Determination of the lower critical solution temperature (LCST) of hydrogels

This experiment was carried out in a thermostatic water bath equipped with heating and cooling systems, using the standard procedure described elsewhere [20]. The hydrogels were placed in water distilled twice at several temperatures, ranging from 5 to 60 °C until the equilibrium swelling ratio of the samples was reached. Then, the excess water on the samples surface was removed as stated above and the samples were weighed. The phase transition temperature associated with the LCST was determined as the inflexion point in the plot of swelling as a function of temperature.

2.8 Characterization of hydrogels

Absorption spectra of TSSNHs were measured in the wavelength range from 250 to 600nm using an ELICO SL 160A Model UV–Vis spectrophotometer (Hyderabad, India). FTIR spectroscopic analyses were carried out by using a Bruker IFS 66V Fourier Transform Infrared Spectrometer (Ettlingen, Germany). FTIR spectrophotometer was connected to a photoacoustic cell in the spectral range from 4000 to 500cm$^{-1}$. The morphological variations were observed (coated with a thin
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layer of palladium gold alloy) by using a JOEL JSM 840A (Tokyo, Japan) scanning electron microscope (SEM), Transmission Electron Microscopy (TEM) Technai F12 TEM (Philips Electron Optics, Holland), (grid size: 97 μm) (Ted Pella, Inc., Redding, CA, USA), Thermogravimetric analyses (TGA) of nano composites were evaluated on a SDT Q 600 TGA instrument (T.A. Instruments-water LLC, Newcastle, DE 19720, USA) at a heating rate of 100°C/min under a constant Nitrogen flow (100 ml/min).

2.9 Antibacterial activity

The antibacterial activity of the developed hydrogels was investigated using disc method.

2.9.1 Disc Method: Nutrient agar medium was prepared by mixing peptone (5.0g), beef extract (3.0g), and sodium chloride (NaCl) (5.0g) in 1000 ml distilled water and the pH was adjusted to 7.0. Finally, agar (15.0g) was added to the solution. The agar medium was sterilized in a conical flask in a autoclave at a pressure of 15lbs for 30min. This medium was transferred into sterilized Petri dishes in a laminar airflow chamber (Microfilt Laminar Flow Ultra Clean Air Unit, India, Mumbai). After solidification of the media, bacillus culture (50-μl) was spread on the solid surface of the media. To this inoculated Petri dish, one drop of gel solution (20 mg/10 ml distilled water) was added using 50μl tip and incubated for 2 days at 37°C in the incubation chamber.

2.10 Biodegradation characterizations

Biodegradation study was performed by using the weight loss (%) method as mentioned below:
Nutrient agar medium was prepared by using the standard procedure described elsewhere [21]. The agar medium was sterilized by autoclaving at 121°C for 30 min at a pressure of 6.8 kg (15 lbs). An Escherichia coli bacterium was inoculated in this medium and the pure culture was maintained separately in the incubator. Then, to 10 ml of sterilized broth, 0.100 g each of the samples, i.e. both P(GT-NIPAM) hydrogel and their Ag° nano composites samples were added aseptically in separate test tubes and each tube of samples was supplemented with inoculums of the bacterial strains separately. The degradation of samples by E. coli was monitored at time intervals of 1, 5, 15 and 30 days. After the required time period, samples were washed repeatedly with deionized water, oven-dried at 40±1°C for 24 hrs. Then, the samples were weighed to determine the weight loss.

2.11 Results and discussion

2.11.1 Swelling studies

The swelling characteristics of the physical gels were investigated twice at ambient temperature. The swelling studies are presented in the form of Fig. 1A illustrates the effect of GT varied formulations on the swelling property of hydrogels, Ag+ ions loaded hydrogels and hydrogel–silver nanoparticle composites. An increase in swelling properties were noticed when GT concentration (hydrophilic groups) was increased in the hydrogels composition. Furthermore, Fig. 1A indicates that the swelling capacity of the Ag+ ions loaded hydrogels and Ag° nanoparticles formed hydrogels, further increased with increase of GT content in the hydrogel system.
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Fig 1 A, Swelling behavior of hydrogels; Fig 1 B and C Temperature dependence of equilibrium swelling ratios (B) P(GT-NIPAM)1 to P(GT-NIPAM)3 and (C) P(GT-NIPAM)+AgO+ to P(GT-IPAM)3+ AgO+ hydrogels

The swelling ratio depends on the amount of Ag\(^+\) ions loading and the Ag\(^0\) nanoparticle formation. The results, shows that the swelling capacity follows in the order: Ag\(^0\) hydrogel >Ag\(^+\) ions hydrogel >pure hydrogel. The reason is being that when Ag\(^+\) ions loaded hydrogels are treated with Azadirachta Indica (AI) solution, they turned into dark coffee colours, indicating the formation of nanoparticles throughout the hydrogel networks. During this step, the uptake of many Ag\(^+\) ions
leads to the formation of the nano particles within the hydrogel, networks thereby expands the gel networks and promotes higher water uptake capacity. A similar phenomenon was also observed by Varaprasad et. al [20, 21].

2.11.2 Effect of feed composition on thermoresponse

The LCST of the P(GT-NIPAM) hydrogels under study was investigated by a temperature dependent swelling method using the standard procedure [20]. Fig 1B and C shows the temperature dependent swelling curves. The effect of feed composition on the swelling ratio was known from its lower critical solution temperature LCST value. (Fig.1B). The LCST value was calculated by a method [20] in which the swelling curve is divided into three segments, and then three tangents were drawn to respective segments. The values of the connecting points of central tangent with other two tangents gives two values (T1, T2) of which the average gives the value of LCST of hydrogels (Table 2). These values indicate that GT concentration is directly proportional to both the swelling capacity and the LCST property of hydrogels. This property is due to linkage of a hydrophilic polymer with NIPAM hydrogel networks thereby increasing the hydrophilicity of hydrogels. Similarly, these types of phenomena were observed in the P(GT-NIPAM) silver nanocomposite hydrogels (as indicated/depicted in Fig 1C). The resulting LCST values are presented in the Table 2. According to this data, it is concluded that the LCST value has increased significantly in the GT added hydrogels, and this effect is vital in potential applications such as biomedical devices or in reversible systems.
Table 2: LCST values Hydrogels & Hydrogel Silver nano composites.

<table>
<thead>
<tr>
<th>Hydrogel Code</th>
<th>Tangent Values</th>
<th>LCST (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(GT-NIPAM)1</td>
<td>30.09 37.85</td>
<td>33.97</td>
</tr>
<tr>
<td>P(GT-NIPAM)2</td>
<td>30.49 40.75</td>
<td>35.62</td>
</tr>
<tr>
<td>P(GT-NIPAM)3</td>
<td>25.70 47.62</td>
<td>36.66</td>
</tr>
<tr>
<td>P(GT-NIPAM)1+Ag°</td>
<td>25.70 37.99</td>
<td>31.68</td>
</tr>
<tr>
<td>P(GT-NIPAM)2+Ag°</td>
<td>25.92 40.15</td>
<td>33.03</td>
</tr>
<tr>
<td>P(GT-NIPAM)3+Ag°</td>
<td>24.88 44.2</td>
<td>34.66</td>
</tr>
</tbody>
</table>
2.11.3 Spectral analyses

UV-Visible spectroscopy is an important tool for structural characterization of inorganic metal nano particles. It is well known that the optical absorption spectra of metal nano particles depends on surface plasmon resonances, which shift to longer wavelengths with increasing particle size. The UV-Visible spectra of all the formulations are shown in Fig 2. In the figure, a strong absorption peak was observed at $\lambda_{\text{max}}$ 405nm due to silver nanoparticles embedded in hydrogels, confirming the formation of silver nanoparticles in the hydrogel network. Probably, one mechanism can be speculated that, with increase of GT content in the hydrogel, the silver salt loading increases, resulting in more silver nanoparticles formation (reduction reaction) in the gel structure, which in turn positively increases the absorption in the UV-visible spectra. This is due to the formation of silver nanoparticles in the P(GT-NIPAM) hydrogels, as confirmed in the FTIR, SEM, TEM micrographs and TGA.

![UV-visible spectra of GT varied silver nanocomposite (P(GTNIPAM)+AgO to P(GT-NIPAM)3+AgO) hydrogels.](image-url)
2.11.4 FTIR analysis

The GT added silver nano composite hydrogels were characterized by FTIR analysis, which is confirms the formation of silver nano particles inside the hydrogel network. The P(GT-NIPAM) hydrogel (Fig. 3) shows absorption peaks at 3446 cm\(^{-1}\) due to overlapping of O-H and amine group stretching bands of AM and GT units present in the hydrogel network. The two bands at 2929 and 2853 cm\(^{-1}\) which are associated with C-H stretchings of P(GT-NIPAM) units, are clearly observed in the spectra. The peaks at 1646 cm\(^{-1}\) and 1548 cm\(^{-1}\) are associated with the C=O stretching vibrations and N-H bending vibration of P(GT-NIPAM).

![FTIR spectra of pure P(GT-NIPAM) and P(GTNIPAM) + AgO nano composite hydrogels.](image)

The most important isopropyl group of hydrogels (due to NIPAM) is observed at 1372 cm\(^{-1}\) [20]. Whereas, silver nano composite has shown all the above characteristic peaks, by shifting their wavelengths slightly (3438 cm\(^{-1}\) corresponding to the overlapping stretching band of NH\(_2\) and -OH functional groups and 1641 cm\(^{-1}\) and 1547 cm\(^{-1}\) relating to C=O stretching and N-H bending vibrations.)
of AM and GT, respectively and 1375 cm$^{-1}$ relating to isopropyl group). This is due to electronic interaction between the nano-silver and electron rich groups present in the hydrogel network. The IR Spectra of, P(GT-PNIAM) hydrogels and silver nano composite hydrogels confirms both the formation of hydrogels and silver nano composite hydrogels.

**2.11.5 Morphology studies: SEM & TEM Analysis**

In order to investigate the morphology of AgNPs, scanning and transmission electron microscopes (SEM and TEM) were used as most accurate techniques and obtained the micrographs as presented in Fig. 4. Fig 4A (SEM) depicts the morphology of TSSNH,(P(GT-NIPAM)3+ AgO) and it shows the formation of silver nano particles and their distribution on the surface area of the hydrogel network. Further, the TEM image (Fig 4B) of the sample also indicates a highly uniform distribution of silver nano particles in the hydrogel network. It is obvious that the silver nano particles have dark spherical in shape at nanoscale levels (ranging 5 to 10 nm) and without aggregation in the cross-linked hydrogel network.

![Fig 4 SEM images of (A) P(GT-NIPAM)3+ AgO+ hydrogels; (B) TEM images of P(GT-NIPAM)3+ AgO hydrogels;](image-url)
2.11.6 Thermal analysis:

The thermal stability of silver-loaded $\text{P(GT-NIPAM)}_3$ hydrogel and neat $\text{P(GT-NIPAM)}_3$ hydrogel were determined by thermogravimetric analysis. Fig 5 illustrates the thermogram of silver-loaded and unloaded dry hydrogel samples. The weight loss was observed for $\text{P(GT-NIPAM)}$ hydrogel sample up to $534^\circ\text{C}$ is 99.85%, whereas $\text{Ag}^0/\text{P(GT-NIPAM)}$ hydrogel shows only 90.02% at the same temperature. The % amount of silver nano particles present in the $\text{P(GT-NIPAM)}$ hydrogel can be calculated from the difference in the weight loss (%) between the pure hydrogel and silver nano composite hydrogel at $534^\circ\text{C}$ (which is 9.83%). The studies indicates the improvement in the stability of silver nano composites hydrogel which might be due to the nano-effect of silver particles, which were formed inside the hydrogels template.

![Graph showing weight loss vs temperature for different hydrogels](image.png)

Fig : 5 Tg curves of $\text{P(GT-NIPAM)}_3$ and $\text{P(GT-NIPAM)}_3+\text{Ag0+Al nanocomposite hydrogels}$
Now a days the research is focused on the biodegradability of hydrogels, because of their superior activity and their biological interaction with body components; hence they are used in biomedical applications [3]. The biodegradation property of pure P(GT-NIPAM) hydrogel and Ag° nanocomposite hydrogels developed, were carried out by weight loss methods. The degradation behaviours of P(GT-NIPAM) hydrogel and Ag° nano composites hydrogel are shown in Fig 6. From the figure, it is observed that pure P(GT-NIPAM) hydrogel shows higher weight loss (%) than P(GT-NIPAM) Ag° nano composite hydrogels. This is due to the fact that Ag° nanoparticles escape from the hydrogel in aqueous medium and might be attached to the negatively charged bacterial cell wall, which causes cell death of the bacteria. Therefore, cells metabolic activity is reduced (degradation is also less). However, this is not the case for pure P(GT-NIPAM) hydrogel which does not have inorganic Ag° nano particles. Therefore, it readily undergoes biodegradation when compared to Ag° nano composaites.
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2.11.8 Antibacterial activity

The potential use of inorganic nanoparticles incorporated in biodegradable hydrogels as functional wound dressings was assessed by observing their antibacterial activity against common bacteria, such as *E. coli* [22]. Ag° nanoparticles have been shown to kill several pathogenic bacteria [23]. Similarly, *Azadirachta Indica* is a highly biologically active compound [24]. It is a naturally occurring, non-toxic and bioactive agent in human scientific life [25]. Owing to their exceptional properties, these materials are used in antibacterial applications.

The bactericidal effects of (P(GT-NIPAM) hydrogel, A1 with P(GT-NIPAM) hydrogel and P(GT-NIPAM) Ag° nanocomposite biodegradable hydrogels are shown in Fig 7. The diameter of the inhibition zone for the P(GT-NIPAM) silver nanocomposite hydrogels [Fig 7 P(GT-NIPAM)3 = 14mm and P(GT-NIPAM)1 = 10mm] is larger than
that of Al with P(GT-NIPAM) hydrogels \[P(GT-NIPAM)_3 = 8mm\] \[P(GT-NIPAM)_1 = 7mm\], whereas the pure P(GT-NIPAM)_3 hydrogels (0mm) showed no inhibition ability. Therefore, GT in combination with Ag° nano composites hydrogels exhibits excellent antibacterial activity.

Fig : 7. Antibacterial activity of plain P(GT-NIPAM)_3, P(GTNIPAM)+Al, P(GT-NIPAM)_3+Al, P(GT-NIPAM)_1+Ag°+Al and P(GT-NIPAM)_3+Ag°+Al nanocomposite hydrogels on bacillus
2.12 Conclusion

In conclusion, silver nanoparticles have been successfully incorporated in hydrogels template. The natural polyamide GT has played excellent properties for anchoring and stabilization of silver nanoparticles. These composites were developed and characterized by spectral, thermal, swelling behaviours, degradation and electron microscopic studies. The P(GT-NIPAM) hydrogel silver nano composites have exhibited strong antibacterial activity against bacillus. Therefore these agents can easily find applications in wound/burn dressings.

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