Supporting Information

Fast-forming BMSCs-encapsulating hydrogels through bioorthogonal reaction for osteogenic differentiation

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**Experimental Details**

**Materials and instruments**

Hyaluronic acid (74 KDa) was purchased from Bloomage Freda Biopharm Co., Ltd.. Tetrazine-amine and TCO-PEG₄-COOH were purchased from Biocon Biotechnology Co., Ltd.. 4-arm-PEG-NH₂ (Mw = 20000) was obtained from Xiamen SINOPEG Biotech Co., Ltd. Isobutyl chloroformate (IBCF) and N-Methyl morpholine (NMP) were purchased from Tokyo Chemical Industry. Fetal bovine serum (FBS), Dulbecco’s modified eagle medium (low glucose) and TrypLE™ express enzyme were obtained from Gibco (Life Technologies, USA). Recombinant bone morphogenetic protein-2 (BMP-2) was obtained from Cloud-Clone Corp.. Alkaline phosphatase (ALP) assay kit was from Beyotime Biotechnology Co., Ltd.. BMSCs were isolated according to the previous literatures¹ and passage 4-6 was used in all the experiments. All the other regents were obtained from domestic suppliers and used as received.

¹H NMR spectra were recorded on a Varian NMR spectrometer at 400 MHz. WST assay was measured on BiotekCytation 3. The morphology of the hydrogel was observed under scan electron microscope (SEM, Quanta FEG 250). The cellular images were observed by confocal laser scanning microscopy (CLSM, Nikon A1). The storage modulus (G’) and loss modulus (G”) of hydrogels were measured using a Haake rotational rheometer (RS6000).

**Synthesis and characterization of HA-T₉ and PEG₄TCO**
Prior to synthesis of HA-Tz, HA was converted to its tetrabutylammonium salt (HA-TBA) according to the previous work.\textsuperscript{2} Briefly, NaHA solution was exchanged with Dowex 50W resin for 8 h. Thereafter, the resin was filtered off and the filtrate was titrated to pH 7 with TBA-OH, followed by lyophilization and stored at -20°C.

HA-TBA and Tetrazine-amine (0.1 times equivalent to the carboxyl groups on HA) were dissolved in DMSO. Afterward, BOP and DIEA (1.2 times equivalent to Tetrazine-amine) were added and stirring overnight. Next, the solution was dialyzed (MWCO = 3500) against distilled water for 1 day, and then purified through SephadexG-15. Finally, the solution was lyophilized to obtain the final product.

TCO-PEG\textsubscript{4}-COOH was dissolved in DMF, then, IBCF and NMP (1.5 times equivalent to TCO-PEG\textsubscript{4}-COOH) were added to activate the carboxyl groups on TCO. After stirring at 4°C for 20 min, 4-arm-PEG-amine (0.9 times equivalent to TCO-PEG\textsubscript{4}-COOH) was added and stirred overnight. Thereafter, the above solution was precipitated into diethyl ether, and the precipitation were dissolved and purified through SephadexG-15, followed by lyophilisation to obtain the final product.

**Preparation of HA hydrogels**

The HA hydrogels were prepared through inverse electron-demand Diels-Alder reaction between T\textsubscript{Z} and TCO in PBS. Briefly, the HA-T\textsubscript{Z} and PEG4TCO
solution (final concentration: 2% and 3% w/v, respectively) was mixed thoroughly. The gelation time was calculated by regarding the gel state when the sample was not flowing.

**Physiochemical and mechanical properties of HA hydrogels**

Briefly, HA hydrogels were prepared according to above described procedure. The as-prepared hydrogel was freezing in the liquid nitrogen, followed by lyophilization. The morphology of hydrogel was imaged by SEM after gold sputtering. The storage modulus (G') and loss modulus (G'') of the HA hydrogels were measured using a Haake rotational rheometer. For swelling ratio experiment, the swollen weight (Ws) was the weight of hydrogel which reached equilibrium in PBS, while the dried weight (Wd) of hydrogel was obtained by totally freeze-dried. The swelling ratio of hydrogel was defined as ((Ws-Wd)/Wd). The porosity of the hydrogels was measured by solution substitution method. The freeze-dried hydrogel (Wd) was immersed in ethanol for 5 min and the total weight of hydrogel and ethanol was recorded as Wt. The ethanol-impregnated hydrogel was then taken out and the residual ethanol was weighted (Ww). The porosity of hydrogel was defined as ((Wt-Ww-Wd)/(Wt-Ww)). The in vitro degradation test was carried out as following: the freeze-dried hydrogel (Wd) was immersed in PBS containing hyaluronidase (HAase, 0, 20, 200 U/mL). Next, the residual hydrogel weight (Wr) was measured at 7th
day and 14\textsuperscript{th} day respectively and the mass loss of hydrogel ((Wd-Wr)/Wd) was obtained. Samples were measured in triplicate.

![Degradation Curves](image)

Fig. S1 \textit{In vitro} degradation curves of hydrogel with HAase (0, 20, 200 U/mL).

**Proliferation of BMSCs within hydrogel \textit{in vitro}**

BMSCs were mixed with two precursor solutions (HA-T\textsubscript{Z} and PEG4TCO solution, total concentration: 5\% w/v, 100 \(\mu\text{L}\)) at the density of 1.5 \(\times\) 10\textsuperscript{6} cells/mL. Thereafter, these two precursor solutions mixed thoroughly. After the hydrogel was formed, the culture medium was added and changed every 2-3 days. Finally, Water Soluble Tetrazolium (WST) was used to test the proliferation of BMSCs at certain times. Furthermore, Live/Dead staining assay was used to detect the survival of BMSCs within the hydrogels.

**Osteogenic differentiation of BMSCs within HA hydrogel \textit{in vitro}**
Briefly, the HA-Tz and PEG4TCO solution were mixed with BMSCs (1 × 10^7 cells/mL) and BMP-2 (100 ng/mL). The obtained hydrogel was then incubated in the inductive medium containing 0.05 mM L-ascorbic acid, 0.1 mM dexamethasone, 10 mM β-glycerophosphate disodium. Meanwhile, the as-prepared hydrogel incubated in the complete medium was used as a control. The hydrogel was taken out at certain times, and rinsed by PBS three times for the following measurement.

Osteogenic differentiation was assessed by measuring the alkaline phosphatase (ALP) activity of BMSCs within HA hydrogel. The samples were taken out at 7th day, 14th day and 21st day, respectively. Afterward, the samples were rinsed with PBS three times to remove as much residual serum as possible. ALP activity was measured according to the manufacturer’s instruction. Data were normalized to the concentration of total DNA.

The mRNA expression levels of osteogenesis-related gene were then assessed at 7th day and 14th day by RT-PCR, including ALP, type I collagen (Col I), osteocalcin (OCN), runt related transcription factor 2 (RunX2). The relative expressions for the target genes were then normalized to that of the reference gene F-actin and the primers for RT-qPCR are listed in Table 1. At each time point, the hydrogel was taken out and rinsed by PBS three times. The total RNA was then extracted according to the protocol and the purity of the RNA was assessed using A_{260/280} nm. Thereafter, 500 ng of RNA was reverse transcribed into cDNA using PrimeScript™ RT Reagent Kit (Takara). RT-PCR
was performed on Mastercycler® nexus (Eppendorf) using SYBR Green I PCR Kit.

Table 1 Primers for RT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Prime sequence</th>
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<tbody>
<tr>
<td>ALP</td>
<td>Forward: CGTCTCCATGGTGGATTATGCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCCAGGCACAGTTGTCAAG</td>
</tr>
<tr>
<td>Col-I</td>
<td>Forward: CTGCCAAGATAATATGTATCACC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAAGCAAGTGTCTCCAAAGACC</td>
</tr>
<tr>
<td>OCN</td>
<td>Forward: GCCCTGACTGCATTGTGCTCTCT</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCACCACCTTACTGCCCCCTCCTG</td>
</tr>
<tr>
<td>Runx2</td>
<td>Forward: TCTTCCCAAGCCAGAGCG</td>
</tr>
<tr>
<td></td>
<td>Reverse: TGCCATTCGAGGTGGTCG</td>
</tr>
<tr>
<td>F-actin</td>
<td>Forward: CACCCGCGAGTACACCCTTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCCATACCCACCACATCACACC</td>
</tr>
</tbody>
</table>

ALP staining and alizarin red S (ARS) staining were performed to further assess the osteogenic differentiation of BMSCs within HA hydrogel. At 7th day, the hydrogel was taken out and rinsed by PBS several times, and then fixed in 4% paraformaldehyde. After that, ALP staining was performed according to the manufacturer’s instructions (Beyotime). For ARS staining, at 14th day, the hydrogel was taken out, washed for several times, and fixed in 95% ethanol.
Then, the sample was incubated with ARS staining solution following the manufacturer’s instructions (Solarbio). The results of staining were observed using an inverted microscope.

Fig. S2 ALP staining (A) and ARS staining (B) of the BMSCS-encapsulating hydrogel after osteogenic culture for 7 and 14 days respectively.

**Statistical analysis**

All the experiment results were reported as mean ± standard deviation for *in vitro* studies. The statistical data analysis was conducted using Origin Pro 8.5 program and *P* values < 0.05 were considered statistically significant.