ORIGINAL RESEARCH ARTICLE

Synthesis and characterization of new derivatives of alginic acid and evaluation of their iron(III)-crosslinked beads as potential controlled release matrices

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Abstract

Context: The excellent gelling and safety profiles of alginic acid combined, however, with drawbacks of its ionotropically crosslinked beads (i.e. their quick release of loaded drugs) prompted us to chemically modify alginic acid.

Objective: Alginic acid was chemically conjugated with four amines of varying hydrophilic-hydrophobic properties (i.e. tris(hydroxymethyl)methyl-, allyl-, benzyl- or pentyl-amines) in an attempt to enhance the drug release profiles from respective metal crosslinked beads.

Materials and methods: Chemical conjugation procedures were performed using dicyclohexylcarbodiimide as a coupling agent and the resulting new derivatives were characterized using proton nuclear magnetic resonance (^{1}H NMR), infrared (IR) spectroscopy and differential scanning calorimetry (DSC). These modified polymers were used to prepare iron (III)-crosslinked beads loaded with folic acid as model drug, which were tested *in vitro* to assess their folic acid release profiles.

Results and discussion: Interestingly, the resulting beads accessed enteric release kinetics, with tris(hydroxymethyl)methyl-amide alginic conjugate producing most pronounced enteric profile.

Conclusion: The results suggest the possibility of achieving controlled drug release from alginate-based beads via facile chemical modification of alginic acid.

Introduction

Alginic acid is a natural anionic biopolymer consists of two uronic acid monomers; namely, mannuronic and guluronic acids[1]. Alginate polymers have been widely used for pharmaceutical and biomedical applications[2–7] owing to their attractive features such as biocompatibility, biodegradability and mucoadhesive properties[8–10]. In addition, alginates can form polymeric networks via simple complexation with divalent and trivalent metallic ions especially with calcium[11,12]. However, Ca-alginate complexes suffer from poor stability in physiologically relevant media (i.e. 0.1 M HCl, simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and 0.1 M NaCl solution) leading to extensive uncontrolled release of loaded drugs[13]. Such drawbacks limit the ability of Ca-alginate complexes as controlled release matrices especially for oral administration[14].

To overcome these drawbacks, researchers have focused on preparing stable complexes either by switching to other metal cations such as zinc, aluminum or iron or by synthetically modifying alginic acid in such a way to enhance the stability of its corresponding complexes[15,16].

Herein, four new derivatives of alginate polymers were prepared in an attempt to improve the stability of metal-crosslinked alginate beads and provide better control over drug release from these matrices. For that purpose, we chemically modified alginate polymers with hydrophilic (i.e. tris(hydroxymethyl)methyl-amine) and hydrophobic (i.e. allyl-, benzyl- or pentyl-amines) moieties and evaluated their potential to form stable complexes via crosslinking with iron (III) metal ions. We envisaged that the presence of tris(hydroxymethyl)methyl-amine will provide additional crosslinking between the hydroxyl groups and central iron ions and hence allow better control on loaded drug release (Figure 1). While the rational for using the hydrophobic substituents (i.e. allyl-, benzyl- or pentyl-amines) is to take advantage of their ability to hinder water diffusion into the polymeric matrix and subsequently modify drug release (Figure 2).

Folic acid was used as a model drug because it is cheap, easy to analyze and commercially combined with iron in dietary supplements[16]. Folic acid is water-insoluble vitamin essential for many biological functions, it alleviates potential risks of neural tube birth defects, colorectal cancer and cardiovascular diseases[17].

Methods

Materials

Sodium alginate (Hayashi Pure Chemical Industries Ltd., Japan), dicyclohexylcarbodiimide DCCI (Fluka, Switzerland),

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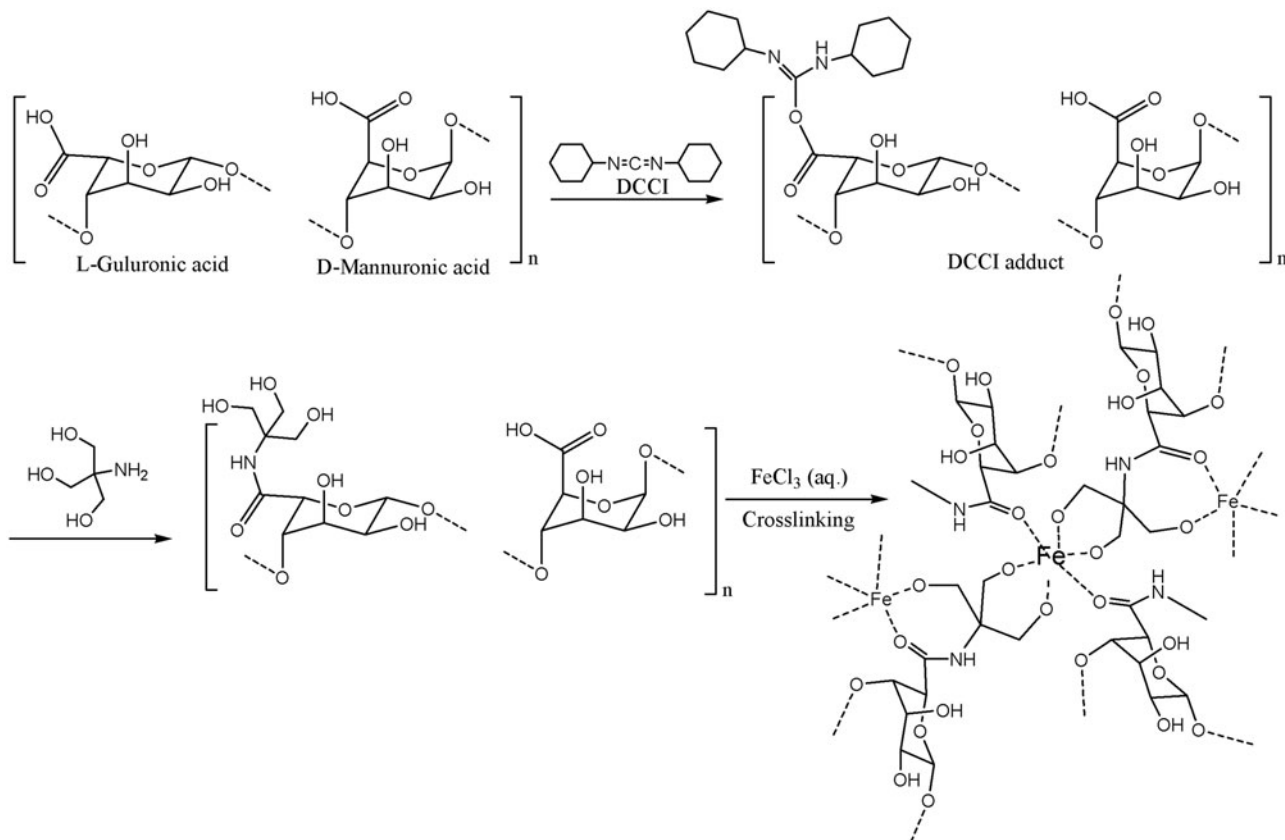
Figure 1. Synthesis of tris(hydroxymethyl)methyl-amide alginate and proposed structure of subsequent crosslinking with iron (III).
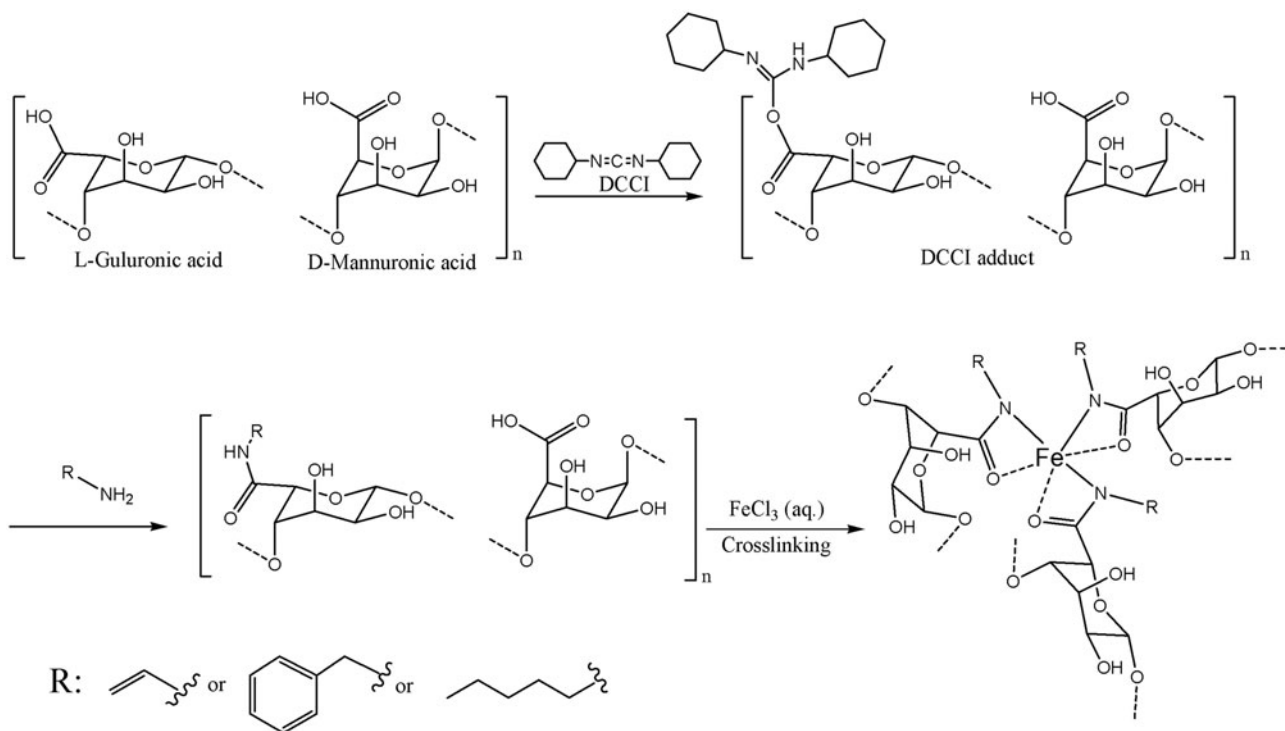

Figure 2. Synthesis of allyl-, benzyl- and pentyl-amide alginates and proposed structure of subsequent crosslinking with iron (III).

tris(hydroxymethyl)methyl-amine (Sigma-Aldrich, St. Louis, MO), allyl-amine (Sigma-Aldrich, St. Louis, MO), benzyl amine (Sigma-Aldrich, St. Louis, MO), pentyl amine (Sigma-Aldrich, St. Louis, MO), ferric chloride anhydrous LR (S.D. Fine-Chem.

Ltd., Boisar, India), potassium dihydrogen phosphate (S.D. Fine-Chem. Ltd., Boisar, India), potassium thiocyanate (Scharlau, European Union), folic acid (Sigma-Aldrich, St. Louis, MO), sodium hydroxide (Lanover House, UK). All solvents

(chloroform, acetone, ethanol, diethyl ether, pyridine, and hydrochloric acid) were of analytical grade (Gainland Chemical Company, UK). All chemicals were used as received.

Synthesis of *N*-allyl-, *N*-benzyl-, *N*-pentyl- and *N*-tris (hydroxymethyl)methyl-amide derivatives of alginic acid and determination of degrees of substitution.

For coupling polymeric carboxylic acids with amines, we used a method similar to those previously described in literature[15,16,18,19]. Sodium alginate (10.40 g, 0.05 mol carboxylate) in 900 ml of distilled water was stirred for one hour, the pH was then adjusted to 3–4 using hydrochloric acid (0.5 M). Thereafter, dicyclohexylcarbodiimide (DCCI, 0.05 mol, 10.32 g) was added. Two hours later, *N*-allyl-amine (0.1 mol, 5.7 g), *N*-benzyl-amine (0.1 mol, 10.7 g), *N*-pentyl-amine (0.1 mol, 8.7 g) or *N*-tris (hydroxymethyl)methyl-amine (0.1 mol, 12.1 g) was added to the reaction mixture. Subsequently, the pH of the reaction mixture was raised to 9 using sodium hydroxide solution (2.0 M). After 24 hours, the reaction was terminated by precipitation with hydrochloric acid (100 ml, 1.5 M) and acetone (1000 ml). The generated precipitate was filtered and thoroughly washed with acetone (3 × 250 ml), ethanol (3 × 250 ml) and diethylether (3 × 250 ml). The resulting mass was left to dry at room temperature overnight.

The degree of amine substitution on alginic acid was determined by back acid–base titration of the corresponding free carboxylate groups of each polymer. The particular polymer (0.1 g) was dissolved in 100 ml of 0.1 M NaOH solution. Subsequently, it was back titrated with 0.5 M HCl using few drops of phenolphthalein as endpoint indicator. The titration was repeated three times for each polymer. The degree of substitution (DS) is calculated from the following equation:

$$DS = 1 - \frac{\left\{ \begin{array}{c} \text{Number of COOH moles in alginic} \\ \text{acid derivatives} \end{array} \right\}}{\left\{ \begin{array}{c} \text{Number of COOH moles in} \\ \text{unsubstituted alginic acid} \end{array} \right\}} \times 100\% \quad (1)$$

where the number of COOH groups = number of moles of NaOH – moles of titrated HCl.

¹H NMR, IR and DSC characterization

Unmodified and modified polymers were characterized using ¹H NMR, IR and DSC. ¹H NMR spectra were recorded at 25 °C on a Varian NMR Spectrometer instrument operating at 300 MHz. Chemical shifts (δ) are reported in parts per million (ppm). The IR spectra were recorded on an 8400S SHIMADZU IR spectrophotometer using KBr discs. Thermograms were recorded on Mettler TOLEDO Star System. The tested samples were placed in aluminum pans and heated at a rate of 10 °C/min.

The corresponding iron-crosslinked polymer matrices were also characterized using IR and DSC as described above. Iron-crosslinked matrices were prepared from alginic acid and synthesized derivatives exactly the same way as their corresponding beads (see the following section), except that they were generated by adding $FeCl_3$ solution to the vigorously stirred polymer solution. The resulting solid masses were filtered, washed with water, and left to dry over few days.

Preparation of folic acid loaded iron-crosslinked polymeric beads

Folic acid-loaded iron-crosslinked alginate beads were prepared using a similar method to that described previously by our group[16,18] with slight modifications. Briefly, folic acid (250 mg) and the particular alginic acid derivative (sodium alginate, benzyl amide-, pentyl amide-, allyl amide- or tris(hydroxymethyl)methyl amide- alginate, 250 mg) were dispersed in 0.1 N sodium hydroxide solution (7.5 ml) and stirred for one hour. The resulting viscous suspension was dropped, using a plastic dropper, into an aqueous solution of ferric chloride (0.5%, w/v, 100 ml). The viscous droplets were left to cure in the ferric chloride solution over 40 minutes to generate dark yellow beads. Subsequently, the beads were filtered, washed with distilled water (2 × 50 ml) and dried at 40 °C over 48 hours. For comparison purposes, unloaded beads were prepared using the same method without the addition of folic acid.

Scanning electron microscopy

Morphology and surface microstructure of the prepared dry beads were examined using scanning electron microscopy (FEI Company – Inspect F50/FEG, the Netherlands). Randomly selected samples of each formulation were mounted onto aluminum stubs using double-sided adhesive carbon tape. After which samples were coated with gold particles for one minute using sputter coater (Quorum Technology, Ashford, UK) and finally examined under SEM using different levels of magnification power.

Determination of folic acid loading in beads

Total amount of folic acid encapsulated into polymeric beads was determined as follows; a specific amount of folic acid-containing beads (35 mg) was dispersed in phosphate buffer saline pH 6.8 (200 ml) and stirred over 24 hours at room temperature. Samples (3 ml) were withdrawn and filtered. The absorbance values of collected folic acid samples were evaluated at the corresponding λ_{max} wavelengths, that is, 283 nm using UV/VIS spectrophotometer (SpectroScan, 80D). The amount of folic acid was determined using an appropriate calibration curve of known concentrations of folic acid in the same media. Unloaded polymeric beads were used as blanks.

Swelling studies of beads

pH-dependent swelling studies were carried out for dry beads. For each class of polymeric beads, accurately weighed samples (ranging from 50 to 100 mg) were immersed in 100 mL of 0.1 M HCl media (pH 1, 37 °C) or phosphate buffer saline (pH 6.8, 37 °C) and shaken in water bath at 100 rpm (Heto Lab Equipment, Denmark) for 24 hours. At predetermined time intervals, beads were withdrawn from the swelling media, wiped gently with papers and weighed. Experiments were done in triplicate and average values were used to calculate dynamic weight change according to the following formula[19]:

$$\text{Weight change } \% = \frac{\text{Final weight} - \text{initial weight}}{\text{initial weight}} \times 100 \quad (2)$$

In vitro release of folic acid from beads

A rotating basket apparatus (Erweka dissolution tester) fitted with a 0.125-mm stainless steel basket was used. For each class of polymeric beads, 140 mg of dried beads were placed in the basket and rotated at 100 rpm. Two dissolution media were used over two subsequent stages: 0.1 M HCl media (pH 1.0, 900 ml, 37 °C) for two hours, followed with PBS media (pH 6.8, 900 ml, 37 °C) for six hours.

Samples (3 ml) were withdrawn at regular time intervals and immediately replaced with an equivalent volume of fresh medium. The absorbance values of folic acid samples collected from 0.1M HCl were measured at $\lambda_{max} = 296$ nm, while samples collected from PBS were measured at $\lambda_{max} = 283$ nm

using UV/VIS spectrophotometer (SpectroScan, 80D). Unloaded beads were used as blanks. The release experiments were done in duplicates and the average released folic acid was reported with the corresponding standard deviations. The concentrations were calculated from calibration curves of known concentrations of folic acid (for each pH medium). The amounts released were plotted against time for each class of polymeric beads.

Determination of iron (III) loading in beads

Total iron content per gram beads was determined as follows; a specific amount of unloaded beads (35 mg) was dispersed in phosphate buffer saline pH 6.8 (200 ml) and stirred over 24 hours at room temperature. Subsequently, the polymeric dispersion was filtered, and 2 ml of the filtrate was treated with potassium thiocyanate (20% v/v, 3 ml) and the resulting solution was diluted to 10 ml by 0.1 M HCl. The absorbance of the end solution was measured at 480 nm using UV/VIS spectrophotometer (SpectroScan, 80D). The amount of iron was determined using an appropriate calibration curve of known concentrations of iron treated with specific concentrations of potassium thiocyanate (20% v/v) in 0.1 M HCl.

Results

Synthesis and characterization of alginate derivatives

The conjugation reactions of alginic acid with different amines were achieved using dicyclohexylcarbodiimide (DCCI) to theoretically activate 100% of the available carboxylic acid groups. Despite the poor water solubility of DCCI, the fact that the reaction was carried out under excess reagent conditions (i.e. DCCI) means that the equilibrium will be driven toward the products despite poor water solubility[15,16,18,20]. The activation was carried out under acidic aqueous conditions (pH 3–4) to improve the reactivity of DCCI via imino-nitrogen protonation. Afterwards, *N*-allyl-amine, *N*-benzyl-amine, *N*-pentyl-amine or *N*-tris(hydroxymethyl)methyl-amine was added in excess to quench the reaction and form the amide bond. The amine reactants were added in excess to ensure the forwardness of the reaction under the sterically hindering environment of the poly-sugar. Later, the pH was raised to 9.0 to regenerate the nucleophilic amine from its hydrochloride salt. The reaction was later terminated by concentrated HCl to neutralize the basic conditions of the reaction medium and to convert a large proportion of the highly hydrated carboxylate anions into the less hydrated carboxylic acid form with the concomitant reduction of polymeric solubility in water. Eventually, the polymers were precipitated out of solution by the addition of acetone. Subsequently, the resulting alginate conjugates were thoroughly washed with ethanol to remove DCC urea, followed by washing with acetone to remove water and ethanol, and then by diethyl ether to remove any remaining water and to facilitate drying of the polymer. Figures 1 and 2 show the synthesis of different amide derivatives.

The degree of substitution for allyl-, benzyl-, pentyl- and tris(hydroxymethyl)methyl-amine conjugates were found to be 44%, 28%, 20%, and 37%, respectively. The resulting amide conjugates were characterized by NMR (Supplementary material), IR (Figure 3A–F) and DSC (Figure 4A–F). All of these findings are thoroughly discussed in the section of Discussion.

Preparation of iron-crosslinked beads loaded with folic acid and characterization of the corresponding iron-crosslinked polymeric matrices

Folic acid-loaded polymeric beads were prepared by dispersing equal amounts of the particular polymer and folic acid in 0.1 M

sodium hydroxide. The resulting viscous suspensions were dropped into ferric chloride solution to generate drug-loaded iron-crosslinked beads which were subsequently filtered, washed with water and dried at 40 °C for 48 hours. The resulting beads were characterized as shown in Table 1 and Figure 5. Detailed examination of surface microstructure of beads was characterized by scanning electron microscopy as shown in Figure 6.

We also implemented IR spectrophotometry and DSC to assess polymer–iron complexes, as in Figure 3(G–K) and Figure 4(G–K). All of these results are explained in the section of Discussion.

Swelling studies of beads

The swelling behavior of dry beads in 0.1 M HCl media and phosphate buffer saline is illustrated in Figure 7(A) and (B), respectively.

Folic acid loading and release profiles

Folic acid-loaded iron-crosslinked beads prepared from different polymers were compared in terms of folic acid loading (Table 1) and drug release profiles as shown in Figure 8.

Discussion

Synthesis and characterization of alginate derivatives

Figures 1 and 2 show the synthesis of different amide derivatives. The degree of substitution for allyl-, benzyl-, pentyl- and tris(hydroxymethyl)methyl-amine conjugates were found to be 44%, 28%, 20% and 37%, respectively. Apparently, the degree of substitution depends on the solubility of the particular amine in the reaction medium. Both allyl- and tris(hydroxymethyl)methyl-amines have higher aqueous solubility compared to benzyl- and pentyl-amines, which explains the higher degree of polymeric substitution for the former amines.

The resulting amide conjugates were characterized by NMR, IR and DSC. Figures in the supplementary materials show the NMR spectra of the new amidic conjugates. The NMR spectra of allyl-amide alginic conjugate showed three new peaks (i.e. compared to sodium alginate) at 4.50, 5.28 and 5.90 ppm corresponding to the newly introduced allylic, geminal and vicinal olefinic protons, respectively. On the other hand, the benzyl-amide alginic conjugate showed two new peaks (sharp and short) at 4.25 and 4.40 ppm corresponding to the geminal benzylic hydrogens, and a new broad band at 7.41 ppm corresponding to the newly introduced aromatic hydrogens. Moreover, the proton NMR spectra of the pentyl- and tris(hydroxymethyl)methyl-amide conjugates exhibited new intense peaks corresponding to the aliphatic chain of the pentyl-amide substituents (0.88, 1.30, 1.44 and 2.58 ppm) and the alcoholic CH_2 of the tris(hydroxymethyl)-methyl (3.52 ppm) moieties, respectively.

Additionally, we probed our semi-synthetic polymers using IR spectrophotometry, as in Figure 3(A–F). Clearly from the figure, the IR spectra of allyl-, benzyl-, pentyl- and tris(hydroxymethyl)-methyl-amide derivatives (Figure 3C–F) maintained the same carbonyl stretching vibrations seen with alginic acid (Figure 3A) (i.e. at 1739–1742 cm^{-1}), indicating the presence of remaining unsubstituted carboxylic acid groups. However, the new amidic conjugates exhibited additional carbonyl stretching bands ranging from 1626–1632 cm^{-1}, which undoubtedly correspond to the newly introduced amidic bonds[21]. These should be distinguished from the stretching vibrations of carboxylate groups seen in sodium alginate at 1617 cm^{-1} (Figure 3B).

Figure 4(A–F) shows the DSC thermograms of alginic acid, sodium alginate and the new amide conjugates. Clearly from the figure, the thermogram of alginic acid exhibits two significant endothermic bands, namely at 85 °C and 195 °C (Figure 4A).
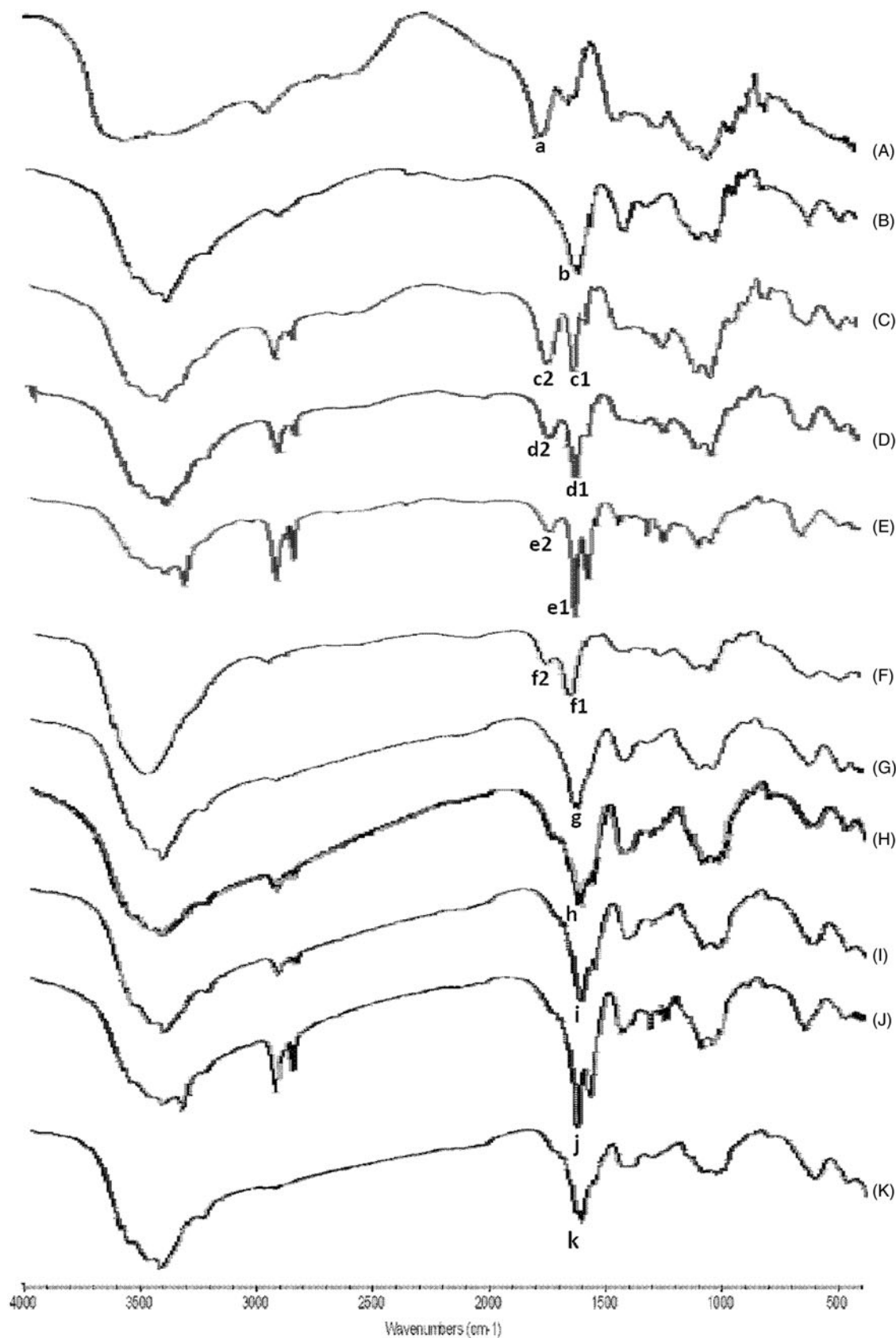
Figure 3. The IR spectrum of (A) alginic acid, $a = 1745\,\text{cm}^{-1}$; (B) sodium alginate, $b = 1617\,\text{cm}^{-1}$; (C) allyl-amide alginate, $c1 = 1627\,\text{cm}^{-1}$, $c2 = 1742\,\text{cm}^{-1}$; (D) benzyl-amide alginate, $d1 = 1627\,\text{cm}^{-1}$, $d2 = 1740\,\text{cm}^{-1}$; (E) pentyl-amide alginate, $e1 = 1626\,\text{cm}^{-1}$, $e2 = 1741\,\text{cm}^{-1}$; (F) tris(hydroxymethyl)methyl-amide alginate, $f1 = 1632\,\text{cm}^{-1}$, $f2 = 1739\,\text{cm}^{-1}$; (G) iron-crosslinked alginate complex, $g = 1617\,\text{cm}^{-1}$; (H) iron-crosslinked allyl-amide alginate complex, $h = 1622\,\text{cm}^{-1}$; (I) iron-crosslinked benzyl-amide alginate complex, $i = 1621\,\text{cm}^{-1}$; (J) iron-crosslinked pentyl-amide alginate complex, $j = 1626\,\text{cm}^{-1}$; (K) iron-crosslinked tris(hydroxymethyl)methyl-amide alginate complex, $k = 1617\,\text{cm}^{-1}$.
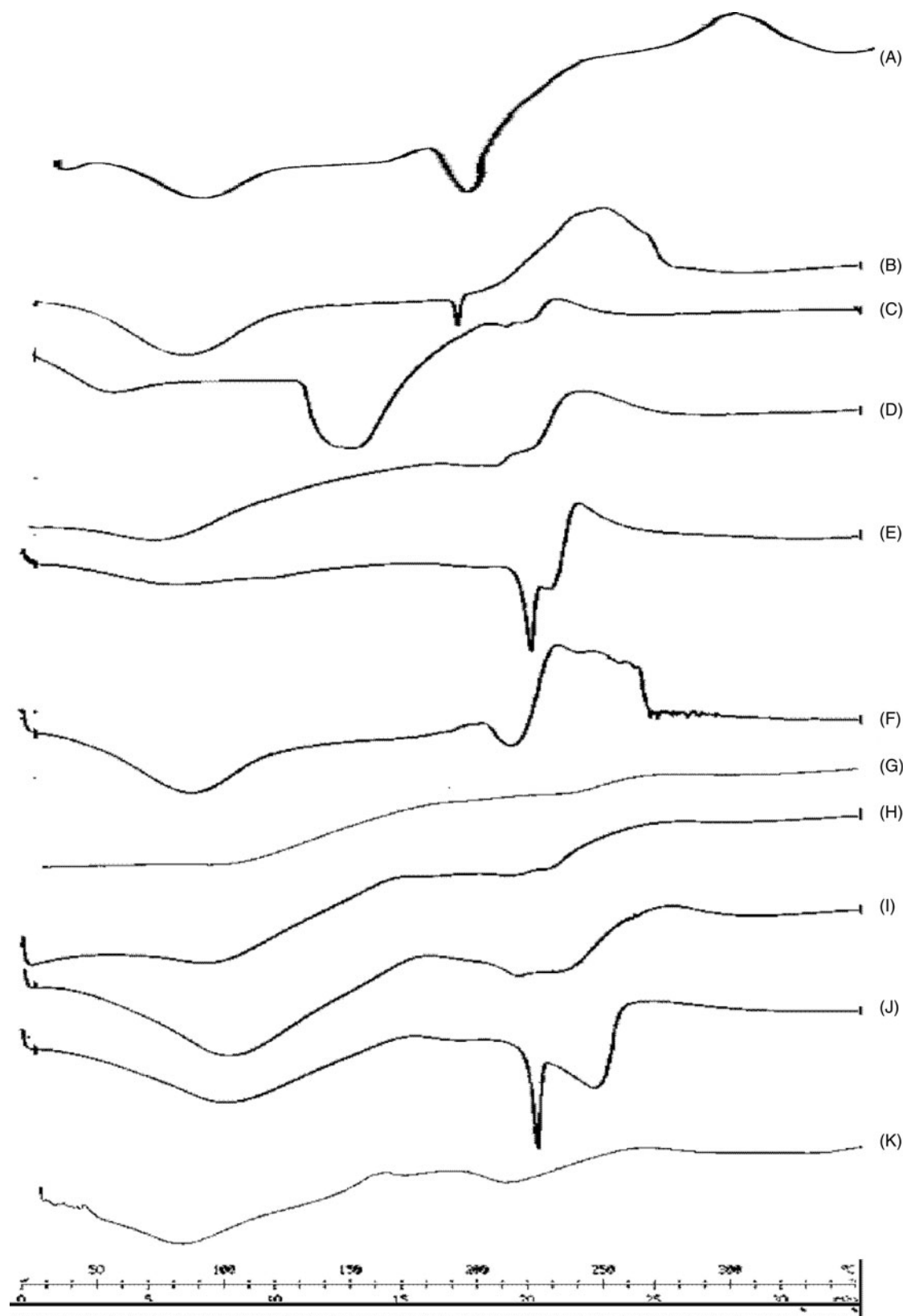
Figure 4. The DSC traits of (A) alginic acid, (B) sodium alginate, (C) allyl-amide alginate, (D) benzyl-amide alginate, (E) pentyl-amide alginate, (F) tris(hydroxymethyl)methyl-amide alginate, (G) iron-crosslinked alginate complex, (H) iron-crosslinked allyl-amide alginate complex, (I) iron-crosslinked benzyl-amide alginate complex, (J) iron-crosslinked pentyl-amide alginate complex, (K) iron-crosslinked tris(hydroxymethyl)methyl-amide alginate complex.

The first band corresponds to the heat of evaporation of the associated water of hydration, while the second band represents the heat required to break various intra- and intermolecular hydrogen bonding within the polymeric matrix (i.e. melting).

A third exothermic band appears at 305 °C related to polymeric degradation (e.g. oxidation)[11,18].

However, upon amidification, certain changes were observed in the thermograms of the semi-synthetic polymers. The first

Table 1. Characterization of folic acid loaded iron-crosslinked polymeric dry beads including color, size and weight with the corresponding folic acid and iron loadings[a].

Cross linked polymer	Color	Size (mm)[b] $\pm$ SD	Weight (mg)[c] $\pm$ SD	Drug loading (mg/g bead mass) $\pm$ SD	Iron loading (mg/g bead mass) $\pm$ SD
Sodium alginate	Black	1.7 ± 0.2	2.4 ± 0.1	610.4 ± 37.8	236.2 ± 14.4
Allyl-amide alginate	Yellow	5.0 ± 1.4	6.8 ± 0.9	596.4 ± 5.3	119.3 ± 22.9
Benzyl-amide alginate	Black	1.7 ± 0.3	3.3 ± 0.1	684.5 ± 2.8	182.7 ± 11.9
Pentyl-amide alginate	Yellow	4.2 ± 0.9	8.8 ± 1.0	595.9 ± 63.9	141.7 ± 19.7
Tris(hydroxymethyl) methyl-amide alginate	Brown	1.9 ± 0.2	5.2 ± 0.2	605.2 ± 3.8	142.2 ± 9.7

[a]Data represent the average and standard deviation of three measurements.
[b]Random sample of 30 beads were hand-picked and their sizes were visually measured using ruler (as in Figure 5). The average size (diameter) per bead was calculated ($\pm$standard deviation).
[c]Three random samples each of 20 beads were weighed and the average weight per bead was calculated. Then the three average weight measurements (per bead) were used to calculate the reported average weight per bead ($\pm$standard deviation).
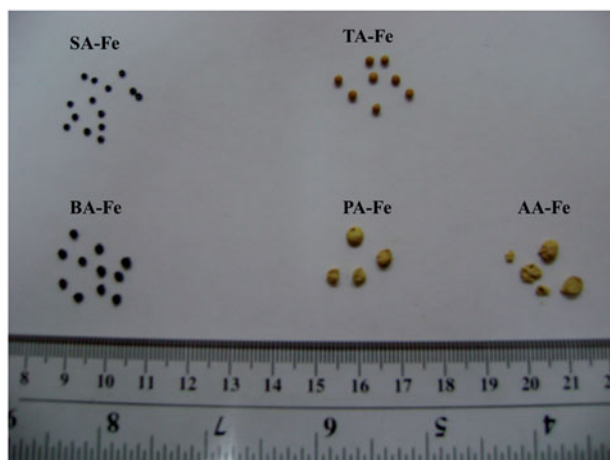
Figure 5. A photograph showing dried folic acid-loaded iron-crosslinked using different polymers. SA-Fe: folic acid-loaded iron-crosslinked Sodium Alginate beads; TA-Fe: folic acid-loaded iron-crosslinked Tris(hydroxymethyl)methyl-amide Alginate beads; BA-Fe: folic acid-loaded iron-crosslinked Benzyl-amide Alginate beads; PA-Fe: acid-loaded iron-crosslinked Pentyl-amide Alginate beads; AA-Fe: folic acid-loaded iron-crosslinked Allyl-amide Alginate beads.

change was the dramatic reduction of the intensity of the first endothermic band in the allyl-, benzyl- and pentyl-amide cases (Figure 4C, D and E). These bands became shallower and shifted to lower temperature values in a manner apparently proportional to their degree of hydrophobicity. In fact, this band nearly completely disappeared in the pentyl-amide conjugate (Figure 4E). On the other hand, this particular band intensified in the DSC profile of tris(hydroxymethyl)methyl-amide conjugate (Figure 4F), which correlates nicely with the hygroscopic nature of this hydrophilic substituent.

The second major change in the DSC profiles of amide conjugates is related to alginic acid's second endothermic band (*ca.* 195 °C). This band became broader and shifted to *ca.* 150 °C in the thermogram of the allyl conjugate (Figure 4C). We believe this happened because the newly introduced allyl groups disrupted hydrogen bonding networks within alginate matrix, leading to weaker and heterogeneous hydrogen bonds. On the other hand, this endothermic band was completely missing in the thermogram of the benzyl-amide conjugate (Figure 4D) presumably because the benzyl moieties greatly disrupted the inner hydrogen bonding networks within alginate matrix.

Surprisingly, pentyl-amide conjugation transformed this band into sharper and more intense peak at 220 °C (Figure 4E). We believe that the combination of pentyl substituents (more

hydrophobic than allyl and benzyl analogues) with ionized polymeric backbone seems to promote the formation of highly ordered, tightly hydrogen-bonded polymeric arrangement reminiscent of hydrophobic polyelectrolytes[22].

Finally, the thermogram of tris(hydroxymethyl)methyl-amide conjugate shared a melting band at *ca.* 215 °C with the pentyl-amide conjugate, albeit at broader temperature rang (Figure 4F). It seems that the flexible tri-hydroxyl substitution of tris(hydroxymethyl)methyl-amide groups promotes extensive, yet heterogeneous inner hydrogen bonding networks within alginate matrix leading to the observed broad endothermic band.

Preparation of iron-crosslinked beads loaded with folic acid and characterization of the corresponding iron-crosslinked polymeric matrices

Iron-crosslinked sodium alginate, tris(hydroxymethyl)methyl-amide and benzyl-amide conjugates succeeded in generating spherical beads of uniform shape that shrank by approximately ten folds upon drying. On the other hand, pentyl-amide and allyl-amide derivatives generated less regular beads in the curing solution that yielded larger matrices after drying (Table 1 and Figure 5). We believe this behavior is related to the observed lower solubility of the pentyl- and allyl-amide conjugates in the preparation solution. Apparently, such lower solubility arises from the hydrophobic nature of pentyl chains (i.e. within pentyl-amide conjugate) while probably due to the higher substitution degree (44%) of allyl substituents (i.e. within ally-amide conjugate). Poor aqueous solubility renders the corresponding aqueous polymeric suspension with low viscosity and tendency to disperse quickly upon dropping into the ionotropic curing solution. These findings came in agreement with the observed sizes and weights of the beads/matrices (Table 1 and Figure 5). The resulting beads/matrices were also different in color. Apparently, beads' colors correlate with iron content: Heavily iron-loaded beads were black (i.e. benzyl-amide and sodium alginate beads), while yellowish-colored beads (i.e. tris(hydroxymethyl)methyl-amide, pentyl-amide and allyl-amide beads) contained notably lesser iron contents.

Morphology and surface topography of dry beads were also evaluated using scanning electron microscopy as shown in Figure 6. SEM micrographs showed reasonable spherical shapes of beads prepared from sodium alginate, benzyl-amide and tris(hydroxymethyl)methyl-amide alginate conjugates (Figure 6A, D and G, respectively). Detailed examination of the surface microstructure revealed holes and cracks on the surface of iron-crosslinked beads prepared from sodium alginate (Figure 6B) and benzyl-amide alginate polymers (Figure 6E). Such cracks are probably due to partial collapse of polymeric network upon
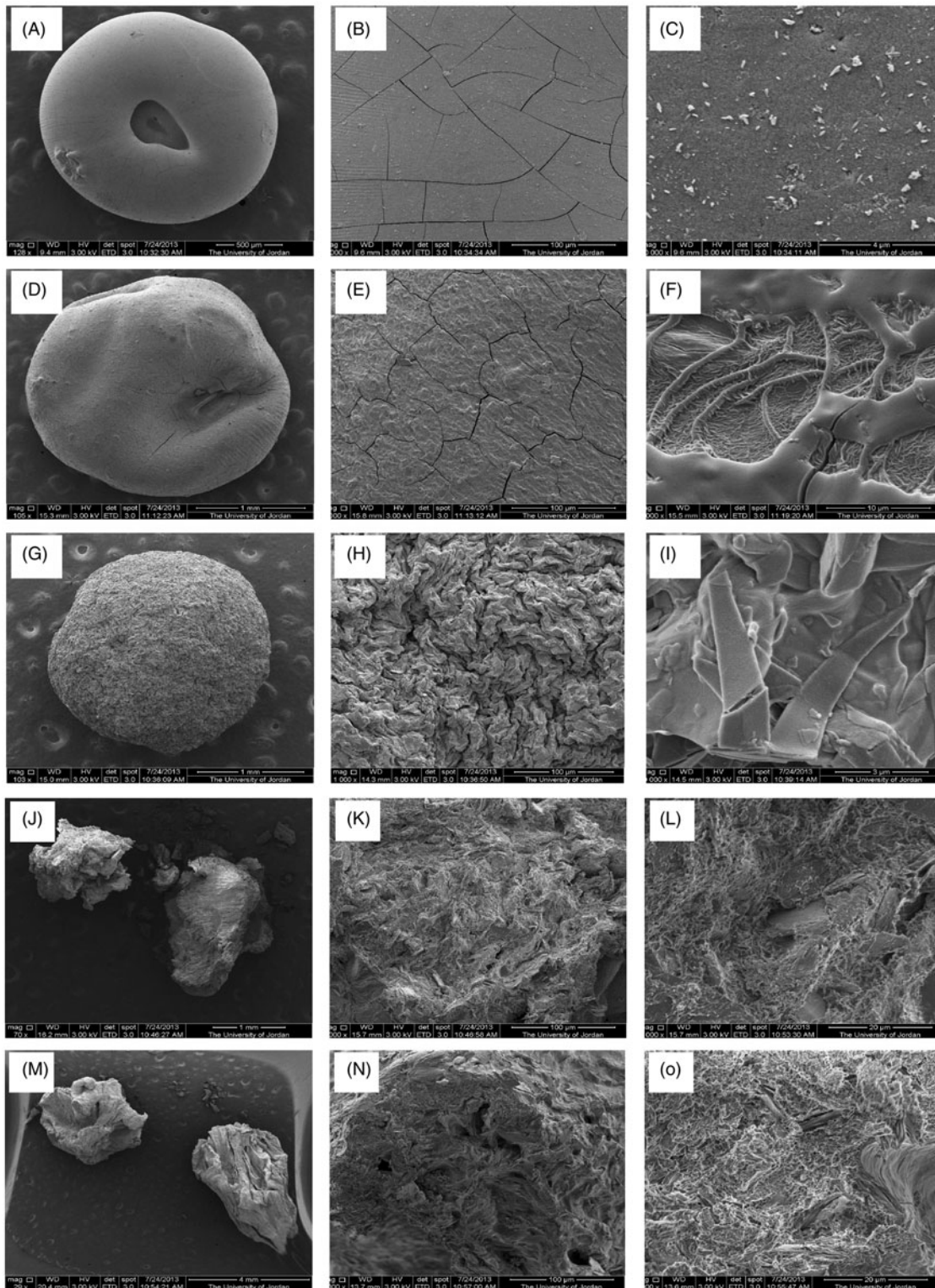
Figure 6. SEM micrographs of dry folic acid loaded iron-crosslinked beads: Sodium Alginate beads (A–C); Benzyl-amide Alginate beads (D–F); Tris(hydroxymethyl)methyl-amide Alginate beads (G–I); Allyl-amide Alginate beads (J–L); Pentyl-amide Alginate beads (M–O). Figures A, D, G, J and M represent individual beads while remaining figures represent corresponding surface microstructures of beads at different levels of magnification power.

dehydration[23]. Beads prepared from tris(hydroxymethyl)methyl-amide alginate polymers presented rough surface with characteristic channels and wrinkles (Figure 6H and I). Such surface microstructure would be due to the hydrophilic/hygroscopic nature of these polymers leading to the tortuous surface upon dehydration in the preparation process. On the other hand, beads prepared from allyl-amide and pentyl-amide alginate derivatives exhibited less regular flake-like matrices with rough surfaces

having micropores (Figure 6G–O). As mentioned above, this irregular shape is related to the lower solubility of these polymers resulting in low viscosity and tendency to disperse quickly upon dropping into the ionotropic curing solution.

We implemented IR spectrophotometry to assess polymer–iron complexes, as in Figure 3(G–K). However, we avoided polymeric beads as probes in the infrared investigation, because iron complexation is expected to take place within the vicinity of the
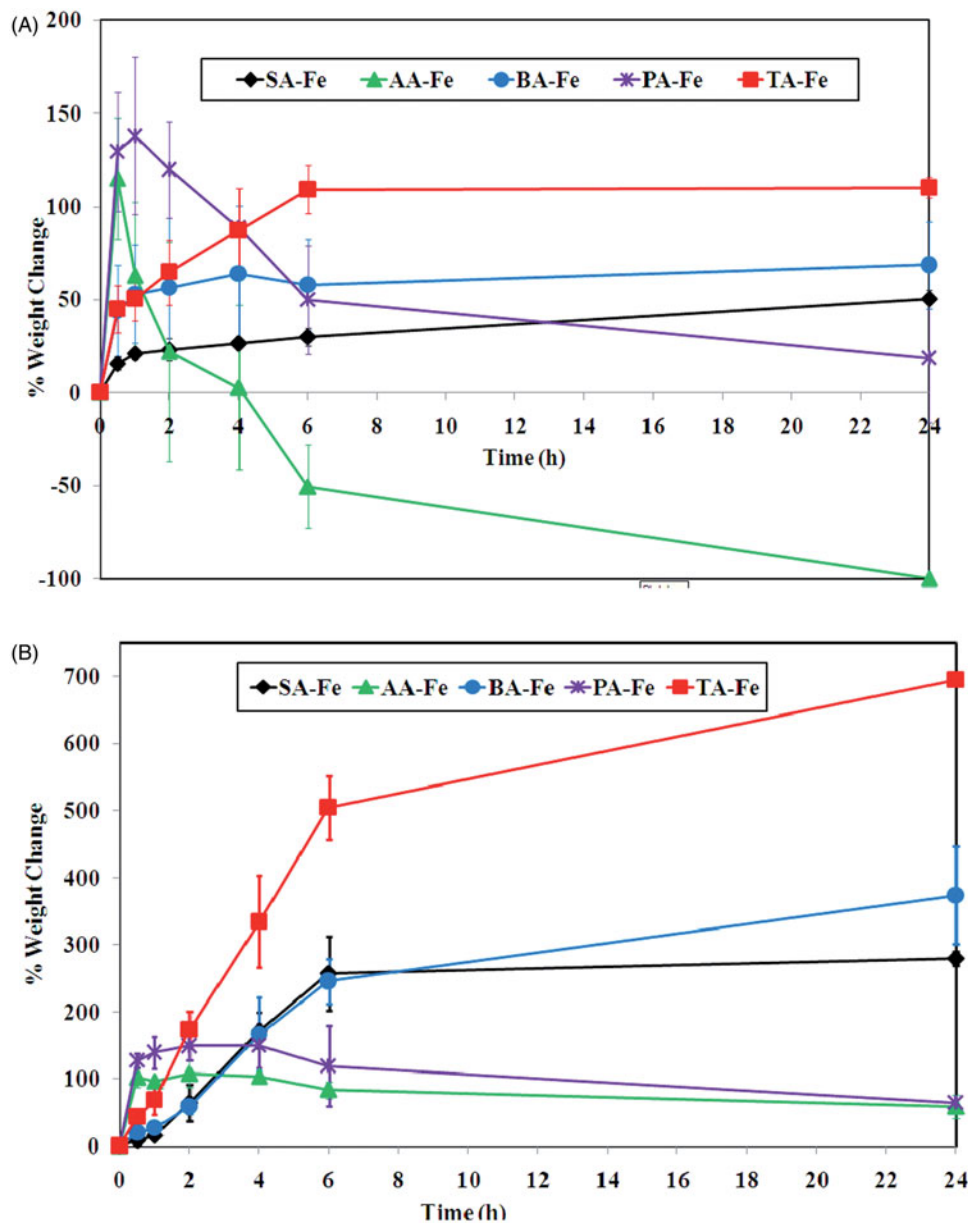
Figure 7. Swelling profiles for dry iron-crosslinked beads in (A) 0.1 M HCl and (B) phosphate buffer saline (pH 6.8) at 37 °C. Each point represents the average of three swelling measurements. Error bars represent the standard deviation of measurements. SA-Fe: folic acid-loaded iron-crosslinked Sodium Alginate beads; AA-Fe: folic acid-loaded iron-crosslinked Allyl-amide Alginate beads; BA-Fe: folic acid-loaded iron-crosslinked Benzyl-amide Alginate beads; PA-Fe: folic acid-loaded iron-crosslinked Pentyl-amide Alginate beads; TA-Fe: folic acid-loaded iron-crosslinked Tris(hydroxymethyl)methyl-amide Alginate beads.

beads' crust, which comprises a small percentage of the generated beads, which will give minimal absorption bands related to iron-complexation hidden by stronger bands resulting from non-complexed core polymers. Furthermore, the polymeric matrices used for infrared exploration were prepared by adding the crosslinking ion to vigorously stirred polymer solutions to maximize the degree of crosslinking, and consequently minimize any absorption bands related to the non-complexed polymers.

Apparently, the amidic carbonyl stretching vibration bands of the new polymeric conjugates (Figure 3H–K) shifted to lower wave numbers upon iron-complexation, that is, on average from $1629 \, cm^{-1}$ to $1621 \, cm^{-1}$, strongly suggesting the formation of iron(III)-amide coordinate bonds within polymeric complexes. Electron transfer from amidic carbonyls to iron(III) should reduce the double bond character of the carbonyl groups resulting in such downward shifts[15,18,20]. On the other hand, the carboxylate stretching band of sodium alginate was not altered by addition of iron(III) (Figure 3B versus 3G) indicating mere electrostatic

attraction between positively charged iron ions and negatively charged carboxylate groups without actual electron transfer-based coordinate bond formation.

It remains to be mentioned that the carboxylic acid stretching vibrations seen in the IR spectra of the new polymers (at *ca.* $1740 \, cm^{-1}$, Figure 3C–F) disappeared from the spectra of the corresponding iron(III)-complexes (Figure 3H–K) probably because we dissolved the polymers in sodium hydroxide media prior to iron complexation, thus converting the carboxylic moieties into carboxylates of stretching vibrations at *ca.* $1617 \, cm^{-1}$ (i.e. similar to sodium alginate), which are probably hidden under the stretching vibrations of iron-complexed amidic carbonyl moieties.

Iron-crosslinked matrices were also characterized by DSC, as in Figure 4(G–K). Evidently from the figure, complexation to iron caused a general upward shift in the endothermic hydration bands of hydrophobically substituted alginates (allyl, benzyl and pentyl), that is, on average from 60 °C to 100 °C (Figure 4H, 4I and 4J
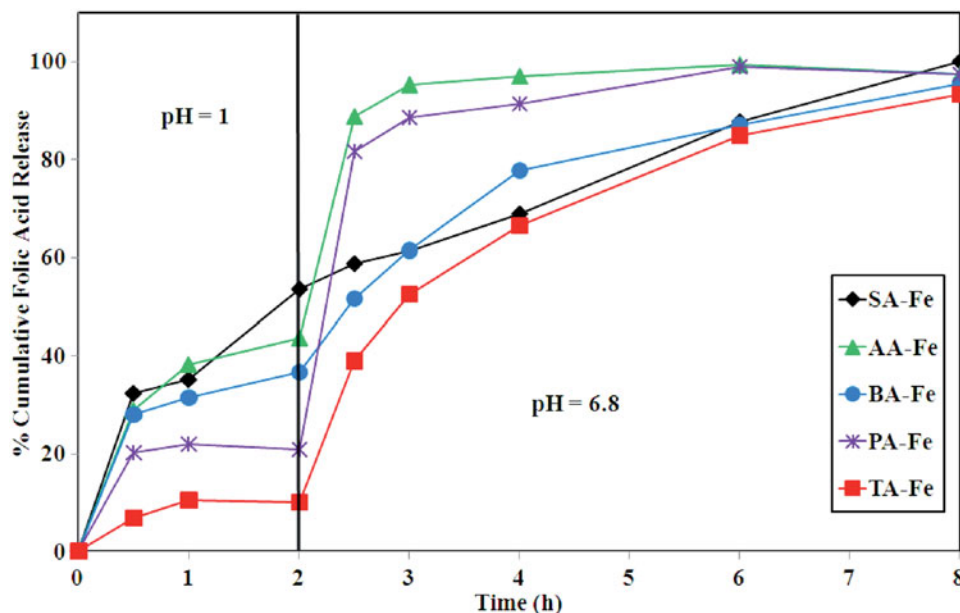
Figure 8. % cumulative release of folic acid from different beads (formulas' numbers are as in Table 1). Each point represents the average of two dissolution measurements. SA-Fe: folic acid-loaded iron-crosslinked Sodium Alginate beads; AA-Fe: folic acid-loaded iron-crosslinked Allyl-amide Alginate beads; BA-Fe: folic acid-loaded iron-crosslinked Benzyl-amide Alginate beads; PA-Fe: folic acid-loaded iron-crosslinked Pentyl-amide Alginate beads; TA-Fe: folic acid-loaded iron-crosslinked Tris(hydroxymethyl)methyl-amide Alginate beads.

versus 4C, 4D and 4E). This can be explained by the formation of strong interactions between iron complexes and water molecules, that is, greater than uncomplexed polymers. Still, this trend is nearly absent in tris(hydroxymethyl)methyl case (Figure 4K versus 4F), which is attributed to the excellent hydrophilic properties of the tris(hydroxymethyl)methyl-amide conjugates regardless to iron complexation.

The second endothermic band, seen in the DSC traits of uncomplexed polymers (at around 200 °C), suffered an apparent reduction in intensity upon iron crosslinking (i.e. became shoulder-like) which is related to the fact that iron-carboxylic and amide chelates are more resistant to heat-induced cleavage compared to hydrogen-bonding networks within uncomplexed polymers[18].

However, iron-crosslinked pentyl-amide alginate represents a noticeable exception as the polymer maintained the same sharp endothermic band after iron complexation (Figure 4J versus 4E). In addition, iron complexation intensified a subsequent endothermic shoulder (at 230 °C, Figure 4E) to full blown endothermic band (245 °C, Figure 4J). These findings suggest that the significant hydrophobicity of pentyl moieties hampers the diffusion of aqueous iron ions into the polymeric matrix during crosslinking, thus conserving hydrogen-bonding interactions observed in the uncomplexed polymer.

Swelling studies of beads

For each class of polymeric beads, accurately weighed amount of dried beads were placed in different media to investigate their swelling behavior. Two swelling media were used: 0.1 M HCl (pH 1) and PBS (pH 6.8) and dynamic weight change versus time was monitored. In theory, swelling of alginate beads results from water uptake, attributed to the inward movement of water molecules into voids within the polymeric network of these hydrogel beads[24].

In acidic media (Figure 7A), iron-crosslinked sodium alginate and iron-crosslinked tris(hydroxymethyl)methyl-amide alginate beads exhibited continuous swelling over 24 hours until reaching 50.3% ± 5.1 and 110.3% ± 5.7, respectively, with no indication of erosion and dissolution. The apparent superior swelling profile of

the latter is probably due to its enhanced hydrophilic nature associated with tris(hydroxymethyl)methyl moieties. Anyhow, we believe both classes of beads resisted erosion and dissolution in acidic media because of their extensive intermolecular hydrogen-bonding networks, which rendered their inner matrices quite coherent.

Interestingly, despite the hydrophobic nature of benzyl-, allyl- and pentyl- groups, their corresponding modified alginate beads exhibited enhanced water uptake compared to unmodified alginate counterparts with maximum swelling values of 58.6% ± 23.2, 115.2% ± 32.6 and 137.8% ± 42.1, respectively. These findings suggest that the newly introduced hydrophobic groups acted as steric appendices that separated alginate polymeric fibers. This would decrease the cohesive intermolecular hydrogen-bonding interactions among modified alginate polymeric fibers leaving them more available to hydration and swelling. Intriguingly, benzyl-amide alginate beads showed no evidence of erosion or dissolution under acidic conditions, while allyl- and pentyl-amide alginate beads quickly disintegrated. We anticipate that the aromatic rings of the benzyl-amide alginate matrix improved the cohesiveness of the corresponding beads through forming tight π–π stacking crosslinks among the polymeric fibers causing the observed physical robustness of the beads and their resistance to erosion. Obviously, lacking such π–π stacking crosslinks in allyl- and pentyl-amide alginate fibers rendered their corresponding beads fragile and easily disintegrated.

Unsurprisingly, all alginate-based beads behaved in PBS media in a similar fashion to their behavior in acidic media (Figure 7B) albeit with greater swelling extent: Iron-crosslinked beads prepared from sodium alginate, tris(hydroxymethyl)methyl-amide alginate and benzyl-amide alginate polymers exhibited continuous swelling over 24 hours reaching 279.5% ± 10.8, 694.3% ± 4.3 and 373.9% ± 72.3, respectively, without disintegration or erosion. On the other hand, beads prepared from allyl-amide alginate polymers showed prompt swelling to 102.6% ± 15.1 within the first 30 minutes followed with rapid weight loss due to disintegration. Similarly, beads prepared from pentyl-amide alginate polymers exhibited fast swelling of 149.1% ± 21.7 within the first two hours followed by disintegration.

Apparently, alginate-based beads (all derivatives) tend to exhibit greater swelling in PBS compared to acidic conditions (i.e. Figure 7A versus B) because crosslinking ferric ions are exchanged with non-gelling sodium ions in PBS media. The resulting ionized alginate polymers strongly attract water molecules; moreover, ionized carboxylates electrostatically repel each other leading to chain relaxation and hence improvement in gel swelling[19]. On the other hand, acidic conditions promote protonation of alginic carboxylates thus reducing their ionization and hydration potential.

Folic acid loading and release profiles

As shown in Table 1, iron-crosslinked beads prepared from different polymers exhibited high and comparable loadings of folic acid of *ca.* 595–685 mg/g bead mass. Such high loadings are probably attributed to the strong coordination between iron (III) ions with polymeric amide and carboxylate moieties (present also in folic acid). This reduces drug leaching during ionotropic curing and hence enhances drug encapsulation within polymeric beads.

The dissolution profiles of folic acid from different bead formulas were studied under simulated gastric conditions (0.1 M HCl, pH = 1.0) followed by simulated intestinal conditions (PBS, pH = 6.8). The dissolution media were maintained at 37 °C under sink conditions. Figure 8 illustrates the dissolution profiles of folic acid from different alginate-based polymers. Clearly from the figure, iron-complexed beads prepared from non-functionalized sodium alginate released more than 53% of the folic acid content within two hours in simulated gastric conditions. In contrast, iron-crosslinked beads prepared from hydrophobically modified polymers, i.e. allyl-, benzyl- and pentyl-amide alginates, reduced folic acid release in simulated gastric media to 44%, 37% and 21%, respectively. More interestingly, iron-crosslinked beads from hydrophilic tris(hydroxymethyl)methyl-amide alginate significantly reduced folic acid release to only 10% under acidic conditions.

These results clearly indicate that the added hydrophobic moieties reduced folic acid release under acidic conditions in a manner proportional to their hydrophobicities (i.e. pentyl > benzyl > allyl) despite their swelling-enhancing effects discussed earlier (''Swelling studies of beads''). The only possible explanation of this paradoxical conduct would be that the added hydrophobic groups held loaded drug molecules via hydrophobic and van der Waals' attractive forces causing the apparent reduction in release rate.

Intriguingly, despite its hydrophilic nature, tris(hydroxymethyl)methyl-amide alginate beads exhibited the most pronounced reduction of folic acid release; possibly due to the formation of tighter crosslinked crust compared to beads prepared from other modified alginates. Such enhanced tightness, despite moderate iron content (Table 1), would result from additional iron crosslinking to the flexible hydroxyl groups of tris(hydroxymethyl)methyl-amide structure[25] (Figure 1). Needless to say that the other hydrophobically modified polymers lack such additional crosslinks (Figure 2). Moreover, the additional trihydroxyl groups seem to capture iron ions and bring them closer to neighboring amidic carbonyl groups thus allowing tighter coordinate bonding and electron transfer between amidic carbonyls and iron ions. This explanation is strongly supported by the significant downward shift of the carbonyl stretching vibrations upon complexation to iron, that is, compared to other modified alginates (from 1632 to 1617 cm^{-1}, Figure 3F versus 3K), which indicates stronger amide-iron coordinate bonds within the polymeric matrix resulting from closer proximity between iron and amidic oxygen atoms as a consequence to coordination to nearby flexible hydroxyl groups (kinetically favored bonding).

In fact, we believe iron ions are captured within cyclic structures between amidic carbonyls and hydroxyl moieties as in Figure 1.

Unsurprisingly, all beads exhibited faster release of folic acid in simulated intestinal media compared to acidic media. This is attributed to the fact that carboxylic acid moieties in alginic acid polymers become ionized and hydrated under alkaline conditions. This should enhance water permeation through the polymeric matrices and hence boosts folic acid diffusion out from the polymeric beads.

Nevertheless, polymeric beads originating from different polymers exhibited significantly different release profiles in simulated intestinal environment: tris(hydroxymethyl)methyl-amide alginate beads showed the slowest release profile (probably for the same reasons mentioned above), while allyl- and pentyl-amide alginates exhibited the fastest profiles. Apparently, the hydrophobicity of pentyl and allyl moieties (boosted in the latter with a high substitution degree of 44%) combined with carboxylate ionization in alkaline pH imparted these polymers with surfactant-like properties, which enhanced the aqueous solubility of folic acid.

For better understanding of the release mechanism from different beads, the results were analyzed using the power law introduced by Peppas and coworkers[26–28]:

$$\frac{M_t}{M_\infty} = kt^n \tag{3}$$

where M_t and M_∞ indicate the cumulative amount of drug release at time t and infinite time, respectively; k is a constant related to the geometric features of the vehicle; and n is the release exponent which indicates the mechanism of drug release. Upon fitting the release data in alkaline media against this equation, the release exponent, n, had values of 0.48, 0.72 and 0.51 for beads prepared from sodium alginate, tris(hydroxymethyl)methyl-amid and benzyl-amide alginate polymers, respectively. Considering the spherical shape of these beads, the results indicate that drug release follow anomalous transport mechanism, indicating the superposition of both diffusion-controlled and swelling-controlled drug release mechanisms[28]. On the other hand, beads prepared from allyl- and pentyl-amide alginate conjugates significantly enhanced the dissolution of folic acid in alkaline media, which rendered their corresponding profiles beyond the limits of Peppas equation applicability. The combined effects of hydrophobic pentyl and allyl substituents with the ionization of carboxylate groups in alkaline pH imparted these polymers with surfactant-like properties that enhanced the aqueous solubility of folic acid.

Conclusions

In this report, four alginate derivatives were prepared, three hydrophobically modified and one hydrophilically modified. These were loaded with folic acid and crosslinked with iron (III) to yield spherical beads, which we evaluated as potential controlled release systems. In contrast to untreated alginate beads, the new functionalized beads succeeded in suppressing the release of folic acid in acidic media to variable extents, while some enhanced folic acid release in subsequent alkaline media. The results suggest the possibility of controlling drug release from alginate-based beads via simple modification of alginate polymers.

Declaration of interest

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