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Dual Stimuli-Sensitive Carrageenan-Based Formulation for Additive Manufacturing

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KEYWORDS: Polyelectrolyte; smart hydrogels; responsive biopolymer; drug delivery; 3D printing.

ABSTRACT: The design and development of controlled release systems of molecules of interest (nutrients, flavors, and drugs) have attracted significant attention over several years. Herein, we report a formulation of dual temperature and electro responsive κ- and ι-carrageenan based hydrogel for efficient food material and drug delivery. The microstructure and the thermal behavior of the hydrogel were characterized. The *in-vitro* drug release from the hydrogel was also studied. Using this carrageenan-based formulation and folic acid as the drug model, a high drug loading, and a sustained release because of either electric field or temperature were observed. In principle, the proposed formulation does not rely on 3D printing to perform its function; however, it adds to the feedstocks for 3D printing in the food and pharmaceutical industries. For the future, this could allow potentially more complex smart structures to be created from this material, further tuning release behavior.

INTRODUCTION: Biopolymer gels are materials composed of a polymer backbone, water, and in most cases, a crosslinking agent. They can absorb water or biological fluids due to their hydrophilic nature and three-dimensional polymeric network[1]. Biopolymer gels can be manufactured in different forms such as films, disks, rods, and microparticles. This will lead to a variety of applications in the medical and pharmaceutical fields and food production industries[1].

Carrageenan is a generic name for a family of linear, sulfated heteropolysaccharide extracted from red algae[2,3]. They are water-soluble[2], hydrophilic[4], anionic[3], and of high-molecular-weight[5]. Carrageenan does not have a single molecular structure but consists of a family of structures with an ester sulfate content of 15- 40% (w/w). [4,6] They are composed of linear chains of D-galactopyranosyl units linked via alternated (1→4)-β-D-and (1→3)-α-D-glucoside, which sugar units have one or two sulfate groups. Some units contain a (3,6)-anhydro ring[1,6–8]. Depending on the presence of the (3,6)-anhydrogalactose on the β(1 - 4)-linked residue, as well as on to the position and the number of sulfate groups [1,3,9], commonly carrageenan is classified into μ, ν, λ, ξ, κ, ι, and θ types (although other types have also been identified)[4,7]. However, the commercially available types of carrageenan are the κ-, λ-, and ι-carrageenan[4,5,8,10,11]. The κ-carrageenan is composed of alternating D-galactose-4-sulfate and (3,6)-anhydro-D-galactose units. The difference between ι-carrageenan and κ-carrageenan is sulfate groups in position two on the (3,6)-anhydro-D-galactose units. λ-Carrageenan has no (3,6)-anhydro-D-galactose units, but it is composed of alternating (1,3)-D- galactose-2-sulfate and (1,4)-D-galactose-2,6-disulfate units (sulfate substitution 32–39%)[12,13]. There is no pure form of any carrageenan, and commercial carrageenan is either a mixture of these types, with the predominating quantity of one type. Alternatively, they are hybrid molecules containing structural components of more than one type[6].

κ- and ι- Carrageenan gel formation is a complex process that depends on galactan structure, concentration, temperature, and the presence of co- and counter-ions[14]. Carrageenan gels form via an ionotropic gelation mechanism coupled with a cold-set mechanism[15]. The gelation is thermally reversible so that gels soften or disintegrate at elevated temperatures[16]. The gelling process in carrageenan solutions is generally accepted as involving a coil-to-helix transition

followed by aggregation of double helices to form a space-spanning network[17,18]. At high temperatures of up to 80 °C, carrageenan exists as random coils[19]. As they are cooled down to room temperature, linkage of the chains (the chain has restricted rotation due to C-O-C link) causes a twist in the molecule resulting in a helical structure which is further associated into double helices[19,20]. A gel network is then formed when these double helices undergo further aggregation[15,19]. This aggregation is mediated by specific binding of the gel promoting cations [21,22]. Certain cations (for example, K^+ for κ-carrageenan and Ca^{2+} for ι-carrageenan) are found to induce conformational changes in the carrageenan with the initial coil-to-helix transition [17,23]. The cations also act as co-ordination sites to bring discrete double helices into proximity to each other[14,19,21]. Due to the anionic nature of the polymer, cations are required to reduce electrostatic repulsion between polymer chains and induce linkages[20].They shield the negatively charged sulfate groups present on the carrageenan backbone from each other[19].

The Food and Drug Administration (FDA) has approved the GRAS (Generally Recognized as Safe) status of carrageenan for food-grade applications[24]. Carrageenan, as a healthy natural product, is widely used in food production as the thickening agent (mainly λ-carrageenan), gelling agent (κ and ι-carrageenan), and stabilizer or combinations of these functions in many food products, standardized with the necessary amounts of sucrose, glucose, salts or gelling aids, such as KCl[4,25]. For instance, carrageenan, mainly κ and ι- types and their salts, are widely used as binders in low-fat meat products during the heating stage[26]. Usually, a mixture of κ- and ι-carrageenan is used in food applications[27]. Due to the weakness of pure ι -carrageenan gels, the κ form is often added to provide strength, without the mixed gel (mixed chains) losing the desired rheological characteristics of pure ι- gels [25].

Carrageenan can also be used in non-food industries as excipients in pharmaceutical pills and tablets or the immobilization of biocatalysts[1]. Pacheco-Quito *et al* [28] have provided a review of research on the various types of carrageenan-based biomedical and pharmaceutical applications. Carrageenan is a common ingredient in dental care products, such as toothpaste and gels[29]. Its antiviral and antimicrobial potential in vitro has already been described[30,31]. Tests (*in vitro*) have demonstrated that ι-carrageenan is a potent inhibitor of the influenza A virus, also importantly of pandemic H1N1/2009[31]. Determined by their ability to form a gel and ability to undergo covalent crosslinking and hydrogen bonding, carrageenan is a suitable candidate for entrapping attributes inside its gelled or crosslinked core or through the formation of hydrogen bonding. Furthermore, chemical modification of carrageenan can improve its affinity for the encapsulated components[24]. Among the biomaterials already studied for the immobilization of enzymes, and with potential use in functional bioactive packages, carrageenan is a very promising material[32]. There are reports that ι- carrageenan increases the aroma retention time and slows down their transfer across the interface. In their systemic review, Guan *et al*. [33] have presented a comprehensive overview of the carrageenan application as dissolution and permeability enhancer and in polyelectrolyte complex formation in sustained-release matrices.

Those biopolymer gels that can exhibit a phase transition (i.e., volume change) in response to change in the external conditions such as electric current, pH, ionic strength, temperature are called "smart" gels[34]. Carrageenan is one of the major components in many stimuli-responsive hydrogels. κ-Carrageenan has been used to fabricate thermal, pH, and magnetic field responsive nanocomposite hydrogels as a drug delivery system with a prolonged-release profile resulting in improved drug release[35]. Geyik and Işıklan[36] have synthesized a binary graft derivative of κ-carrageenan with dimethylaminoethyl methacrylate and acrylic acid that shows temperature- and

pH-sensitive swelling behavior. Daniel-da-Silva et al.[37] fabricated crosslinked κ-carrageenan gel nanoparticles for the sustained release of methylene blue. They found that the volume of gel nanoparticles is changed by a temperature-trigger, and therefore methylene blue release profile and delivery kinetics are temperature-responsive. They have reported that the release was attained 60% at 25 °C and 37 °C; however, it increased to 95% at 45 °C.

Fortification with folic acid in one or more of the commonly consumed dietary items is now regarded as the best method to ensure that increased folate intake reduces the risks associated with folate deficiency. Folic acid is relatively stable between pH 5.0 and 12.0 in an aqueous solution when protected from light, even when it is heated (e.g., 100°C for 10 h)[38]. However, it has limited water solubility[39]. A useful feature of carrageenan gels is their high capacity for water absorption, which improves drug dissolution[33]. Edible gums in Cheddar cheese (alginate, pectin, xanthan gum, ι-carrageenan) were evaluated for folic acid encapsulation efficiency as single and mixed polymers[40]. Folic acid incorporated in alginate and combinations of alginate and pectin were reported to improve stability and high encapsulation efficiency[40,41]. In the same report, although the encapsulation efficiency of folic acid in ι-carrageenan was relatively low, by using a mixture of ι-carrageenan and alginate or pectin, the encapsulation efficiency is increased by more than 1.5 fold[40]. There is no report on encapsulating folic acid in a carrageenan mixture.

There is a need to develop new techniques for enhancing folate content, stability, and bioavailability in food products. It has also been reported that carrageenan gels are electrically conductive[42]. Considering that one of the major application areas for carrageenan is dairy products, and also one of the uses of pulsed electric fields (PEFs) is for pasteurization and possibly sterilization [43], carrageenan gel can be formulated to encapsulate folic acid for use in dairy products manufacturing as an alternative medium for delivery of the vitamin. Moreover, it has

been suggested that the application of a weak DC potential on hydrogels and hydrosols can be used as a method of laminar sanitization[1,44]. We have recently reported the drug release from 3D printed formulations of κ-carrageenan and agar [45] as well as tamarind and gellan mixtures [46]. This study aims to explore the potential of a 3D printable mixture of κ and ι-carrageenan to encapsulate and potentially enhance the stability of hydrophilic moieties. Folic acid was used as the model drug to investigate the capacity of this formulation in modifying folic acid release triggered by the temperature or by the direct electric current (DC).

Materials and Methods

Materials and preparation of samples: κ- and ι-carrageenan, potassium chloride, and folic acid were purchased from Sigma-Aldrich (Dorset, UK). All materials were used as received. The concentration of κ- and ι-carrageenan for all samples are 1 wt. % and 1 wt. %. Following samples were prepared; Carrageenan sols (κ-, ι- and mixed, without salt or with salt (0.5 %, 1.0%, 2.0%), without folic acid or with 0.05% folic acid) were prepared by dispersing powdered formulation in deionized water (80 °C) in a sealed bottle in a water bath (for 30 min for TD-NMR and DSC samples), mixing continuously using a magnetic mixer. Gels were prepared by pouring the aforementioned sols (80 °C) into the gel reservoirs, covering to prevent moisture loss, and allowing the samples to cool in the refrigerator (4 °C). Gels were stored overnight before the experiment. All material contents in the formulations were prepared on a weight/volume basis.

Time-domain NMR (TD-NMR): The experiments were conducted using TD-NMR, Minispec 20 Hz, (Bruker BioSpin GmbH, Karlsruhe, Germany) to monitor the mobility and state of aggregation of the polysaccharide chains. T_2 values were recorded using the software application "t2_cp_mb" a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence provided by Bruker. For each measurement, 200 data points were collected. Pulse separation between the 90° and the 180° pulse

was 0.5 ms, and the recycle delay was set to 2 s. Data were accumulated with eight scans. For each measurement, the sample was placed in a small glass tube and then in an NMR-glass tube (outside diameter 10 mm) at 25 °C. Four samples were prepared, and each sample was measured once.

Differential scanning calorimetry (DSC): To determine the extent of molecular ordering, the measurements were carried out on a Setaram Micro DSC 3 Evo (Seteram, France) using stainless-steel cells. Deionized water was used as a reference. Initially, the sample was cooled to 20 °C and then held there for 20 min. A cooling ramp was applied at a scanning rate of 1.0 °C/min up to 10 °C and then stayed there for 20 min. A heating ramp was used at a scanning rate of 1.0 °C/min up to 80 °C, where it was held for another 20 min, and then a cooling ramp at the same rate back to 5 °C. This cycle was repeated another time so that two heating and two cooling curves were obtained in total (Figure S2, Supporting Information). The data collected from the second cycle are reported as the results. Peak onset and offset temperatures on cooling and heating were obtained from the intersection of tangents to the baseline and peaks, or of two peaks in the case of mixtures. The sol-gel temperatures were determined as the peak maximum of the heating and cooling curves. One sample (~ 750 mg) was prepared for each formulation. The system of carrageenan w/without KCl has already been comprehensively studied by DSC, and the results have already been reported (*e.g.,* [10], [47], and [48]). We found our results consistent with the literature. Therefore, each sample was measured once. Selected samples were run twice, and no noticeable variations were seen.

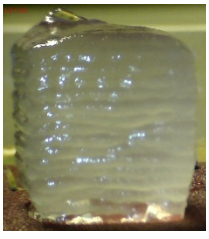
Rheology: To determine the phase behavior and the viscoelastic properties of the formulations, rheological characterization of the materials was performed on a Kinexus Rheometer (Pro or Pro+, Malvern Panalytical, Malvern, UK) using serrated parallel plates geometries (upper plate diameter: 20 mm, lower plate diameter: 65mm) with a gap size of 1 or 2 mm (depending to the gel

thickness). All measurements were repeated three times. Gel samples were loaded in the form of discs (diameter: 20mm and with the height of the gap).

Amplitude sweep: To determine the linear viscoelastic (LVER) region and phase angle, oscillation amplitude sweep (strain controlled) measurements were performed in the range of 0.01-100 % at the frequency of 1 Hz at 25 °C.

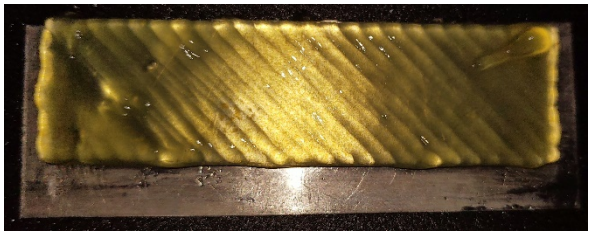
Frequency sweep: To get the mechanical spectrum and the relaxation exponent of the sample, frequency sweep (at the shear strain of 0.1 % of LVER maximum limit) measurements were performed in the range of 10-0.1 Hz at 25 °C.

3D printing: A custom-built food 3D printing system was used in this study (Supporting Information). 3D digital design of the object was generated with Cura 15.04.6 (Ultimaker B.V., Netherlands). A 10 mL syringe (covered with one layer of insulation) and a 22G needle (inner diameter 0.413 mm) were used for all samples. The syringe was filled with sol samples (80 °C). All samples were printed at the flow level of 60%.[49] The cubes (15 mm ×15 mm ×15 mm), the doughnuts (ID: 9mm, OD:30 mm, height: 4mm) and rectangles (70 mm ×20 mm ×3mm) were printed for printability assessment, for thermal release and electric stimuli drug release respectively (Fig. 1. a-c). The cubes were printed on sandpaper (3M™ Utility Cloth Sheet 314D, R.S. Components Limited, Corby, UK), doughnuts were printed on aluminum foil and rectangles were printed on stainless steel strips (76mm ×26 mm×1mm). All objects were printed at the printing bed temperature of 50 °C.



(a)

(b)



(c)

Figure 1. 3D printed objects for the experiments (a) the cube (15 mm ×15 mm ×15 mm) out of κ- and ι-carrageenan (1wt. %, 1 wt. %) in the presence of KCl (2 wt. %) (b) doughnut (ID: 9mm, OD:30 mm, height: 4mm) and (c) rectangle (70 mm ×20 mm ×3 mm) printed over stainless steel out of κ- and ι-carrageenan (1wt.%,1 wt.%) in the presence of KCl (2 wt.%) and folic acid (0.05 wt.%)

***In vitro* Release studies- drug loading:** The drug loading for each formulation was determined as follows: three doughnut-shaped printed samples were weighed and subsequently were separately placed in 40 ml of deionized water at 37 °C at 150 rpm inside the benchtop-shaking incubator (Incu-Shake MIDI, SciQuip, Shropshire, UK) for 24h. Then, 1 ml of the medium was analyzed by a UV-Vis spectrophotometer (Orion™ AquaMate 8000, Thermo Fisher Scientific, UK) at λ_{max} of 298 nm at room temperature. The drug loading was calculated as the average calculated amount of released folic acid over the average weight of the printed samples. It is used as the reference value both for thermal and electric stimuli release.

Thermal release: Three doughnut-shaped printed samples were separately placed in 40 mL (60 mL for 1wt.% of KCl). At the time intervals of 5, 10,15, 30, 45, 60, 90, 120, 180, and 240 min, 1ml of the medium was taken out (and replaced by 1ml of deionized water) and was analyzed by a UV-Vis spectrophotometer (Orion™ AquaMate 8000, Thermo Fisher Scientific, UK) at λ_{max} of 298 nm at room temperature. The reported drug release data are the average value for the three samples. Blank experiments were conducted using similarly prepared but drug-free samples.

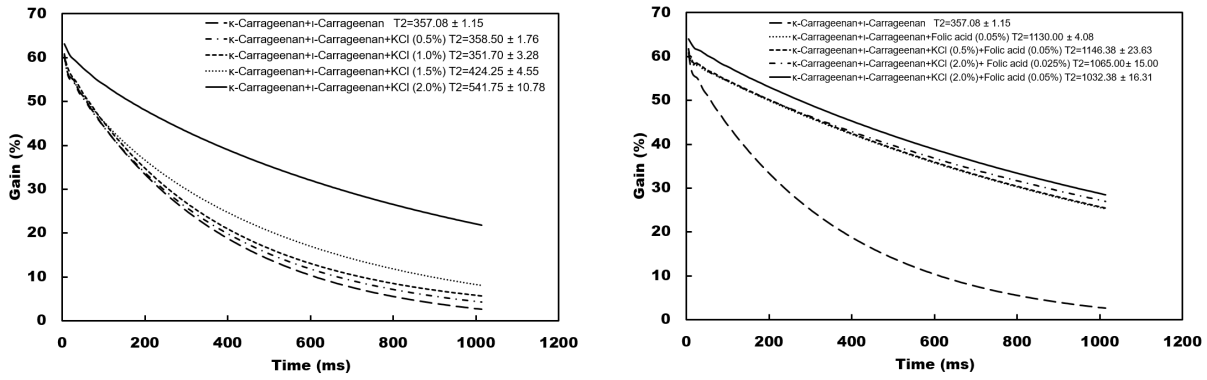
Electric stimuli drug release: For studying the release of folic acid from the 3D printed sample, it was connected to the positive terminal while a stainless steel strip was connected to the negative

terminal of a power supply. Both electrodes were placed at a distance of 25 mm in a small glass staining jar. The release medium was deionized water (Figure S3, Supporting Information).

To study the release without any trigger, 1 mL of solution was sampled (and replaced with 1ml of deionized water each time) from the jar every 5 min during the first quarter of an hour. To study the release as the result of the electrical trigger, at 15 min (just after sampling), a potential difference of 5V (BaseTech, BT-305) was applied continuously, and sampling continued at time intervals of 20, 25, and 30 min (and replaced with 1ml of deionized water each time). At 30 min, the potential was stopped, and more 1ml samples were taken out at 35, 40, and 45 and 50 min (and replaced with 1ml of deionized water each time). All samples were analyzed by a UV-Vis spectrophotometer (Orion™ AquaMate 8000, Thermo Fisher Scientific, UK) at λ_{max} of 298 nm. Three replicates were used for each formulation. Blank experiments were conducted using similar, but drug-free 3D printed samples.

Results and Discussion

TD NMR is a non-destructive method and is used to determine the transverse relaxation time (T_2) of water via the transverse relaxation of ^{1}H protons[50].The value of the relaxation time at the end of the measurement (T_2) and its decay was used as an indicator for water-binding capacity. The results are presented in Figures 2. a and b.



(a) (b)

Figure 2. The value of the relaxation time at the end of the measurement (T_2) as well as its decay for κ- and ι- carrageenan (a) in the presence of salt and (b) in the presence of salt and folic acid

T_2 decay is represented according to different models: exponential, Gaussian, Abragamian, and stretched or compressed exponentials[51]. For liquids or systems with high mobility, exponential decays describe the system rather well[51]. In other words, T_2 decay for liquids or systems with high mobility is commonly fitted by an exponential decay function. All T_2 decay curves in Figures 2.a and b are fitted by an exponential equation (Table S1, Supporting Information). Therefore, all formulations presented here are systems with high mobility. Moreover, by adding salt and/or folic acid, the T_2 decay curves are changed from 'stretched exponentials' to 'compressed exponentials' that indicate inhomogeneous distributions of exponential decays, interpreted as inhomogeneity of the sample itself[51]. This is the characteristic of multiphase systems that usually generate multi-modal decays[51]. This microheterogeneity is most probably a result of a microphase separation process[52]. We can conclude that by adding a relatively large amount of salt (KCl ~2%), the system behaves as a multiphase system.

However, only a slight amount of folic acid makes the system multiphase, and it is relatively irrespective of the amount of salt present. A decrease in water mobility is reflecting in a rise in T_2 and hence a lesser degree of microphase separation. Therefore, in release studies, we expect a lower folic acid release in formulations containing a higher amount of salt. There may also be additional intermolecular associations at higher ionic strength. These could arise because of higher concentrations of salt and folic acid and increased electrostatic repulsions due to less shielding by counter-ions.

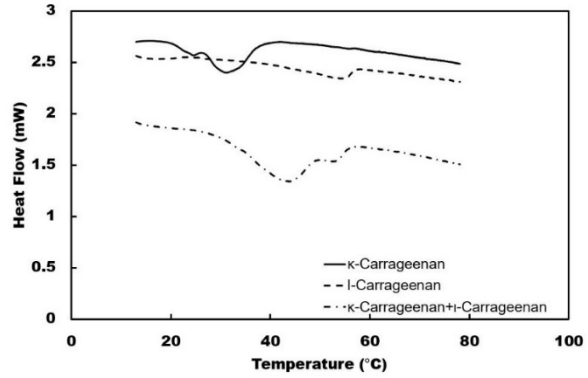
DSC heating and cooling traces are shown in Figure 3 and Table S2 (Supporting Information). The results obtained here being consistent with, and complementary to, those previously reported[53]. For κ-carrageenan, gelling is accompanied by a sharp transition, while melting involves a broader transition at a higher temperature. The melting of both κ-carrageenan and ι-carrageenan takes place at a slightly higher temperature (~ 31 °C and ~ 54 °C) than their gelling (~24 °C and 50 °C). The setting of the gel is associated with the conformational transition of the molecules attributed to double helix formation. The melting transition is attributed to the disruption of helix-helix aggregates[48]. It has already been reported that κ-carrageenan forms aggregates, whereas ι-carrageenan forms fewer or no aggregates[10,25]. Gel melting occurs at a higher temperature because it requires the melting of helical aggregates (possibly even crystallites). The κ-carrageenan / ι-carrageenan mixture shows two-step gelation in both heating and cooling (Figure 3a and d). Each step is almost coinciding with that observed for κ-carrageenan and ι-carrageenan, on cooling (Figure 3d and Table S2 (Supporting Information))[10]. On heating (Figure 3a), one step is coinciding with that seen for ι-carrageenan; however, the corresponding step with κ-carrageenan is displaced by ~ 20 °C due to thermal hysteresis. Network interaction types can be interpenetrating, coupled, or phase-separated in mixed gels[27]. Our results show that the two

components gel independently of each other in mixed κ-carrageenan / ι-carrageenan. A KCl concentration dependence of the onset, offset, and peak maximum temperatures of the κ-carrageenan / ι-carrageenan mixture in the presence of salt was observed on cooling and heating (Figure 3b and e and Table S2(Supporting Information)). In the presence of 1 g KCl (1%, 0.013 mol), the κ-carrageenan and ι-carrageenan networks melt and gel at the same temperature. It shows that the two different aggregation steps now occur over a similar range. It might also imply that independent κ-carrageenan and ι-carrageenan networks are no longer present. However, on further increase in salt concentration, the first exotherm (already assigned to κ-carrageenan) moves to higher temperatures. The second exotherm (previously assigned to ι-carrageenan) remains approximately in the same position but becomes progressively smaller (Figure 3b and e). The reason is that with K^+, there is evidence of ion-pair formation, with stronger binding to κ-carrageenan than to ι-carrageenan[54].

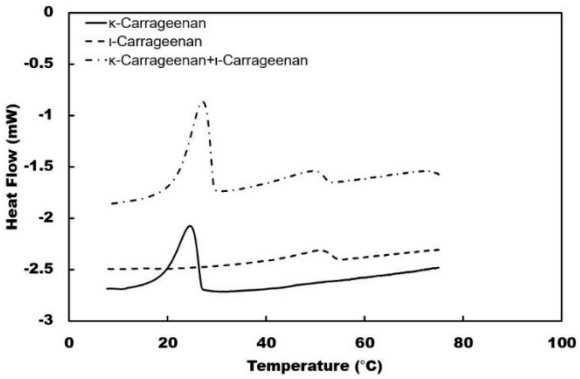
As illustrated in Figure 3f, after adding folic acid, the DSC cooling scans again show two exotherms that further increase in salt concentration the first exotherm (κ-carrageenan) moves to higher temperatures. In contrast, the second exotherm (ι-carrageenan) remains approximately in the same position. Broad (as opposed to sharp, it does not imply the peaks are broadening) endothermic peaks (Figure 3 a-c) might be indicating the possible involvement of a kinetically limited conformational change and the difficulty of coil-to-helix transition because of the presence of folic acid. Therefore, our data suggest a direct binding between folic acid and carrageenan, particularly ι-carrageenan. Nitrogen atoms on the folic acid molecule could interact with sulfate groups of carrageenan. ι-Carrageenan has an extra SO_3^- group, which explains why it is more bound to folic acid.

1

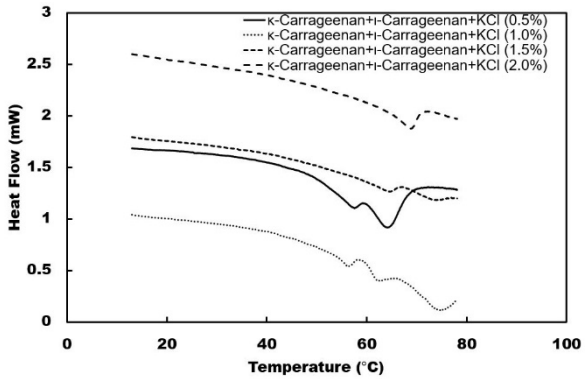
2


Heat Flow (mW)
Temperature (°C)
κ-Carrageenan
ι-Carrageenan
κ-Carrageenan+ι-Carrageenan

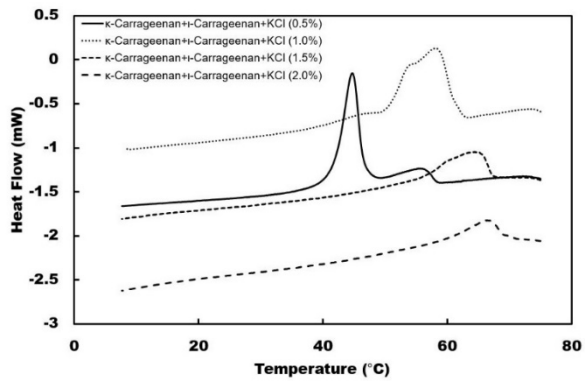
(a)


Heat Flow (mW)
Temperature (°C)
κ-Carrageenan
ι-Carrageenan
κ-Carrageenan+ι-Carrageenan

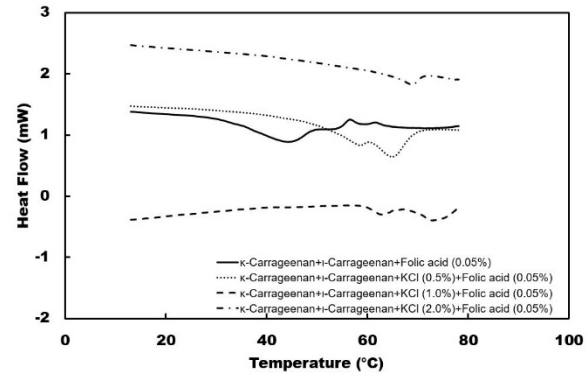
(d)


Heat Flow (mW)
Temperature (°C)
κ-Carrageenan+ι-Carrageenan+KCl (0.5%)
κ-Carrageenan+ι-Carrageenan+KCl (1.0%)
κ-Carrageenan+ι-Carrageenan+KCl (1.5%)
κ-Carrageenan+ι-Carrageenan+KCl (2.0%)

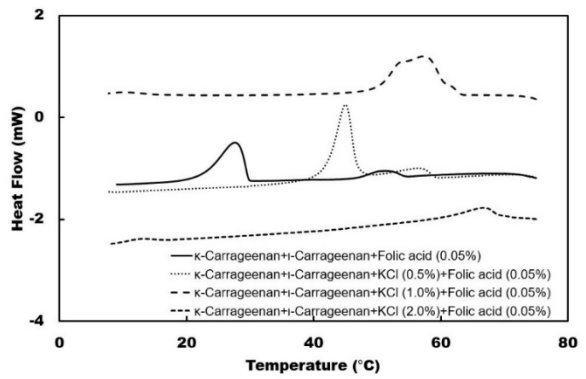
(b)


Heat Flow (mW)
Temperature (°C)
κ-Carrageenan+ι-Carrageenan+KCl (0.5%)
κ-Carrageenan+ι-Carrageenan+KCl (1.0%)
κ-Carrageenan+ι-Carrageenan+KCl (1.5%)
κ-Carrageenan+ι-Carrageenan+KCl (2.0%)

(e)


Heat Flow (mW)
Temperature (°C)
κ-Carrageenan+ι-Carrageenan+Folic acid (0.05%)
κ-Carrageenan+ι-Carrageenan+KCl (0.5%)+Folic acid (0.05%)
κ-Carrageenan+ι-Carrageenan+KCl (1.0%)+Folic acid (0.05%)
κ-Carrageenan+ι-Carrageenan+KCl (2.0%)+Folic acid (0.05%)

(c)


Heat Flow (mW)
Temperature (°C)
κ-Carrageenan+ι-Carrageenan+Folic acid (0.05%)
κ-Carrageenan+ι-Carrageenan+KCl (0.5%)+Folic acid (0.05%)
κ-Carrageenan+ι-Carrageenan+KCl (1.0%)+Folic acid (0.05%)
κ-Carrageenan+ι-Carrageenan+KCl (2.0%)+Folic acid (0.05%)

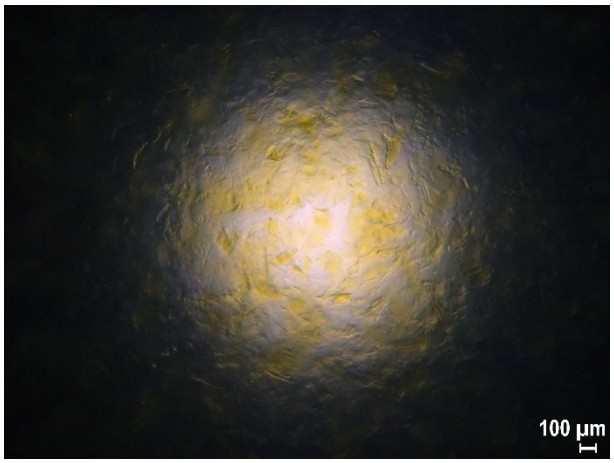
(f)

Figure 3. DSC traces for solutions of κ- and ι-carrageenan (1 wt. %, 1 wt. %), (a, c) in the presence of salt (b, d), in the presence of salt and folic acid (c, f) on heating (a, b, c) and cooling (d, e, f). Salt and folic acid concentrations are indicated in the figures. The scanning rate is 1.0 °C/min.

1

2 This semi-opaque quality of the gels also suggests some form of microheterogeneity, and
3 examination with a light microscope reveals a granular structure on a distance scale of 1-100 μm.
4 Figure 4. displays the microscope image for the printed sample of κ- and ι-carrageenan in the
5 presence of KCl (2.0%)and folic acid (0.05%), which might highlight this folic acid and ι-
6 carrageenan binding. The other samples (printed or non-printed) were transparent (under the
7 microscope), and we could not take any images.

8

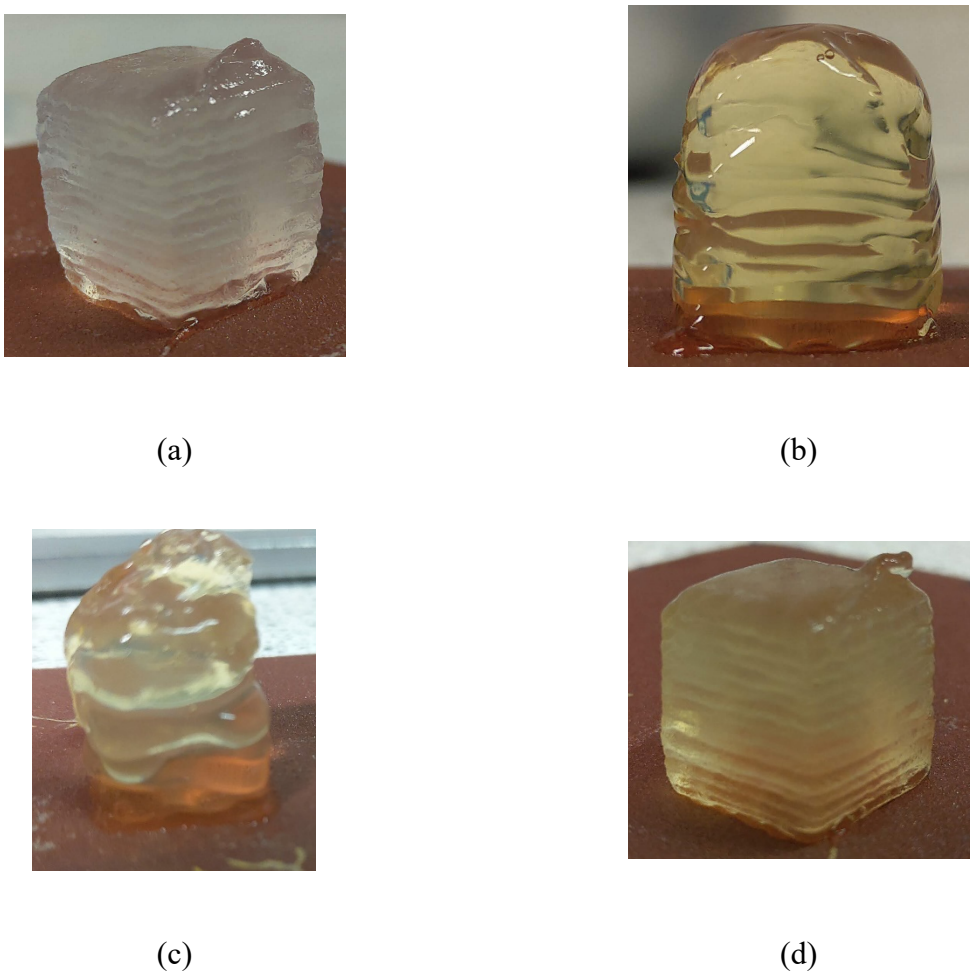


9

10 **Figure 4.** Microscope image of κ- and ι-carrageenan (1 wt. %, 1 wt. %) in the presence of KCl
11 (2.0%) and folic acid (0.05%). The sample was printed in rectangular form (70 mm ×20 mm ×3
12 mm) over a glass slide. The image was taken using an optical microscope (DM 2500 LED, Leica®,
13 CH) with a charge-coupled device camera (DFC450 C, Leica®, CH)

Several pieces of research have shown that κ- carrageenan / ι- carrageenan mixtures undergo two-step gelation, meaning that two independent polysaccharide gel networks are formed[10]. However, the gel stiffness of mixed gels was found to be much larger than the sum of the elastic moduli of the individual gels[55]. This means that the two types of carrageenan do not form independent homogeneously distributed networks with the same structure as in the individual systems[55]. An overview of the literature shows that the formation of separate interpenetrated networks in mixtures of κ- carrageenan / ι- carrageenan is excluded[56]. It has been shown that the different types of gel production and the different ionic concentrations determine the structural dissimilarity[27]. This leads to the conclusion that the structures are highly dependent on gelation history[27].

Figure 5. displays the image of printed cubes for each sample. It can be seen that the printability (shape fidelity) of κ- and ι-carrageenan depends only on the KCl concentration. κ- and ι-carrageenan in the presence of KCl 2.0% (with or without folic acid (0.05%)) has an excellent printability (shape fidelity). Nevertheless, the lower concentration of salt gives rise to lower printability (shape fidelity). Even the printability (shape fidelity) of κ- and ι-carrageenan in the presence of folic acid (0.05%) and KCl (0.5%) is higher than that of KCl (1.0%). Routine rheology measurements (Table S3 (Supporting Information)) show that both gels are printable (self-supporting)[57]. However, the gel with KCl (1.0%) has a complex shear modulus much higher than with KCl (0.5%). Therefore, the material that is extruded from the syringe is a strong, already robust gel, where the layers cannot be fused together. We think this high rigidity of the gel with KCl (1.0%) could be related to the origin of some state of new physical associations and ionic interactions in the gel matrix.



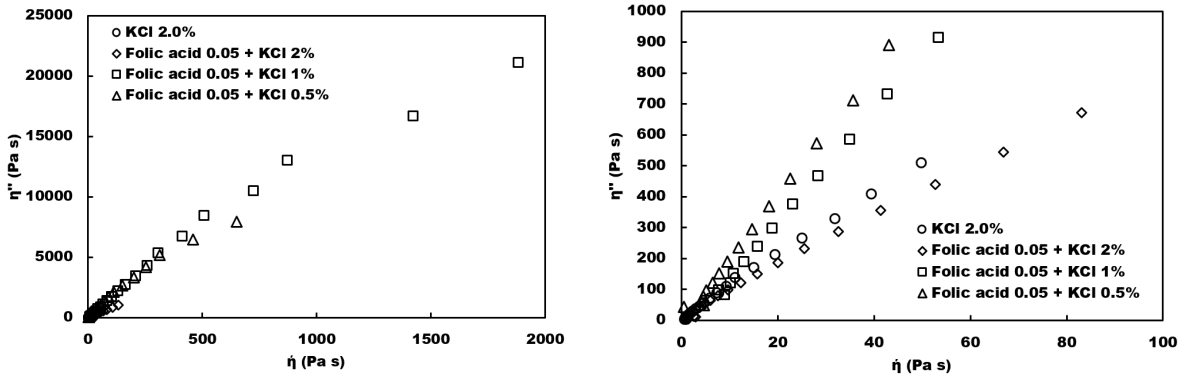
(a) (b)

(c) (d)

Figure 5. The printability of ϰ- and ι-carrageenan in the presence of (a) KCl 2.0% (b) Folic acid 0.05% and KCl 0.5% (c) Folic acid 0.05% and KCl 1.0% (d) Folic acid 0.05% and KCl 2.0%. The cube size is 15 mm ×15 mm ×15 mm.

Figure 6. gives plots of log η'' (out of phase component of shear viscosity) *versus* log η' (dynamic component of shear viscosity), also known as the Cole-Cole plot[58]. The Cole-Cole plot can be used for analyzing the miscibility of polymer blends; Miscible blends (similar to single-component viscoelastic systems) have only one semicircular arc, while two-phase blends exhibit two arcs. Those two-phase systems with liquid-like behaviors produce a smooth curved arc (with an additional arc in some cases), while those with solid-like behaviors produce an arc that deviates

from the semicircular shape[59]. Here, the Cole-Cole plot of all samples shows one arc. The formulations with folic acid, KCl (1.0 %), and KCl (0.5 %) show a high viscosity tail that reflects a long-term relaxation mechanism due to the dynamics of ionic aggregates that become more significant by increasing the salt concentration (neutralization intensity)[23]. However, as the salt concentration increases, the viscosity tail (long-term relaxation) decreases that may be due to the aggregation of ions. The re-association of anions and cations is responsible for the existence of salt aggregation in the polymer electrolytes. The aggregation of salt suppresses the number density of free mobile ions, which leads to a decrease in viscosity[60].



(a) (b)

Figure 6. Cole-Cole plot for ϰ- and ι-carrageenan in the presence of folic acid 0.05% and KCl (different concentrations) (a) full range (b) focus on the shorter range

Polymeric matrices of ι-, ϰ- and λ-carrageenan and their blends (without salt), prepared by simple mixing or solvent evaporation technique, have been used for controlled release drug delivery. It has been reported that although ι-, ϰ- and λ-carrageenan cannot form miscible blends, they can still be used in the development of a controlled drug release formulation due to their

ability to form hydrogen bonds with the drug[61]. The release behavior of folic acid from ϰ-carrageenan / ι-carrageenan mixture in the presence of salt under *in vitro* conditions in deionized water at 37°C has been shown in Figure 7. Cumulative release of the folic acid follows a steady, continued-release profile, without a burst release phenomenon, and the time for 50 % release is 30 min (0.5% KCl) and 60 min (2% KCl). The release behavior of folic acid from sodium alginate-pectin electrospun fibers under *in vitro* conditions has been reported[62]. In this report, 97% of folic acid was released from the electrospun fibers in an aqueous solution (pH 7.8) within 1 h[62]. Similarly, Madziva *et al* [41] reported about 90 % release of folic acid from alginate-pectin microcapsules in phosphate buffer in 80-120 min. These findings provide practical information for designing a carrageenan-based system from which folic acid can be released in response to temperature. The release data were analyzed by using the generalized model [63] (Equation 1) where ($\frac{M_t}{M_\infty}$) is the folic acid fractional release;

$$\frac{M_t}{M_\infty} = kt^n \qquad \text{(Equation 1)}$$

M_t is the amount of molecule released up to any time t

M_∞ is the final release amount of molecule

k is a structural/geometric constant for a particular system

n is designated as the release exponent representing the release mechanism

Nanaki *et al* [61] have reported that carrageenan-based formulations consisting of ι/λ and ϰ/λ blends (without salt) released the drug over a 5 h period while formulations consisting of ϰ/ι mixtures released the drug over a 9 h period. However, the release seemed to follow 1st order kinetics.

Here, the release from both formulations fitted the generalized model well, with a correlation coefficient R^2 of 0.9421(0.5%KCl) and 0.9818 (2% KCl). Values of n (0.4746 for 0.5% KCl and 0.4797 for 2% KCl) close to 0.5 indicate that diffusion of the folic acid through the carrageenan matrix is rate-controlling[64]. It can also be seen that the release of folic acid is faster with 0.5% KCl than with 2% KCl. Factors that affect the release profile are the proportion of carrageenan in the complex and the concentration of the cross-linker (here, salt). Our TD-NMR results suggest that the size of the aggregate (as the basis of the gel) decreases as the salt concentration is increased. Higher cross-linker (K^+) concentration could strengthen crosslinking, increase the density of the polymer matrix, and decrease the inner space for drug diffusion[65]. As a result, the drug diffusion coefficient decreases and slows the release rate [66]. The slower folic acid release in the presence of a higher concentration of salt might also be the result of increased ionic interactions hindering the release of molecules. Another factor that affects the release profile is the carrageenan–drug interaction that increases the drug loading. We have already found that folic acid binds to κ- and ι-carrageenan. However, in all formulations, the amount of κ- and ι-carrageenan is the same. The highest loading of folic acid for these formulations was found to be in the order of KCl (2%) > KCl(0.5%) > KCl (1%). Electrostatic interaction between carrageenan and folic acid may increase drug solubility[65]. However, due to the meager amount of solids content in the gel system, binding of the drug to the gel matrix is limited, but at the same time, the diffusion path of the drug is less obstructed by the gel matrix[67].

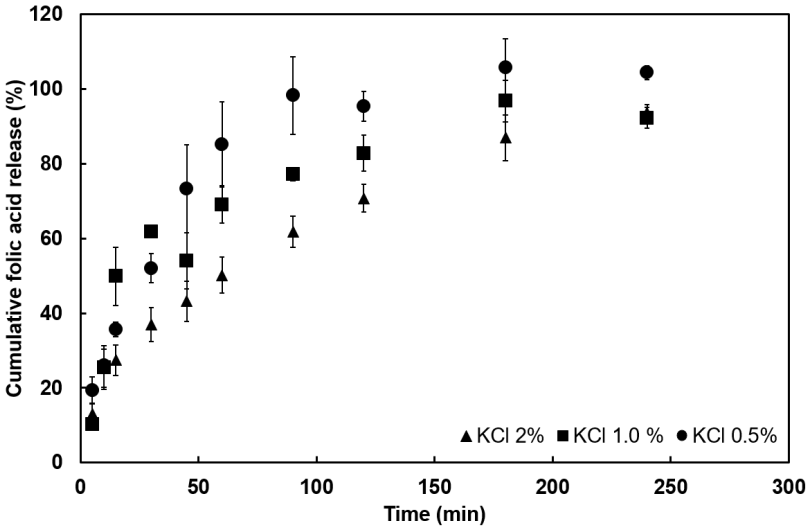
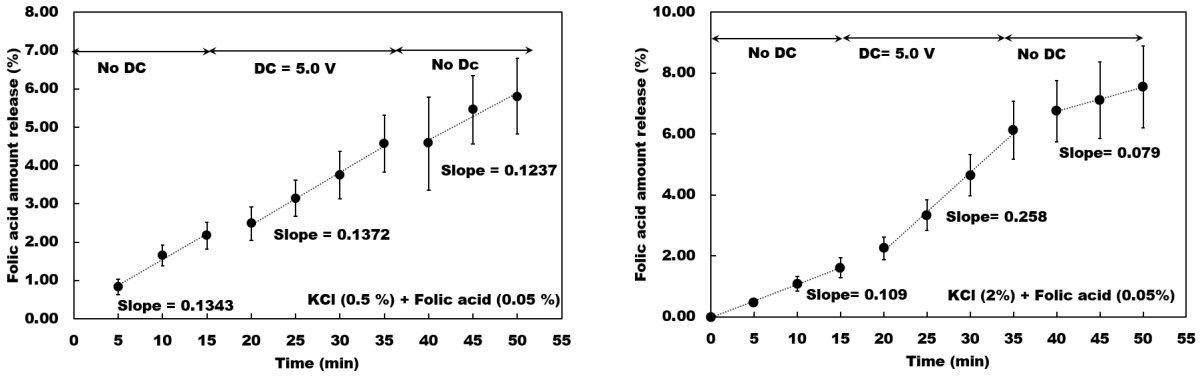


Figure 7. The release behavior of folic acid from κ-carrageenan / ι-carrageenan mixture in the presence of salt under in vitro conditions in deionized water at 37°C. The reported drug release data are the average value for the three doughnuts (ID: 9mm, OD:30 mm, height: 4mm) samples, and the error bars show the standard deviation.

When a polyelectrolyte gel is placed within an electric field exhibiting a potential gradient, such as by placing between two electrodes with an applied voltage, the hydrogel swells or contracts depending on the charge of the hydrogel. This responsive behavior occurs through a combination of Coulombic, electrophoretic, piezoelectric, electroosmotic, and electrostrictive interactions[68]. Electrically controlled drug release has also been investigated for carrageenan gels. The release of the folic acid with electric stimulation in deionized water is shown in Figure 8. We could not obtain well-shaped 3D printed electrodes for κ- and ι-carrageenan in the presence of folic acid (0.05 %) and KCl (1.0 %). When the electric field is applied to the gel, folic acid releases from the gel at a constant rate, whereas the release slows down shortly after stopping the applied field. This is because the potential creates a uniform electric field that causes the hydrogel to shrink. Shrinking the gel and changing the network size pushes the drug molecules with the liquid phase at the same

time from the hydrogels[69]. A delay of around 5min is observed in the release upon applying the potential, which is due to conducting the experiment in deionized water. A short time is needed for KCl to be released in the medium, make it conductive, and later folic acid is released. That is why the rate of the release is higher when KCl (2.0 %) than when KCl (0.5 %). However, when considering the electrical response of gels, the electrochemistry of the system must be taken into account. The catalytic activity of the electrodes or other electrolytic processes will be superimposed on the electrical response of most gels[70].



(a) (b)

Figure 8. The release of the folic acid from ϰ-carrageenan / ι-carrageenan mixture in the presence of (a) KCl (0.5 %), (b) KCl (2.0 %) with electric stimulation (DC: 5.0 V) in deionized water at ambient temperature. The release slows down shortly after stopping the applied field. The delay of around 5min is due to conducting the experiment in deionized water. The reported drug release data are the average value for the three samples, and the error bars show the standard deviation.

The ϰ- and ι-carrageenan in the presence of folic acid (0.05 %) and KCl (2 %) might also be pH-responsive. It is even bending while in the electric field (Figure S4 (Supporting Information)). This

bending response, which is the result of osmotic pressure difference around the gel [70], places this formulation under the category of biodegradable soft (edible) actuators, which have potential applications as less invasive electronic capsules in monitoring the internal organs[71]. These are a promising area for future research.

Conclusion: Designing multi-responsive formulations is highly desirable for many food engineering and health applications. However, the available materials are rare. In this paper, we used a formulation based on κ- and ι-carrageenan and potassium chloride to obtain different properties, among them printability, thermal release, and electrical stimuli drug release. All formulations presented here are systems with high mobility and multiphase structures. Microheterogeneity and microphase separation were also observed. The drug release profiles (thermal and electrical) can be controlled by modifying the formulation parameters of the gel and therefore increasing or decreasing the density of the aggregate. Moreover, when the electric field is applied to the gel, folic acid releases from the gel at a constant rate, whereas the release slows shortly after stopping the applied field. Although in principle, the proposed formulation does not rely on 3D printing, the food and drug industry needs new feedstocks [72], and this formulation is a new addition that could offer exciting applications for the future. Considering the widespread use of carrageenan in dairy products and pulsed electric fields (PEFs) for pasteurization and possibly sterilization [43], the developed carrageenan-based gel can be used as an alternative medium for enriching dairies by folic acid.

Associated content

Supporting Information

The Supporting Information is available free of charge at

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