

Characterization of a Multi-responsive Magnetic Graphene Oxide Nanocomposite Hydrogel and Its Application for DOX Delivery

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Abstract Nanocomposite hydrogels are one of the most important types of biomaterials which can be used in many different applications such as drug delivery and tissue engineering. Incorporation of nanoparticles within a hydrogel matrix can provide unique characteristics like remote stimulate and improved mechanical strength. In this study, the synthesis of graphene oxide and graphene oxide nanocomposite hydrogel has been studied. Nanocomposite hydrogel was synthesized using carboxymethyl cellulose as a natural base, acrylic acid as a comonomer, graphene oxide as a filler, ammonium persulfate as an initiator, and iron nanoparticles as a crosslinking agent. The effect of reaction variables such as the iron nanoparticles, graphene oxide, ammonium persulfate, and acrylic acid were examined to achieve a hydrogel with maximum absorbency. Doxorubicin, an anti-cancer chemotherapy drug, was loaded into this hydrogel and its release behaviors were examined in the phosphate buffer solutions with different pH values. The structure of the graphene oxide and the optimized hydrogel were confirmed by Fourier-transform infrared spectroscopy, Raman spectroscopy, X-ray diffraction, scanning electron microscopy, and atomic force microscopy.

Keywords Nanocomposite hydrogel; Carboxymethyl cellulose; Graphene oxide; Doxorubicin; Drug delivery

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INTRODUCTION

Polymeric hydrogels, consisting of a three-dimensional hydrophilic polymer network and water filling at the interstitial space of the network, have attracted much attention of scientists as functional soft materials.^[1] The network structure of the hydrogels, which can be formed by physical cross-linking (*i.e.*, hydrogen bonding, electrostatic, hydrophobic, or dipole-dipole interactions) or chemical cross-linking, leads to a 3D environment with high water retention^[2,3] and their hydrophilic nature is because of the presence of polar chemical moieties such as hydroxyl (—OH), carboxyl (—COOH), amide (—CONH—), and sulfonic (—SO₃H) groups in the polymer backbone. This characteristic structure has enabled them to resemble biological tissues and as biocompatible materials that can be used for a wide range of applications, such as biosensors, bioseparation, drug release vehicles, and tissue engineering scaffolds.^[4] Three methods to prepare the hydrogels with high strength are as follows: topological gel, double network gel, and nanocomposite gel. The topological gel is different from physical and chemical gels by using the supramolecular architecture with topological characteristics. Double network gels are prepared by incorporating a solution of high-molar-mass linear polymer within a moderately cross-linked but fully

swollen charged polymer network.^[5] For nanocomposite hydrogels, inorganic fillers such as clays and carbon nanotubes are usually involved in the preparation of the hydrogels as the crosslinkers.^[6] Among these methods, double network gels and nanocomposite gels are considered to be typical and effective methods to prepare the hydrogels with improved mechanical properties.^[7]

Graphene oxide (GO) is an oxidized derivative of graphene^[8] which has initiated an eruptive interest for developing functional and structure-reinforced nanocomposite owing to its extraordinary thermal stability, excellent dispersion in water, premiere electronic conductivity, remarkable structural flexibility, high specific surface area (~2630 m²/g), and widespread potential applications in nanoscience and nanotechnology.^[9] Because of the layered structure that contains hydroxyl, epoxide, and carboxyl groups on its surface which are introduced as a result of oxidation, it can be easily exfoliated into monolayer sheets stably dispersed in water.^[10,11] GO has aroused great interest as nanofillers for improving the mechanical and physical properties of polymers, mainly due to its large theoretical specific surface area and layered structure.

Magnetic iron oxide nanoparticles (MIONs), with diameters between about 1 and 100 nm were found to have various applications, ranging from environmental analysis and separations to medicinal imaging.^[12,13] The size and surface characteristics of the nanoparticles are crucial factors that deter-

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ine the success of the particles when used *in vivo*. To improve biocompatibility properties and reduce aggregation, the nanoparticle surfaces must be tailored. Iron oxide nanoparticles can be coated with various polymers,^[12] especially biopolymers such as polysaccharides.^[14] Polymers with more than one group capable of binding to particle surfaces (multidentate ligands) provide mechanical and chemical stability to the MIONs.^[12]

Polysaccharides and their derivatives have attracted much interest in biomedical fields due to a set of unique characteristics such as non-toxicity, high biocompatibility, biodegradability, low cost, renewability and availability. Sodium carboxymethyl cellulose (CMC) is a water-soluble anionic linear polysaccharide and semi-synthetic derivative of cellulose that is produced by the partial substitution of the 2, 3, and 6 hydroxyl groups of cellulose with hydrophilic carboxymethyl groups. It has been widely used in several areas such as drug delivery, textile printing, paper industry, nanocomposite materials, detergents, food and oil well drilling operations. CMC-based hydrogels can be considered as very good matrices thanks to the high-water content, environmentally friendly, and in combination with the low cost of CMC.^[15]

Because of the excellent properties of GO, MION, CMC, and hydrogels, their combination is suitable for real applications. In this study, we report the synthesis of CMC-based nanocomposite hydrogel by grafting acrylic acid (AA) onto CMC in the presence of MION as a crosslinker and GO as a mechanical reinforcement. Systematically, the effect of reaction variables on the water absorbency of the nanocomposite hydrogel was investigated. The swelling behavior of MION/GO/CMC-*g*-PAA hydrogel in various salt and pH solutions was also studied. Also, the absorbency under load (AUL) of the nanocomposite hydrogel was determined. The microstructure and properties of the hydrogels were characterized with scanning electron microscopy (SEM), Fourier-transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), thermogravimetric (TGA), and differential scanning calorimetry (DSC). Furthermore, their biological properties, *in vitro* antibacterial activity, and cytotoxicity were evaluated. On account of MION/GO/CMC-*g*-PAA hydrogel properties and a facile fabrication procedure, the hydrogel is expected to have marvelous prospects as a carrier for drugs and scaffolds for the biological field.

EXPERIMENTAL

Materials

CMC (Sunrose F300HC, viscosity of 1% solution at 25 °C: 2500–3500 MPa·s, degree of substitution (DS): 0.8–1) and industrial grade graphite powder were purchased from a supplier in Iran. Ammonium hydroxide (NH₄OH, from Merck, 25%, V/V), iron (II) chloride tetrahydrate (FeCl₂·4H₂O, from Merck), anhydrous iron (III) chloride (FeCl₃, from Merck) and acrylic acid (AA, from Merck) were analytical grades and used without further purifications. Fetal bovine serum, penicillin, streptomycin, and RPMI1640 powder were obtained from GIBCO. Doxorubicin was received from Osveh pharmaceutical Co., Tehran, Iran, as a gift. Mouse fibroblast (L929) cells were purchased from National Cell Bank of Pasteur Institute of Iran. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide

tetrazolium was purchased from Sigma-Aldrich. Double distilled water (DDW) was used throughout the experiments. All other chemicals were analytical grades and used as received.

Instrumental Analysis

FTIR spectra were recorded by a FTIR spectrophotometer (Equinox55) using the KBr method. SEM model (Seron technology AIS 2300 C) was used to examine the surface morphology of hydrogels which is first sputtered with gold. The thermal stability of the hydrogel was investigated using a thermogravimetric analysis instrument (Perkin Elmer Pyris 1) from room temperature to 700 °C under a nitrogen atmosphere at a heating rate of 20 °C/min. To measure the *T_g* of the synthesized hydrogel, DSC model DSC-500B was used. Atomic force microscopy (AFM) imaging was carried out at room temperature using an Auto Probe CP from Park Scientific Instrument in contact mode. The magnetic measurement of the sample was carried out in a vibrating sample magnetometer (VSM) (Meghnatis Daghigh Kavir Co.; Kashan Kavir; Iran). Raman analysis of the graphite and GO were carried out with a Senterra Raman spectrometer equipped with a CCD detector at a wavelength of 785 nm from 200 cm⁻¹ to 3500 cm⁻¹. Wide-angle XRD patterns of the graphite, GO and optimized hydrogel were recorded with a Philips (PW1800) X-ray diffractometer using Cu, and data from the 2θ angular regions between 4° and 60° were collected.

Preparation of Graphene Oxide

Graphene oxide was synthesized using the Hummers method from natural graphite powder.^[16] In a 2000 L beaker, concentrated H₂SO₄ (138 mL) was slowly added to a mixture of graphite powder (6 g, 1 wt. equiv.) and NaNO₃ (3 g, 0.5 wt. equiv.) with vigorous mechanical stirring while the mixture was cooled to 0 °C in an ice-bath. Then, KMnO₄ (18 g, 3 wt. equiv.) was added in portions so that the temperature of the reaction mixture remained below 20 °C. The reaction mixture was warmed to 35 °C and stirred for 30 min. After that, water (276 mL) was added slowly into the beaker and the mixture temperature was increased to 98 °C due to the exothermic dissolving of H₂SO₄. The reaction temperature was maintained at 98 °C for 15 min by external heating, then the heat was removed and the reaction was cooled by a water bath for 10 min. Finally, additional water (840 mL) and 30% H₂O₂ (6 mL) were added to the suspension to stop the reaction. The resulting black precipitate was filtered and washed in succession with 200 mL of water, 200 mL of 30% HCl, and 200 mL of ethanol (twice) and then was followed by repeated washing with distilled water until the pH of the solution reaches neutral. Finally, GO was dried at 60 °C for 24 h in an oven.

Preparation of MION/GO/CMC-*g*-PAA Hydrogels

For making hydrogel, briefly GO powder (0.05–0.3 g) with carboxymethyl cellulose (1 g) was first sonicated and dispersed in 60 mL of distilled water in an ultrasonic bath for 15 min. The mixture was added to a three-neck reactor equipped with a mechanical stirrer and set in a warm water bath at the desired temperature (80 °C). Then, the initiator solution (2.18–7.67 mmol APS in 5 mL of H₂O) was added to the mixture and after stirring for 5 min, the monomer (346.93–1040.80 mmol AA) was added to the reaction vessel and allowed to polymerize for 60 min. After cooling to room temperature, a premixed

solution of $\text{FeCl}_3/\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ in 10.0 mL of DDW was added dropwise over a period of 5 min to the reaction mixture at room temperature with vigorous stirring. After stirring for 30 min, in order to form MIONs, 10 mL of ammonium hydroxide was added to the reaction mixture and allowed stirring for an additional 30 min. After 15 min, the hydrogel was poured into 200 mL of nonsolvent ethanol and remained for 2 h to dehydrate. Then ethanol was decanted and the product was chopped into small pieces (diameter ~5 mm). Again, 200 mL of fresh ethanol was added and the hydrogel was soaked for 24 h. The dewatered gel particles were filtered and dried in an oven (at 60 °C) for 24 h. After grinding, the obtained powder was stored in the absence of moisture, heat, and light for further experiments. The amounts of the materials used for the preparation of various hydrogels are summarized in Table 1.

Table 1 Reaction contents in different hydrogel preparations.

Entry	GO (g)	APS (mmol)	AA (mmol)	$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (mmol)	FeCl_3 (mmol)	Swelling (g/g)
1	0.10	5.46	867.33	9.62	31.21	231.46
2	0.10	5.46	867.33	11.70	37.45	262.38
3	0.10	5.46	867.33	15.66	50.01	233.80
4	0.10	5.46	867.33	19.56	62.50	172.20
5	0.10	5.46	867.33	23.46	74.98	75.40
6	0.10	5.46	867.33	29.31	93.71	53
7	0.10	5.46	867.33	39.12	125	18
8	0.05	5.46	867.33	11.70	37.45	174.46
9	0.15	5.46	867.33	11.70	37.45	298.29
10	0.2	5.46	867.33	11.70	37.45	146.65
11	0.25	5.46	867.33	11.70	37.45	93.71
12	0.3	5.46	867.33	11.70	37.45	75.50
13	0.15	2.18	867.33	11.70	37.45	161.37
14	0.15	3.29	867.33	11.70	37.45	286.41
15	0.15	4.38	867.33	11.70	37.45	290.20
16	0.15	6.57	867.33	11.70	37.45	318.23
17	0.15	7.67	867.33	11.70	37.45	188.44
18	0.15	6.57	346.93	11.70	37.45	85.52
19	0.15	6.57	520.40	11.70	37.45	127.56
20	0.15	6.57	693.87	11.70	37.45	178.58
21	0.15	6.57	1040.80	11.70	37.45	188.41

Swelling Measurements

The teabag method was used for swelling measurements.^[17] The bag containing the powdered sample of MION/GO/CMC-g-PAA hydrogel (0.05g with average particle sizes ~250–400 μm) was immersed in 250 mL of distilled water completely for 2 h at room temperature and then was hung up for 15 min, to remove the excess water. The equilibrium swelling was calculated based on the following equation:

$$\text{ES (g/g)} = \frac{W_2 - W_1}{W_1} \quad (1)$$

where W_1 and W_2 are the weights of dried and swollen gel, respectively.

Swelling Kinetics

To study the kinetics of water absorbency, a certain amount of optimized MION/GO/CMC-g-PAA hydrogel with different particle sizes were poured into numbers of weighed tea bags and immersed in 250 mL of distilled water.^[18] At specified time intervals, the tea bags were removed from the water and the absorption of the hydrogels was measured according to Eq. (1).

All measurements were also evaluated under the external magnetic field.

Swelling in Salt Solutions

According to the above method, MION/GO/CMC-g-PAA hydrogel absorption was examined in different solutions of NaCl, CaCl_2 and AlCl_3 in order to evaluate the salt sensitivity of the prepared samples.

Absorption at Various pH Values

Since the MION/GO/CMC-g-PAA hydrogel contains carboxyl groups, it may operate as a pH-sensitive hydrogel. To evaluate this character, specific solutions ranging from pH=1 to 12 were prepared by dilution of NaOH and HCl solutions. The pH values were checked exactly by the pH meter.^[18] Then 0.05 g of optimized hydrogel was used for swelling measurements according to Eq. (1). All measurements were also evaluated under the external magnetic field.

On-off pH Switching

Responsibility of the optimized hydrogel to pH makes it possible to use as a switch. This was evaluated in terms of swelling and deswelling in two acidic (pH=2) and basic (pH=8) phosphate buffer solutions. The buffers were prepared by mixing stock standard solutions of H_3PO_4 and NaH_2PO_4 and adjusted to the desired pH with 0.1 mol/L HCl or NaOH. To adjust the ionic strength of solutions to 0.1 mol/L, the proper amount of NaCl was added. The swelling capacity of the MION/GO/CMC-g-PAA hydrogel at each pH was measured according to Eq. (1) at consecutive time intervals and for each cycle, a fresh solution was used. The interval between the pH changes was approximately 35 min. All measurements were also evaluated under the external magnetic field.

Absorbency Under Load (AUL)

This test was carried out as described in the literature^[18] in order to determine the swelling behavior of nanocomposite under pressure of 6205 Pa (0.9 psi) in a 0.9% saline solution. The 0.05 g of dried MION/GO/CMC-g-PAA hydrogel was uniformly put into a glass cylinder, which has a screen fabric as the bottom. A cylindrical weight giving the desired pressure was placed on top. The cylinder arrangement is then placed on a liquid source in a Petri dish ($d=118$ mm, $h=12$ mm). After certain time intervals, the swollen particles were weighed again, and AUL was calculated according to Eq. (1). All measurements were also evaluated under the external magnetic field.

Drug Release Study

For this study, 0.5 mL of aqueous doxorubicin solution (2 mg/mL) as an anti-cancer drug and 0.05 g of optimized MION/GO/CMC-g-PAA hydrogel were dispersed in 2 mL of distilled water using a rotary shaker (200 r/min) at 37 °C. Then, the loaded MION/GO/CMC-g-PAA hydrogel was dried in an oven at 50 °C. In a rotary shaker (200 r/min), the dried gel was dispersed in 100.0 mL of phosphate buffer (pH=5.4 and 7.4) at 37 °C. At given intervals, 1.00 mL of filtered samples were withdrawn to assay the drug release as a function of time using a calibration curve constructed from a series of drug solutions with known concentrations. Finally, the amount of drug-releasing was measured at 500 nm by UV-Vis spectrophotometer.^[19] All measurements were evaluated under an external magnetic field.

Cytotoxicity and Cell Proliferation Assay

The cytotoxicity and cytocompatibility of MION/GO/CMC-*g*-PAA hydrogel were evaluated by using mouse fibroblast cell line L929 obtained from National Cell Bank of Iran (NCBI Code C161). The cells were cultured in RPMI 1640 medium supplemented with 100 u/mL of penicillin (Sigma, USA), 100 µg/mL of streptomycin (Sigma, USA), and 10% fetal bovine serum (Gibco, USA).

To evaluate the viability of treated cells and cytocompatibility of hydrogel, the MTT assay was performed. Briefly, 10000 cells/well were seeded in 96 well culture plates and incubated at 37 °C in 5% CO₂ for 24 h. The powdered MION/GO/CMC-*g*-PAA hydrogel was weighed in 0.01 gr portions and sterilized in a UV box for 1 h. Then, 2 mL of RPMI 1640 medium was added to form the hydrogel. Different amounts of hydrogel (50 and 100 µL) were added to wells. Three wells were marked for each concentration and a control. The viability assay was performed after incubation for 24 h.

After the appropriate incubation period, the media were removed and the wells were washed with PBS buffer for once. Then, 100 µL of MTT 5 mg/mL (Sigma, USA) solution was added to each well and the plate was incubated in a CO₂ incubator for 2 h. Subsequently, 50 µL of isopropanol was added to each well and the resultant formazan was dissolved by pipetage. The absorbance wavelength of each well was read using an ELISA Reader at a test wavelength of 595 nm.

RESULTS AND DISCUSSION

Synthesis Mechanistic Aspect

The schematic synthetic route to MION/GO/CMC-*g*-PAA hydrogel is suggested in Scheme 1. In the first step, the APS initiator is decomposed under high temperature and the produced sulfate anion radicals were abstracted hydrogen from functional groups of sodium carboxymethyl cellulose and GO to generate corresponding radicals. Then, the resulting radicals initiate graft copolymerization of AA monomers to provide

GO/CMC-*g*-PAA copolymer.

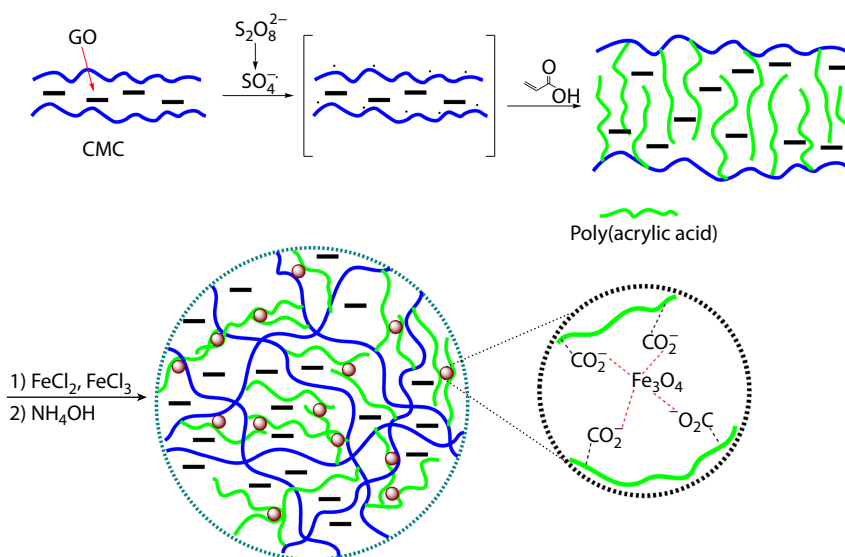
Next, MIONs were prepared *in situ* by adding a premixed solution of Fe²⁺/Fe³⁺ to a GO/CMC-*g*-PAA solution at room temperature with stirring. The GO/CMC-*g*-PAA copolymer has two key roles: stabilizer for fabricated MIONs and splitting medium for MION clusters, and act as chelating sites with existing functional groups on the GO/CMC-*g*-PAA that can form complexes with Fe³⁺ and Fe²⁺ ions. After this so-called nucleation stage, the aqueous solution of NH₄OH was added to grow MIONs in this step. The growth was controlled by the grafted polymer. Since MIONs can chelate to several polymer chains simultaneously, they act as crosslinkers and form 3D network structures.^[20]

Raman Spectroscopy

To distinguish the carbon-based materials, Raman spectroscopy is a useful method.^[21] Fig. 1 shows the Raman spectra of graphite and GO. As shown in Fig. 1(a), graphite showed three characteristic bands at 1306.40 cm⁻¹ (D-band, C—C), 1577.19 cm⁻¹ (G-band, C=C), and 2611.59 cm⁻¹ (2D-band). The spectrum of graphene oxide (Fig. 1b) exhibits two prominent bands at 1312.28 and 1587.53 cm⁻¹, which correspond to the well-documented D and G bands, respectively.^[22,23]

XRD Patterns

X-ray diffraction (XRD) technique was used to determine the interlayer spacing of the graphite, graphene oxide, and nanocomposite. Results are shown in Fig. 2. Compared with graphite, the interlayer spacing of graphene oxide increased from 3.34 Å to 7.84 Å, indicating that the presence of oxygen-containing functional groups increased the thickness of the carbon plates. The XRD pattern of graphene oxide shows a strong peak at 2θ=11.27°, corresponding to a basal spacing of 7.84 Å (Fig. 2a).^[24,25] The XRD pattern of the optimized hydrogel is depicted in Fig. 2(b). There is a shift peak 2θ=11.27° in GO corresponding to the position at 2θ=32.89° observed in the optimized hydrogel pattern. This could be explained by a rearrangement in the morphology of the polymeric chain after grafting GO to hydrogel.^[26]



Scheme 1 Suggested synthetic route to MION/GO/CMC-*g*-PAA hydrogel.

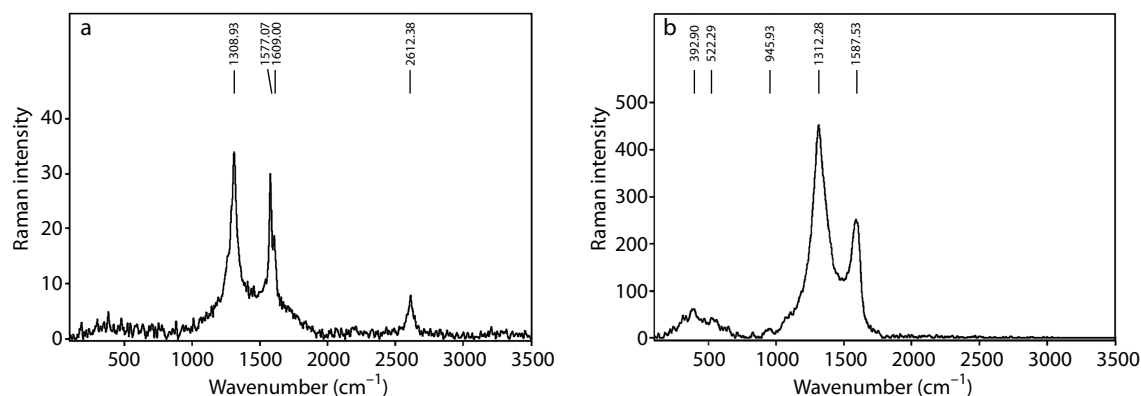


Fig. 1 Raman spectra of (a) graphite and (b) graphene oxide.

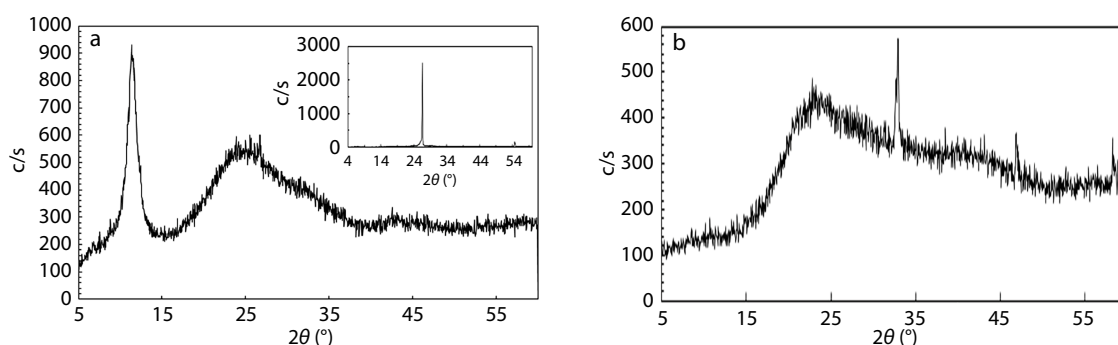


Fig. 2 XRD patterns of (a) graphene oxide and (b) MION/GO/CMC-g-PAA hydrogel.

FTIR Results

The FTIR spectrum of GO is shown in Fig. 3(a). The bands at 1184.08 and 1265.97 cm^{-1} correspond to C—O stretching vibration. The presence of a strong absorption band at 1623.77 cm^{-1} confirms the presence of non-oxidized C=C groups. The bands around 1710.55 and 3444.24 cm^{-1} are due to C=O and O—H stretching vibration, respectively.^[27,28] The FTIR spectrum of CMC is shown in Fig. 3(b). The band at 2921.63 cm^{-1} corresponds to a C—H stretching vibration, while the presence of a strong absorption band at 1625.70 cm^{-1} confirms the presence of —COO[−] groups. The bands at around 1430.92 and 1328.71 cm^{-1} are assigned to —CH₂— and —OH bending vibrations, respectively, and the band at 1106.94 cm^{-1} is due to CH—O—CH₂ stretching vibration.^[29,30]

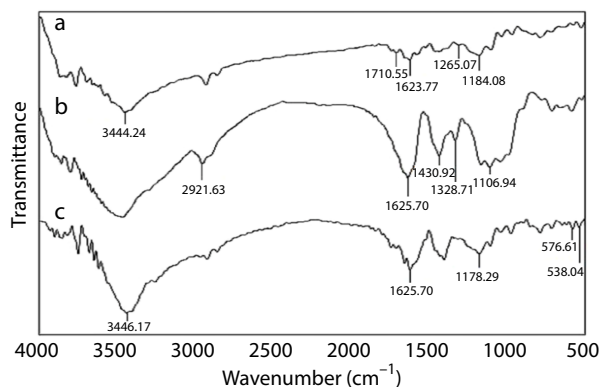


Fig. 3 FTIR spectra of (a) GO, (b) CMC and (c) MION/GO/CMC-g-PAA hydrogel.

As seen in Fig. 3(c) for optimized nanocomposite, the band at 580.47 cm^{-1} is the stretching mode due to the interactions of Fe—O—Fe in Fe₃O₄. The band at 1039.44 cm^{-1} displays the stretching vibrations of the C—O—C bond. The Fe—O stretching mode (539.97 cm^{-1}), O—H stretching vibration (3446.17 cm^{-1}), and O—H deformed vibration (1623.77 cm^{-1}) were observed for optimized hydrogel.^[31]

Thermal Gravimetric Analysis

TGA curves of CMC, GO, graphite, and MION/GO/CMC-g-PAA hydrogel in nitrogen atmosphere are shown in Figs. 4(a) and 4(b). As seen in Fig. 4(a), the graphite shows almost a flat TGA curve, exhibiting little mass loss from room temperature to 700 °C. In contrast, during the same temperature range, GO lose 60% of its initial mass. The first mass loss of 10% is found below 100 °C, which is attributed to the loss of water molecules absorbed into the GO bulk material. The main peak observed at around 210 °C, representing the major mass loss due to pyrolysis of the labile oxygen-containing functional groups, yielding CO, CO₂, and steam.^[32] The pursuant mass loss at above 250 °C is due to the elimination of remaining functional groups. Therefore, one can estimate the oxygen-containing functional groups of the dried GO to be nearly 58% after the deletion of water from GO (Fig. 4a).^[33]

The main decomposition of the polysaccharides (like CMC) usually starts above 250 °C. As seen in Fig. 4(b), the first stage was attributed to the desorption of water molecules as humidity from the polysaccharide structure. The second and third stages of decomposition occur at 250 and 340 °C, respectively, possibly owing to depolymerization with the formation of H₂O, CO, CO₂, and CH₄. Furthermore, as there are

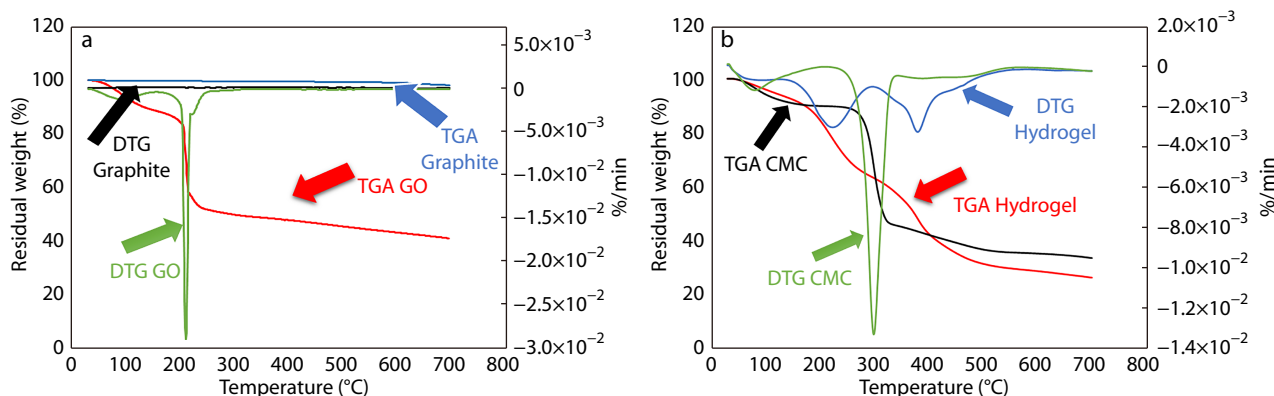


Fig. 4 TGA curves of (a) graphite and GO, and (b) CMC and MION/GO/CMC-g-PAA hydrogel.

many COO^- groups in the CMC chain, it could also be decarboxylated in this temperature range.

Fig. 4(b) also shows the thermogravimetric curve of MION/GO/CMC-g-PAA hydrogel. At the primary stage, the minor weight loss of about 7% from 63 °C to 154 °C is again attributed to the dehydration of hydrogel nanocomposite. The weight loss of about 19% within the temperature of 154–238 °C might be due to the elimination of CO_2 molecules from the polymeric backbone.^[34] The temperature at which maximum degradation occurs, T_{max} has been found to be 380.22 °C.

Differential Scanning Calorimetry

As is well-known, DSC experiments could reveal first- and second-order thermal transitions of the polymers such as glass transition (T_g), crystallization (T_c), and melting (T_m) temperature.^[35] As shown in Fig. 5, the first point at 94 °C in both NaCMC and MION/GO/CMC-g-PAA hydrogel curves are related to the complete loss of water molecules. The second transition at 280 °C in the NaCMC is possibly owing to the partial oxidation of the sugar ring on the polymer chains.^[36] In MION/GO/CMC-g-PAA hydrogel, the second peak (220 °C) corresponds to the liberation of water due to the chemical transformation of acid groups to esters *via* cyclization. The third endothermic peak (380 °C) may be related to the decarboxylation of grafted polyacrylate chains.

Vibrating Sample Magnetometer

For measuring the magnetic properties of the nanoparticles,

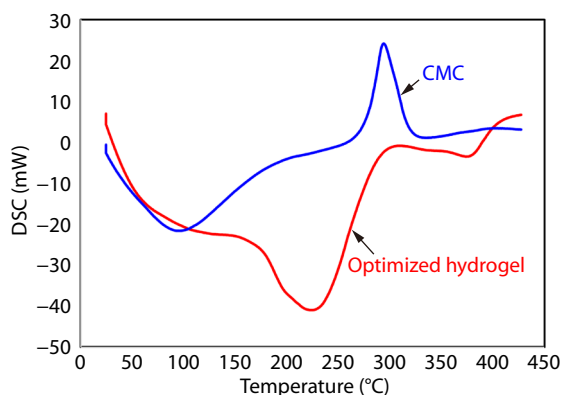


Fig. 5 DSC thermograms of CMC and MION/GO/CMC-g-PAA hydrogel.

a vibrating sample magnetometer (VSM) was used. Fig. 6 shows the hysteresis loops of the MION/GO/CMC-g-PAA hydrogel at room temperature. A symmetrical sigmoidal shape of the magnetization curve without any hysteresis in the magnetization demonstrated that the nanocomposite hydrogel showed superparamagnetic behavior. These results suggest that the MION/GO/CMC-g-PAA hydrogel matrix acts as an effective stabilizer and prevents agglomeration phenomena during nanoparticles synthesis, which could have led to the formation of larger particles, resulting in a deviation from superparamagnetism. These magnetic properties are critical for application in biomedical and bioengineering.^[37,38]

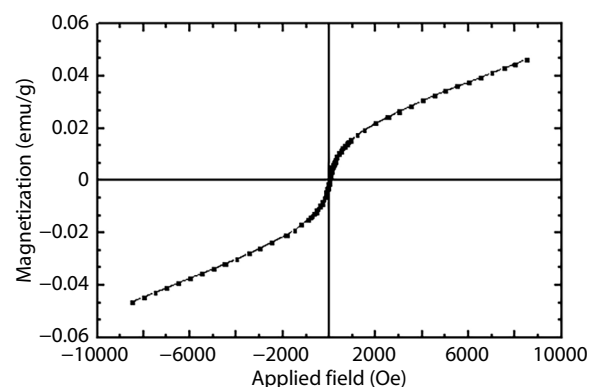


Fig. 6 Magnetizations curve for MION/GO/CMC-g-PAA hydrogel.

Scanning Electron Microscopy

The surface morphology of the MION/GO/CMC-g-PAA hydrogel is shown in Figs. 7(a)–7(d) at four magnifications. The porous structure of the nanocomposite allows better interaction of the hydrophilic groups of the polymer with external stimuli and consequently better permeation of water molecules within the hydrogel. At higher magnifications, some nanosized species can be seen at the surfaces of the hydrogel.

Because of the large-scale bar of SEM images, they were not able to show MIONs on the surface of the nanocomposite. Therefore, for further confirmation of the presence of the Fe_3O_4 in the nanocomposite, the energy-dispersive spectra (EDS) from the sample were recorded. The EDS spectrum in Fig. 7(e) shows the presence of Fe and O elements in the sample which further confirms that the nanoparticles at-

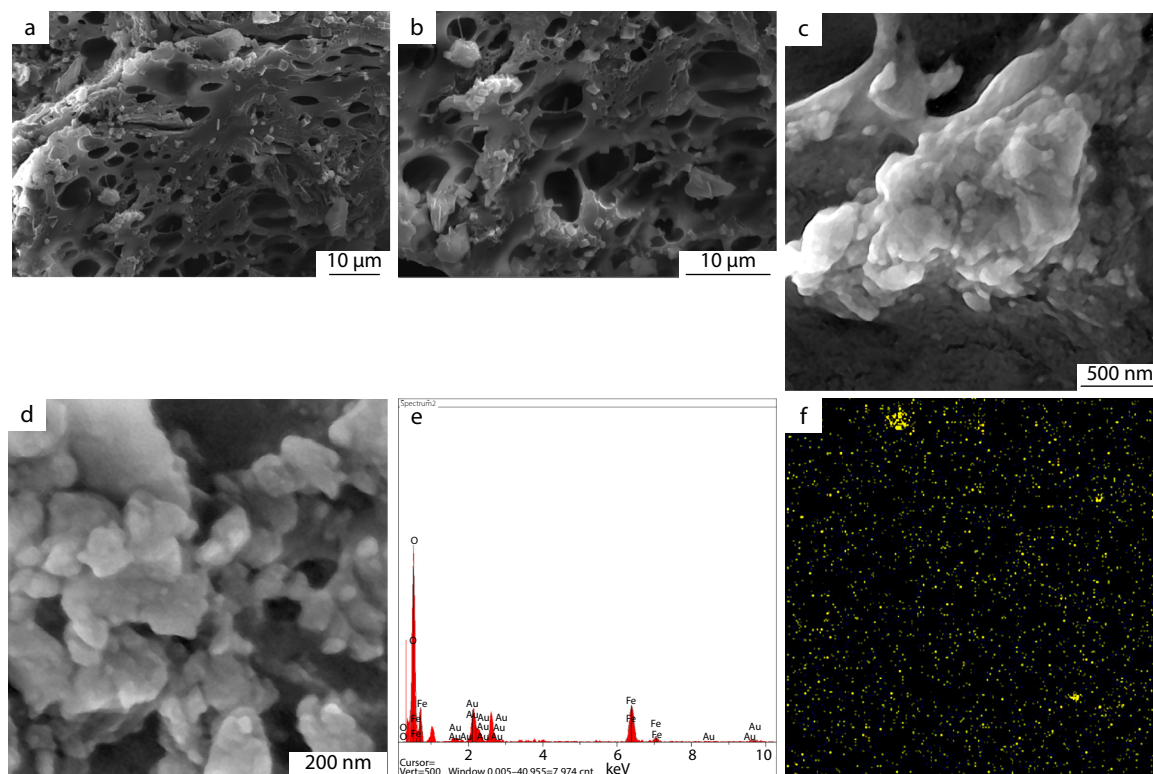


Fig. 7 SEM images in four magnifications $\times 3$ k (a), $\times 5$ k (b), $\times 60$ k (c) and $\times 120$ k (d), EDX spectrum (e) and EDX mapping of Fe element (f) of MION/GO/CMC-g-PAA hydrogel.

tached to the MION/GO/CMC-g-PAA hydrogel surface are Fe_3O_4 .^[29,39] In order to confirm the dispersion of Fe_3O_4 nanoparticles in the optimized hydrogel framework, we performed an elemental mapping analysis of Fe for the nano-composite hydrogel shown in Fig. 7(f).

Atomic Force Microscopy (AFM)

AFM imaging was used to provide more reliable measures of the morphology and size distribution. Figs. 8(a) and 8(b) show contact-mode AFM images of GO and MION/GO/CMC-g-PAA hydrogel. As is seen, they represent the spherical morphology with a smooth surface. The three-dimensional AFM micrographs of the surface of GO and MION/GO/CMC-g-PAA hydrogel are shown in Figs. 8(c) and 8(d). The porous structures of the hydrogel in comparison with GO are supported by AFM images. From the histogram curve, the average particle sizes for graphene oxide and optimized hydrogel were about 35 and 27 nm, respectively (Figs. 8e and 8f).

Optimization of Hydrogel

Effect of crosslinker concentration on the swelling

As is well known in hydrogels, higher crosslinking density decreases the space between the copolymer chains, and therefore, the resulted highly crosslinked rigid structure cannot be expanded and hold a large quantity of water. According to Table 1, 11.7 mmol of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ provides the best values for swelling. At a lower amount, the gel content is diminished appreciably because of the very loosely crosslinked network formation, resulting in swollen hydrogels with poor dimensional stability.^[40]

Effect of initiator concentrations

Water absorbency values of hydrogels can be affected by initiator concentration. This was studied by varying the APS concentration from 2.18 mmol to 7.67 mmol. The maximum swelling (318.23 g/g) was obtained at 6.57 mmol of APS (Table 1). Less than that, the resulted polymers were completely soluble. An increased number of active free radicals on the carbohydrate backbone may be ascribed to initial enhancement in water absorption. Following the decrease in swelling is originated from an increase in terminating reaction step and the free radical degradation of carbohydrate backbones by sulfate radical-anions.^[17]

Effect of monomer concentration

The relationship between monomer concentration and water absorption was studied by varying the AA concentration from 346.93 mmol to 1040.80 mmol. The maximum swelling (318.23 g/g) was obtained at 867.33 mmol of AA (Table 1). With increasing the AA concentration, the initial increment in swelling values took place due to the higher hydrophilicity of the hydrogel. The swelling-loss after the maximum may be originated from (a) the increased chance of chain transfer to AA molecules, (b) an increase in viscosity of the reaction that restricts the movement of the reactants and deactivates the macro radical growing chains soon after their formation, and (c) the enhanced homopolymerization reaction over graft copolymerization. Similar conclusions were reported by other investigators.^[17]

Swelling Kinetics

In practical applications, not only a higher swelling capacity is required, but also a higher swelling rate is needed. Fig. 9

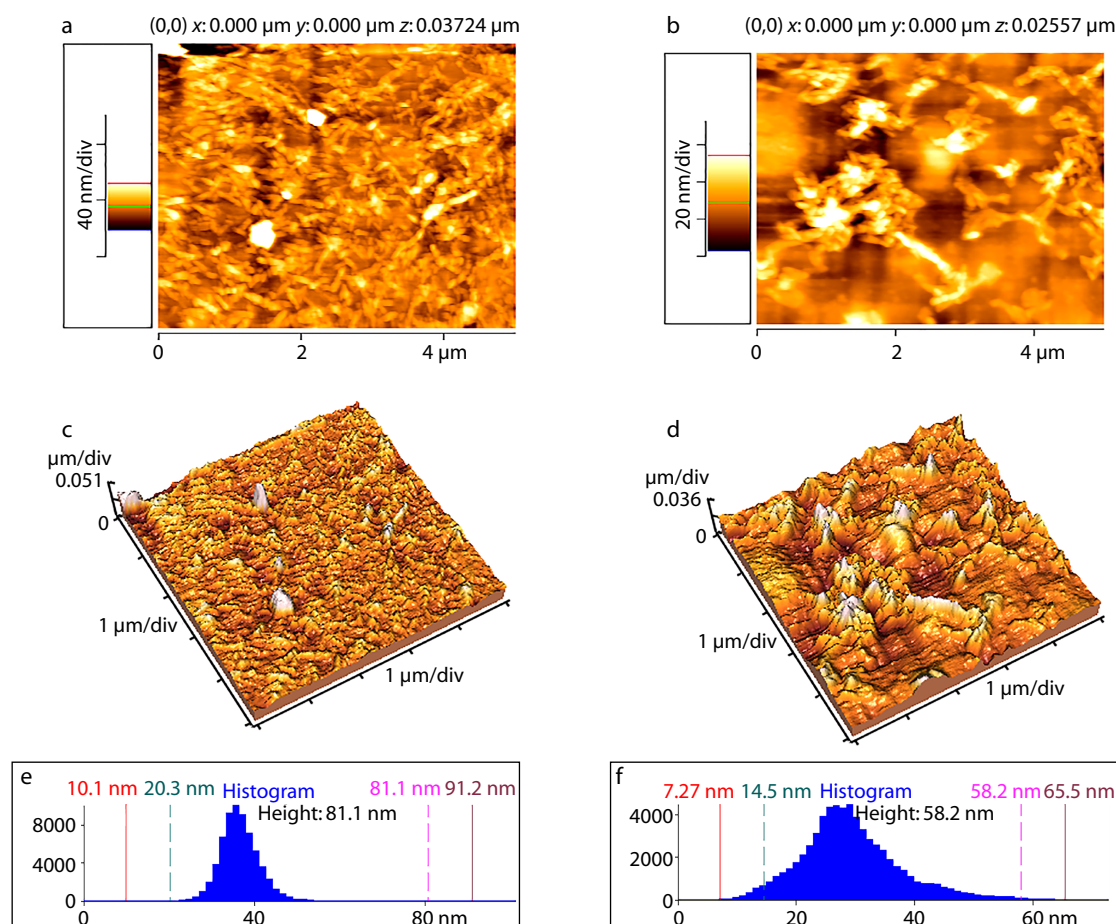


Fig. 8 (a, b) AFM topographical and phase images, (c, d) three dimensional topographic images and (e, f) histogram curves of GO and MION/GO/CMC-g-PAA hydrogel, respectively.

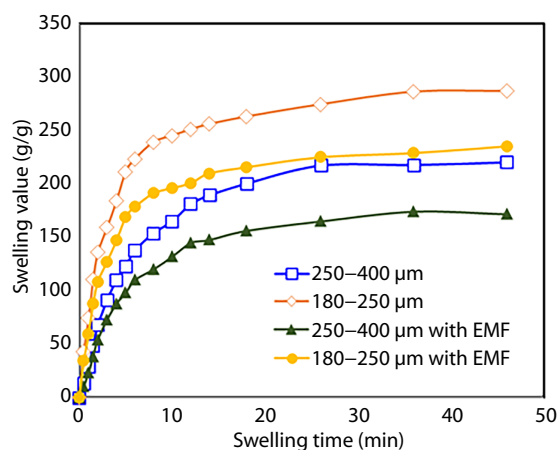


Fig. 9 Swelling kinetic of MION/GO/CMC-g-PAA hydrogel with different particle sizes in the absence and presence of EMF.

represents the dynamic swelling behavior of MION/GO/CMC-g-PAA hydrogel with various sizes in the absence and presence of the external magnetic field (EMF). Initially, the rate of water uptake sharply increases and then begins to level off. The time required to reach equilibrium swelling capacity was ~ 20 min. Power-law behavior is obvious from this figure. The data may be well fitted with a Voigt-based equation (Eq. 2):

$$S_t = S_e \left(1 - e^{-\frac{t}{\tau}} \right) \quad (2)$$

where S_t (g/g) is swelling at time t ; S_e , equilibrium swelling (power parameter, g/g), is swelling at the infinite time or maximum water-holding capacity; t is time (min) for swelling S_t , and τ (min) stands for the rate parameter. To find the rate (τ) and power (S_e) parameters for the sample, the data obtained from swelling of the nanocomposite hydrogel at consecutive time intervals were fitted to Eq. (2) using Origin 2015 software.^[2] The τ and S_e parameters for MION/GO/CMC-g-PAA hydrogel in absence of EMF are found to be 6.06 min, and 215 g/g for a sample with particle sizes of 250–400 μm , and 3.27 min, and 269 g/g for a sample with particle sizes of 180–250 μm , respectively. These parameters in the presence of EMF are 7.21 min, 163 g/g for a sample with particle sizes of 250–400 μm , and 4.02 min, 221 g/g for a sample with particle sizes of 180–250 μm , respectively.

As can be seen, in the presence of EMF, the swelling capacity of MION/GO/CMC-g-PAA hydrogel is lower, which is probably due to attract of ION adjacent neighbors under EMF. This can be reduced distances between the IONs as a result of attraction force induced by a given EMF. This phenomenon cause production of close-packed shells of ION which can block the diffusion of water into MION/GO/CMC-g-PAA hydrogel.^[41]

Swelling in Salt Solutions

The swelling behavior of the MION/GO/CMC-*g*-PAA hydrogel in different concentrations of NaCl, CaCl₂, and AlCl₃ was executed and results are represented in Fig. 10. As is well-known, the swelling capacity of MION/GO/CMC-*g*-PAA hydrogel in these salt solutions decreased with increasing the salt concentration. The charge screening effect is the main explanation for the intense loss of swelling due to the osmotic pressure discrepancy between the hydrogel and exterior solution. Also, due to the complexation of carboxylate groups with multivalent cations, which could increase the crosslinking density of hydrogel, and swelling capacity decreases rapidly as the hydrogel nanocomposite was transferred into the aforementioned saline. This might cause a hydrogel network shrinking.^[26]

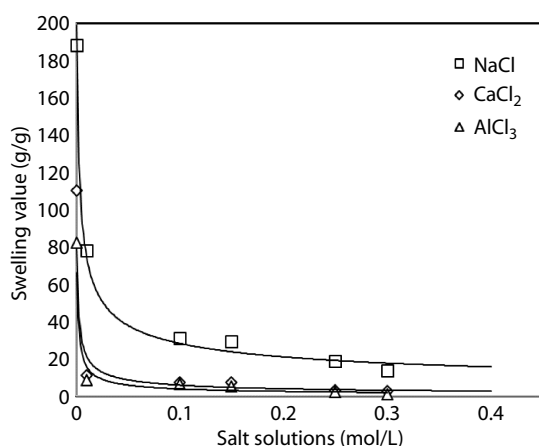


Fig. 10 Swelling capacity of MION/GO/CMC-*g*-PAA hydrogel in different salt solutions.

Effect of pH on Equilibrium Swelling

The effect of pH on the swelling behavior of the MION/GO/CMC-*g*-PAA hydrogel was also tested in pH=2–12 (Fig. 11). As can be seen, the absorption of the hydrogel increases extremely as the pH increases from 2 to 4 and markedly decreases in the pH range of 10–12. Also, the changes in the water absorbency are inconsiderable from pH 4 to 10. In acidic pHs, most of the —COOH groups are in their protonated form and hydrogen bonds between ether and carboxyl groups can promote polymer collapse.^[34] This can limit the water absorption by the carboxyl groups leading to diminution of swelling. At higher values of pH (5–7), most of the carboxylate groups are ionized and the electrostatic repulsion between —COO[−] groups causes an enhancement of the swelling capacity. Consequently, the charge screening effect of the counterion on the polyanion chain leads to a reduction in the expansion of the network at pH >8. Therefore, water absorbency is reduced.^[18]

To more investigation, this experiment was also repeated in the presence of EMF, and as shown in Fig. 11, the same swelling behavior of MION/GO/CMC-*g*-PAA hydrogel has happened. Again, the effect of attraction of ION adjacent neighbors under EMF can reduce the swelling capacity of MION/GO/CMC-*g*-PAA hydrogel in any pHs in the presence of EMF.

pH-Sensitivity

Regarding the main difference of swelling of MION/GO/CMC-*g*-PAA hydrogel at pH=2 and pH=8, the pH-dependent swelling

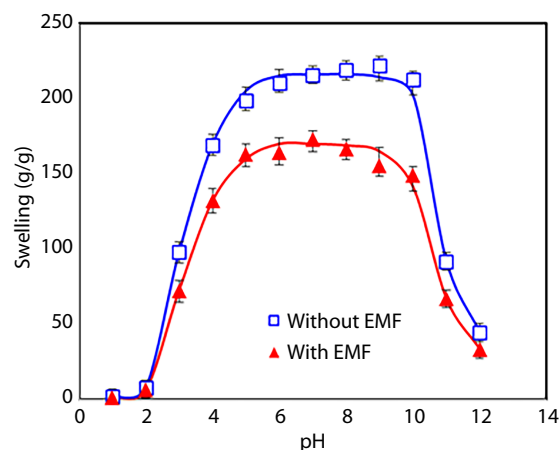


Fig. 11 Swelling equilibrium of MION/GO/CMC-*g*-PAA hydrogel in solutions with different pH values in the absence and presence of EMF.

reversibility was investigated (Fig. 12). MION/GO/CMC-*g*-PAA hydrogel was first swollen at pH=8 due to anion-anion repulsive electrostatic forces and then, at pH=2, it collapsed within a few minutes due to protonation of the carboxylate and formation of the hydrogen bond between —OH and —COOH groups. After three on-off cycles, the hydrogel still shows a good response to environmental pH stimulants. This behavior makes the MION/GO/CMC-*g*-PAA hydrogel a good candidate for drug delivery.^[26] For more investigation, this experiment was also repeated in the presence of EMF, and as expected, the same swelling behavior of MION/GO/CMC-*g*-PAA hydrogel has happened. Again, the effect of attraction of ION adjacent neighbors under EMF can reduce the swelling capacity of MION/GO/CMC-*g*-PAA hydrogel in any pHs in the presence of EMF.

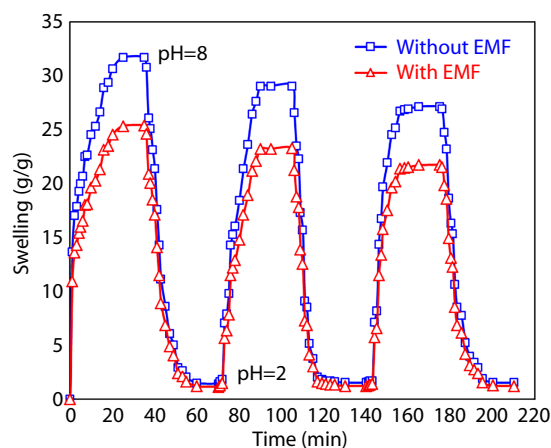


Fig. 12 On-off switching behavior of MION/GO/CMC-*g*-PAA hydrogel as reversible pulsatile swelling (pH=8) and deswelling (pH=2) in the absence and presence of EMF.

Absorbency under Load (AUL)

The swelling behavior of the MION/GO/CMC-*g*-PAA hydrogel under load is an important characteristic for its potential application in the soil medium. To determine the swollen gel strength, the swelling kinetics were measured under different loads in NaCl solution as swelling medium (Fig. 13). For further

investigation, this experiment was also repeated in the presence of EMF, and the same behavior of swelling of MION/GO/CMC-*g*-PAA hydrogel happened. Due to the greater depletion of absorbed water at applied pressure, the swelling of MION/GO/CMC-*g*-PAA hydrogel is reduced in both cases. The obtained data were fitted to Eq. (2) using Origin 2015 software and the τ and S_e parameters for MION/GO/CMC-*g*-PAA hydrogel are found to be 1.27 min, and 24.23 g/g in absence of EMF and 1.36 min, and 19.41 g/g in presence of EMF, respectively.^[17]

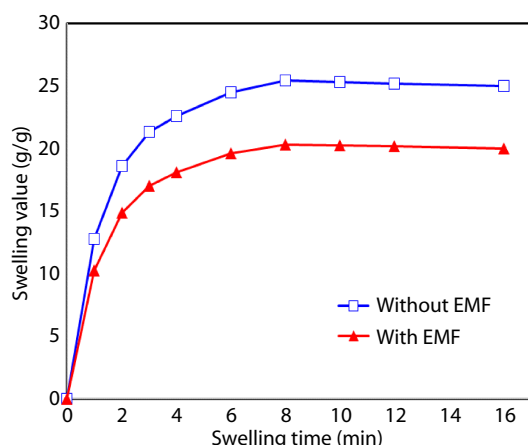


Fig. 13 Time dependence of the AUL values for MION/GO/CMC-*g*-PAA hydrogel swollen in saline solution without and with EMF.

DOX Release

In this work, the release of DOX was studied as an anticancer drug at different pHs with and without magnetic fields at 37°C. The release of DOX from MION/GO/CMC-*g*-PAA hydrogel in pH=5.4 and 7.4, is shown in Fig. 14. As can be seen in Fig. 14, the amount of released DOX from MION/GO/CMC-*g*-PAA hydrogel in pH=5.4 solution is higher than the release of drug in pH 7.4 solution (19.5% release at pH=7.4 compare to 70% at pH=5.4 during 12 h). Due to the production of lactic acid inside cells, the extracellular pH of cancerous cells is acidic,^[42] and therefore the higher release at pH=5.4 is a good sign for the successful application of entitled MION/GO/CMC-*g*-PAA hydrogel as a carrier for DOX in cancer treatment. Considering the unique magnetic responsibility of MION/GO/CMC-*g*-PAA hydrogel, the effect of EMF on the DOX release was investigated in both pHs at 37 °C. The DOX release from MION/GO/CMC-*g*-PAA hydrogel in the presence of EMF is lower than that in the absence of EMF which indicates the diffusion of the drug from MION/GO/CMC-*g*-PAA hydrogel is controlled by its swelling behavior (Fig. 14). In general, the higher swelling behavior of MION/GO/CMC-*g*-PAA hydrogel can be connected with the more drug release.

To analyze the release mechanism, a simple semi-empirical equation, presented by Peppas, had been adopted:^[41]

$$\log\left(\frac{M_t}{M_\infty}\right) = \log k + n \log t \quad (3)$$

where M_t/M_∞ represents the amount of drug diffused into the solution at time t and infinite time (at equilibrium), respectively, k is the rate constant, and n is the diffusion exponent characteristic of the release mechanism. The values of k and n are found by fitting the data to the above expression. The

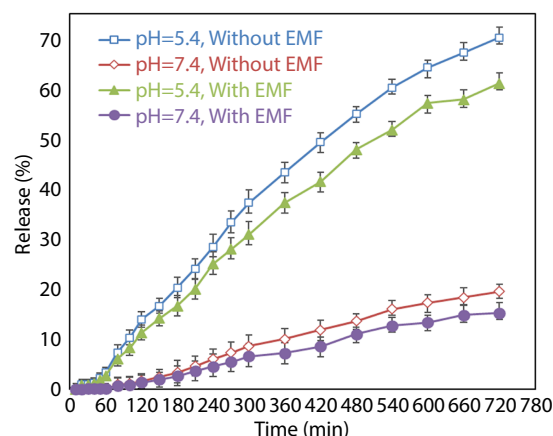


Fig. 14 The release behaviors of DOX from DOX-loaded MION/GO/CMC-*g*-PAA hydrogel at pH=5.4 and pH=7.4.

classification of drug release from a cylindrical geometry is as follows: (a) values of n of 0.45 (or less) correspond to a purely Fickian diffusion mechanism; (b) n values between 0.45 and 0.89 indicate an anomalous diffusion mechanism, and (c) values of n greater than 0.89 indicate a relaxation-controlled mechanism. The constant $\log k$, the exponent n estimated, and coefficient of determination (R^2) from MION/GO/CMC-*g*-PAA hydrogel in pH=5.4 at 37 °C with and without EMF were obtained and the results are shown in Table 2. It can be seen that the diffusion exponents, n , for the MION/GO/CMC-*g*-PAA hydrogel in both cases (in the presence and absence of EMF) are greater than 0.89. So, the drug release mechanism is relaxation-controlled.

Table 2 Release exponent (n), rate constant ($\log k$), and correlation coefficient (R^2) for DOX release from MION/GO/CMC-*g*-PAA hydrogel in pH=5.4 at 37 °C in the presence and absence of EMF.

Condition	$\log k$	n	R^2	Drug release mechanism
Absence of EMF	-6.158	1.37	0.968	Controlled by polymer relaxation
Presence of EMF	-6.252	1.389	0.969	Controlled by polymer relaxation

Cytotoxicity and Cell Proliferation Results

The cytotoxic effect of the hydrogel in L929 cells was investigated by the MTT assay. L929 is regarded as one of the most suitable cell lines^[43] for cytotoxicity studies. L929 cells were incubated with MION/GO/CMC-*g*-PAA hydrogel for 24 h and examined for any signs of cytotoxicity by the MTT assay. The results show that no toxicity occurred even with relatively high concentrations of MION/GO/CMC-*g*-PAA hydrogel. Moreover, after 24 h the cell viability in the presence of hydrogel proved to be significantly (8 times to 10 times) higher than that in the control sample. Also, increasing the amount of MION/GO/CMC-*g*-PAA hydrogel enhanced cell proliferation, but not significantly (Fig. 15).

Fibroblast cells were found in the majority of tissues but exhibit different gene expression patterns.^[44] It has been reported that allogeneic fibroblast cells are silently replaced by host cells. Moreover, there is no evidence of rejection clinically or immunologically in the patients which received allogeneic cells.^[45]

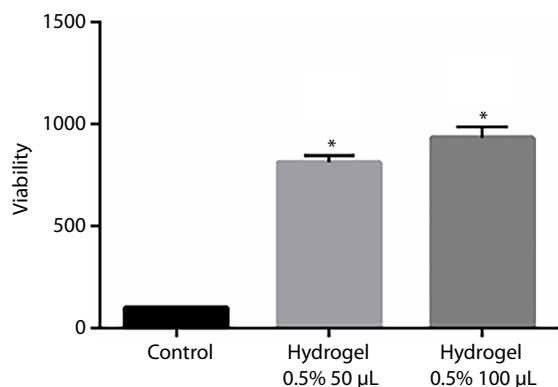


Fig. 15 Viability evaluation in fibroblast L929 cells after treatment with hydrogel (data were analyzed by one way ANOVA). Hydrogel 0.5% elevated cells proliferation significantly compared to control ($p < 0.05$). Increasing the amount of hydrogel increased proliferation however was not significant ($p < 0.05$).

On the other hand, fibroblast density is an important factor in the differentiation and development of the epidermal layer.^[46] According to this study, this factor can be crucially enhanced in the presence of MION/GO/CMC-*g*-PAA hydrogel. In conclusion, the introduced hydrogel in this study, also a drug delivery agent, can provide the necessary elements for tissue regeneration in wound healing and tissue engineering.

CONCLUSIONS

In this study, MIONS-CMC hydrogel nanocomposites were successfully prepared in a facile one-pot strategy without using the usual chemical crosslinking. These novel hydrogel nanocomposites could be potentially applied to tissue engineering and biomedical fields for their good biocompatibility, biodegradability, and structural properties. SEM photographs showed that the MIONS-CMC hydrogel nanocomposite has regular porous networks of sponge-like structure. The *in vitro* biocompatibility test of the MIONS-CMC hydrogel showed that the MIONS-CMC hydrogel has good biocompatibility and can be applied in biomedical areas as suitable soft tissue filler and as an appropriate matrix for stimuli sensitive delivery of doxorubicin anticancer drug.

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