CHINESE JOURNAL OF ANALYTICAL CHEMISTRY
Volume 45, Issue 9, September 2017
Online English edition of the Chinese language journal

Available online at www.sciencedirect.com
ScienceDirect

Cite this article as: Chin J Anal Chem, 2017, 45(9), 1259–1263.

RESEARCH PAPER

Synthesis of Boronic Acid-functionalized Soluble Dendrimers and its Application in Detection of Human Liver Microsomal Glycoprotein Based on Enzyme-linked Immunosorbent Assay

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Abstract: It is difficult for analysis of glycoproteins in the biological sample due to the low abundance, susceptiblility to be inhibited and interfered by other non-glycoproteins. An enrichment step is usually required before glycoprotein analysis, but the operation steps of conventional solid-phase based glycoprotein enrichment methods are difficult to be compatible with the most classical enzyme-linked immuno sorbent assay (ELISA) technique. In this study, a novel water-soluble dendrimer based boronic acid capture (DBC) method was developed using PAMAM 4.0 as carrier and boronic acid as affinity group. The method was applied to detection of glycoproteins in human liver microsomes using ELISA. The DBC enrichment conditions were optimized by model glycoprotein, and then its sensitivity and anti-interference ability were investigated. The optimized method was applied to enrich glycoproteins in human liver microsomal. The results showed that the enrichment selectivity of DBC for glycoprotein could be up to 100,000 folds, and the signal of glycoprotein could be improved by 100 times. In conclusion, using DBC as a novel enrichment material for glycoprotein, combined with ELISA analysis, high sensitivity and selectivity detection could be achieved in biological samples with only one simple incubation step, which makes it a useful tool for glycoprotein research.

Key Words: Glycoprotein; Boronic acid; Dendrimer; Enrichment

1 Introduction

Liver is the largest detoxification organ in the body and has enormous amount of metabolic enzymes, which can catalyze the transformation of various types of drugs[1]. Human liver microsomes (HLM) are widely used in the study of drug metabolism *in vitro* because of their low preparation cost, good reproducibility, easy transport and preservation[2]. The metabolic enzymes in HLM usually are glycoproteins, with glycosylation sites, such as carboxylesterase 1 (CES1) and uridine diphosphate glucuronosyltransferase 2B7 (UGT2B7). Human CES1 is a class I metabolic enzyme involved in detoxification and metabolism of exogenous substances in the liver, primarily responsible for biotransformation of various ester-containing drugs and pre-activated drugs such as antineoplastic agents and anesthetics[3]. These drugs were reported to be closely related with the occurrence and development of liver cancer[4]. UGT2B7 is an important member of phase-metabolic enzyme uridine diphosphate glucuronosyltransferase (UGT) family and plays a key role in a variety of drug metabolism[5]. However, due to the low abundance of glycoprotein, and severe interference from

Received 20 April 2017; accepted 10 June 2017
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This work was supported by the National Natural Science Foundation of China (No. 21505015), the Program of the Key Project in Liaoning Province Education Department of China (No. LZ2016001), the High Level Talent Innovation and Entrepreneurship Program of Dalian, China (No. 2016RQ041), and the Natural Science Foundation of Liaoning Province, China (No. 20170540286).

DOI: 10.1016/S1872-2040(17)61035-1

non-glycoprotein during the analysis and testing process, it is necessary to enrich glycoprotein before analysis to improve detection sensitivity.

Boronic acid could bind glycoprotein with 1,2- and 1,3-*cis*-*o*-dihydroxy groups to form a five- or six-membered cyclic ester under basic conditions. The cyclic ester could dissociate under acidic conditions. This reversible reaction does not alter structure of the sugar chains in glycoproteins[6,7]. In recent years, boronic acid functionalized materials developed for the selective separation and enrichment of glycoproteins has attracted more and more attention, such as affinity chromatography materials[8,9], nanoparticles[10], magnetic beads[11,12], mesoporous materials[13,14] and microplates[15,16]. However, these glycoprotein detection methods were based on solid-phase materials, which were difficult to be used as water-soluble materials, and showed poor compatibility with the most classical enzyme-linked immuno sorbent assay (ELISA) technique. Besides, the operation was complex, and harsh conditions had to be applied.

Soluble dendrimers, with hyperbranched surface groups, could be easily surface functionalized[17–19]. In addition, the high solubility of the dendrimer helps the material to chelate glycoprotein in solution, achieving homogeneous enrichment, and extends the application range of the protein enriched material.

In this study, a novel water-soluble dendrimer based boronic acid capture material (DBC) was developed using dendrimer (PAMAM 4.0) as the carrier and boronic acid affinity technique. Different from the previous solid-based glycoprotein capture materials, such as monolithic columns, beads etc, DBC exhibited high solubility and dispersibility in aqueous solution. Thus DBC could be applied in the classic ELISA analysis as a time-saving, simple protocol, which could be carried out in any biochemical laboratory. Moreover, DBC was easily synthesized and showed high glycoprotein enrichment selectivity and sensitivity in analysis of human liver microsomes (HLM) glycoprotein.

2 Experimental

2.1 Instruments and reagents

Multifunctional Microplate Reader (Thermo, USA) was used in the analysis of glycoproteins. 4-Formylphenylboronic acid, dendrimers (PAMAM4.0), bovine serum albumin (BSA) were bought Sigma-Aldrich). Human liver microsomes (HLM) was obtained from Research Institute for Liver Diseases Co. Ltd (Shanghai). UDP-glucuronosyltransferase 2B7 (UGT2B7), carboxylesterase 1 (CES1b) were purchased from Becton, Dickinson and Company, USA. Primary and secondary antibodies of UGT2B7 and CES1b were purchased from Abcam. Ultrafiltration membrane (MWCO 3000 Da) was obtained from Millipore, USA) Ultra-pure water was used throughout the experiments.

2.2 Experimental methods

2.2.1 Synthesis of DBC

Approximately 20 μL of PAMAM 4.0 was added in 500 μL of anhydrous methanol, and 1 mg of 4-formylbenzeneboronic acid was dissolved in 500 μL of anhydrous methanol. The above two solutions were thoroughly mixed in a microreactor, and incubated at 60 °C oil bath for 24 h. 2 μL glacial acetic acid was added as a catalyst every 8 h. The unreacted materials were removed by ultrafiltration membrane and the obtained product (DBC) was lyophilized.

2.2.2 Optimization of coating concentration of DBC for glycoprotein enrichment

(1) Coating The freeze-dried DBC was dissolved in 1 mL of PBS (pH 7.4) as the mother liquor. The mother liquor was diluted as 1: 4000, 1: 3000, 1: 2000, 1: 1000, 1: 500 and 1: 100 with sodium carbonate buffer (pH 9.6). The microplate was coated with the diluted DBC at 100 μL per well, and incubated at 4 °C overnight. Each concentration of DBC was repeated in parallel three wells.

(2) Blocking The coating solution was removed, and the microplate was washed twice with ammonium acetate buffer (pH 8.8) for 3 min. The plate was blocked with 200 μL blocking buffer (pH 8.8 ammonium acetate buffer containing 1% BSA) per well and incubated at 37 °C for 1 h.

(3) Capture of glycoprotein The blocking buffer was discarded, then 100 μL CES1b solution was added per well and incubated at 37 °C for 1 h.

(4) Incubation with primary antibody The unbound protein was discarded. 100 μL of anti-CES1b antibody solution was added per well and incubated at 37 °C for 1 h. The anti-CES1b antibody was diluted with blocking solution as 1: 1500.

(5) Washing Unbound anti-CES1b antibody was discarded. The plate was washed with ammonium acetate buffer (pH 8.8) for 3 min and 5 times.

(6) Incubation with secondary antibody 100 μL of the secondary antibody was added per well and incubated at 37 °C for 1 h. The secondary antibody was diluted with blocking solution as 1: 2000.

(7) Washing The plate was washed with ammonium acetate buffer (pH 8.8) for 3 min and 5 times.

(8) Detection The TMB substrate A and B were mixed at the ratio of 1: 100. 100 μL TMB substrate mixture was added to each well and incubated at 37 °C for 20 min. Approximately 100 μL of stop solution (1 M H_2SO_4) was added to each well. The absorbance was measured at 450 nm