Nanoengineered Methylcellulose bioinks *via* Tetrazine-Norbornene Click Chemistry

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Statement of Purpose: Surgical treatments are required to mitigate the progression of cartilage degeneration in afflicted patients because native cartilage does not possess regenerative capacity. Furthermore, oral or intra-articular administration of therapeutic agents showed little effect on chondrocytes because or rapid clearance by the synovium and the avascular matrix in the cartilage. Within the field of tissue engineering, 3D bioprinting enables the fabrication of cell-laden hydrogel scaffolds with anatomically relevant structures and patient-specific geometries, improving the prospects for tissue repair and regeneration [1]. An important factor to the success of 3D bioprinting is the development of bioink. In this regard, inverse electron demand Diels-Alder (iEDDA) click reaction between tetrazine (Tz) and norbornene (NB) moieties has emerged as a promising strategy for formulating bioink due to its bio-orthogonal reactivity and biocompatibility. The electron-rich NB and electron-poor Tz form covalent bonds efficiently without the need of initiator or external stimuli and produce nitrogen gas as the only by-product, allowing *in situ* cell encapsulation and injectable delivery to tissues [2, 3]. Laponite (LP) is a 2D disk-shaped synthetic nanosilicate that is increasingly used as an additive for biofabrication because of its unique rheological property, biodegradability, and bioactivity (i.e. cell adhesion, osteogenic/chondrogenic differentiation property) [4]. We have recently developed bioink formulations based norbornene-functionalized MC (MCNB). The NB groups on MCNB permit thiol-norbornene crosslinking, while the thermo-sensitivity of MC backbone affords excellent printability [5]. In this work, we developed an initiator-free bioink system using MCNB and Tz-modified macromers that crosslink into hydrogels via iEDDA click reaction. Additionally, we incorporated LP in the MCNB/PEGTz bioink to improve the gelation kinetics and bioactive properties.

Methods: MCNB was synthesized by first dissolving MC in distilled (D.I.) water for overnight. Controlled amount of carbic anhydride and triethylamine were then added to the MC solution and the reaction was performed for 24 h. After reaction, MC solution was purified via dialysis and lyophilized. After modification, MCNB/LP mixture was prepared by dissolving in D.I. water, respectively. MCNB hydrogels were fabricated by reacting with 4-arm PEG-Tz or PEG-methyltetrazine (PEG-mTz) *via* iEDDA click chemistry. In order to prepare Tz-modified macromer, 4-arm PEG aspartic acid was dissolved in DMF and O-(7-azabenzotriazole-1-yl)-N,N,N,N'-tetramethyluronium, hexafluorophosphate were then added to solution. Dissolve tetrazine amine or methyltetrazine amine in DMF and dropwise into solution. Then, add N,N-Diisopropyl-ethylamine in solution and reaction was performed for 16 h. After reaction, solution was purified via dialysis and lyophilized. Gelation kinetics and printability of iEDDA MCNB hydrogels in the absence or presence of LP were characterized by rheometer. An extrusion 3D bioprinter (Cellink BioX) was used to test the printability of MCNB bioink. Live/dead staining was performed to assess the viability and morphology of the encapsulated C28 human chondrocytes.

Results: The NB groups in MCNB react with Tz crosslinker rapidly without using initiators (Fig. 1A). We tested *in situ* gelation of MCNB as a function of temperature and tetrazine types (i.e. Tz or mTz) (Fig, 1B). As expected, sol-gel transition did not occur with MCNB with low concentration (4 wt%, data not shown). However, gelation occurred with addition of PEG-Tz or PEG-mTz crosslinker, with a faster gel point at 37°C than at 25°C (from 15 min to 6 min). In addition, gel point was faster when PEG-Tz was used as the crosslinker (6 min for PEG-Tz and 18 min for PEG-mTz). Interestingly, the incorporation of nanosilicate LP further accelerated the Tz-NB reaction (Fig. 1C), potentially by increasing physical interactions between MC chains and LP. At 4 wt%, MCNB had low printability because LCST of is higher than 37°C (Fig. 1D. without LP). The printability was improved by incorporation of LP (Fig. 1D. with LP). Finally, MCNB based clickable hydrogels were highly cytocompatible, as shown by the high viability of the encapsulated C28 chondrocytes (Fig. 1E).

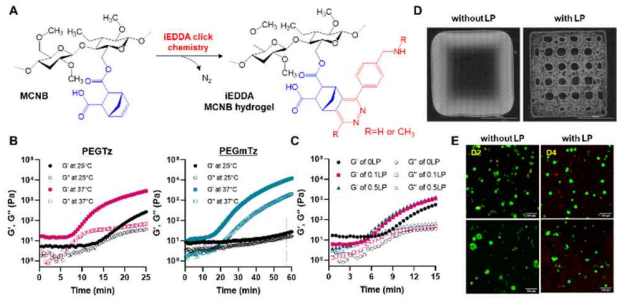

Figure 1. (A) Reaction scheme for MCNB modification and gelation. (B) *In situ* gelation behavior of MCNB with PEG-Tz or PEG-mTz. (C) Effect of LP on gelation behavior of MCNB. (D) Microscopic images of 3D printed MCNB construct with/without LP. (E) Live/dead images of encapsulated C28 chondrocyte in iEDDA MCNB hydrogel.

Conclusions: In summary, we have developed an initiator-free MC derivative capable of orthogonal crosslinking using iEDDA click chemistry. MC-based nanoengineered hydrogel system showed fast gelation behavior and high cytocompatibility. Future work will focus on exploring the optimal conditions of printing and on creating 3D bioprinted Rheumatoid arthritis and osteoarthritis models or cartilage tissue engineering.

References:

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