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Thermosensitive injectable in-situ forming carboxymethyl chitin hydrogel for three-dimensional cell culture

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ABSTRACT

Injectable hydrogels have gained great attentions for cell therapy and tissue regeneration as a result of the applications in minimally invasive surgical procedures with the ease of handling and complete filling of the defect area. Here, a novel biodegradable, thermosensitive and injectable carboxymethyl chitin (CMCH) hydrogel was developed for three-dimensional (3D) cell culture. The obtained CMCH solution remained transparent liquid flowing easily at low temperatures and gelled rapidly at 37 °C. The gelation time of CMCH hydrogels could be easily tuned by varying temperature and the degree of carboxymethylation, which facilitates the cell encapsulation process at room temperature and in-situ forming hydrogel at body temperature. Moreover, the CMCH-14 hydrogels in PBS buffer remained stable and continuous porous structure and could be degraded in the presence of lysozyme or hyaluronidase. HeLa cells proliferated sustainably and self-assembled to form 3D multicellular spheroids with high cell activity on the surface of CMCH-14 hydrogel. Encapsulation of COS-7 cells within the in-situ forming CMCH hydrogel demonstrated that CMCH hydrogels promoted cell survival and proliferation. *In vivo* mouse study of the CMCH hydrogels showed good in-situ gel formation and tissue biocompatibility. Thus, the biodegradable thermosensitive injectable CMCH hydrogels hold potential for 3D cell culture and biomedical applications.

Statement of Significance

Biodegradable hydrogels have been widely studied for cell therapy and tissue regeneration. Herein, we report a novel thermosensitive injectable carboxymethyl chitin (CMCH) hydrogel for 3D cell culture, which was synthesized homogeneously from the bioactive natural chitin through the "green" process avoiding using organic solvent. The CMCH solutions exhibited rapid thermoresponsive sol-to-gel phase transition behavior at 37 °C with controllable gelation times, which facilitates the cell encapsulation process at room temperature and in-situ forming hydrogel at body temperature. Importantly, *in vitro* 3D cell culture and *in vivo* mouse study of the CMCH hydrogel showed promotion of cell survival and proliferation, good in-situ gel formation and biocompatibility. We believe that such thermosensitive injectable CMCH hydrogels would be very useful for biomedical applications, such as tumor model for cancer research, post-operative adhesion prevention, the regeneration of cartilage and central nervous system and so on.

1. Introduction

Three-dimensional (3D) cell culture is well-known to better represent the *in vivo* environment than classic two-dimensional (2D) cell culture, as cells in 3D environments are much more similar to cells in a living organism [1–3] 3D cellular spheroids, which take advantage of the natural tendency of many cell types to aggregate, appear like *in vivo* tissue in terms of cellular communication and the development of extracellular matrices [4]. Multicellular spheroids represent the most common use of *ex vivo* 3D cultures, particularly useful in cancer research [5,6]. Recently, multicellular spheroids are found to hold promising potential in promoting the self-renewal and the stemness

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maintenance of stem cells in the long-time culture [7–10]. The most common 3D scaffold materials used for tissue engineering are natural or synthetic polymers [11] or their combination to overcome respective shortcomings [12].

Hydrogels are three-dimensional hydrophilic polymer-based networks with high water content resembling the native extracellular matrix [13]. Because of their remarkable characteristics, such as high porosity, unique biocompatibility, and adjustable physical, chemical, and biological properties, polymeric hydrogel systems have been extensively explored for 3D cell culture [11]. Due to the ease of administration, simple cell encapsulation, the minimally invasive treatment, and the possibly enhanced patient compliance, in-situ forming injectable hydrogels have been renowned to generate appropriate environment for 3D cell culture in biomedical applications [14]. Recently, a variety of injectable hydrogel systems have been developed in 3D cell culture, including injectable hydrogels prepared using chemical crosslinking [15–17], photocrosslinking [18], enzymes for biological crosslinking [19,20] and physical interactions [21–24]. Injectable hydrogels prepared by chemical crosslinking demonstrate good mechanical properties, but *in vivo* applications have been limited due to the possible cytotoxicity of the reactive chemical crosslinkers. In contrast, injectable hydrogels prepared by physical crosslinking can be formed easily without reactive chemical reagents, resulting in excellent biocompatibility. Various physical crosslinking injectable hydrogels including naturally based hydrogels such as Matrigel [25], collagen [26,27], alginate [28,29], hyaluronan [23,30], chitosan [11,22,31] and stimuli-responsive synthetic polymers such as PEG-PLGA-PEG [32,33], PEO-PPO-PEO [14], poly(*N*-isopropylacrylamide) (PNIPAAm) and its related polyacrylamides [34] have been extensively studied. The commercially available Matrigel is very widely used in cell culture research studies and all the organoid cultures reported made use of it because it contains laminin, collagen IV and a variety of growth factors in varying proportions, gelling rapidly and irreversibly between 24 °C and 37 °C [11,25]. However, Matrigel has some disadvantages, such as heterogeneous composition, compositional diversity between different batches, unknown protein component and the tumor rich source, which makes it unsuitable for studies into the effect of special component and for *in vivo* applications [11]. The alginate/divalent cation (e.g., Ca^{2+}) system suffers from the rapid and poorly controlled gelation rate due to the high solubility of divalent ions in aqueous solutions and the limited long-term stability in physiological conditions due to release of divalent ions into the surrounding media [14,35]. The PEO-PPO-PEO gel has undesired characteristics including short residence time, weak mechanical properties and non-biodegradablity [36]. PNIPAAm and other carbon–carbon backbone based thermosensitive injectable polymers are non-degradable, resulting in limitation of clinical applications. It is of great interest to synthesize new biodegradable and thermosensitive in-situ-forming injectable polymer hydrogels for 3D cell culture and tissue regeneration.

Our strategy is to construct biomedical materials based on bioactive polysaccharide from natural resources. Chitin is structurally similar to glycosaminoglycan and its analogs, which are widely distributed throughout the extracellular matrix (ECM) of all connective tissues in human and other animals [37]. Chitin can be enzymatically degraded by lysozyme [38,39], which widely exists in various human tissues [40,41]. Chitin has been generally recognized to be nontoxic, biocompatible and biodegradable, and shows promise in biomedical applications [37]. However, chitin has poor solubility in physiological solvents due to its strong intermolecular hydrogen bonding, which is the major obstacle in applications and few has been reported on the use of chitin for the preparation of hydrogels as cell carriers for tissue engineering applications [42]. It has been reported that chitin can be dissolved in NaOH/urea aqueous solution at −30 °C, and biomaterials can be fabricated directly from the chitin solution [43–45]. In our previous work [46], carboxymethyl chitins (CMCHs) were synthesized homogeneously in this "green" solvent and some CMCHs with lower degree of carboxymethylation were shown for the first time to undergo thermosensitive sol-to-gel transition under physiological condition. Herein, we work further to investigate the potential of this novel biodegradable, thermosensitive and in-situ forming injectable CMCH hydrogel for 3D cell culture and clinic application in minimally invasive in-situ tissue repair.

2. Materials and methods

2.1. Synthesis of carboxymethyl chitin

Carboxymethyl chitin (CMCH) was prepared in aqueous homogeneous NaOH/urea solution according to our previous work [46]. Briefly, a certain amount of sodium monochloroacetate was added slowly into chitin solution (2 wt%, 11 wt% NaOH/4 wt% urea aqueous solution) at 2 °C. The reaction system was maintained at 15 °C for 24 h, and then neutralized with HCl in an ice-bath and the product mixture was dialyzed against distilled water for one week and freeze dried. The degree of substitution and degree of acetylation for the obtained CMCHs were characterized by ^{1}H NMR in 20% DCl according to the method described in our previous work [46].

2.2. In-situ hydrogel formation and gelation time determination

CMCH hydrogel was obtained by dissolving the CMCH sample in NaOH aqueous solution and adjusting the pH of the CMCH solution with HCl. For example, 0.1 g of CMCH was dissolved in 5 mL of 0.15 M aqueous NaOH solution. Thereafter, 125 μL of 6 M HCl was added to the solution slowly under magnetic stirring in an ice bath, to obtain a clear and homogeneous solution with the pH of 7.4 and NaCl concentration of about 0.9 wt%. Then, 2 mL of the obtained CMCH solution was loaded in a 5 mL centrifuge tube and incubated in a water bath at different temperatures. The gelation time was determined using the inverted tube test. No visible flow within 60 s was regarded as the criteria for gel formation when the vial was vertically inverted. Triplicates were measured for each sample, and the data are shown as the mean value plus a standard deviation (±SD).

2.3. Rheology characterization

The rheological property of CMCH solutions was evaluated using a controlled stress rheometer (HAAKE Rheo Stress 6000, Thermo Scientific, Germany) equipped with a temperature-controlled water bath. The CMCH solution was loaded on a cone-on-plate apparatus (conical degree = 2°, cone diameter = 20 mm, fixed gap = 0.105 mm). A temperature sweep was conducted between 10 °C and 42 °C at a heating or cooling rate of 0.5 °C/min. The storage modulus (G′) of CMCH hydrogels were determined by a time sweep at 37 °C until G′ reached a plateau. Triplicates were measured for each sample and the data are shown as the mean value plus a standard deviation (±SD). The experiments were performed with a frequency of 1 Hz and 5% strain in the linear viscoelastic region. The sample was sealed by a thin layer of silicon oil to prevent the evaporation of water.

2.4. Compressive modulus

Dynamic mechanical analysis (DMA, TA instrument Q800 series, USA) was used to determine the compressive modulus of CMCH hydrogels. CMCH solution was injected into a cylinder mold and

ID	Title	Pages
41	Thermosensitive injectable in-situ forming carboxymethyl chitin hydrogel for three-dimensional cell culture	10