Novel Cholesterol Lowering Polymeric Drugs Obtained by Molecular Imprinting

Chad C. Huval, Mathew J. Bailey, William H. Braunlin, S. Randall Holmes-Farley, W. Harry Mandeville, John S. Petersen, Steven C. Polomoscanik, Robert J. Sacchiro, Xi Chen, and Pradeep K. Dhal*

GelTex Pharmaceuticals, Inc., 153 Second Avenue, Waltham, Massachusetts 02451

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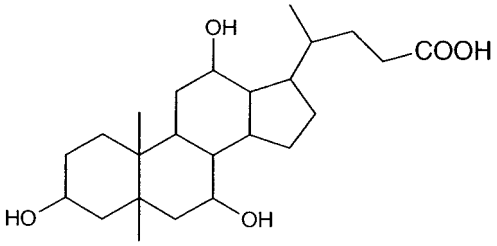
The use of polymeric bile acid sequestrants for lowering gastrointestinal bile acid concentrations is an established approach for treating high cholesterol related diseases.[1] Being nonabsorbed, these polymeric drugs do not exhibit systemic side effects that are associated with other well-known cholesterol lowering agents such as HMG-CoA reductase inhibitors (e.g., statins).[2] However, potent bile acid sequestrants need to be selective toward bile acids, have high capacity factors, and tightly bind the bile acids in the complex chemical milieu of the gastrointestinal (GI) tract. In general, these sequestrants are polymers bearing ammonium groups, and their primary mode of sequestration is based on electrostatic interaction between the cationically charged polymers and anionically charged bile acids. In recent years, the rational design of polymer architectures incorporating various functional groups has led to the discovery of a number of highly effective bile acid sequestrants.[3] Nevertheless, given the importance of this therapy, the design and synthesis of more potent and selective bile acid sequestrants which can be used at lower therapeutic doses is an ongoing activity.

"Molecular imprinting" is a novel technique to incorporate specific substrate recognition sites into polymers.[4] Molecular recognition characteristics of these polymers are attributed to complementary size, shape, and binding sites imparted to the polymers by the template molecules.[5] As a part of our ongoing efforts to develop novel polymeric pharmaceuticals,[6] we have been interested in discovering potent bile acid sequestrants. Application of the molecular imprinting technique to recognize steroids such as cholesterol has been modestly successful.[7−9] In the present communication, we describe the application of the molecular imprinting technique to prepare novel bile acid sequestrants that have been tested in both in vitro and in vivo experiments.

Our approach to prepare molecularly imprinted bile acid sequestrants involves the incorporation of cationic groups in hydrogel matrices in a substrate specific manner. Unlike traditional imprinting procedures which involve template-mediated polymerization of functional monomers,[4] the present material synthesis is based on the cross-linking of polymer−template assemblies at ambient temperature.

We selected poly(allylamine) as the functional polymer. This polymer has good water solubility and presents a high density of amino groups, which can be used for subsequent chemical modification. Furthermore, in its cross-linked form this polymer possesses low toxicity and is biocompatible.[10] The sodium salt of cholic acid (**1**), which is one of the bile acids, was used as the



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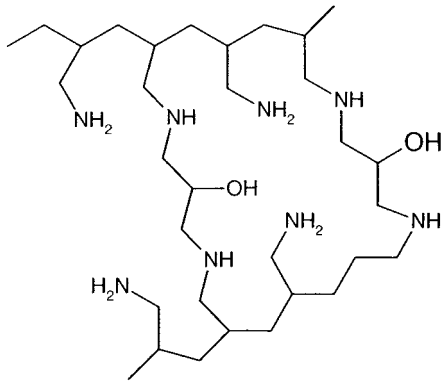
template. Epichlorohydrin was used as the cross-linking agent. Imprinted polymer networks were obtained by cross-linking partially neutralized poly(allylammonium chloride) with epichlorohydrin in the presence of sodium cholate template. This cross-linking reaction was quite efficient, and virtually no residual epichlorohydrin was found at the end of the reaction. After the completion of the cross-linking reaction, the removal of the sodium cholate template from the polymer network was accomplished by subjecting the polymer to a series of washing cycles. The polymer was dried, ground, and sieved to appropriate particle size (<106 μm), yielding the desired imprinted polyammonium salt network (**2**).



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Quantitative removal of the embedded template from the polymer matrix was established by HPLC analysis of the washed liquids. In addition to the control polymer **P-1** (with a statistical distribution of binding sites was prepared in the above manner without any template), we have prepared two imprinted polymer networks **P-2** and **P-3** using two different concentrations of the template. The polymer **P-2** was prepared using 0.043 mmol of template per milliequivalent of amine, and the polymer **P-3** was prepared using 0.216 mmol of template per milliequivalent of amine. The compositions of the reaction mixtures and the results of the physicochemical characteristics of these polymers are summarized in Table 1.

These cholic acid imprinted polymer networks are expected to contain binding sites complementary to the carboxyl group as well as to the shape of the steroid skeleton of the template. Bile acid sequestration properties of these imprinted polyammonium networks were assessed by in vitro and in vivo experiments. Compari-

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Table 1. Compositions, Physical Characteristics, and Bile Acid Binding Properties of Molecularly Imprinted Polyammonium Networks

polymer	amine (mequiv)	epichlorohydrin used (mmol)	Na cholate used (mmol)	SI[a]	association const (K) (mM^{-1})	Q_{max}[b]	cooperative parameter (n)
P-1	53.42	37.5		0.44	0.29	1.30	1.92
P-2	53.42	37.5	2.31	0.38	0.33	1.07	2.54
P-3	53.42	37.5	11.53	0.39	1.97	1.97	3.58

[a] SI is the swelling index of polymer (wet weight − dry weight/dry weight) in deionzied water. [b] Q_{max} is the maximum binding capacity expressed as mmol of ligand/g of polymer.

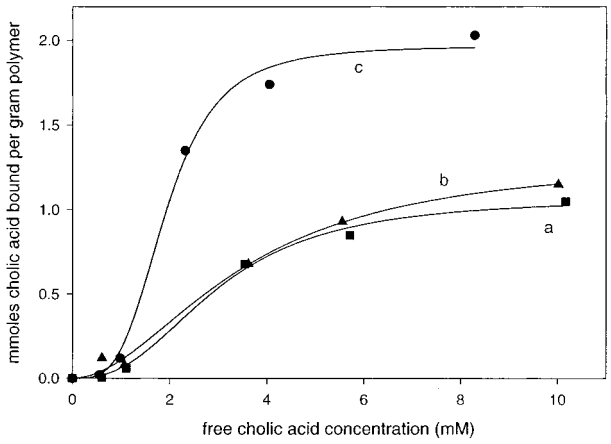


Figure 1. Bile acid binding isotherms of cross-linked polyammonium salts: (a) control polymer **P-1**; (b) imprinted polymer **P-2**; (c) imprinted polymer **P-3**.

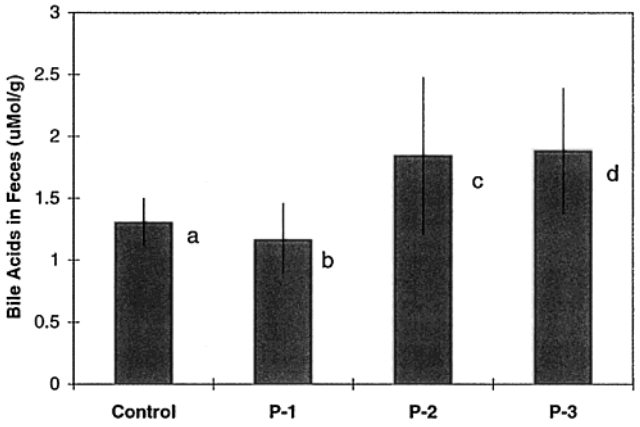


Figure 2. Results on the in vivo bile acid excretion after treatment with polymeric bile acid sequestrants: (a) control group without any polymer; (b) control polymer **P-1**; (c) imprinted polymer **P-2**; (d) imprinted polymer **P-3**.

sons were made with the results obtained using the control polymer, which enabled us to discern the role of imprinting from the usual electrostatic interaction between the polymers and bile acids.

In vitro bile acid sequestration experiments were performed by a batch equilibrium binding procedure. In these experiments, predetermined amounts of imprinted and control polymer particles (20 mg) were allowed to interact with solutions of cholic acid of varying concentration. These binding experiments were carried out in duplicate at 37 °C in BES buffer. The pH of the solution was maintained at 6.8. The amounts of cholic acid bound to polymer particles were determined by HPLC analyses of aliquots, after filtration of polymers. The bile acid binding isotherms obtained from these experiments are shown in Figure 1. The curves shown in this figure are theoretical fits to the Hill equation.[11]

$$\mathrm{LS} = S_{\max}\frac{K^n(L)^n}{1 + K^n(L)^n}$$

In this equation, K is the intrinsic binding constant in mM^{-1}, LS is the density of bound sites (mmol per g of polymer), S_{max} is the total density of sites (mmol per g of polymer), L is the free bile acid concentration in mM, and n is a measure of the cooperativity of binding. The value of $n = 1$ corresponds to noncooperative binding (Langmuir isotherm), and n increases with corresponding increases in cooperativity.

Binding parameters such as association constants, maximum binding capacities, and cooperativity parameters for the imprinted and nonimprinted polymers were computed from the binding isotherms, and the results are presented in Table 1. The imprinted polymer prepared using higher amounts of cholic acid (**P-3**) exhibits significantly higher capacity and cooperativity for the template compared to the control polymer (Figure 1c vs Figure 1a). On the other hand, substrate binding properties of the imprinted polymer prepared using a low template concentration (**P-2**) is marginally better than the control polymer. The amount of template used to prepare **P-3** is nearly 5 times more than that used for **P-2** (vida supra). Such a variation in template concentration during imprinting may have led to less imprinted sites for **P-2** and hence lower substrate binding property.

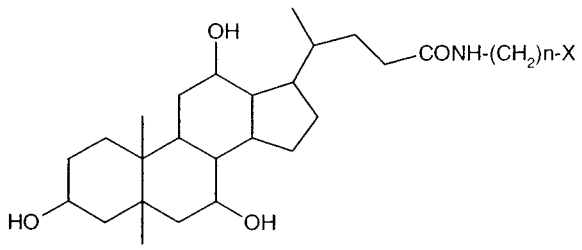
Since both imprinted and control polymers exhibit a similar degree of swelling and contain the same number of amine groups (see Table 1), they should also contain a similar number of accessible ionic interaction sites. Therefore, the higher capacities of the imprinted polymers over the control polymer may be attributed to the generation of substrate selective, high affinity binding sites in the polymer matrices during the template-mediated cross-linking reaction.

Additional evidence supporting the role of imprinting in creating high affinity bile acid binding sites in the polymers can be obtained from in vivo studies. In vivo bile acid sequestration characteristics of these polymers were evaluated using hamsters as the animal model. These hamsters were fed the polymers in their diet, and the bile acids excreted in their feces (after normal digestive cycle) were analyzed. In a typical experiment, three groups of hamsters (four hamsters per group) were selected. Each group of hamsters was fed one of the three polymers along with the diet. Additionally, there was a control group of hamsters that received similar treatment without any polymer in the food. The amounts of food and polymer were kept identical for each group. The total bile acid contents of the animal feces (polymer bound and free) were quantitatively estimated by an enzymatic method.[12]

The results on the bile acids excreted in the feces of hamsters for each polymer (averaged for each group) are shown in Figure 2. There is no significant difference

in the fecal bile acid content of the control group of animals and the animals receiving the nonimprinted polymer **P-1** (Figure 2a,b). On the other hand, higher concentrations of bile acids were found in the feces of animals receiving the imprinted polymers **P-2** and **P-3** (Figure 2c,d).

Despite containing the same number of cationic sites and possessing similar physicochemical properties, the abilities of these imprinted polymers to exhibit superior in vivo bile acid sequestration properties to the nonimprinted polymer are noteworthy. It is known that, in general, bile acids are presented in the biological system as glycine and taurine conjugates (**3a** and **3b**). Furthermore, there are various kinds of bile acids present in the GI tract that are formed in vivo by the enzymatic dehydroxylation of cholic acid derivatives.[13] Therefore, the abilities of cholic acid imprinted polymers to sequester different bile acids in vivo is significant. Furthermore, the lack of bile acid sequestration properties of the nonimprinted statistical polymer, which showed measurable in vitro sequestration, is interesting. By contrast to in vitro conditions, the GI tract contains a gradient of pH and salt concentration and a transporter mediated active hepatic reuptake of bile acids.[14] Therefore, mere ionic interactions between the nonimprinted polymer and the bile acids may not be adequate to withstand these desorbing forces. It appears that molecular imprinting has imparted additional binding properties to the polymers, which enable them to better compete with these desorbing forces in the GI tract.



3a: n =1 X = COOH; 3b: n =2, X = SO_3H

At the present time it is difficult to identify precisely the factor(s) governing the improved bile acid sequestration properties of these imprinted polyammonium networks. Some plausible mechanisms can be put forth, however. One possibility is that, besides the electrostatic interaction, shape selective fitting of steroidal skeletons of bile acids into complementary cavities created in polymer matrices during the imprinting procedure may lead to improved retention of the bile acids within the polymer matrices. While traditional imprinted polymers have low swelling indices, these polymers swell to more than twice their volume. This would impart induced fit properties to polymers (analogous to enzymes), thereby enabling them to accommodate similar compounds with modest structural variations. Alternatively, the imprinted polymers would initially bind small amounts of bile acids that favorably fit to a few imprinted, strong binding sites. These initial polymer bound bile acids would subsequently improve the binding of additional bile acid molecules through hydrophobic interactions, producing aggregates or micelles of bile acids that are stable to competing in vivo desorbing forces. Bile salts are known to self-aggregate in water with increasing concentration.[13,15] This phenomenon would manifest in higher capacity of the polymer for bile acids than those based on 1:1 complex. This possibility is supported by higher cooperativity parameter values (from in vitro experiments, Table 1) of imprinted polymers than the control polymer. Whatever the mechanism of binding, this report is the first demonstration of the application of the molecular imprinting technique to produce polymeric drugs, which have shown their effectiveness in both in vitro and in vivo studies.

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