Trans Aconitic acid based biopolymeric hydrogel with Antibacterial, Antioxidant and Anticancer property

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ABSTRACT

A novel biopolymeric hydrogels have been synthesized using trans aconitic acid, ethylene glycol and glutamic acid without a cross linker. The biopolymeric hydrogels were characterized by analytical techniques like FT-IR. The hydrogel shows a pH sensitive swelling behaviour with increased swelling in acidic media followed by gradual decreased in basic media. The thermogram (TGA) of the hydrogel shows two stage of degradation. The obtained hydrogels were also subjected to antibacterial activity against gram positive S. aureus and gram negative E.Coli by using zone inhibition method, and antioxidant activity by DPPH radical assay at different concentration compared with the standard Ascorbic acid and anticancer activity by MTT assay using HT29 colan cancer cell line. The results exhibit excellent antibacterial, antioxidant and anticancer property with respect to their standards respectively.

Keywords: pH – sensitive hydrogel – Trans aconitic acid – Glutamic acid – antibacterial - Antioxidant activity – cytotoxicity

Introduction

Hydrogels had drawn more attention, because of their natural porous structure and high-water content could absorb abundant exudates or blood. Hydrogel has act as good barricade to microorganisms, maintain a comparable moist environment at the skin defect site, maintain...
good O₂ and water permeability [1-3]. Gulrez et al (2011) also deals an important characteristic which are water holding capacity and permeability, dissolution rate, and its biocompatibility. Out of these three unique properties, the biocompatibility needs to be understood to ensure that the hydrogel could be degraded within the body without emitting any toxic substances [4]. In recent years, natural biopolymers had frequently been utilized to prepare intelligent hydrogels against controlled drug delivery systems which is due to their excellent properties as well as non-toxicity, biodegradability, biocompatibility, renewability and ability of chemical modification [5,6]. The antioxidant material is widely used in biomedicine, bio-pharmaceutics, cosmetics, food industry, plastic materials, nano engineering and wound healing applications [7]. Free-radical scavengers(Antioxidant) are substances which can prevent or attenuate oxidative damages to cells caused by ROS/RNS [8]. They can be endogenous antioxidants (i.e., formed within human body) or exogenous antioxidants (i.e., from outside the body). Nitric oxide, a primary reactive nitrogen species (RNS), was first identified as a crucial endothelium-derived relaxing factor in the cardiovascular system [9]. Nitric oxide could act as a diffusible radical and a key messenger in cell signalling to regulate a series of cellular processes, and get involved in nitrosylation of various biomolecules [10]. Reactive oxygen species (ROS) is a kind of by-product of cellular metabolism. The high concentration of ROS can disrupted the cellular oxidant/antioxidant balance, and critically injure human cells when the skin is damaged, and even cause a second injury [11]. Reactive oxygen species (ROS) are to be deactivated before they damage cells. The renewable resource based monomers has been focused in recent years, because of antibacterial, antioxidant and anticancer behaviour with non-toxic nature. Recently, natural resource centred material subsidized for the synthesis of biopolymeric hydrogels [6]. Trans Aconitic acid(TAA) has been used as a non-toxic, biodegradable, and biocompatible monomer, has shown potential application in biomedical fields because it possess significant properties
such as anticancer, antioxidant, antibacterial and so on [12,13]. In addition to that TAA is also a versatile reactive functional monomer which containing three terminal carboxylic groups, and it can also be collected by dehydration of citric acid [14, 15]. It is naturally present in sugar cane and can be isolated from by-products of the sugar industry (molasses, vinasse) [16,17]. Kar et al. (1993) has also noticed the effects of trans-aconitic acid (TAA) an aconitase inhibitor, against L. donovani due to its action in the fatty acid oxidation. They observed that the TAA inhibited the in vitro growth of promastigotes and amastigotes in macrophages and in vivo (i.e., infected hamsters) without toxicity to host cells. [18, 13]. Suzan et al., 2007 has also been have confirmed the aconitic acid and its derivatives are very efficient antioxidants in biological systems. TAA has been exhibit the highest activity with lowest side effects [19] which is due to TAA hold an antioxidants and free radical scavenger behaviour.

Diol(ethylene glycol) is a di-functional linear monomer which enhance the performance of hydrogels due to its linearity, flexibility and biocompatibility nature [20]. Poly(ethylene glycol)-cross linked poly(methyl vinyl ether-co-maleic acid) hydrogels have been provided good mechanical and biological property which could be used for human ovarian cancer cell culture [21]. Chitin/1, 4-butane diol has also been shown the modified surface characteristics of biocompatible blends and it is used for various applications [22]. The radical thiol-ene reaction has been utilised for the facile synthesis of poly(ethylene glycol) (PEG) based cyclodextrin containing hydrogels and it can provide drug delivery applications.[23] Glutamic acid (GA) and its derivatives are naturally active material with significant characteristics such as antioxidants, antimicrobial and non – toxic in nature [24,25]. GA is used as a conjugate material which is due to it increases the efficacy of anticancer drug and decreases its toxicity toward normal cells. Polyglutamic acid is a biodegradable, edible and nontoxic behaviour toward humans [26]. Aminopterin (4-aminopteroic acid), a 4-amino analogy of folic acid, is an antineoplastic drug with immune suppressive properties used in
High intake of folate may reduce the risk of colon cancer [28]. Folate intake has been decreased the risk of pancreatic cancer in women but not in men [29]. Previously, citric acid, itaconic acid, sodium alginate and indole-3-acetic acid based pH sensitive biopolymeric hydrogels have been reported by our research group with detailed spectral, thermal and swelling properties have also been discussed [30-33]. However, there has been no report found in the literature on trans aconitic acid (TAA) for the fabrication of hydrogels with superior biomedical properties. The scope of this work towards the biomedical applications, and there is a continuous demand for improved the biopolymeric and their which possess pH sensitivity, antibacterial, and antioxidant as well as anticancer properties at reduced costs. Such requirements need to form a combination of properties difficult to attain with existing materials. Biopolymeric materials having a maximum number of applications are mandatory. Hence, the present investigation aimed to develop biomedical hydrogels (especially antibacterial, antioxidant, cytotoxicity) under simple organic solvent-free reaction conditions with short duration via condensation polymerization. The percentage of swelling equilibrium at various pH from acidic to basic media have also been studied. The synthesized hydrogel was characterized using various instrumentation techniques, FT-IR spectroscopy, thermo gravimetric analysis, and biological screening like antibacterial activity by zone of inhibition method, antioxidant activity by DPPH radical method and NO radical method and Cytotoxicity by MTT assay method. In order to inspect as biomaterial, the ensuing aconitic acid based biopolymeric hydrogel subjected to various pathogens. Antimicrobial studies were parallel to the positive control. The thermal stability of hydrogel has been increased compare to the monomers. Trans Aconitic acid based biomaterials has been noticed efficient antioxidants properties when compared with standard rutin, protecting both lipids and proteins from peroxidation. Trans aconitic acid and glutamic acid structures has been influenced the antioxidant efficacy in biological systems. Due to its free radical scavenger and antioxidant
properties, synthesis of aconitic acid and glutamic acid based biomaterials are under investigation to determine which exhibit the highest activity with the lowest side effects. Hence, the prepared biopolymeric hydrogel can be useful for wound healing, anticancer and antioxidant applications.

**Experiment**

**Materials and methods**

Trans Aconitic acid (TAA) was purchased from Sigma Aldrich (Mumbai, India). Ethylene glycol (EG) and Glutamic acid (GA) were purchased from Mark (India). Demineralized water was used for throughout polymerizations process and for the preparation of various range of buffer solutions.

**Preparation of the biopolymeric hydrogel**

Trans aconitic acid (0.0 25mol) was dissolved in ethanol (5 mL) using a round bottom flask closed with guard tube and stirred with magnetic stirrer. ethylene glycol (0.025 mol) was added dropwise and the mixture was stirred for 3 hours in silica oil bath at 160 °C. The formation of glassy sticky gel was formed named as pre-polymer. In addition, Glutamic acid (0.025 mol) which has dissolved in 10 ml of ethanol, added into pre-polymer and then heated continuously for 6 hours in nitrogen atmosphere. The high viscous reddish brown glassy gel was formed. Further, the gel was immersed in distilled water for 24 hours to remove the unreacted monomers and impurities. Then the sample was dried in an oven for 48 h at 35 °C. The scheme of the preparation has shown below,

![Figure 1. Preparation of biopolymeric hydrogel (AEG)](image-url)
Fourier Transform Infrared (FTIR) spectroscopy
The Fourier Transform Infrared (FTIR) spectra were recorded to know the functional group present in a hydrogel with an FTIR spectrophotometer (Shimadzu, 8400, Japan) using KBr pellet. The powdered sample was mixed with KBr pellet. The scans recorded were the average of 100 scans and the selected spectral range between 400 and 4000 cm\(^{-1}\).

Swelling Equilibrium
A known weight of the hydrogel was immersed in swelling media (Distilled water, pH solutions pH 3, PH 7, pH 9) for 24 hour at ambient temperature. After that the samples were removed and blotted on a filter paper to remove the excess water solution present on the surface and then weighed. The swelling equilibrium were calculated according to the following swelling equilibrium equations was given by

\[
S_{eq} \% = \frac{W_{eq} - W_d}{W_d} \times 100
\]  

(1)

\(W_{eq}\) - Weight of swollen hydrogel

\(W_d\) - Weight of dry hydrogel

TGA/DTA
Thermal stability of hydrogel was evaluated by SDT Q 600 and Q20 TGA instrument at heating rate of 20\(^\circ\)C/min at \(N_2\) atmosphere. TGA thermogram was recorded between ambient and 800\(^\circ\)C.

Antibacterial activity
The synthesised hydrogels were inoculated in tubes with sterile saline solution (3 ml) for 24 hours at 37\(^\circ\)C. The selected microorganisms were gram positive \textit{Staphylococcus aureus} (MTCC430), and gram negative \textit{Escherichia coli} (MTCC739). The test was performed in Nutrient Agar (NA) plates seeded with an 8 h broth culture of different bacteria. In each of these plates, wells were cut out using a sterile cork borer. The samples were carefully added with different concentrations (10, 50, 100 and 250 \(\mu\)g) into the wells by using a sterilized dropping pipette. The wells were allowed to diffuse at room temperature for 2 hours. Later the plates were then incubated in a closed container at 37\(^\circ\)C for 18–24 h. \textit{Gentamicin} (10\(\mu\)g) was chosen as a positive control and distilled water was used as a negative control. The
The antimicrobial activity of the synthesised hydrogels was evaluated by measuring the diameter of the inhibition zone.

**Antioxidant activity**

**Free radical scavenging activity on DPPH.**
The antioxidant activity of the hydrogel was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH. The various concentrations of hydrogels (100-500 µg) were taken and the volume was adjusted to 125 µL with DMSO. Then added methanolic solution of DPPH and allowed to stand for 20 min at 27 ºC. The absorbance can be measured at 517 nm. Percentage radical scavenging activity of the sample was calculated as follows:

\[
\text{DPPH radical scavenging activity(%) } = \frac{A-B}{A} \times 100 \rightarrow (2)
\]

where,

\[ A = \text{Control optical density}, \ B = \text{Sample optical density}. \]

**MTT Assay – Cytotoxicity**
The relative cytotoxicity of hydrogel was determined by MTT viability assay developed for high throughput screening (HTS). The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.2 - 0.5mg/ml, and incubated for 1 to 4 hours. The quantity of formazan (presumably directly proportional to the number of viable cells) was measured by recording changes in absorbance at 570nm using a plate reading spectrophotometer. Viable cells with an active metabolism, which convert MTT into a purple coloured formazan product. When cells die, they lose the ability to convert MTT into formazan and DMSO was added to dissolve formazan crystals. Untreated cells were taken as the control with 100% viability.

**Results and Discussion**

**Fourier Transform Infrared (FTIR) spectroscopy**
The FTIR spectroscopy was used to confirm the formation, nature of bonding and chemical structure of the synthesised hydrogel. The spectral character of synthesised prepolymer and biopolymeric hydrogel (AEG) are obtainable in Fig 1. The prepolymer AE has showed a broad absorption peak around 3419. 28cm\(^{-1}\), which belongs to the hydrogen bonded O–H stretching vibration. The -CH symmetric stretching vibration occurs at 2957.3cm\(^{-1}\) [34]. The most distinctive peak at 1726.94 cm\(^{-1}\) which can be corresponds to the C=O stretching vibration and C–O stretching were observed at 1185.04 cm\(^{-1}\) of ester groups. The peak at 1650.77 cm\(^{-1}\)
indicates the C=C stretching vibration of trans aconitic acid. This shows that, the prepolymer contains the ester linkage in its hydrogel network. The increase of the intensity bands around at 2876 and 2931 cm\(^{-1}\) (C–H stretching), which apparently were attributed to the introduction of the poly ethylene glycol crosslinker into the hydrogel. The sharp peak at 3389 cm\(^{-1}\) can be recognized to either the hydrogen bonded -OH in diol and -NH bond or overlapped [35]. The absorption band appeared at 1620 cm\(^{-1}\) attributed to -COO- stretching in the hydrogel [36].

![Figure 2. FTIR spectroscopy analysis of prepolymer and Biopolymeric hydrogel](image)

**Equilibrium swelling studies**

In general swelling ability of the pH- and temperature-sensitive hydrogels plays vital role in biomedical applications [37]. Equilibrium swelling studies were performed on a hydrogel equilibrated in a buffer with pH values ranging from 4, 7 and 9 at room temperature. At each of the pH values, the equilibrium swelling Seq % was determined according to equation 1. Fig 2 has showed a summary of the experimental results of swelling equilibrium as a function of various pH of Aconitic acid, Ethylene glycol and Glutamic acid based different composition of AEG hydrogel.

**Equimolar composition of TAA and GA with variable composition of EG**

The Seq% value of AE1G, AE2G, AE3G and AE4G hydrogels at pH 4.0, 7.0, and 9.0 were Shown in Fig 3. The maximum swelling has been attained at pH 4 was due to high osmotic pressure and electrostatic repulsion between protonated amino groups (-NH) of polymer chains which leads to increased swelling of hydrogel [38]. On the other hand, at higher pH, the decreasing nature of swelling equilibrium due to the protonated amino groups were
transformed into unionized amino groups due to hydrogen bonding, weakening of the intermolecular interactions and disruption of physical cross links between the polymer chains, smaller crosslink density and a larger rigid network. This results shown to be in good agreement with the FTIR analysis, which confirms the presence of hydrogen bonding interaction between -OH and –COOH groups [39,40]. Hence, the decline of osmotic pressure and electrostatic repulsion between protonated amino groups (NH) lead to progressively decreasing swelling equilibrium of the hydrogel.

![Bar chart showing swelling equilibrium percentage at pH 4, 7, and 9 for different compositions of TAA and GA with variable composition of EG.](image)

**Figure 3.** Equimolar composition of TAA and GA with variable composition of EG

**Thermo gravimetric studies**

Thermal gravimetric studies have been performed to identify the changes in thermal decomposition nature of hydrogel. The weight loss of the hydrogel network during the increase in temperature was recorded. Hydrogels are hydrophilic materials which consisting of three types of water: non-freezing water, freezable bound water, and free water. The distribution of these components which can alter the properties and functionality of hydrogels. The characteristics of hydrogel network are attributed to the large number of molecules of freezable bound water, which is weakly bound to the polymer chain by hydrogen bonding and undergoes a thermal phase transition at temperatures lower than 0°C [41]. They weakly interact with non-freezing water and hydrogel molecules. This determines the mechanical properties of a hydrogel. Based on the quantity of freezable bound water, a hydrogel becomes tough or soft. Freezable bound water determines the swelling behaviour of a hydrogel. Thermal degradation
behaviour of the AEG hydrogel was given in Fig 4. The thermal properties of the hydrogel were assessed by thermo gravimetric analyses, in the temperature range from ambient to higher temperature at the flow rate of 20°C, under a dynamic N2 atmosphere. The TGA profile of pure hydrogel has showed that three stages of thermal degradation. The initial weight loss percentage detected at about 250°C which is attributed to moisture presented in the hydrogel network. This stage can be assigned to the loss of the residual water present in the sample. [41]. The destruction of functional groups containing oxygen atoms led to the weight loss around 221°C. The weight loss has been observed 4%. This step was mainly due to the decomposition and degradation of the functionalized moieties such as ester and amine bonds. [42, 43] At the second stage degradation started at 420°C with the weight loss of 75% might be the carbonisation of the polymeric network (Fig 4). At high temperatures (above 600 °C), no significant weight loss was observed, which could be explained by aconitic acid content (60 wt%) of hydrogel [44]. In wound dressing applications, a hydrogel should absorb wound exudates, which is pivotal to keeping a dry wound and hastening the healing process [45]. Nonetheless, a 700 °C, there was no significant difference in the percent of char remaining for all the polymers. Notwithstanding, the polymers all demonstrated good thermal stability, especially in air. TGA results has showed that the thermal stability of the aconitic acid based hydrogels was higher than other, therefore have potential applications as high performance materials.

Figure 4. Thermogram of the synthesised hydrogel
Antibacterial activity

Antibacterial activity is a very well-intentioned property for biological applications and has been described for many polymers. To know the qualitative determination of antibacterial calculation the synthesized hydrogels subjected to antibacterial analysis. The inhibition zones of hydrogels are shown in Fig 5(a, b). The results of antibacterial activity against *Staphylococcus aureus* (Gram +ve), *Escherichia coli* (Gram -ve) are shown in Table 1. *Gentamicin*(10µg) was used as a standard in its broad spectrum of antibiotics. The Aconitic acid moieties can interact with a particular constituency of the cytoplasmic membrane of bacteria, causing degradation and structural changes lead to the death of anti-bacterial cells [46]. The inhibition zones of the hydrogel against *E. coli* were 4.3 mm, 8.6 mm, 12.2mm, and 18.5 mm and inhibition against *S. aureus* were 3.7 mm, 6.3 mm, 10.3mm and 15.6mm for the concentration increases from 10µg, 50µg, 100µg and 250µg respectively. Further results of hydrogel have been exhibited better inhibition activity towards *E. Coli*. Hence TAA based hydrogel shows better anti-bacterial activity against Gram positive bacteria due to the acid proportion in the hydrogel which increases the diameter of the antimicrobial inhibition zone(mm). The zone of inhibition against anti-bacterial pathogens is signicantly characterized for wound dressings, particularly in protecting the wound from further infection [47,48]

Table 1. Anti bacterial activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>10µg</th>
<th>50µg</th>
<th>100µg</th>
<th>250µg</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>4.3</td>
<td>8.6</td>
<td>12.2</td>
<td>18.5</td>
<td>25</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>3.7</td>
<td>6.3</td>
<td>10.3</td>
<td>15.6</td>
<td>25</td>
</tr>
</tbody>
</table>

Figure 4a. E. Coli
Figure 4b. S. aureus
Antioxidant property
Antioxidants are compounds that inhibit oxidation. Oxidation is a chemical reaction that can produce free radicals, thereby leading to chain reactions that may damage the cells of organisms. Antioxidants such as thiols and ascorbic acid (vitamin C) terminate these chain reactions. An antioxidant plays a vital role due to stop the oxidative potentiality by destroying the free radical which is produced during the oxidation process. Recently, some progresses have been achieved in the evaluation of the antioxidant activity of materials [49]. The hydrogel causes considerable antioxidant activity which is due to -NH group present in this hydrogel network [50]. The increasing concentration (shown in Table 2) of this sample promoted the antioxidant ability in vitro. DPPH radical scavenging activity increased from 33, 48, 57, 64 and 74 % when increase the concentration from 25-500 µg compared with standard ascorbic acid. Additionally, the IC_{50} value of the hydrogel found to be around 158 µg/mL. IC_{50} parameter, which indicates the ability to scavenge free radicals. A higher IC_{50} parameter means that a given antioxidant is more reactive.

Table 2. Antioxidant activity of biopolymeric hydrogel by DPPH radical

<table>
<thead>
<tr>
<th>Radical</th>
<th>Con(ug)</th>
<th>Scavenging Activity(%)</th>
<th>IC_{50}</th>
<th>IC_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AEG</td>
<td>Std.</td>
<td></td>
</tr>
<tr>
<td>DPPH</td>
<td>25</td>
<td>38</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>48</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>57</td>
<td>49</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>64</td>
<td>66</td>
<td>23.11</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>74</td>
<td>85</td>
<td></td>
</tr>
</tbody>
</table>
Anti-Cancer Application of Biopolymeric Hydrogel

Nowadays, cancer is one of the most common diseases in the world, affecting different parts of the body such as lung, breast, prostate, colon, etc [52,53]. Beside chemotherapy, hydrogels and nanocomposite hydrogels are used as effective devices for hyperthermia and radiation therapy. Most researches focused on stimuli-responsive hydrogels especially pH-sensitive hydrogel and nanocomposite hydrogels for the treatment of cancers. They can undergo a fast and sharp swelling or shrinkage changes in response to a small change in the environmental pH. Using these pH-responsive systems, the release of the anticancer drugs from hydrogel could be controlled in a smart way by changing the pH of the medium.

Table 3. % of cell viability of AEG hydrogel

<table>
<thead>
<tr>
<th>Tested concentration(µg/ml)</th>
<th>% of cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>38.20</td>
</tr>
<tr>
<td>250</td>
<td>46.42</td>
</tr>
<tr>
<td>100</td>
<td>61.27</td>
</tr>
<tr>
<td>50</td>
<td>73.21</td>
</tr>
<tr>
<td>25</td>
<td>79.84</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3 and Fig 6(a-f) the cell viability of the biopolymeric hydrogel at various concentration (25, 50, 100, 250 & 500 µg/ml) are 79.84%, 73.21%, 61.27%, 46.42% and 38.20% respectively for Colon Cancer (HT29) cell line were shown. Colon cancer is a type of cancer that begins in the large intestine (colon). The Colon is the final part of the digestive tract. As the concentration increases % of cell viability decreases, so this kind of biopolymeric hydrogel can be used for destroying the cancer cells [54]. The % of cell viability contracts when the concentration is boosted. It can be concluded as cytotoxic at low concentrations was more cell viability, but its high concentrations lower cell viability and cell proliferation compared to the controls. Similar observation noted in Michiko et al, where the cells were exposed to various concentrations and their viability decreased.
Conclusion

During the last decades, biobased renewable polymers from various resources have been attracted attention of research community which is due to their merits viz., eco-friendly, less time, cheap, availability, simple and convenient etc. In this study, the biopolymeric Acotinic acid based hydrogels were synthesized by condensation polymerization using Aconitic acid, Ethylene glycol and Glutamic acid. The spectral techniques such as FT-IR supported the formation hydrogels. Swelling behaviours of hydrogels were also studied for pH 4.0, 7.0 and 9.0 in phosphate buffer solution revealed higher swelling in acidic medium compared to basic medium. The obtained hydrogels shows antioxidant activity by DPPH radical assay at different concentration compared with the standard Ascorbic acid. The results exhibit excellent antioxidant property with respect to the standards. Hence the synthesized biopolymeric hydrogels may have a great opening for industrial and biological applications, such as metal
ion removal, controlled release of drugs to pH sensitive parts of human being and also possess good antioxidant property.

References


[Date]


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