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Encapsulation of liver microsomes into a thermosensitive hydrogel for characterization of drug metabolism and toxicity

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ABSTRACT

This study reported the encapsulation of liver microsomes into a thermosensitive hydrogel to characterize drug metabolism and predict drug effects. Pluronic®F-127 (F127) and acrylamide-bisacrylamide (Acr-Bis) were utilized as the two precursors. After chemical crosslinking catalyzed by ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylenediamine (TEMED), the resulting Pluronic F127-acrylamide-bisacrylamide (FAB) hydrogel could encapsulate microsomes at 4 °C and facilitate metabolic reactions at 37 °C. The gel morphology at different Acr-Bis concentrations was characterized using field emission scanning electron microscopy (FE-SEM). Higher concentrations of Acr-Bis could lead to higher degrees of cross-linking of the gel. A fluorescent staining assay was subsequently used to demonstrate successful encapsulation of microsomes into the gel as well as the free diffusion process of micromolecular substrates. The thermosensitivity of the FAB gel was studied using swelling ratio and protein release assay to verify its ability to encapsulate microsomes. The metabolic activity of microsomes encapsulated in gels was investigated by detecting the metabolites of FDA-approved substrates, including dextromethorphan, chlorzoxazone and testosterone. Compared with the traditional method of microsomal incubation, the FAB gel maintained 60%–70% of microsome activity. Lastly, the classic anticancer prodrug cyclophosphamide (CTX) was chosen as a model drug for the study of drug metabolism and the prediction of drug effects. When the microsomes encapsulated in the FAB gel were used in the cell culture system, CTX induced a higher level of apoptosis in MCF-7 cells compared with traditional microsomes.

1. Introduction

Cell culture is an extremely important and widely used method for *in vitro* pharmaceutical research. However, this method cannot comprehensively mimic the real physiological situation because most cultured cells, including liver cells, lack or express low levels of the most important drug-metabolizing enzymes [1,2]. Overlooking the transformation of drugs into their reactive metabolites and intermediates leads to incorrect conclusions. For example, the hepatotoxicity of acetaminophen only occurred when it was metabolized to the reactive intermediate N-acetyl-p-benzoquinone imine (NAPQI) [3]. Excessive NAPQI depleted glutathione (GSH) and covalently bound to protein, finally leading to liver damage [4]. This issue is particularly important for prodrugs such as cyclophosphamide (CTX), which must be metabolized to the reactive anticancer agent nitrogen mustard [5]. Thus, drug metabolism should not be neglected during the evaluation of drug effects, and integrating metabolism into traditional cell culture methods would be a significant step towards improving the reliability of *in vitro* research.

Previous studies have suggested some methods to investigate drug toxicities and the effects of drug metabolism, including transgenic cells, independent discrete multiple organ co-culture, 3D cell culture and high throughput devices [6–9]. However, these methods suffer from several drawbacks. For example, transgenic cells only express one or a few isozymes, making them an inaccurate reflection of the *in vivo* situation [2]. Although 3D cell culture might be a suitable tool for the prediction of hepatotoxicity, other organs exhibit little or no biotransformation ability, precluding the prediction of drug metabolism induced effects or toxicities [10]. High throughput devices are generally time-consuming and require highly complex preparation processes. Also, the

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comprehensive evaluations necessary to enable these methods to estimate microsomal metabolic ability and concrete stages of cell death have not been designed [11–14]. Here, we have proposed a simple and accurate method to predict drug metabolism induced effects or toxicities. We propose that comprehensive results can be obtained by adding Pluronic®F127- acrylamide-bisacrylamide (FAB) hydrogels containing microsomes into traditional 2D cell culture systems.

Because of their good biocompatibility, microporous 3D structure, permeability to oxygen and nutrients, and tissue-like elastic properties, hydrogels have been widely applied in various fields, including cell culture, tissue engineering, and drug delivery [15]. Various macromonomers have been designed to polymerize into hydrogels, and the thermosensitive material Pluronic®F-127 (F127) has been frequently studied and applied in recent years [16]. These polymers have been crosslinked to form thermosensitive hydrogels for multiple applications but have not been used to generate metabolism reaction vectors [17–19]. Thus, we chose F127 as the hydrogel material and used it to encapsulate microsomes for the first time. When at 4 °C, F127 was soluble and the swollen hydrogel was able to adsorb microsomal proteins. At 37 °C, F127 turned into a more rigid gel, and the compaction of the polymer chains locked the microsomes in place, preventing cytotoxicity caused by direct contact between microsomes and cells. Enzymatic activity was maintained at both temperatures, enabling realistic metabolic reactions. However, the mechanical strength of F127 was not high enough to form a stable vector. Thus, we introduced acrylamide-bisacrylamide (Acr-Bis) to the final gel system to increase its mechanical strength without affecting its thermosensitivity and other functions.

Microsomes extracted from the liver are widely used to mimic the drug metabolism process *in vitro* [20]. This analysis is particularly important in cases such as the classic anticancer prodrug CTX, which must be catalyzed by cytochrome CYP 3A isoforms from microsomes to induce its anticancer effects [21]. In this study, we used the FAB gel as the metabolism reaction vector, liver microsomes as the drug catalyzer and CTX as the model drug to verify the functionality of this system in studying drug metabolism and predicting drug effects.

2. Materials and method

2.1. Materials

F127, dextromethorphan hydrobromide, dextrorphan tartrate solution, 3,3′-dioctadecyloxacarbocyanine perchlorate (Vybrant DiO), and CTX monohydrate were purchased from Sigma–Aldrich (St. Louis, MO, USA). 30% Acr-Bis (29:1), Ammonium persulfate (APS), and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from the Beyotime Institute of Biotechnology (Nanjing, China). Chlorzoxazone, 6-hydroxy chlorzoxazone and 6β-hydroxy testosterone were purchased from J&K CHEMICA (Beijing, China). The Annexin V-FITC/PI Apoptosis Assay Kit was purchased from KeyGEN (Nanjing, China). The BCA Protein Assay Kit was obtained from Becton Dickinson Co., Ltd. Testosterone was kindly provided by Shuguang Hospital (Shanghai, China). Tetraarylazadipyrromethenes (BODIPY) was kindly provided by Dr. Li in Fudan university (Shanghai, China).

2.2. Cell lines

Human breast cancer MCF-7 cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured at 37 °C in a humidified 5% CO_2 atmosphere. The cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Microsome preparation and quantitation

Rat liver microsomes were prepared by differential centrifugation as previously described [22]. The protein content was quantified by the BCA Protein Quantitation assay. All animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University.

2.4. Synthesis of thermosensitive macromer

The central part of F127′ (the macromer in this work is denoted as F127′) is a triblock copolymer, poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO_{99}-PPO_{65}-PEO_{99}). Both ends of the F127 polymer are modified with acryl to create double bonds. Briefly, 10 g F127 was dissolved in 50 ml N,N-Dimethylformamide (DMF) at 50 °C until the solution became clear, and 258 μl acryloyl chloride (molar ratio 1:4) was then added to react overnight at room temperature with constant stirring. The product F127′ was precipitated in diethyl ether and dried under vacuum.

2.5. FAB hydrogel preparation

A stock solution of F127′ was prepared with 30 wt % macromer in deionized water and stored at 4 °C. The hydrogel system consisted of Acr-Bis solution (0.3% or 1%), F127′ (10%), APS (1%), TEMED (0.5%) and phosphate buffered saline (PBS, 0.1 M, pH 7.4). Then, 50 μl or 100 μl of this hydrogel solution were added to a 96-well plate (as a template) at 4 °C and polymerization proceeded at 37 °C for 20 min. The shaped FAB hydrogels were then washed with 5% mannitol solution 5 times at room temperature and then freeze-dried under vacuum. The hydrogels were stored at 4 °C until use.

The FAB hydrogels were immersed in microsome solutions for 48 h at 4 °C before metabolism tests. The concentration of microsome solutions was determined according to the following formula:

$$v \times c = x \times y$$

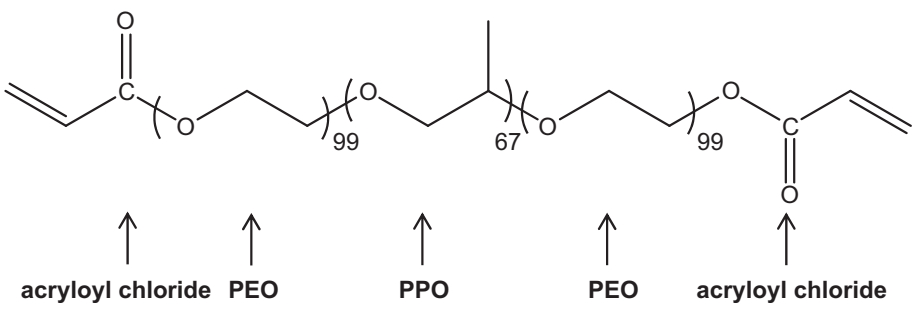
where v was the final working volume of the whole metabolism system, c was the final working concentration of microsomes, x was the volume of the FAB hydrogels and y was the concentration of microsomes contained in the gels, which was equal to the concentration of the microsome immersion solution.

2.6. FAB hydrogel morphology

The inner network morphology of the cross-linked hydrogel was studied using Field Emission Scanning Electron Microscopy (FE-SEM). Two blocks of 50 μl freeze-dried FAB hydrogels (one was a 0.3% Acr-Bis–10% F127′ gel and the other was a 1% Acr-Bis–10% F127′ gel, denoted as 0.3%–10% FAB gel and 1%–10% FAB gel respectively) were sputter coated with gold for 240 s (E1030 Ion sputter). The surface and interior of the hydrogel were imaged by FE-SEM (Hitachi model 4800) at 1.0 kV.

2.7. Swelling ratio of FAB hydrogel

A completely dry, pre-weighed, disc-shaped FAB hydrogel (both 0.3%–10% FAB gel and 1%–10% FAB gel) was weighed and then immersed in excess of 5% mannitol solution at 4 °C and 37 °C respectively. At 1, 8, 16, 24, 32, 40 and 48 h, the hydrogel



Scheme 1. Chemical structure of the F127′ macromer.