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RESEARCH ARTICLE

Humic acid particle embedded super porous gum Arabic cryogel network for versatile use

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Super porous gum Arabic (GA) cryogels were synthesized by crosslinking of natural GA with divinyl sulfone at cryogenic conditions, −20°C for potential biomedical applications. Humic acid (HA) nanoparticles were also prepared by using degradable and biocompatible crosslinkers such as trimethylolpropane triglycidyl ether, poly(ethylene glycol) diglycidyl ether, and trisodium trimetaphosphate in a single step and then entrapped within GA cryogel network as GA/HA particle cryogel. Furthermore, GA/HA cryogel was used as a template for Ag, Cu, and Fe nanoparticle preparation, and their antimicrobial properties were tested against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* strains. The minimum inhibition concentration values of Ag and Cu nanoparticle-loaded GA/HA cryogel composites were determined as 10 mg mL^{-1}. Furthermore, the blood compatibility tests such as hemolysis and blood clotting indexes were determined for GA cryogels and found to be more compatible with 0.08 ± 0.01% hemolysis and 89.4 ± 6.1 blood clotting values, whereas the hemolysis of the Ag, Cu, and Fe nanoparticle-loaded GA/HA Ag, Cu, and Fe metal nanoparticle cryogel composites decreased in the order of Fe > Cu > Ag nanoparticles.

KEYWORDS

cryogel/microgel, GA/HA natural polymer, gum Arabic cryogel/hydrogel, hemocompatible cryogel composite, humic acid micro/nanogel

1 | INTRODUCTION

Humic acid (HA) is a natural macromolecule mainly including phenolic, carboxylic acid, quinone, enolic, and ether functional groups. It can be obtained from natural sources such as terrestrial soil, natural water, and sediment.[1] Humic acid has many uses: In cosmetic, pharmaceutical, and medicinal applications, it can be used for wound healing, cancer therapy, drug carrier, and prion disease therapy because of antimutogenic/desmutogenic,[2] antibacterial,[3] antifungal, antioxidant,[4] antiinflammatory, and antiestrogen[1,5] and antiviral activities that are related to its phenolic, carboxylic, and quinone functional groups into the structures. Previously, the crosslinked HA particle synthesis was reported for the first time by using different crosslinkers such as divinyl sulfone (DVS), adipoyl chloride, and gluteraldehyde and utilized in environmental applications.[6] In this study, HA particles were prepared by using epoxy and phosphate groups containing crosslinkers such as trimethylolpropane triglycidyl ether (TMPGDE), poly(ethylene glycol) diglycidyl ether (PEGGE), and trisodium trimetaphosphate (STMP) as more biocompatible and biodegradable structure in comparison with the previously reported ones.[7,8]

Over the last 2 decades, preparing 2 different polymers has attracted great attention in material science and technology due to the combination of properties of both materials in 1 polymer network with potentially new and synergetic effects.[9,10] Lately, cryogel networks are of great interest for a wide range of biomedical applications such as separation and immobilization of cell,[11,12] enzyme, or biomacromolecules; drug-delivery purposes[13]; and tissue engineering.[14] Cryogels possess many superior properties in comparison with conventional hydrogel because of their low density, high and interconnected porosity, and higher surface areas.[15] In the literature, different types of natural-based polymers such as hyaluronic acid, chondroitin sulfate,[16] agarose, chitosan, silk-fibroin,[11] gelatin,[17] alginate, cellulose,[18] rubber, and DNA-derived cryogels have been reported as versatile biomaterials for different potential applications.[14,19]

The aim of this study was to synthesize and characterize gum Arabic (GA) cryogel crosslinked with DVS and the embedding of HA particles within the super porous GA cryogel network to prepare natural GA/HA cryogel. Therefore, HA particles from a natural source with inherently excellent properties were implanted into GA cryogel network with sponge-like super porous polymer network structure.

Furthermore, HA is well known for adsorption of metal ions and stabilization agent for metal nanoparticles (MNPs) because of its high complexation affinity with metal species.[1,20] Metal nanoparticles, such as Fe, Ag, and Cu NPs, can be introduced into the prepared GA/HA cryogels as GA/HA-M (M: Fe, Ag, and Cu) cryogel composites that render increased antimicrobial activity for the resultant materials.[21-23] Morphological and physicochemical characterizations of the particles and cryogels were evaluated by using scanning electron microscope (SEM), Fourier transform infrared (FT-IR), dynamic light scattering (DLS), and zeta potential measurements. The maximum swelling capacity, moisture content of GA cryogel, and GA/HA cryogel were determined at physiological conditions such as pH 5.4, 7.4, and 9; the pore volume (Vp) of the cryogels was determined. In addition, antimicrobial effects and blood compatibility of GA cryogel, GA/HA cryogels, and GA/HA-M cryogel composites were also determined by in vitro tests.

2 | EXPERIMENTAL

2.1 | Materials

Humic acid sodium salt technical grade and GA from acacia tree (MW ~ 250000) were purchased from Sigma-Aldrich. Divinyl sulfone (98%, Merck), TMPGDE (technical grade, Aldrich), STMP (≥95% Aldrich), PEGGE (M_n: 500, Aldrich) as crosslinkers, triethylamine (99.5%, Sigma-Aldrich) as an accelerator, L-alpha-lecithin (granular, 98%, Acros Organic) as surfactant, and gasoline (95 octane, local vender) as solvent were used as received. Silver nitrate (>99.5%, Fluka), copper (II) chloride ($CuCl_2{\cdot}6H_2O$; 98%, Sigma), and iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$; >99%, Acros Organics) were used as metal ion sources. Ethyl alcohol (99%, Kimetsan) and ultra-pure distilled water 18.2 MΩcm (Millipore-Direct Q UV3) were used as solvents. Nutrient agar (for microbiology, Merck) and nutrient broth (NB, for microbiology, Merck) were used as microbial growth media. *Escherichia coli* ATCC 8739, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 6538 strains were obtained from the Microbiology Department of the School of Medicine at Canakkale Onsekiz Mart University.

2.2 | Synthesis of humic acid particles

The HA particles were synthesized according to the earlier procedure with some modification.[6] In short, 0.5 g HA was dissolved in 10 mL 1 M NaOH solution. Then, 600 μL of HA solution was dispersed into 50 mL 0.1 M lecithin-gasoline solution at 50°C under 800 rpm mixing rate. The crosslinkers as TMPGDE, PEGGE, and STMP (300% mol of HA) were added to the reaction medium, and the reaction was carried out for 12 hours. The synthesized particles were precipitated by centrifugation at 35,544 g for 2 minutes. The prepared particles were washed with gasoline and cyclohexane 1 time and ethanol 3 times. Humic acid particles were dried with a heat gun.

2.3 | Synthesis of gum Arabic cryogel and gum Arabic/humic acid cryogel

The GA and GA/HA cryogels were synthesized by cryopolymerization technique. Separately, 0.04 g/mL of GA solution in water and 0.02 g of synthesized HA particles containing 0.04 g/mL of GA solution in water were prepared. These solutions were placed in a deep freezer for about 3 minutes. Then, DVS as crosslinker (225% based on mole ratio of GA) was added into these solutions and vortexed. Then, quickly, the cryogel precursors were put into ~8 mm diameter of plastic straws. For cryopolymerization, these plastic straws were placed in a freezer at −20°C for 24 hours. The prepared GA and GA/HA cryogels were cut into cylindrical shapes, washed with distilled (DI) water several times, and dried in an oven at 50°C.

2.4 | Iron, silver, and copper nanoparticles containing gum Arabic/humic acid-metal cryogel composites

For synthesis of GA/HA-M (M: Fe, Ag, and Cu) cryogel composites, 0.5 g of GA/HA cryogel was placed into 1000 ppm of 30 mL iron (III) chloride hexahydrate, silver nitrate, and copper (II) chloride solutions at 500 rpm mixing rate for 12 hours. The corresponding metal ion-loaded p(GA/HA) cryogel was treated with 0.2 M 100 mL $NaBH_4$ until no more evolution of gas was observed. Finally, these MNPs embedding GA/HA-M cryogel composites were washed with DI water and dried in an oven at 50°C.

2.5 | Characterization of humic acid particles and gum Arabic-based cryogels

The functional groups of the prepared materials were measured by using FT-IR spectroscopy (Nicolet iS10, Thermo) in the spectral range of 4000 to 650 cm^{-1} at a resolution of 4 cm^{-1} by using the attenuated total reflectance technique. The surface charge of the HA particles was determined by using Zeta-pals zeta potential analyzer (BIC) in 1 mM KCl solution. The size of the HA particles was also determined by using DLS (90 plus, Brookhaven Instrument Corp) analyzer with 35 mW solid-state laser detector with an operating wavelength at 658 nm. The morphological characterization of the materials was imaged by SEM (Jeol JSM-5600 LV) with an operating voltage of 20 kV. The materials were placed on carbon tape that was attached on aluminum SEM stubs and coated with gold to a few nanometer thicknesses in a vacuum before taking the images. The thermal degradation of the cryogels was determined by using a thermogravimetric analyzer (SII TG/DTA 6300) in the range of 50 to 1000°C at a heating rate of 10°C min^{-1} under a dry flow of N_2 of 100 mL min^{-1}. The metal content of GA/HA cryogel composites was also determined via TGA analysis.

2.6 | Swelling ratio, moisture content, and pore volume of gum Arabic-based cryogels

One piece of dry GA-based cryogel with known weight was placed into 3 different pH solutions, pH 5.4 (citrate buffer), pH 7.4, and pH 9 (phosphate buffer) and incubated for 2 hours. The swollen cryogels were taken from the solutions, placed on the filter paper to remove excess water, and reweighed. For measuring the Vp of the HA-based cryogels, the cryogels were swollen in cyclohexane solution for 2 hours.[19] The percentage swelling (S%), moisture content (M%),

and Vp of the cryogels are calculated via the following equations 1, 2, and 3:

$$S\% = \left[\left(M_{swollen} - M_{dry}\right)/M_{dry}\right] \times 100 \quad (1)$$

$$M\% = \left[\left(M_{swollen} - M_{dry}\right)/M_{swollen}\right] \times 100 \quad (2)$$

$$Vp = \left(M_{cyclohexane} - M_{dry}\right)/\left(M_{dry} \times d\right) \quad (3)$$

where M_{dry} and $M_{swollen}$ are the weight of dry and swollen cryogels, respectively. $M_{cyclohexane}$ is the swollen cryogel in the poor solvent, such as cyclohexane, and d is the density of solvent. All the swelling experiments were done 3 times, and the average values were reported with the standard deviations.

2.7 | The antimicrobial properties of gum Arabic-based cryogels

The antimicrobial susceptibilities of GA, GA/HA, and Ag, Cu, and Fe NPs containing GA/HA-M cryogel composites were investigated by broth microdilution technique against 3 common bacterial strains: *E. coli* ATCC 8739 (Gram negative), *S. aureus* ATCC 6538 (Gram positive), and *B. subtilis* ATCC 6633 (Gram positive). In brief, the bacteria stock cultures were revived every day and the stock solutions were adjusted to nearly 100×10^5 CFU mL^{-1}. The cryogels were irradiated for 2 minutes under UV light at 420 nm for sterilization. Five different amounts of the sterilized cryogel: 100, 50, 25, 10, and 1 mg were placed in 9.9 mL of NB, and 0.1 mL stock culture was inoculated in NB medium. This broth medium was incubated at 35°C for 24 hours. The minimum inhibitory concentration (MIC) values were identified as the lowest concentration of antimicrobial material showing no visible growth. Then, the cryogel containing culture medium was diluted 10×10^4-fold by using sterilized FTS solution. For enumeration, 100 μL of each diluted solution was plated on nutrient agar and incubated for 18 to 24 hours at 35°C, and the growing colonies were counted.

2.8 | In vitro blood compatibility assay of gum Arabic-based cryogels

Blood compatibility of GA, GA/HA, and Fe, Ag, and Cu NPs containing GA/HA-M cryogel composites was investigated by hemolysis and blood clotting tests according to the earlier procedure.[24] Fresh human blood, taken by healthy volunteers recognized by the Human Research Ethics Committee of Canakkale Onsekiz Mart University (KAEK-2015-13), was used in the blood compatibility tests.

2.9 | Hemolysis

Fresh blood (2 mL) was placed in test tubes containing ethylenediaminetetraacetic acid as an anticoagulant, and then this solution is diluted with 2.5 mL of 0.9% saline solution to prepare blood testing solution. First, 1 piece of cryogel nearly 10 mg was incubated in a shaking bath for 10 minutes in 10 mL saline solution at 37.5°C. Then, diluted fresh blood (2 mL) was added to the cryogel containing solution and incubated at 37.5°C for 1 hour. After the incubation, the suspensions were centrifuged at 100*g* for 5 minutes. The absorbance of the supernatant solutions was measured by UV-Vis spectrophotometer at 542 nm, which is the absorbance value of the releasing hemoglobin. The hemolysis ratio was calculated according to Equation 4:

$$\text{Hemolysis ratio}\% = \left(A_{sample} - A_{negative}\right)/\left(A_{positive} - A_{negative}\right) \times 100 \quad (4)$$

A_{sample} represented the absorbance of cryogel containing blood solution. $A_{positive}$ and $A_{negative}$ represented the absorbance of 0.2 mL diluted blood in 10 mL DI water and 0.2 mL diluted blood in 10 mL saline solution, respectively. The test was repeated at least 6 times.

2.10 | Blood clotting

The fresh blood (2 mL) placed in a test tube containing ethylenediaminetetraacetic acid as an anticoagulant mixed with 0.2 M 0.24 mL of $CaCl_2$ solution for blood clotting study. One piece of cryogel, about10 mg, was swollen in 0.9% saline solution and was put into 50 mL centrifuge tube, and 0.27 mL of blood solution was placed on the cryogel surface by slow dropping to completely cryogel. Then, the tubes were incubated in a shaking bath at 37.5°C for 10 minutes. After the incubation, 10 mL of DI water was slowly added and centrifuged at 100*g* for 30 seconds. The supernatant solution was taken, diluted with 40 mL DI water, and incubated at 37.5°C for 1 hour. The absorbance of this solution was measured by UV-Vis spectrophotometer at 542 nm. The blood clotting was calculated according to Equation 5:

$$\text{Blood clotting index} = \left(A_{sample+blood}/A_{blood}\right) \times 100 \quad (5)$$

$A_{sample + blood}$ and A_{blood} represented the absorbance of blood solution contact with the cryogel and the blood solution diluted in 50 mL DI water, respectively.

3 | RESULTS AND DISCUSSION

The HA particle synthesis by use of DVS, adipoyl chloride, and glutaraldehyde as crosslinkers for using environmental applications was reported earlier.[6] In this study, to improve the biocompatibility and biodegradability of HA particles, 3 different crosslinkers such as TMPGDE, PEGGE, and STMP known as nontoxic and biodegradable crosslinkers were used. As shown in Figures 1A and Sure S1, the degradable epoxy or phosphate groups containing crosslinkers can be used and directly bind with hydroxyl or carboxylic acid groups of HA in basic conditions in a single step via water-in-oil microemulsion system, 0.1 M lecithin-gasoline solution. The reaction was carried out at 50°C to accelerate the crosslinking of HA. The prepared HA particles crosslinked with STMP were embedded to macroporous GA cryogel network during the cryogelation of GA with DVS crosslinker to prepare GA/HA cryogel. The cryogel of GA was prepared under the freezing conditions by crosslinking of linear GA molecules. The schematic representation of crosslinking reaction of GA with DVS was demonstrated in Figure Sure S1. Under the cryogenic conditions, eg, −20°C, the GA cryogel was formed in the nonfrozen liquid region that is situated around the ice crystal known or act as porogen. At end of the reaction,

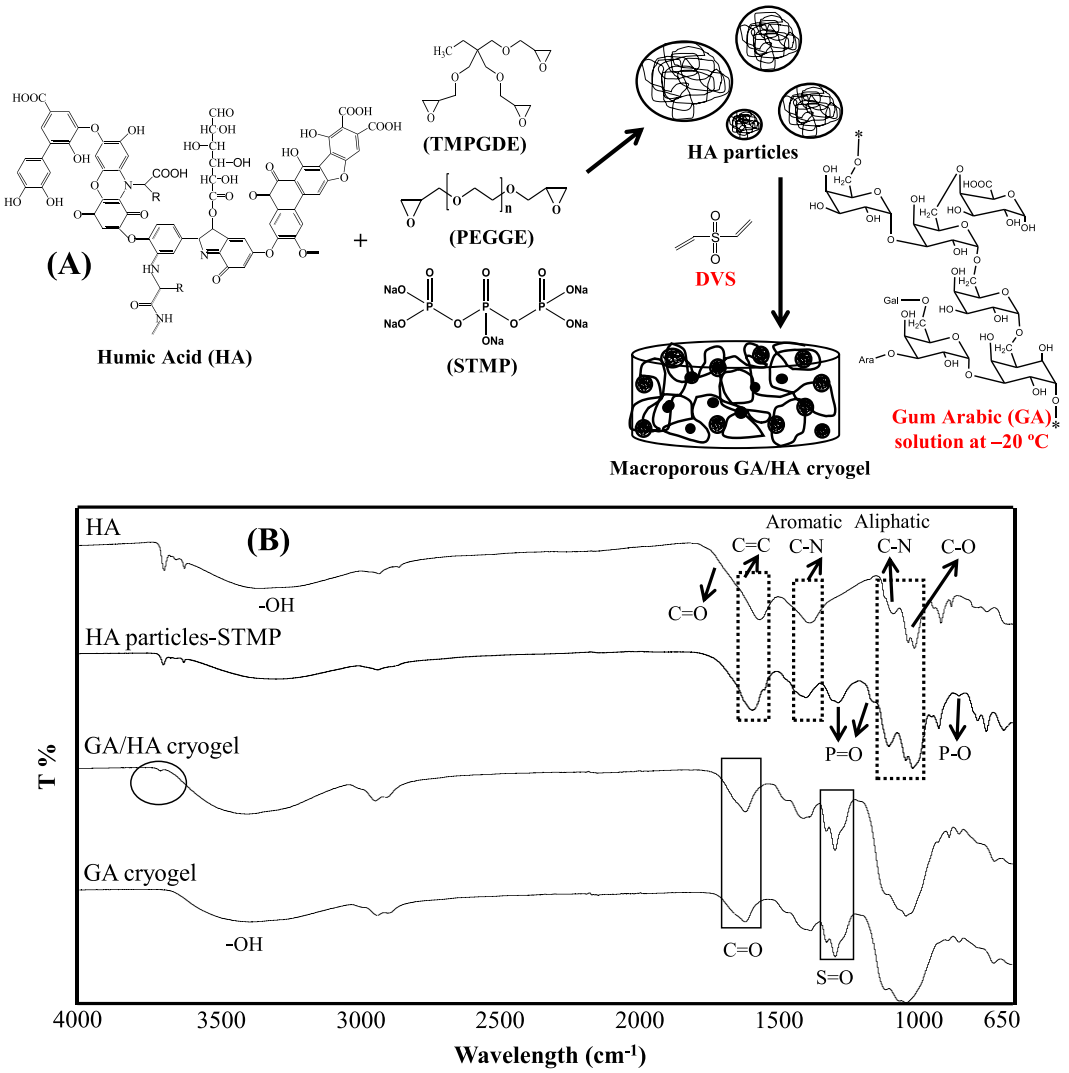

FIGURE 1 (A) Schematic representation of synthesis of humic acid (HA) particles and gum Arabic (GA)/HA cryogel and (b) Fourier transform infrared (FT-IR) spectra of HA particles crosslinked with trisodium trimetaphosphate (STMP), GA cryogel, and GA/HA cryogel [Colour figure can be viewed at wileyonlinelibrary.com]

thin walls and large pores of GA-based cryogels were generated by the crosslinking of GA with DVS. The FT-IR spectra of HA, HA particles crosslinked with STMP, GA cryogel, and GA/HA cryogel composites were shown in Figure 1B. As can be seen from the spectrum of HA, the broad peak at 3350 cm^{-1} can be attributed to hydroxyl group stretching vibrations that belong to phenol, carboxylic acid, and alcohol groups of HA. The broad peak seen at about 1700 to 1600 cm^{-1} can be assigned to nonbonded C O stretching vibrations of HA. This peak was shifted toward lower stretching frequency upon crosslinking the HA chains with STMP crosslinker. The other specific peaks at 1570, 1385, 1090, 1031, and 1009 cm^{-1} can be ascribed to the aromatic C C aromatic C—N amines, aliphatic C—N amines, and alkoxy C—O groups of HA, respectively. The new absorption peaks at 1278, 1150, and 847 cm^{-1} for HA particles can be assigned to P O and P—O groups due to crosslinking of HA with STMP. The FT-IR spectra of GA cryogel that exhibit the broad absorption peak between 3500 and 3100 cm^{-1} can also be attributed to hydroxyl groups of GA, and the other peaks at 2930 to 2890, 1610, 1377, and 1031 cm^{-1} are also due to —CH, —C O, and —C—O stretching vibrations of GA. Additionally, the specific peaks at 1314 and 1280 cm^{-1} show that stretching vibration belongs to S O groups due to the DVS crosslinker. Compared with the FT-IR spectrum of GA/HA cryogel, the new absorption peaks nearly 3650 to 3620 cm^{-1} ascribed to the vibration of primary, secondary, and tertiary alcohol groups of HA particle.

Figure 2A shows typical SEM images of HA particles crosslinked with TMP, PEGGE, and STMP crosslinkers. It is obvious from SEM images that the synthesized HA particles crosslinked with TMP and PEGGE are spherical shaped, and the particle size range is from 0.5 to 5 μm, whereas HA particles crosslinked with STMP are also spherical and generally nanosized. Particle size distribution and zeta potential results of HA particles crosslinked with TMP, PEGGE, and STMP were listed in Table 1 after filtering the reaction mixture with a syringe filter of 1.2 μm pore sizes. The size distribution of HA particles crosslinked with TMP, PEGGE, and STMP were measured as 461 ± 39, 431 ± 26, and 791 ± 36 nm, respectively. Therefore, all types of HA particles are assumed nanosized range. In addition, the surface potential of the HA particles was also measured, and the results were found as −36.75 ± 0.69, −32.19 ± 1.71, and −41.04 ± 0.71 for TMP, PEGGE, and STMP crosslinkers, respectively. It is apparent that there are no significant differences in the surface charge of HA particles regardless

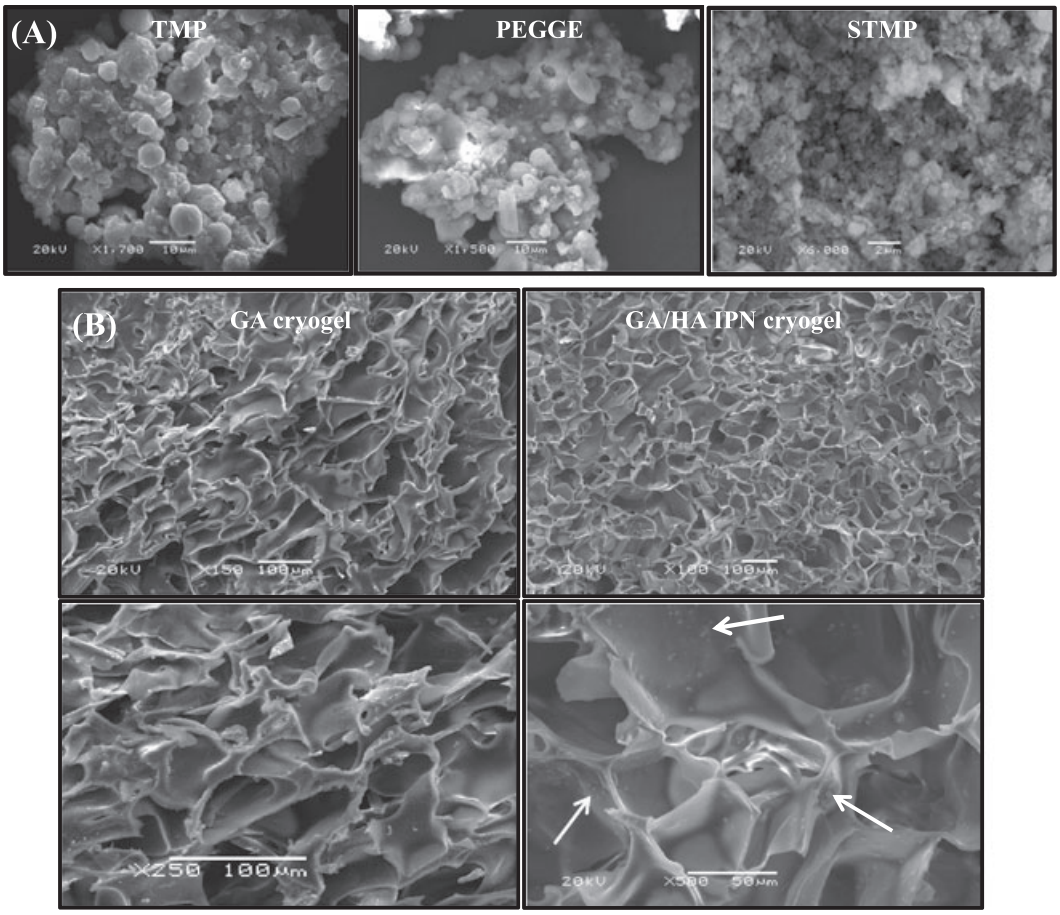

FIGURE 2 Scanning electron microscope (SEM) images of (A) humic acid (HA) particles prepared by using different crosslinkers and (B) gum Arabic (GA) and GA/HA cryogels

TABLE 1 Particle size distribution and zeta potential of humic acid (HA) particles prepared crosslinked with trimethylolpropane (TMP), poly(ethylene glycol) diglycidyl ether (PEGGE), and trisodium trimetaphosphate (STMP) crosslinkers

Materials	HA Particle TMP	HA Particle PEGGE	HA Particle STMP
DLS (nm)[a]	461 ± 39	431 ± 26	791 ± 36
Zeta potential (mV)[b]	−36.75 ± 0.69	−32.19 ± 1.71	−41.04 ± 0.71

[a]Size distribution of the particles was measured after the filtration of the particle with 1.2 μm pore sizes of filter.

[b]Measured in 1.0 mM KCl solution.

of the used crosslinked observed. The SEM images given in Figure 2B for GA cryogel and GA/HA cryogel clearly reveal the highly and interconnected porous morphology. Both cryogel forms were composed of large pore structure in the range of 50 to 150 μm with thin inner wall of network. It was also apparent that the surface morphology of GA cryogel was smooth, whereas the surface structure of GA/HA cryogel was rougher because of the uniformly distribution of HA particles entrapped within the GA cryogel network, illustrated with white arrows in Figure 2B.

Swelling capacity and moisture content of GA cryogel and GA/HA cryogel were determined at different physiological pH conditions: pH 5.4 (acidic), pH 7.4 (neutral), and pH 9 (basic), and the results are given in Figure 3. The digital camera images of swelling capacity of GA/HA cryogels at different times were demonstrated in Figure 3A. As can be seen in the images, GA/HA cryogels were saturated with water only in about 5 seconds due to their super porous structure. Water can be quickly absorbed by the hydrophilic functional groups and fill the interconnected macroporous network of the cryogel. The maximum swelling of GA cryogel was found as 745 ± 177%, 502 ± 140%, and 1320 ± 182% at pH 5.4, 7.4, and 9 of buffer solutions, respectively. There were no significant differences obtained at acidic and neutral conditions, whereas GA-based cryogels were shown highly swollen characteristics at the basic conditions because of the ionization of the hydroxyl groups on GA moieties. The GA cryogels were almost swollen 2-fold more than GA/HA cryogel at the same conditions. These results suggest that HA particles cause to decrease the hydrophilicity of the GA cryogels. Similarly, as shown in Figure 3C, the moisture content of GA and GA/HA cryogel at different pH conditions demonstrated no significant difference in moisture content depending of the pH of the environments. Both cryogels have the moisture content value of between 80 and 90%. Swelling abilities and the moisture content properties of the cryogels are strongly based on the Vp and the crosslinker density of the cryogel matrix. Therefore, total Vp% of the GA cryogel and GA/HA cryogel were measured as 0.85 ± 0.2 and 0.23 ± 0.02 mL of pores for 1 g dry cryogel network, as the same amounts of crosslinker for each cryogel system were used. Gum Arabic cryogel has more porous structure than GA/HA cryogel as it was confirmed from the swelling studies of the cryogels. The swelling, moisture

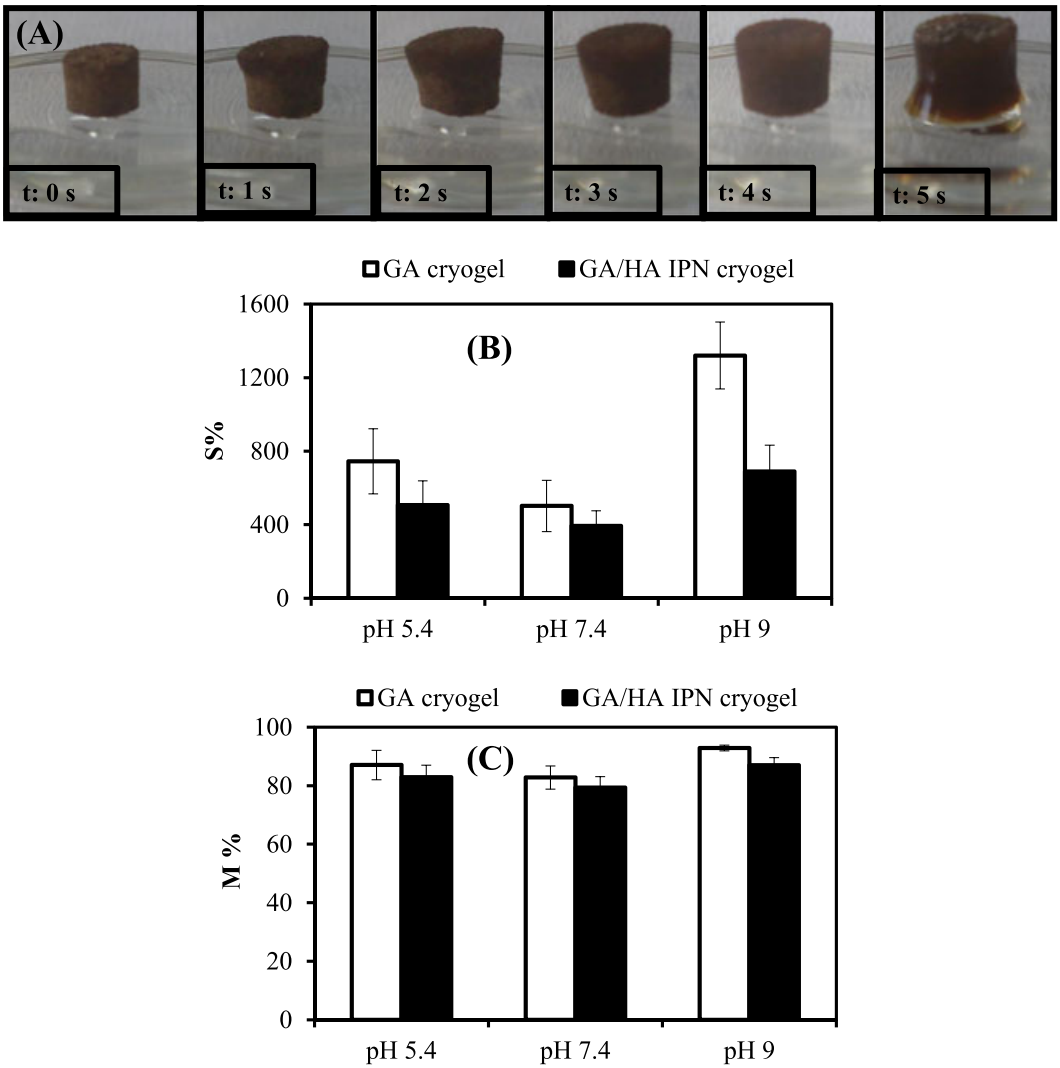

FIGURE 3 (A) Digital camera images of swelling properties of gum Arabic (GA)/humic acid (HA) cryogel, (B) maximum swelling percent (S%), and (C) maximum moisture content (M%) of GA cryogel and GA/HA cryogel at different pH buffer solutions: pH 5.4, 7.4, and 9 [Colour figure can be viewed at wileyonlinelibrary.com]

content, and Vp of the cryogels depend on the concentration of polymers and crosslinker. Here, as the polymer concentration and crosslinker amount were the same for GA cryogel and GA/HA cryogel, the pore filling effects of HA particles in GA/HA cryogel can decrease the Vp of the corresponding cryogel and interpenetration of the polymeric chains can further restrict swelling ability of structures. All these results demonstrated that the natural GA cryogels possess highly swelling nature, moisture content, and interconnected porous structure that provide great advantages for supporting cell growth, proliferation and migration, and in tissue engineering for biomedical applications. Therefore, these natural polymeric-based cryogels possess great environments for cells, enzymes, and for biomolecules such as DNA and protein carrier/support materials or for specific column materials for determination and separation of different analytes.

It was reported that HA can be used as reducing and stabilizing agents for MNPs because of the interaction ability of the functional groups on HA with various metal ions. The shape, size, and stability of the MNPs were independent on the used reducing agent.[1] The schematic representation of MNP preparation within the GA/HA cryogel network was illustrated in Figure 4A. As demonstrated in the figure, 3 different metal ions such as Ag(I), Cu(II), and Fe(III) were loaded into GA/HA cryogel network by absorption from the corresponding metal ion solutions. The in situ MNP within GA/HA-M cryogel composites was accomplished by treating the metal ion-absorbed cryogels with $NaBH_4$ solutions for the reduction of the metal ions. The existence of HA particles in the cryogel network can increase the metal ion absorption capacity and enhance the stability of NPs by preventing their aggregation.[25] Digital camera images of the prepared dried GA cryogel, GA/HA cryogel, and MNP containing GA/HA-M cryogel composites were illustrated in Figure 4B. The white colored and spongy GA cryogel was turned into dark brown colored upon the addition of HA microgels into the cryogel network for the preparation of GA/HA cryogel. After the MNP preparation process, the colors of the cryogel changed as can be seen such as brown, green, and light brown colors for Ag, Cu, and Fe MNP within cryogel composites, respectively. The thermal degradation of GA cryogel, GA/HA cryogel, and MNP containing GA/HA-M (M: Ag, Cu, and Fe) cryogel composite was investigated to determine the MNP content within the polymer network. As can be seen in Figure 4C, the degradation started between 140 and 210°C for each form of cryogel due to the bounded water.

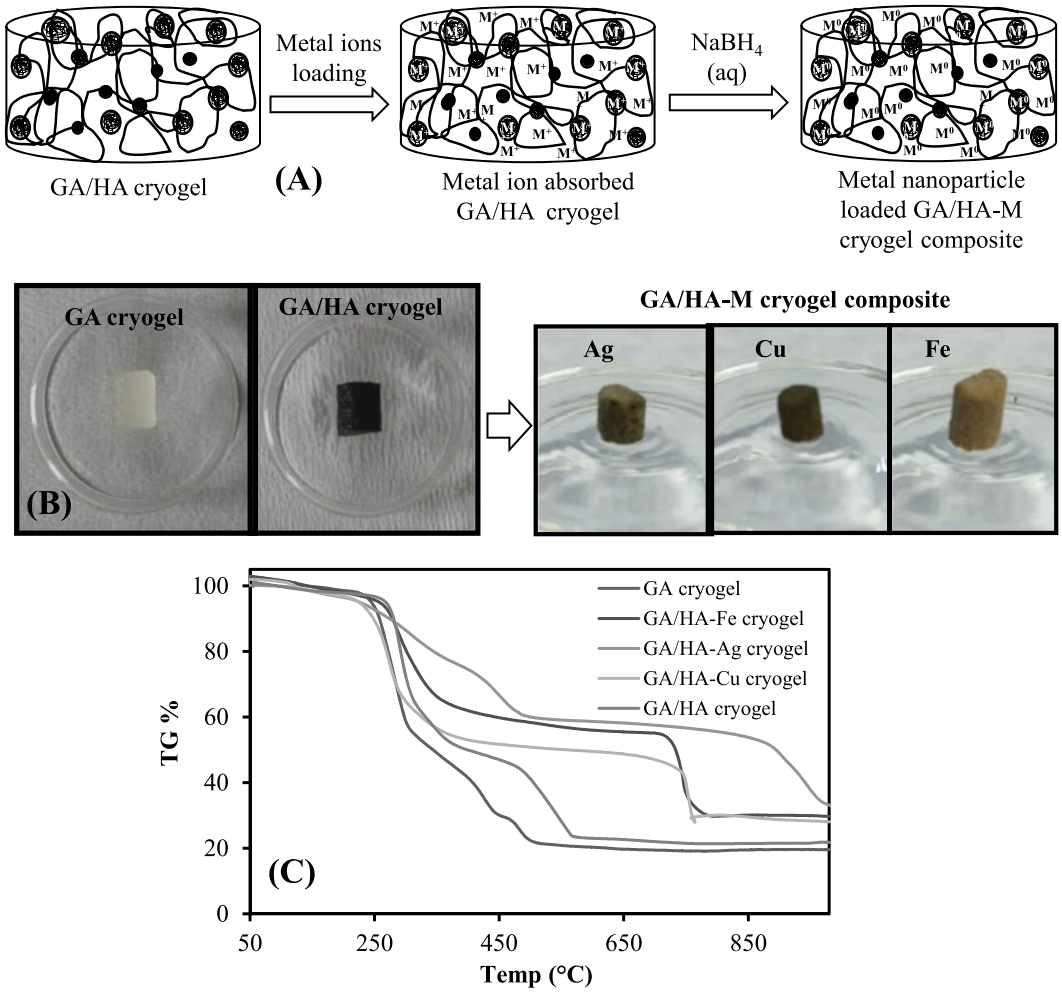

FIGURE 4 (A) The schematic representation of preparation of metal nanoparticle as gum Arabic (GA)/humic acid (HA)-M (M: Ag, Cu, or Fe nanoparticles) cryogel composites; (B) digital camera images of GA cryogel, GA/HA cryogel, and GA/HA-M cryogel composites; and (C) their thermal degradation curves [Colour figure can be viewed at wileyonlinelibrary.com]

The main degradation was observed between 220 and 500°C as 2-step degradation profile with 72% weight loss for GA cryogel. The weight loss at 990°C was measured as 19.4%, 21.6%, 27.7%, 29.3%, and 30.1% for GA cryogel, GA/HA cryogel, GA/HA-Cu, GA/HA-Fe, and GA/HA-Ag composite cryogels, respectively. The TG analysis indicated that the metal content of GA/HA-M cryogel composites are about 6.1, 7.7, and 8.5 wt% for Cu, Fe, and Ag NPs, respectively.

Metal nanoparticles such as Ag and Cu are known for their ability to inhibit the growth of microorganisms due their highly antimicrobial effects.[21-23] Thus, Ag, Cu, and Fe MNP-loaded GA/HA-M cryogel composites were prepared to enhance the killing effects of the bare GA/HA cryogel. The MIC values of GA cryogel, GA/HA cryogel, and GA/HA-M cryogel composites against *E. coli* ATCC 8739 (Gram negative), *S. aureus* ATCC 6538 (Gram positive), and *B. subtilis* ATCC 6633 (Gram positive) strains were given in Table 2. It is apparent from Table 2 that GA cryogel, GA/HA cryogel, and GA/HA-Fe cryogel composite do not show any effect against the mentioned bacteria strains. On the other hand, MIC values of GA/HA-Ag and GA/HA-Cu cryogels were determined as 10 mg mL^{-1} against Gram negative and positive bacteria. These results confirm that there are no differences between Ag and Cu NPs. Interestingly, as HAs are well-known antimicrobial materials against a wide range of bacteria strains, however, after the crosslinking reaction, its antimicrobial susceptibility decreased due the existence new functional groups and generated size of cryogels (mm size range) that is much more than that bacteria. Therefore, Ag and Cu NPs embedding in the cryogel can increase the interactions between the cryogel and the microorganism as some of MNPs are located on the surface of HA particles and on the surface wall of GA cryogels inhibiting the microorganism growths.[23] All these results strongly demonstrated that sponge-like GA/HA cryogels can be used

TABLE 2 The minimum inhibition concentration (MIC) values (mg mL^{-1}) of gum Arabic (GA) cryogel, GA/humic acid (HA) cryogel, and GA/HA-M (M: Ag, Cu, or Fe nanoparticles) cryogel composites against *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, and *Bacillus subtilis* ATCC 6633 strains

	MIC (mg mL^{-1})		
Materials	*E. coli* (Gram −)	*S. aureus* (Gram +)	*B. subtilis* (Gram +)
GA cryogel	–	–	–
GA/HA cryogel	–	–	–
GA/HA-Ag cryogel composite	10	10	10
GA/HA-Cu cryogel composite	10	10	10
GA/HA-Fe cryogel composite	–	–	–

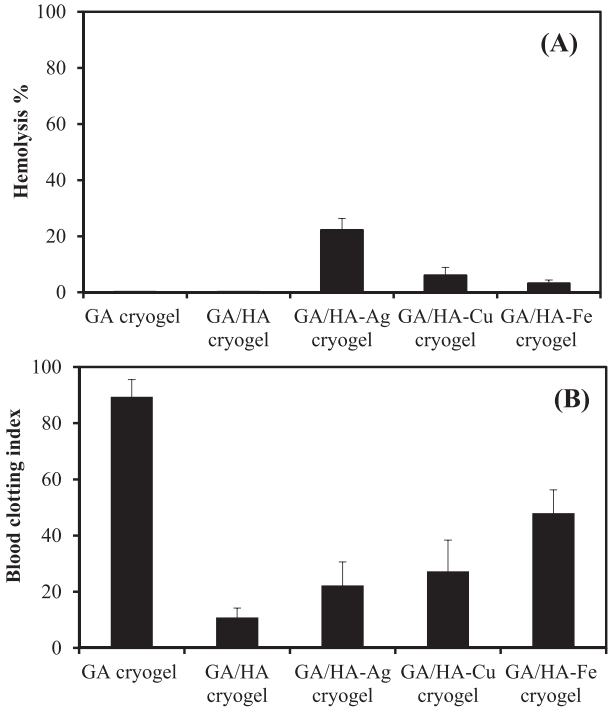

FIGURE 5 (A) Hemolysis % and (B) blood clotting indexes of gum Arabic (GA) cryogel; GA/humic acid (HA) cryogel; and Ag, Cu, and Fe nanoparticle (NP) embedding GA/HA-M (M: Ag, Cu, or Fe nanoparticles) cryogel composites

as a template for MNP preparation, and these composites are promising materials in the design of multitask materials for medical applications.

Material with biomedical potential necessitates a test for blood compatibility as blood can be in contact with those materials. These materials can destroy the red blood cells, which is the meaning of hemolysis, and can affect the thrombosis and coagulation mechanisms upon contacting with blood. Therefore, the blood compatibility, such as the hemolysis ratio, and the blood clotting ability for the materials need to be investigated for any blood contacting materials. Hemolysis is an extremely important matter to assess the blood compatibility of materials where the destruction of erythrocyte cells is determined. According to the standard, 0 to 2%, 2 to 5%, and 5 to 100% hemolysis ratios are considered as nonhemolytic, slightly hemolytic, and hemolytic materials, respectively.[26] Hemolysis values of GA, GA/HA, and Ag, Cu, and Fe NPs containing GA/HA-M cryogel composite were determined as shown in Figure 5A. As can be seen, no significant differences were observed between GA and GA/HA cryogels with 0.08 ± 0.01 and 0.11 ± 0.05% hemolysis ratios, and they can be considered more hemocompatible from hemolysis test. After the preparing of Ag, Cu, and Fe NPs within GA/HA cryogel network, the hemolysis ratio was increased to 22.15 ± 4.12, 6.03 ± 2.87, and 3.17 ± 1.24%, respectively. These results show that only GA/HA-Fe cryogel composite is slightly hemolytic, whereas red blood cells were badly influenced by the presence of Ag and Cu NPs within GA/HA-M cryogel composites. These results are in accordance with antimicrobial susceptibilities of GA/HA-M cryogel composites. Another blood compatibility test is blood clotting, and the results for this test for GA, GA/HA, and Ag, Cu, and Fe NPs containing composite GA/HA-M cryogels were demonstrated in Figure 5B. Gum Arabic cryogel has good biocompatibility with high blood clotting index as 89.4 ± 6.1. As seen in Figure 5B, the blood clotting mechanism was affected by the introduction of HA particles into the GA cryogel network that resulted in 10.8 ± 3.3 blood clotting index for GA/HA cryogel. The functional groups of HA particles such as phenolic, carboxylic, and/or quinine groups can be induced the clotting mechanism on the blood. The hemocompatibility of GA/HA-M cryogel composites was increased in the order of Ag < Cu < Fe NPs with blood clotting indexes of 22.2 ± 8.3, 27.3 ± 11.1, and 48.0 ± 8.3, respectively. According to the literature, green-synthesized Ag, Cu, and Fe NPs can be used for in vivo biological applications with high hemocompatibility.[22] These MNPs were loaded via the interaction of the functional groups of HA particles into the cryogel network, and the blood compatibility, especially antitrombogenic activity, was increased by the loadings of these MNPs into the structure. Because MNPs and the functional groups can combine within the HA particles due to the electrostatic interactions, they may affect the clotting mechanism in the blood, preventing the blood from clotting.

4 | CONCLUSIONS

In this study, HA particles from natural source were successfully synthesized by using 3 different biocompatible crosslinkers, TMP, PEGGE, and STMP, and included into super porous GA cryogel network to prepare GA/HA cryogels for potential biomedical applications. It was also demonstrated here that GA/HA cryogels were used as a template for in situ Ag, Cu, and Fe NP preparations. It was found that Ag and Cu NP-loaded GA/HA-M cryogel composites are effective antimicrobial materials against Gram negative and Gram positive bacteria strains such as *E. coli*, *S. aureus*, and *B. subtilis*, whereas blood compatibility revealed that the existence of MNP in the cryogel network decreased the biocompatibility of the GA/HA-M cryogel composites. Furthermore, the results for in vitro blood compatibility suggested that GA cryogels are blood compatible with 0.08 ± 0.01% hemolysis and 89.4 ± 6.1 blood clotting values. Therefore, the prepared microgel and microgel-embedded cryogel networks that were prepared from natural sources such as HA and GA are versatile materials and have great potential to be used in many biomedical applications.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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