Magnetothermal heating facilitates the cryogenic recovery of stem cell–laden alginate–Fe$_3$O$_4$ nanocomposite hydrogels†

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Constructs of magnetic nanocomposite hydrogels microencapsulated with stem cells are of great interest as smart materials for tissue engineering and regenerative medicine. Due to the short shelf life of such biocomposites at an ambient temperature, their long-term storage and banking at cryogenic temperatures are essential for the “off-the-shelf” availability of such biocomposites for widespread clinical applications. However, high-quality cryogenic recovery of stem cell–nanocomposite hydrogel constructs has not yet been achieved due to the damage to cells and/or microstructures of hydrogel constructs caused by ice formation, particularly during warming from cryogenic temperatures. Herein, stem cell–magnetic nanocomposite hydrogel constructs, which have an inherent magnetothermal property provided by embedded magnetic nanoparticles, are explored to achieve ultra-vapid cryogenic warming. The binding of water molecules by the hydrogel combined with the magnetothermal heating greatly suppressed ice formation during both cryogenic cooling and warming. Thus, the cryogenic recovery of nanocomposite hydrogel constructs with intact microstructures and fully functional stem cells from ultra-low temperatures was successfully achieved. We further demonstrated that magnetic nanocomposite hydrogels microencapsulated with stem cells could be conveniently manipulated for a self-assembled 3D culture. Together, we have developed a highly efficient and easy-to-perform approach for the cryogenic recovery of stem cell-encapsulated magnetic nanocomposite hydrogel constructs. Our results will facilitate the applications of such stem cell–magnetic nanocomposite hydrogels in regenerative medicine and tissue engineering.

Introduction

Nanocomposite hydrogels (NCHs) consisting of polymer matrices and nanomaterials are of great interest as smart materials for applications in tissue engineering, targeted drug release, cancer therapy, and enzyme immobilization.1–7 NCHs combine the hydrogel’s similarity to the native extracellular matrix (ECM) and special functionalities of nanomaterials for different biomedical applications.8–10 Previous reports suggest that magnetic NCHs can be fabricated as porous scaffolds to stabilize growth factors or other biological agents bound to magnetic nanoparticles (MNPs), providing a suitable environment for cell adhesion and proliferation.11 Magnetic NCHs have also been used in cancer hyperthermia therapy and drug delivery via an external magnetic field (MF).12 Enzymes can also be immobilized on MNPs in NCHs to improve their stability before being released via heating through an external MF.13,14 Moreover, NCHs in various shapes and forms (e.g., microbeads, core–shell microcapsules, microfibers) can be conveniently fabricated by different methods (e.g., electrostatic spraying and microfluidic device).15–18

Recently, constructs of NCHs microencapsulated with stem cells have been of interest in tissue engineering and regenerative medicine.19–21 Mesenchymal stem cells (MSCs) have great prospect in clinical medicine, because they can self-renew and differentiate into multiple lineages.22 As extracellular matrices, hydrogels provide a microenvironment for stem cell
proliferation, migration and differentiation, while permitting the diffusion of nutrients and cell metabolites via a porous structure.23,24 Cell–laden NCHs impregnated with MNPs can be further used as building blocks and assembled to form sophisticated tissue constructs via MF, which is convenient to manipulate without complex equipment and have the potential to influence many fields including pharmacology and stem cell research.25 However, to avoid continuous cultivation that is expensive, time-consuming and a source of uncontrollable differentiation, banking stem cells and their constructs at cryogenic temperatures is essential to provide the “off-the-shelf” availability of cell-based clinical applications on demand.26,27 Presently, slow-freezing and vitrification methods are used for cryopreservation.28,29 Vitrification can convert a liquid to a glassy state without ice formation at vapor-phase nitrogen temperatures (−160 °C) and thus does not cause cell damage.30,31 Thus, intact structures of cell–laden NCHs can be obtained post cryopreservation using vitrification.32 Additionally, cooling is fast and simple in vitrification.33 However, to the best of our knowledge, vitrification has not yet been reported for stem cell–laden NCHs impregnated with MNPs, in which the inherent magnetothermal property provided by embedded MNPs can be explored to achieve ultrarapid cryogenic warming.

We have previously found that magnetothermal heating and microencapsulation can suppress ice recrystallization for the vitrification of stem cell–alginate hydrogel constructs.34 Alginate hydrogels can inhibit devitrification by decreasing free water in networks, and as such, may be used as an ideal material for warming vitrified samples. However, in this previous study, magnetothermal heating was achieved by adding MNPs into external solutions surrounding the hydrogel constructs. Herein, we have further developed alginate–Fe₃O₄ based magnetic NCHs to achieve the efficient recovery of microencapsulated MSCs using magnetothermal heating post vitrification. The incorporation of MNPs within alginate hydrogels helped establish an inherent magnetothermal property to facilitate rapid and uniform cryogenic warming, enhancing cell viability and the function of cryopreserved biological samples from vitrification. Compared with other NCHs, magnetic NCHs impregnated with MNPs have unique functionalities, such as rapid responses to an external MF.35–38 MNPs incorporated in NCHs can also serve as controllable “on/off” switches to facilitate the release of drugs or growth factors for biomedical and pharmaceutical applications.39 Magnetic responses of MNPs can also be more desirable for remote manipulation and control, compared with other general stimuli, such as pH and temperature.40,41 For magnetic NCHs impregnated with MNPs, heat production can be modulated by not only MF frequency but also MNP abundance.42 In addition, magnetic NCHs impregnated with MNPs can have good mechanical properties, useful for cell growth and differentiation for tissue regeneration.43,44 In this work, we have successfully demonstrated the magnetothermal heating of stem cell–magnetic NCH constructs to effectively suppress ice formation and facilitate rapid and uniform cryogenic warming.

Moreover, stem cell–magnetic NCH constructs with core–shell structures were fabricated to support patterned heating. Magnetic NCH constructs microencapsulated with stem cells recovered from vitrification were further applied for 3D magnetic levitation culture and self-assembly using different MF patterns. Together, our results will facilitate the cryogenic storage and recovery of stem cell-encapsulated NCHs, facilitating their applications in regenerative medicine and tissue engineering.

Materials and methods

Chemicals

All chemicals were acquired from Sigma (St Louis, MO, USA), except otherwise noted. Two CPA (cryoprotective agents) solutions were prepared in this study. CPA #1 consisted of 1 mol L⁻¹ EG (ethylene glycol), 1.5 mol L⁻¹ PROH (1,2-propanediol), 1 mol L⁻¹ trehalose (Sinozyme Biotechnology Co., Ltd, China) and 10% (w/v) dextran T50 (≃0.002 mol L⁻¹). CPA #2 consisted of CPA #1 and 0.5% (w/v) Fe₃O₄ NPs. The CPA solutions were prepared by dissolving in 20% (v/v) culture medium and 80% (v/v) fetal bovine serum (FBS; Hyclone, Logan, Utah, USA).

Cell culture

MSCs were supplied by Anhui Medical University Corporation (Hefei, Anhui, China). Cells were isolated from neonatal umbilical cords from the Second Affiliated Hospital of Anhui Medical University, and the cells used were obtained after the informed consent of patients before parturition. Isolated MSCs were seeded into a T25 culture flask (Eppendorf, Hamburg, Germany), and cultured in DMEM (Hyclone, Logan, Utah, USA) containing growth factors and 10% (v/v) FBS. Then the cells were cultured in a humidified incubator at 37 °C with 5% CO₂. The medium was replaced every 2 to 3 days until the cells reached 80–90% confluency. The cells were washed with PBS (Hyclone, Logan, Utah, USA) and digested with 0.25% trypsin-EDTA (Gibco, Carlsbad, CA, USA) for 2 to 3 min. After the cells floated, serum was added to terminate digestion, and the cell suspension was collected and centrifuged at 100g for 5 minutes to obtain cell pellets. Finally, the cells were resuspended with medium for later use. Cells at passages 1–6 were adopted for subsequent experiments.

Synthesis of Fe₃O₄ nanoparticles

Fe₃O₄ NPs were prepared using a chemical co-precipitation method. 2.71 g FeCl₃·6H₂O and 1.0 g FeCl₂·4H₂O were mixed in distilled water under the protection of nitrogen gas at 70 °C; then, an ammonia solution was added to the mixture solution dropwise. The mixture was continually stirred for 1.5 h to finish the reaction. Afterwards, Fe₃O₄ NPs were collected by a magnet after cooling to room temperature and were washed with distilled water at least three times. Finally, the sample was dried in vacuo and stored at 4 °C for later use.
Fabrication of the electrostatic spraying device

An electrostatic spraying device for microencapsulation consisted of a high voltage generator (Dongwen High Voltage Power Supply Co., Ltd, Tianjing, China), a syringe pump (High-Precision Programmable Syringe Pump, WK-101P, Nanjing Anerke Electronics Technology Co., Ltd, Nanjing, China), a needle (diameter 300 μm) or a tube-in-tube device, an iron support and a gelling bath (0.15 mol L⁻¹ CaCl₂ solution). The syringes, needles (or tube-in-tube devices) and other accessories were sterilized before use.

Fabrication of alginate–Fe₃O₄ nanocomposite hydrogels

Firstly, 2% (w/v) sodium alginate (Sangon Biotech, Shanghai, China) was dissolved in 0.25 mol L⁻¹ d-mannitol solution to maintain isotonic pressure. In addition, 0.5 mol L⁻¹ trehalose was added. The solution was filtered with a 0.22 μm pore size filter prior to use. Later, 0.05, 0.1 and 0.5% (w/v) Fe₃O₄ NPs were incorporated into the solution with/without stem cells (cell density of 8.3 × 10⁶ mL⁻¹). Then, the solutions were introduced in 5 mL syringes and pumped at a flow rate of 10 μL min⁻¹. During extrusion, the distance between the needle tip and the surface of the calcium chloride solution was 3 cm, and the voltage applied between electrodes was 10 kV. Droplets of mixture solutions were generated on the tip of the needle, sprayed into calcium chloride solution and gelled to form ~300 μm microcapsules. Finally, alginate–Fe₃O₄ nanocomposite hydrogels were collected and stored at 4 °C for later use.

Microcapsule size was controlled by adjusting the flow rate, voltage and distance between electrodes. Moreover, double-layer Fe₃O₄ hydrogel microcapsules with stem cells encapsulated interiorly or exteriorly could be fabricated with a tube-in-tube device. Similarly, the size and exterior thickness can be adjusted by the methods mentioned above.

Characterization of alginate–Fe₃O₄ nanocomposite hydrogels

The morphology of alginate–Fe₃O₄ nanocomposite hydrogels was observed under an inverted microscope, and microcapsule diameters were counted using Image-Pro Plus 6.0. The magnetic property of the alginate–Fe₃O₄ nanocomposite hydrogels was measured using X-ray diffraction (XRD; Philips X’Pert PRO, Netherlands) with an angle range of 2θ = 10–90°. Moreover, alginate–Fe₃O₄ nanocomposite hydrogels with/without cooling–warming were freeze-dried using a Linkam FDCS196 cryomicroscope (Linkam, Surrey, UK). Microcapsules were first cooled to −60 °C at 100 °C min⁻¹. After equilibrium for 5 min, the microcapsules were freeze-dried at −60 °C, 10 Pa for 4 h. The freeze-dried microcapsules were sputtered with gold, and their internal and external images were obtained using a scanning electron microscope (SEM; JEOL Model JSM-6390 LA, Japan).

Cytotoxicity of the alginate–Fe₃O₄ nanocomposite hydrogels

Fluorescence staining was used to measure cell viability. The toxicity of the alginate–Fe₃O₄ nanocomposite hydrogels was assayed at 4 °C using an acridine orange/ethidium bromide (AO/EB) staining kit (KeyGen Biotech Co., Ltd, Nanjing, China). MSCs were encapsulated with different concentrations (0.05, 0.1 and 0.5% (w/v)) of the alginate–Fe₃O₄ nanocomposite hydrogels and were cultured at 4 °C for 0.5, 1 and 2 h. Later, cells were released from the hydrogels using 75 mmol L⁻¹ sodium citrate (Sangon Biotech, Shanghai, China) and collected using an Eppendorf 5424R centrifuge (Eppendorf, Hamburg, Germany). Cells resuspended were incubated with AO/EB solution (1 : 1 v/v) in the darkroom at room temperature for 3 min. Fluorescence was observed with an inverted fluorescence microscope (Ti-U, Nikon, Japan); and live cells stained green; dead cells stained red. Fluorescence images of MSCs were taken using a CCD camera (DS-Ri1, Nikon, Japan).

Uptake of Fe₃O₄ nanoparticles

For the cell uptake experiment, MSCs were incubated within alginate–Fe₃O₄ nanocomposite hydrogels for 2 h at 4 °C. Then cells were released from microcapsules and collected. Afterwards, the MSCs were washed with PBS three times, and fixed with glutaraldehyde at 4 °C overnight for analysis. Images were obtained using a transmission electron microscope (TEM; Hitachi, Ltd, Tokyo, Japan).

MTH system

The MTH device (Shenzhen Shuangping Power Supply Technology Co., Ltd, China) consisted of an induction heating machine connected with an intermediate frequency generator (SPG-10 (A)B-II), a water pump, a water tank, a 37 °C water bath and a 6-loop coil (4.5 cm in diameter). Cells resuspended were incubated within alginate–Fe₃O₄ nanocomposite hydrogels for 2 h at 4 °C. Then cells were released from microcapsules and collected. Afterwards, the MSCs were washed with PBS three times, and fixed with glutaraldehyde at 4 °C overnight for analysis. Images were obtained using a transmission electron microscope (TEM; Hitachi, Ltd, Tokyo, Japan).

Vitrification of MSCs

In this study, CPA solutions were penetrating and nonpenetrating CPAs in 80% (v/v) FBS. Penetrating CPAs were EG and PROH, the nonpenetrating CPAs were trehalose and dextran T50.

For cooling, a multi-step method was used to add CPA solutions. Cell–laden alginate–Fe₃O₄ nanocomposite hydrogels were equilibrated in the pre-cooled 1.0 mol L⁻¹ EG and 1.0 mol L⁻¹ PROH for 20 min at 4 °C and centrifuged at 300g to remove the penetrating CPA solution. CPA #2 was added to resuspend the microcapsules and incubated for 15 min. The solution was slightly vortexed using a Vortex-Genie 2 mixer (Scientific Industries, New York, USA) and rapidly loaded into a 250 μL plastic straw (PS, FHK, Japan), and the PS was placed under liquid nitrogen for 5 min.

Warming using MTH

For warming, PS without MTH post vitrification was quickly plunged into a 37 °C water bath and shaken quickly. CPA was subsequently removed from culture medium containing 0.75, 0.5 and 0.25 mol L⁻¹ trehalose stepwise. For the MTH group,
vitrified PS was rapidly transferred to a coil device which was placed in a water bath at 37 °C with a frequency of 375 kHz. Different currents (5, 15 and 25 A) were used for adjusting the MF. After warming with MTH, cell–laden alginate–Fe3O4 nanocomposite hydrogels were collected in an Eppendorf tube and the CPA was removed stepwise as above. Nanocomposite hydrogels were dissolved in 75 mmol L−1 sodium citrate for 10 min and the encapsulated cells were released. Residual Fe3O4 NPs were thoroughly removed using a magnet. Then the solution was centrifuged for 5 min and the supernatant was removed. Finally, the cells were resuspended in medium for subsequent experiments.

MTH was also evaluated using a Fluke Ti25 thermal imager (Fluke, Everett, Washington, USA). CPA solution and 0.05, 0.1, 0.5 and 1% (w/v) of alginate–Fe3O4 nanocomposite hydrogels were loaded in an Eppendorf tube. The initiation temperature for the samples was 37 °C. The samples were plunged into the coil device for 1 min MTH and were quickly taken out. Infrared thermograms of samples were recorded using an imager and the maximum temperature of each sample was indicated.

Cell viability and attachment efficiency

MSC viability post vitrification was evaluated using an AO/EB staining kit as described. MSCs released from microcapsules were resuspended in 20 μL of fresh medium and 1 μL of the fluorescent dye, then incubated for 5 min at room temperature. The fluorescence images of the cells were taken using a Nikon Ti-FL inverted microscope with a DS-Ri1 (Nikon, Japan) camera (10× objective). Red (i.e., EB) and green (i.e., AO) fluorescence represent dead and live cells, respectively. Cell viability was calculated as the percentage of live cells to the total number of cells.

Cell attachment was assessed by cell counting. MSCs post vitrification were incubated with fresh medium overnight; then, the attached (live cells) and suspended cells (dead cells) were counted using a cell-counting chamber. Fresh MSCs without vitrification were used as the controls. Attachment efficiency was calculated as the ratio of attached cells to total cells.

Proliferation and functional tests of MSCs

For the proliferation study, MSCs post vitrification were seeded in 96-well plates at 1 × 10^4 mL−1, and the fresh cells were seeded at the same density as controls. After incubation for 1, 2, and 3 days in 37 °C incubators, the cells in each well were assayed with a CCK-8 assay. 10 μL of the CCK-8 (Beyotime Inst Biotech., China) reagent were added to each well in a 100 μL system after the removal of the medium, and 96-well plates were incubated in 37 °C and 5% CO2 incubator for 4 h. Absorbance at 450 nm was measured with plate reader (Diagnostics Pasteur, Marne la Coquette, France) to evaluate cell activity. Proliferation was calculated as relative absorbance on days 2 and 3 to absorbance on day 1.

To study the functional survival of MSCs post vitrification, immunofluorescence staining was performed for CD44+, CD90+ and CD45− surface markers. MSCs post vitrification were seeded onto sterile coverslips and incubated overnight for attachment. Fresh cells were seeded as controls. Cells on the coverslips were washed with PBS three times and fixed with Immunostaining Fixed Solution (Beyotime, Haimen, China) overnight at 4 °C. Fixed cells were incubated with Immunostaining Blocking Solution (Beyotime, Haimen, China) for 1 h at room temperature to block nonspecific binding. Then the cells were incubated with primary antibodies of mouse anti-human CD44 monoclonal antibody (1 : 100, Proteintech, Wuhan, China), purified mouse anti-pig CD90 (1 : 50, BD Pharmingen, San Diego, CA, USA) and purified mouse anti-human CD45 (1 : 50, BD Pharmingen, San Diego, CA, USA) overnight at 4 °C. On the next day, the cells were washed with PBS three times and then were incubated with a secondary antibody coupled to Alexa Fluor 488 antibody (1 : 50, Thermo Fisher Scientific, Waltham, MA, USA) in the dark for 1 h at room temperature. After washing to remove redundant secondary antibodies, DAPI (Beyotime, Haimen, China) was used for staining nuclei at room temperature for 10 min. Fluorescence images were studied using an inverted fluorescence microscope (Nikon Eclipse Ti-U, Tokyo, Japan).

CD44+, CD90+ and CD45− surface marker expression was measured using flow cytometry (BD FACSVerse, NJ, USA). Fresh MSCs and MSCs post vitrification were detached using trypsin-EDTA and washed with PBS three times. Then the cells were stained with CD44-FITC (1 : 100, BD Pharmingen, San Diego, CA, USA) and CD45-PE (1 : 50, BD Pharmingen, San Diego, CA, USA) in the dark for 1 h at 4 °C. After removal of redundant antibodies, the cells were analyzed with FACSuite software.

For multilineage potential assays, 1 × 10^5 mL−1 were seeded in 12-well plates and cultured until 60–80% confluency was reached. The adipogenic or osteogenic differentiation medium (ThermoScientific, USA) was added into the plates after the removal of the culture medium and the cells were cultured for 14 d and 21 d, respectively. During this time, the medium was changed every 3 days. After the end of induced differentiation, the cells were washed with PBS after the removal of medium and fixed with 4% paraformaldehyde for 30 min. The fixed cells were stained with Oil Red O for 30 min and Alizarin Red S for 10 min, respectively. Nuclei were stained with DAPI. The stained cells were examined under an inverted fluorescence microscope (Nikon Eclipse Ti-U, Nikon, Japan) to evaluate adipogenic and osteogenic differentiation.

3D levitated culture of cell–laden alginate–Fe3O4 nanocomposite hydrogels

To assess the viability of stem cells in nanocomposite hydrogels after a 3D levitated culture, cell–laden alginate–Fe3O4 nanocomposite hydrogels post vitrification were cultured in a culture dish for 0, 3 and 5 days with a magnet over the cap. Viability was evaluated with AO/EB staining.

Self-assembly of cell–laden alginate–Fe3O4 nanocomposite hydrogels

To explore the application of cell–laden alginate–Fe3O4 nanocomposite hydrogels, hydrogels were self-assembled in different
patterns in MF. Magnetic rods were placed in a “cell” shape under the culture dish to cause the nanocomposite hydrogels to assemble in this shape. In addition, when the magnetic rod was immersed in the culture medium, the nanocomposite hydrogels assembled in a spheroid shape for construction.

Cryomicroscopy studies of ice formation

Cryomicroscopy studies were performed to investigate ice formation during cooling and warming. To study the CPA solution during cooling and warming, one drop of the CPA solution was added in the center of the silicone sheet and coverslipped to ensure a thickness of 200 μm. Alginate slices were prepared as follows: a silicone sheet (200 μm) with a hole in the center was placed on a glass. Then, a drop of the sodium alginate solution was added in the center and was sandwiched with a coverslip. Subsequently, this sandwich-like structure was immersed in a culture dish containing CaCl2 solution for crosslinking. After 5 min immersion and removal of the coverslip, a calcium alginate slice was obtained (200 μm). Meanwhile, alginate slices containing Fe3O4 NPs were prepared in the same way. Moreover, the CPA solution was loaded by incubating the alginate slice in the CPA solution for 30 min at 4 °C. Ultimately, the slices were placed on a Linkam FDCS196 cryomicroscope for programmed cooling and warming. Samples were first cooled to −4 °C (initial temperature) and held for 2 min to achieve a thermal equilibrium (without seeding), then started to cool until −120 °C at 100 °C min−1 to emulate a rapid cooling process. After equilibrium at −120 °C for 3 min, the samples were warmed back to room temperature (25 °C). Ice formation and growth were recorded using a QImaging (Surrey, Canada) Retiga CCD color camera during cooling and warming.

Statistical analysis

All data are presented as means ± standard deviation from at least three independent experiments. Statistical analysis was performed with a Student’s two-tailed unpaired t-test between groups (p < 0.05 was considered statistically significant).

Results and discussion

Fabrication of alginate–Fe3O4 nanocomposite hydrogels

Fig. 1a shows an electrostatic spray system consisting of an iron stand, syringe pumps, a high voltage regulator, a needle (or a tube-in-tube device) and a gel bath. Cell–laden alginate–Fe3O4 nanocomposite hydrogels were produced by crosslinking sodium alginate in microdroplets with Ca2+ in a CaCl2 solution to form calcium alginate. Cell–laden alginate–Fe3O4 nanocomposite hydrogels had diameters of ~310 μm. Afterwards, microbeads were collected for vitrification and recovery studies (Fig. 1b). For double-layer microcapsules, a tube-in-tube device was used with the core fluid of MSCs (or Fe3O4 NPs). Shell fluid contained Fe3O4 NPs (or MSCs), as shown in Fig. 1c. These diversified microcapsules can be tailored for patterned heating.

Characterization of alginate–Fe3O4 nanocomposite hydrogels

Alginate–Fe3O4 nanocomposite hydrogel microcapsule sizes are depicted in Fig. 2a. The diameter of alginate–Fe3O4 nanocomposite hydrogels was 313.2 ± 4.8 μm, and the morphological traits were observed under an inverted microscope. Alginate–Fe3O4 nanocomposite hydrogels were almost spherical particles and uniform. In addition, the magnetization curves measured by a vibrating sample magnetometer indicated that magnetic hydrogels at various concentrations of Fe3O4 had different magnetic properties (Fig. 2b). Saturation magnetizations of 0.1% and 0.5% (w/v) alginate–Fe3O4 nanocomposite hydrogels and Fe3O4 alone were 3.6, 22.6, and 65.4 emu g−1, respectively. Moreover, alginate–Fe3O4 nanocomposite hydrogels had a good magnetic response under an external MF. X-ray diffraction (XRD) patterns of alginate–Fe3O4 nanocomposite hydrogels are shown in Fig. 2c. Diffractogram data for Fe3O4 coincide with the standard diffraction pattern, and data for the alginate showed two wide bands at 13.5° and 21.5° of low intensity which is consistent with previous studies.43,46 Hydrogel–Fe3O4 has characteristic peaks of Fe3O4 and the alginate. SEM images of internal and external morphology of alginate–Fe3O4 nanocomposite hydrogels appear in Fig. 2d. The interior of alginate–Fe3O4 nanocomposite hydrogel without cooling–warming showed that NPs were dispersed uniformly inside the hydrogels, and the exterior indicated that NPs were embedded in the hydrogels. Protuberances on the hydrogels may be caused by freeze-drying. The alginate–Fe3O4 nanocomposite hydrogels which were not cooled/warmed were similar to those that were cooled/warmed, which indicates that they have mechanical strength and can maintain structures post-vitrification. Thus, the alginate–Fe3O4 nanocomposite hydrogels can be vitrified with intact structures and can be recycled for later use.

Cytotoxicity of the alginate–Fe3O4 nanocomposite hydrogels

Cell viability was evaluated for the cytotoxicity of the alginate–Fe3O4 nanocomposite hydrogels. Since all experiments were
kept at 4 °C within 2 h, we tested the cytotoxicity of the alginate–Fe₃O₄ nanocomposite hydrogels at 4 °C for 2 h to explore the optimal condition for avoiding toxic injury. Cell viability showed that the alginate–Fe₃O₄ nanocomposite hydrogels did not have cytotoxicity in MSCs at various concentrations at 4 °C, as described in the Methods section (Fig. 3a, Table S1†). Thus, cytotoxicity is observed in a time and concentration-dependent manner. Fluorescence images of MSCs after incubation within the alginate–Fe₃O₄ nanocomposite hydrogels at 4 °C at different time points are shown in Fig. 3b. Fig. 3c shows that few Fe₃O₄ NPs were observed after incubation in 0.5% alginate–Fe₃O₄ nanocomposite hydrogels for 2 h at 4 °C.

Vitrification and warming of stem cell–laden alginate–Fe₃O₄ nanocomposite hydrogels

Vitrification and recovery processes of cell–laden alginate–Fe₃O₄ nanocomposite hydrogels from cryogenic temperatures are illustrated in Fig. 4a. Warming using MTH is a more attractive way of recovering high-quality samples. Fig. 4b shows typical images during cooling and warming using PSs loaded with different solutions. Three PSs containing culture medium, CPA #1 and CPA #2 before cooling are shown in Fig. 4b (i). During cooling, PS with culture medium was opaque, indicating ice crystal formation, whereas CPA #1 and #2 were transparent in appearance and no visible ice formation

Fig. 2 Characterization of alginate–Fe₃O₄ nanocomposite hydrogels. (a) Size distribution and typical differential interference contrast (DIC) images of cell–laden alginate–Fe₃O₄ nanocomposite hydrogels. (b) Magnetization curves of 0.1 and 0.5% (w/v) alginate–Fe₃O₄ nanocomposite hydrogels and Fe₃O₄ NPs measured using a vibrating sample magnetometer at room temperature. Insets are images of alginate–Fe₃O₄ nanocomposite hydrogel dispersions with deionized water and its magnetic response. (c) X-ray diffraction (XRD) pattern of alginate–Fe₃O₄ nanocomposite hydrogels. (d) SEM images of interior and exterior morphology of alginate–Fe₃O₄ nanocomposite hydrogels with (W/) or without (W/O) cooling–warming. Red arrows indicate Fe₃O₄ NPs.

Fig. 3 Cytotoxicity of alginate–Fe₃O₄ nanocomposite hydrogels at 4 °C. (a) Cell viability of alginate–Fe₃O₄ nanocomposite hydrogels with different concentrations at 0.05, 0.1 and 0.5% (w/v) for 0.5, 1 and 2 h. (b) Fluorescence images of MSCs after incubation within 0.05, 0.1 and 0.5% (w/v) alginate–Fe₃O₄ nanocomposite hydrogels for 0.5, 1, and 2 h. (c) TEM images of MSCs incubated within 0.5% (w/v) alginate–Fe₃O₄ nanocomposite hydrogels for 2 h.
occurred. Thus, they can be vitrified in LN₂ (Fig. 4b (ii)). However, devitrification/recrystallization of PSs appeared during warming, as shown in Fig. 4b (iii). Typical observations of ice crystals appearing, reaching maximum recrystallization and thawing completely were captured. The warming of PS containing CPA #1 via a conventional water bath is shown in Movie S1. PS containing CPA #2 with MTH showed reduced ice-crystal growth compared to PS without MTH, and this shortened the ice crystal duration (Movies S2 and S3†). Recovery via MTH also shortened the ice crystal duration compared with the conventional water bath. So, cells may experience harmful temperatures more quickly that reduce ice injury. MTH may suppress devitrification/recrystallization during warming and may facilitate the recovery of cell–laden alginate–Fe₃O₄ nanocomposite hydrogels.

To assess the MTH effect, an infrared thermogram was used to record data that appear in Fig. 4c. The maximum temperature increased with increasing Fe₃O₄ in hydrogels, suggesting that alginate–Fe₃O₄ nanocomposite hydrogels have an MTH effect.

Cell viability and attachment efficiency of MSCs
Viability data for MSCs post vitrification are shown in Fig. 5a. Unencapsulated cells were less viable without MTH (16.9%), and encapsulated cells were more viable (72.1%). Perhaps the hydrogel networks can reduce freezable water and inhibit devitrification during warming. When 0.05% (w/v) Fe₃O₄ NPs was added to a CPA solution with unencapsulated cells and under 15 A current intensity, survival increased compared to warming without MTH (35.8% vs. 16.9%) (Table S2†). However, survival was still poor, perhaps because MTH was insufficient to overcome ice crystal formation produced by devitrification in the bulk solution during warming, causing severe intracellular ice damage. To explore the optimal conditions for the cell viability of encapsulated cells using MTH, we studied Fe₃O₄ NP concentration and current intensity, as indicated in the Methods section. Encapsulated cell viability did not increase significantly when 0.05% and 0.1% Fe₃O₄ NPs were incorporated into constructs at different current intensities, indicating that heating at lower concentrations of Fe₃O₄ NPs was not obvious. When Fe₃O₄ NPs in the construct was 0.5%, cell survival increased, and optimal viability was obtained with 15 A (82.8%) (Table S3†). To decrease the Fe₃O₄ NPs inside the hydrogels to avoid cytotoxicity, Fe₃O₄ NPs in the CPA solution were studied for the synergistic recovery of cell–laden 0.05% Fe₃O₄ nanocomposite hydrogels (Fig. 5b).
Fe₃O₄ NPs inside and outside constructs can generate heat synergistically with MF to reduce ice crystals and improve warming efficiency. In addition, MTH can be regulated by the concentration of Fe₃O₄ NPs as well as by current intensity. To evaluate cell survival after recovery from 0.5% Fe₃O₄ in CPA solution, attachment efficiency was studied, and the data appear in Fig. 5c.

**Functional properties of MSCs with MTH**

The functional properties of MSCs with MTH post vitrification were assessed. There were no significant differences in functional properties of MSCs with MTH post vitrification.
between recovered and fresh cells in proliferation (Fig. 6a, Table S5†), and their morphologies were similar (Fig. 6b). In addition, typical MSC surface glycoproteins/receptors such as CD44, CD90, and the negative marker CD45 were detected by immunofluorescence staining and flow cytometry. The results indicate that the expression of CD44 and CD90 with MTH post vitrification were similar to fresh cells, while CD45 was not expressed on both groups (Fig. 6c and d). Furthermore, adipogenic and osteogenic differentiation data appear in Fig. 6e. Vitrified and fresh cells had similar potentials for multilineage differentiation. Thus, MSCs maintain their functional properties after recovery from the MTH effect.

**3D levitated culture of stem cell–laden alginate–Fe₃O₄ nano-composite hydrogels**

We studied the application of cell–laden alginate–Fe₃O₄ nano-composite hydrogels post vitrification, and a 3D levitated culture appears in Fig. 7a. An illustration of the set-up appears in Fig. 7b. Conventional culture shows two-dimensional cell growth with gene expression, signal transduction and morphology that are different from *in vivo* tissues. However, previous studies have reported that magnetic-levitated culture systems closely resembled an *in vivo* 3D environment which exhibits a similar protein expression profile and molecular mechanism, and thus can be a useful model for biotechnology.
As shown in Fig. 7c, fresh microcapsules and microcapsules recovered with MTH indicated that there was no obvious difference between fresh microcapsules and microcapsules post vitrification, and cells maintained good viability for 5 days after recovery from cryogenic temperatures and levitated culture.

Patterned assembly of stem cell–laden alginate–Fe3O4 nanocomposite hydrogels

Furthermore, nanocomposite hydrogels can be self-assembled to fabricate complex constructs, as shown in Fig. 7d. Also, nanocomposite hydrogels can adsorb onto the magnetic rod to form a spheroid when placed in a culture medium vertically (Fig. 7e). Therefore, cell–laden alginate–Fe3O4 nanocomposite hydrogels can be used post vitrification for 3D tissue constructs and tissue engineering.

Possible mechanism for the recovery of alginate–Fe3O4 nanocomposite hydrogels

To investigate alginate–Fe3O4 nanocomposite hydrogels during cooling and warming, cryomicroscopy experiments were performed. The preparation process of 200 μm alginate hydrogel slices is shown in Fig. 8a. Fig. 8b shows ice crystal formation with different CPA solutions, Fe3O4 NPs and alginate hydrogels during cooling and warming. For the CPA group, ice crystals appeared at −69.9 °C during cooling and grew until −88.9 °C. Recrystallization occurred at −24.0 °C during warming (Movie S4†). In a CPA solution with 0.1% (w/v) Fe3O4 NPs, ice crystals appeared earlier (−63 °C) during cooling compared with the CPA solution alone, and maximal crystallization was faster (−74.2 °C) (Movie S5†). When the concentration of Fe3O4 NPs increased to 0.5% (w/v), ice crystals appeared much earlier (−44.0 °C) and completely crystallized early (−47.0 °C) (Movie S6†). Thus, introduction of Fe3O4 NPs improves ice crystal formation. For the alginate hydrogel group, tiny ice crystals appeared at −90.2 °C, and these crystals did not grow remarkably until −120 °C and during warming, demonstrating that alginate hydrogels can inhibit the formation and growth of ice crystals during cooling and warming (Movie S7†). For alginate hydrogels with the 0.1% and 0.5% (w/v) Fe3O4 NP group, ice crystals appeared at −83.9 °C and −64 °C, and these crystals did not grow notably afterwards (Movies S8 and S9†). It is likely that alginate hydrogels are key to decreasing freezable water, and nanocomposite hydrogel materials only behave tiny ice crystals during cooling and warming. The addition of CPA solution in 0.1% and 0.5% (w/v) Fe3O4 hydrogel did not promote visible ice crystals during cooling and warming (Movies S10 and S11†). Thus, alginate–Fe3O4 nanocomposite hydrogels can be used post vitrification for 3D tissue constructs and tissue engineering.

Fig. 7 3D levitated culture and patterned assembly of stem cell–laden alginate–Fe3O4 nanocomposite hydrogels post vitrification. (a) Images of 3D levitated cell–laden alginate–Fe3O4 nanocomposite hydrogels under MF. (b) Illustration of the 3D levitated culture of cell–laden alginate–Fe3O4 nanocomposite hydrogels post vitrification after culture for 0, 3 and 5 days. (d) Images of self-assembled cell–laden alginate–Fe3O4 nanocomposite hydrogels with “cell” pattern by magnets. (e) Images of self-assembled cell–laden alginate–Fe3O4 nanocomposite hydrogels with spheroids using a magnetic rod.
hydrogels can suppress devitrification and may be used to recover biological samples from cryogenic temperatures.

How MTH may improve recovery of MSCs is illustrated in Fig. 9. For cells without encapsulation, no ice crystals are visible in the CPA solution during vitrification, but devitrification occurs during warming, triggering intracellular ice formation and cell death. Despite MTH with Fe₃O₄ NPs minimize devitrification via a thermal effect and rapid warming, but this still does not prevent cell damage. The use of alginate hydrogels and Fe₃O₄ NPs may enhance cell survival and attachment. Alginate hydrogels can suppress devitrification during warming, and Fe₃O₄ NPs homogenously dispersed around cells generate heat in MF and accelerate warming. The presence of Fe₃O₄ NPs in a CPA solution can reduce the concentration of Fe₃O₄ NPs in microcapsules and facilitate recovery of the intact stem cell-nanocomposite constructs with high quality and may have other uses. Ideally, this process can be used to recover stem cells from cryogenic temperatures.

**Conclusions**

In this study, a novel approach integrating magnetic NCH constructs and magnetothermal heating was developed for the high-quality recovery of stem cell–Fe₃O₄ magnetic NCH constructs from cryogenic temperatures. Uniformly dispersed MNPs inside hydrogel constructs effectively suppressed ice
nucleation and ice crystal growth via microscale thermal disturbances and macroscopic enhancement of warming. Moreover, alginate hydrogel decreased freezable water through binding with water molecules during cooling and warming. Patterned heating was further demonstrated using patterned magnetic NCH structures. A quick, controllable thermal response was achieved via the coordinated regulation of MNP concentration and distribution and external magnetic fields. Thus, stem cells and NCH constructs were well preserved and of high quality (intact structures and full function), which can be further applied for drug release, magnetic thermal therapy, and tissue repairing. Recovered magnetic NCH constructs microencapsulated with stem cells could be further utilized for 3D magnetic levitation culture and subsequent magnetic self-assembly. This work provides a new approach for the cryogenic storage and recovery of NCH constructs microencapsulated with stem cells, and as such, will be useful for tissue engineering and regenerative medicine applications.

Author contributions

GZ, JF and ZXZ conceived and designed the experiments, analyzed data and wrote the manuscript. ZXZ, YC, Zh and MW performed the experiments. All authors commented on the manuscript.

Conflicts of interest

There are no conflicts to declare.

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