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2-Thiophene ethylamine modified hyaluronic acid with its application on hepatocytes culture

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ABSTRACT

Hyaluronic acid (HA) is a component of extracellular matrix, which is important for cell functions and tissue integrity. Biosynthesized HA, as well as its derivatives, is widely used in cosmetics industry, biochemical medicine and medical surgery. In this research, we report a new hyaluronic acid derivative synthesized by amidation of hyaluronic acid with 2-thiophene ethylamine (2TEA). 2-chloro-dimethoxy-1,3,5-triazine (CDMT) served as the activating agent of the carboxylic groups. Primary mouse hepatocytes cultured with this derivative HA-2TEA maintained their epithelial morphology and showed better hepatic functions. This result was confirmed by the higher expression levels of hepatic functional genes in primary hepatocyte cultured with HA-2TEA derivative. Moreover, the protein levels of several hepatic genes were further confirmed by immunofluorescence staining. Thus HA-2TEA(2-thiopheneethylamine) derivative demonstrated good capacity on hepatocytes culture, and maintained hepatocyte functions *in vitro*.

1. Introduction

Hyaluronic acid (HA), also known as hyaluronan, is a non-sulphated glycosaminoglycan (GAG) and major constituent of the extracellular matrix (ECM) [1]. Hyaluronic acid is a linear polysaccharide made of repeated disaccharide units of d-glucuronic acid and *N*-acetyl glucosamine in extracellular matrix of the body [1–3]. Regular HA gel is widely used in cell cultures to improve cell survival and proliferation, or to establish 3D culture system. Particularly, HA gel is often used to model *in vivo* tumor environment [4]. To better mimic *in vivo* niche, many HA derivatives have been developed and investigated from cosmetic material to culture scaffolds [5–17]. Introduction of new function group into polysaccharide chain can alter the physical and biological properties of original HA [14]. For example, the HA backbone can be modified with furan functionalities to enable gel formation with peptide crosslinkers *via* a single-step Diels–Alder click reaction. Such an ECM mimetic gel has been used as a platform to study breast cancer [4]. HA can also be crosslinked by methyl cellulose to provide a minimally invasive and localized drug delivery to injured spinal cord and brain [18]; HA derivatives carrying complementary aldehyde (HAALD) and hydrazide (HAADH) groups were synthesized as 3D matrices for *in vitro* evaluation of chemotherapeutic drugs [3].

Primary hepatocytes isolated from liver donors are widely used in academic research and drug development [19]. Previous studies have also demonstrated the promising potential of hepatocytes in cell therapy and bio-artificial liver device [20]. However, currently the major challenge in the application of hepatocytes is to maintain the gene expression pattern and functions of primary hepatocytes *in vitro* for a long time. Several *in vitro* culture systems based on the optimization of culture condition have been developed. Among them, optimization of extracellular matrix, such as HA, was of great interest due to the critical roles of HA in supporting hepatocyte behavior *in vivo* and *in vitro*. Thus, we introduced a HA derivative

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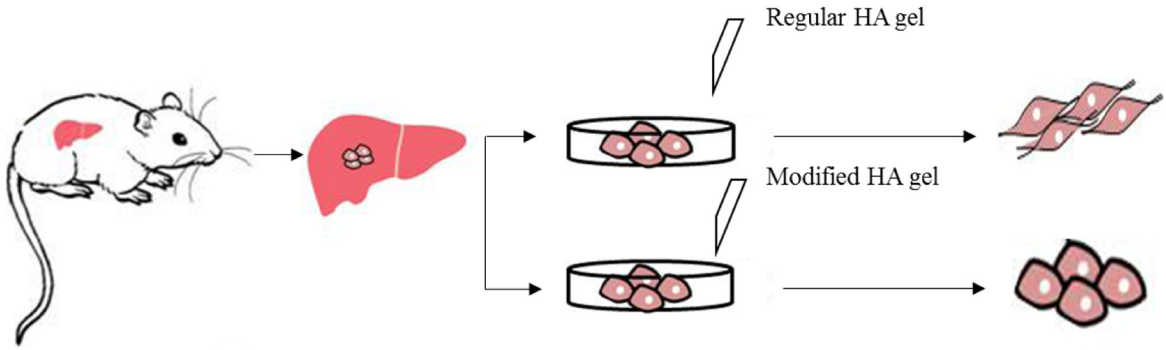

Fig. 1. Schematic of *in vitro* culture of hepatocytes.

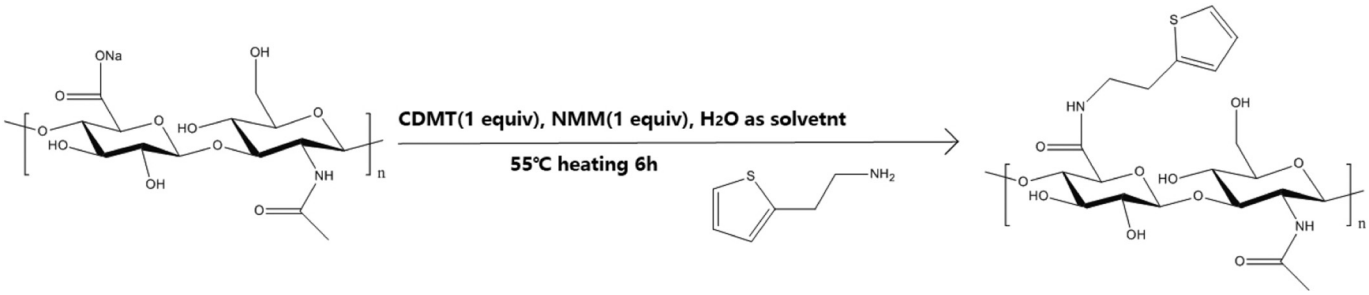

Fig. 2. Chemical formulation of modified hyaluronic acid.

Table 1
Primers for hepatic genes.

Primers	Sequence
Alb-forward	CACCTTTATGGGACACTA
Alb-reverse	CTGACGGACAGATGAGA
Hnf4α-forward	AGGTCAAGCTACGAGGACA
Hnf4α-reverse	GAAGGAGTTCGCAGAAGG
Foxa2-forward	TATGCTGGGAGCCGTGAA
Foxa2-reverse	AGCGCCCACATAGGATGA
Cyp1a2-forward	AGACTCTGGACCCGTGTG
Cyp1a2-reverse	GATGGTTAGCCTCCTTGC
Cyp3a13-forward	GACGATTCTTGCTTACCAGAAGG
Cyp3a13-reverse	CCGGTTTGTGAAGGTAGAGTAAC
Cdh1-forward	GGTCTCCTCATGGCTTTGCC
Cdh1-reverse	GCTTTAGATGCCGCTTCACT
Bsep-forward	TCTGACTCAGTGATTCTTCGCA
Bsep-reverse	CCCATAAACATCAGCCAGTTGT
Gapdh-forward	TGTGAACGGATTTGGCCGTA
Gapdh-reverse	ACTGTGCCGTTGAATTTGCC

modified by 2-thiopheneethylamine (HA-2TEA) gel for hepatocyte *in vitro* culture as Fig. 1. HA-2TEA supported the primary hepatocyte culture for a longer time, while maintained the hepatic functional gene expressions in RNA levels and protein levels. Overall, this chemical modification of HA by HA-2TEA (2-thiopheneethylamine) provided a better culture microenvironment for primary hepatocytes which better mimicked the *in vivo* niche. Moreover, HA-2TEA based hepatocyte culture provided a better platform for exploring the mechanisms of liver diseases and drug development in the future.

2. Materials and methods

2.1. HA-2TEA preparation

As shown in Fig. 2, sodium hyaluronate as the backbone raw material (1 equiv., 1.00 g = 2.5 mmol of COONa available for reaction, Energy Chemical) was dissolved as a 1% hyaluronate solution in DI water. Then, 2-thiophene ethylamine (1 equiv., Sigma Aldrich or TCI America) was added to the mixture along with the coupling activation agent 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 0.5 equiv., 225 mg, Adamas) and 4-methylmorpholine (NMM, 1 equiv., 280 µl, Sigma Aldrich). The mixture was stirred at 55 °C for 6 h and then dialyzed against a 14,000 MWCO dialysis membrane for 3 days with DI water to further purify the polymer. The resulting solution was then lyophilized (Labconco freezone 2.5) to get HA-2TEA.

2.2. HA-2TEA characterization

The chemical structure of modified hyaluronic acid was studied by Fourier transform infrared (FTIR) and NMR. All the FTIR data were collected and analyzed with Spectrum Two type spectrometry (Perkin Elmer, USA) in the range of 4000 to 600 cm^{-1} with 1 cm^{-1} resolution. And the NMR spectra was collected by using an AVANCE III HD 500 MHz NMR (Bruker, Germany) with D_2O as the solvent.

2.3. Hepatocytes isolation and culture

The C57Bl6/J mice were maintained in specific pathogen-free husbandry. Use of animals was approved by Shanghai Research Center for Model Organisms (IACUC No. 2017-0003). For isolation of hepatocytes, C57Bl6/J mice were narcotized by intraperitoneal injection of