Construction of Molecularly Imprinted Polymers for Cholesterol by Semi-covalent Imprinting Approach and Nitroxide Mediated Radical Polymerization

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Abstract

Molecularly imprinted polymers (MIPs) for cholesterol were successfully constructed using a combination of semi-covalent imprinting approach and nitroxide mediated radical polymerization. Cholesteryl (4-vinyl) phenyl carbonate was firstly synthesized and applied as a template-monomer adduct. Nitroxide initiator, 3-(4-butylphenol)-1, 1'-dimethyl-3-(2,2',6,6'-tetramethylpiperidinooxy) propyl cyanide, was synthesized and added to the reaction mixture to assist polymerization of the functional monomer (divinylbenzene; DVB) in the vicinity of the template molecule. Subsequent to polymerization, the cholesterol molecule was hydrolyzed from the polymer matrix by refluxing in 1*M* NaOH and then neutralized with HCl. Binding capability of the MIP-H (hydrolyzed form) to cholesterol was assessed by radioligand binding analysis. Our results revealed that uptake of cholesterol into the binding cavity of the MIP-H was estimated to be up to 6 and 3 times higher than those of the unhydrolyzed form (MIP-UH) and control polymer (NIP-H), respectively. All these findings have opened up a potential approach for a specific ligand recognition of biological macromolecules, which could constitute another promising trend of sensor development.

Keywords: Molecular imprinting technique, molecularly imprinted polymer, cholesterol, nitroxide-mediated radical polymerization

1. Introduction

Cholesterol determination is an important routine requirement for prediction of relative risk of cardiovascular diseases as well as for health promotion [1]. Nowadays, many methods based on different principles have been established. These include using a combination of enzyme catalysis (e.g. cholesterol oxidase) together with color or sensor detection systems [2,3]. In such cases, an enzyme is the most frequently used due to its specific catalytic reaction. However, in many circumstances, disadvantages arise because of the multiple assay steps, the use of carcinogenic or toxic substrates and short half-life. Therefore, the development of a robust and simple analytical assay is needed.

In recent years, molecular imprinting is a newly developed methodology which provides

molecular assemblies of desired structure and properties. The technique engages pre-organization of functional monomers adjacent to a template molecule in which shape and size of the template are resembled by either covalent or non-covalent interactions. Polymerization of the supramolecular assembly in the presence of excess amounts of cross-linker has been performed. Then, removing of the template molecule to retain the specific orientation of functional groups within the cavity of the polymer, is needed [4,5,6,7]. Therefore, in this way a highly selective recognition site in synthetic polymers can be employed for many applications including separations, analysis and catalysis [8,9].

In this study, we report a new method for construction of a molecularly imprinted polymer providing the ability of the molecular recognition of cholesterol. A combination of a semi-covalent imprinting approach and nitroxide mediated radical polymerization has been used to prepare the polymer [10,11]. By this approach, semi-covalent imprinting has been applied by high temperature utilization during polymer synthesis. The rate of polymer synthesis could then be controlled owing to the low amount of free radical generation in the reaction. This may maximize the polymer architecture as well. Capability of the synthesized polymer to bind to cholesterol has been investigated using radioligand binding analysis.

2. Materials and methods

2.1 Materials

Cholesteryl chloroformate (98%), 4-acetoxystyrene (96%), 2,2',6,6'-tetramethyl-1-piperidinyloxy (TEMPO; 99%), divinylbenzene (DVB; technical mixture of isomers, 80%), 4-*tert*-butylstyrene (TBS; 93%), 2,2'-azoisobutyronitrile (AIBN; 98%), and *m*-xylene (anhydrous, 99+%) were purchased from Aldrich. TBS was purified by vacuum distillation. AIBN was purified by re-crystallization from methanol. Prior to use, DVB was passed through an aluminium oxide column to remove the stabilizer (4-*tert*-butylcatechol). [1α, 2α-^{3}H(N)]Cholesterol (specific activity of 41.3 Ci mmol^{-1}) was supplied by Sigma. Scintillation liquid Ecoscint A was obtained from National Diagnostics (Atlanta, GA, USA). The nitroxide initiator, 3-(4-*tert*-butylphenyl)-1,1'-dimethyl-3-(2, 2', 6, 6'-tetramethylpiperidinooxy) propyl cyanide, was synthesized using Abrol's method as described elsewhere [12]. The template-monomer adduct of cholesteryl (4-vinyl)phenyl carbonate was synthesized according to Whitcombe's protocol [10]. Other solvents and reagents were analytical grade unless otherwise stated.

2.2 Semi-covalent imprinting of cholesterol imprinted polymers via nitroxide mediated polymerization

Cholesterol-imprinted polymers (MIPs) and non-imprinted polymers (NIPs; control polymer without template) were synthesized using the nitroxide initiator (Figure 1). Compositions and chemical processes of the imprinting reaction are summarized in Table1. Briefly, the monomers and initiators were dissolved in 0.88 mL of *m*-xylene. The solution was then purged with a gentle flow of Argon for 5 min. After that, the reaction mixture was heated in an oil bath at 125°C for 48 h. After polymerization, the polymer monolith was gronded to small particles, washed with methanol, hexane, and dried in a vacuum chamber. To remove the covalently-linked cholesterol, half of the imprinted polymers were refluxed in 20 mL of 1*M* NaOH for 48 h (referred to as MIP-H). After neutralization with 1*M* HCl, the polymer particles were collected by centrifugation, washed with methanol, hexane, and dried in a vacuum chamber. For comparison, the non-imprinted polymers were subjected to the same hydrolysis treatment (referred to as NIP-H).

2.3 Cholesterol binding analysis

The cholesterol imprinting polymers were incubated in 1 mL of [1α, 2α -^{3}H(N)]cholesterol solution in hexane (1.4 n*M*), with and without addition of non-labeled cholesterol, at 20°C for 16 h. A rocking table was used to provide gentle mixing. After incubation, the samples were spun to sediment polymer particles. A fraction of the supernatant (200 µL) was taken and mixed with scintillation liquid Ecosint A (10 mL), and counted for 1 min in a Rackbeta 2119 liquid scintillation counter (LKB Wallac, Sollentuna, SE). The percentage of [1α, 2α -^{3}H(N)]cholesterol bound to polymers was further calculated using the following equation:

Bound (%) = (Total – Free) × 100/Total

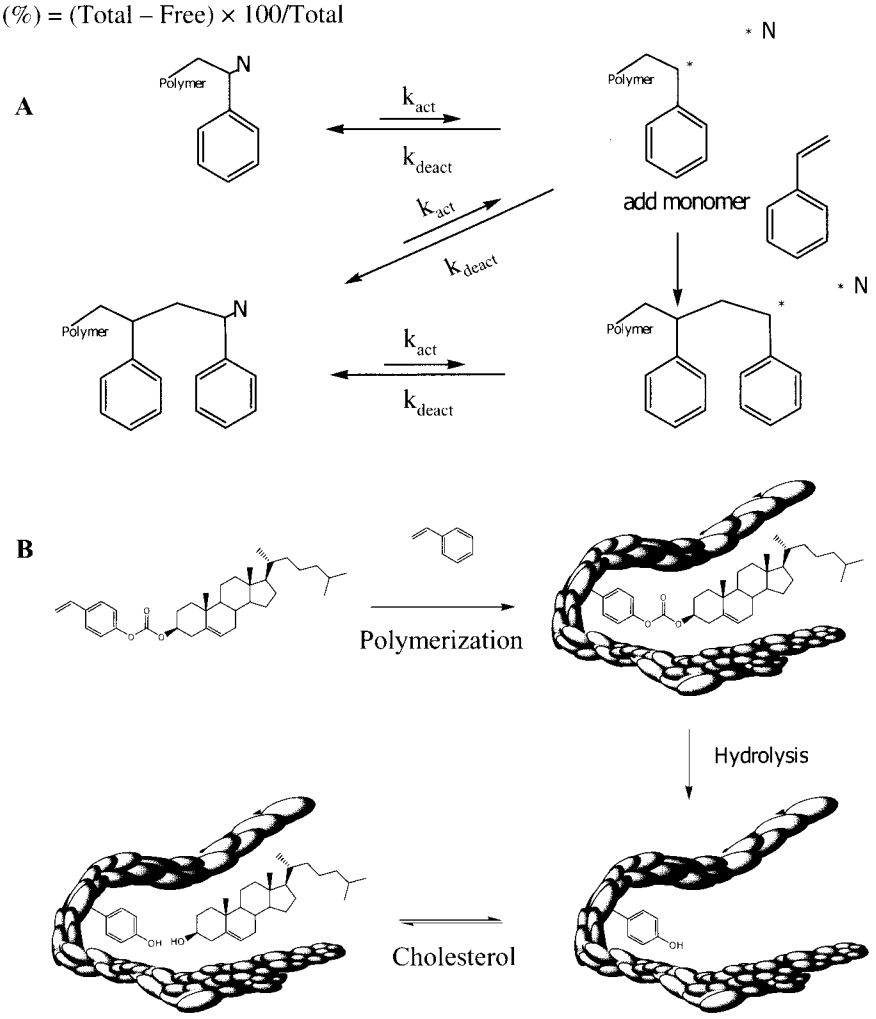

Figure 1 General principle of nitroxide mediated radical polymerization (A) and sacrificial or semi-covalent imprinting strategy (B)

Table 1 Compositions and reaction condition for polymer preparation

Polymer	Monomer (mmol)		Porogen (ml)	Temperature (°C)	Hydrolysis treatment
	Cholesterol (4-vinylphenyl) carbonate	DVB	*m*-xylene		
MIP-UH	0.29	5.51	0.88	125	No
MIP-H	0.29	5.51	0.88	125	Yes
NIP-H	-	5.81	0.88	125	Yes

3. Results and discussion

Using semi-covalent imprinting in combination with nitroxide mediated radical polymerization, the molecularly imprinted polymer possessing cholesterol binding pockets has successfully been constructed. Binding of cholesterol to the imprinting polymer was analyzed by radioligand binding assay. Table 2 summarizes the percentage of binding activity to ^{3}H-cholesterol of the hydrolyzed form (MIP-H), unhydrolyzed form (MIP-UH) and the non-imprinted polymer (NIP-H). At 25 mg, the MIP-H provided a high affinity binding approximately 3-fold greater than that of the NIP-H. Meanwhile, no significant difference on cholesterol binding was observed at a lower amount of polymer (5 mg). However, it is worth noting that an increase of binding capability up to 6-fold was achieved upon removal of the template molecule from the polymer via hydrolysis.

To further confirm that the binding affinity to cholesterol of the MIP-H was attributable to the selective binding in the imprinted pockets, a competitive binding analysis was performed. Various amounts of non-labeled cholesterol ranging from 1.4 n*M* – 14 m*M* were added into the reaction mixture between MIP-H and 1.4 nM ^{3}H-cholesterol. After equilibrium was reached, supernatant was subjected to liquid scintillation counting. As shown in Figure 2, the amount of bound radioisotope was decreased in the presence of a large excess of competitors. In some circumstances a decrease of up to 30 to 40% in the signal was observed in the presence of millimolar level competitor. This finding indicated a high binding avidity and accessibility of the binding site in the imprinted polymer. However, it should be noted that more than 48 hours is required to attain the solid state of polymer. This infers the possibility of a low amount of free radicals in the reaction system.

In conclusion, our findings suggest that semi-covalent imprinting along with nitroxide initiator can be a promising approach for the construction of effective cholesterol imprinting polymers. Incorporation of the imprinting polymer onto sensor devices, e.g. quartz crystal microbalances, lend support to a feasible utilization of the imprinted polymer for the detection of cholesterol in the future.

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Table 2 Cholesterol binding capability of molecularly imprinted polymers

Polymer (mg)	Binding capability to cholesterol (%)		
	NIP-H	MIP-UH	MIP-H
5	3.0	-1.2	2.93
25	8.7	4.09	25.33

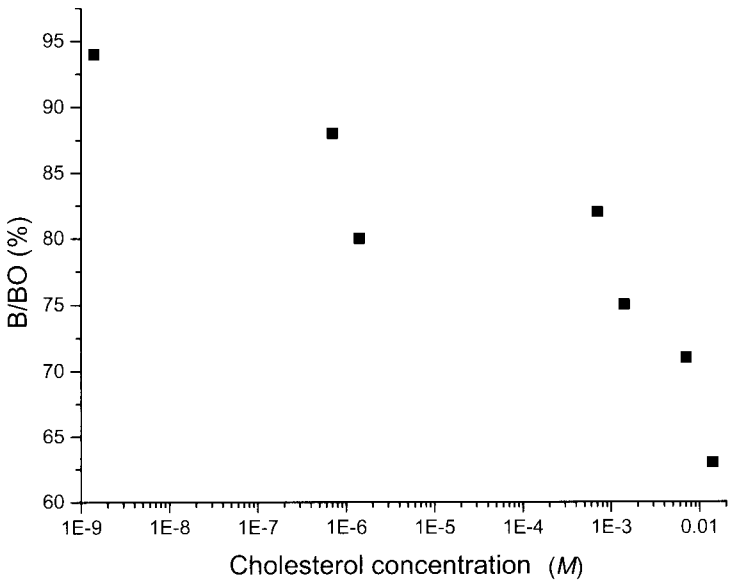

Figure 2 Competitive binding analysis of cholesterol imprinting polymer; B/BO is the ratio of the amount of radioligand bound in the presence of displacing ligand (B) to the amount bound in the absence of displacing ligand (BO).

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