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Ammonium and Guanidinium Functionalized Hydrogels as Bile Acid Sequestrants: Synthesis, Characterization, and Biological Properties

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ABSTRACT

Novel cationic polymers as bile acid sequestrants (BAS) have been considered to be an attractive long-term therapy for the treatment of hypercholesterolemia. Due to the poor in vivo efficacy of the first generation of BAS like cholestyramine and colestipol, there is a need for discovering new generations of potent BAS. As part of our polymeric drug discovery efforts, we have developed a facile route to prepare functional hydrogels bearing pendant amine and guanidinium groups. The polymeric amines were prepared either by direct polymerization of amine containing monomers or by chemical modification of suitable polymeric precursors. Polymers bearing guanidinium groups were obtained by a polymer analog reaction on crosslinked polymeric amines (primary or secondary) using a readily available guanylating agent in aqueous medium. Incorporation of guanidinium groups to these polymers occurs under mild reaction condition. A numbers of polymer structures with pendant amine and guanidinium groups located at varying distances from the polymer backbone were obtained. These

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polymeric ammonium and guanidinium salts were evaluated in vivo as BAS and hence cholesterol lowering agents.

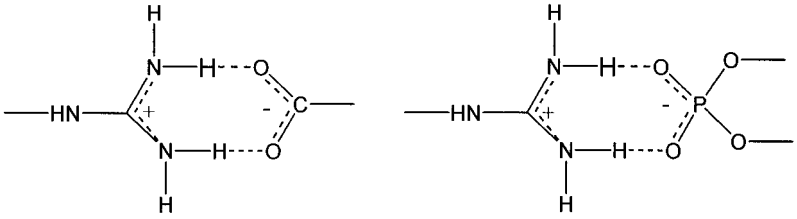
Key Words: Cationic hydrogels; Polyguanidinium salts; Polymeric drugs; Bile acid sequestrants.

INTRODUCTION

Crosslinked, cationic polymers that bind anionic bile acids in the gastrointestinal (GI) tract have been considered as novel nonabsorbed therapies for the treatment hypercholeterolemia.[1,2] The mode of action of these polymeric sorbents, known as bile acid sequestrants (BAS) involves sequestration and removal of bile acid (a metabolic outcome of cholesterol) from the digestive tract. This event triggers increased synthesis of bile acids in the liver utilizing the plasma cholesterol pool and their subsequent transfer to the bile pool. The net outcome of the process is the overall lowering of the serum cholesterol. The use of cationically charged hydrogels for sequestration of bile acids is indeed an established approach for treating hypercholesterolemia.[3] These polymeric drugs are nonabsorbed and hence, do not exhibit the systemic side effects that are associated with more widely used hypercholesterolemic drugs such as statins.[4] Until recently cholestyramine and colestipol have been the two FDA approved BAS on the market.[5] In spite of their appealing safety profiles, these two first generation BAS exhibit low clinical potency and require high daily doses to bring about desired cholesterol lowering effects. For example, the doses of cholestyramine and colestipol required for a 20% reduction in serum cholesterol are typically 16–24 g/day. This has led to reduced patient compliance thereby limiting their viability for chronic treatment. This low clinical efficacy of BAS (in spite of better in vitro potency) has been attributed to a competition between the polymeric sequestrant and active bile acid reuptake transporter system of the GI tract towards bile acids.

It appears that for a cationic polymer to be a potent BAS, it must have high binding capacity, strong binding strength, and selectivity towards bile acids in the presence of competing desorbing forces of the GI tract. Therefore, a clinically successful BAS needs to exhibit slow off-rates of bound bile acids from the polymer resin to effectively overcome the transporter-mediated active reuptake of bile acids from the GI tract. In recent years, the rational design of novel functional polymer structures has resulted in the discovery of numerous potent BAS. In general, polyammonium salts with optimum density of cationic groups (and optionally hydrophobic groups) constitute key features of the most potent BAS.[6] Incorporation of these features in cationic hydrogels has led to the discovery of a number of BAS over the last decade from our laboratories, as well as from other groups.[7,8]

As a part of our efforts to design and synthesize new classes of cationic hydrogels as potent BAS, we have considered novel polymeric hydrogels bearing primary amine groups and guanidinium groups as possible cholesterol lowering agents. Although hydrogels containing different cationic groups such as ammonium and phosphonium groups have been investigated as potential BAS,[9,10] use of guanidinium functionality as binding sites to sequester bile acids has not been reported to date. The use of guanidinium groups as recognition and binding of oxyanions has attracted increasing interest in recent years.[11] The unique combination of cationic and hydrogen bonding properties of guanidinium group makes it a very interesting anion binding functional group. As shown in Sch. 1, guanidinium



Scheme 1. Structure of Zwitterionic bonding between guanidinium groups and oxyanions.

groups possess the ability to form a pair of strong Zwitterionic hydrogen bonds with oxyanions such as carboxylate and phosphate ions.[12] Because of its high p*K*a value (13.5), the guanidinium group remains protonated over a much wider pH range than amines.[13] This property of guanidinium groups has been exploited to biomimetic catalysts, biomaterials, etc.[14–16]

For the sequestration of bile acids by polymeric amine gels, the nature of the linker arm connecting the backbone and amine groups may influence the binding behavior as a consequence of steric effects. Towards this end, we have synthesized a series of crosslinked polymers bearing amine groups separated from the polymer backbone by varying spacer lengths using a facile chemical modification method. These amine containing polymers, as well as other polymeric amines, were used as the precursors for the synthesis of poly(guanidinium) salts by a polymer analog reactions. Evaluation of these polymers as BAS would provide a possible insight on the role of the natures of the cationic functional groups as well as steric effects on anion complexation. Bile acid sequestration properties of these polymeric amines and poly(guanidinium) salts have been evaluated under in vivo conditions using hamsters as animal models.

EXPERIMENTAL

Materials

Unless stated otherwise, all reagents and solvents were obtained from Aldrich Chemical Company, Milwaukee, WI, and were used as received. Elemental analyses were carried out at QTI Laboratories. Polyvinylamine was obtained from Air Products, Allentown, PA. Polyallylamine and polydiallylamine were synthesized as their hydrochloride salts following reported procedures.[17,18]

Synthesis of Crosslinked Polymeric Amines

Epichlorohydrin crosslinked polymeric amine gels were prepared by reacting aqueous solutions of different polymeric amines with appropriate amounts of epichlorohydrin following the reported procedure.[19] As a representative example, detailed experimental procedure for the synthesis of polyallylamine gel is given. Thus, in a 500 mL beaker was taken 60 g of 50% aqueous solution of polyallylamine · HCL. The polymer solution was further diluted with 90 mL of deionized water. While stirring, 17.6 g of 50% (w/w)

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aqueous solution of NaOH was slowly added to the polymer solution and the stirring continued for an additional 2 hr. After this time, 0.75 mL of epichlorohydrin was added to the polymer solution and stirring continued. The polymer solution slowly became viscous and finally became a soft gel after about 20 min. After the formation of the gel, the stirring was stopped and the reaction mixture was left at room temperature for 48 hr. Subsequently, the gel was broken into small pieces, stirred with 400 mL of deionized water for 40 min, and filtered. This process was repeated three times and the filtered polymer was dried at 60°C, yielding 22.0 g of the polymer gel as an off-white solid.

Synthesis of Crosslinked Poly{*N*-(ω-Amino)alkyldiallylamine} Gels

Using polymer modifcation procedures, these aminoalkyl substituted polydiallylamine gels were obtained in two steps. Epichlorohydrin crosslinked polydiallylamine was used as the polymeric precursor. The modification reactions include: (i) alkylation of polydiallylamine gel using *ω*-phthalimido alkyl bromide as the alkylating agent and (ii) deprotection of phthalimide groups to generate the polymers with pendant amino groups. As a representative example, detailed experimental procedures are given for the synthesis of poly{*N*-(3-amino)propyl diallylamine}.

Synthesis of Poly{*N*-(3-Pthalimido)propyl Diallylamine}

To a suspension of 16.75 g of polydiallylamine gel (prepared in the presence of 4.5 mol equivalent of epichlorohydrin) in 60 mL of methanol was added 1.8 g of 50% (w/w) aqueous NaOH solution. After stirring the suspension for 20 min, 7.0 g of *N*-(3-bromo)propyl phthalimide was added. The resulting reaction mixture was stirred at 60°C for 24 hr. After cooling down to room temperature, the suspension was filtered and was washed with 200 mL of methanol and 200 mL of deionized water (thrice each). The polymer gel was filtered and dried at 60°C yielding 4.5 g of an off-white solid.

Synthesis of Poly{*N*-(3-Amino)propyl Diallylamine}

To a supension of 4.0 g of poly{*N*(3-pthalimido)propyl diallylamine} in 1 : 1 mixture of ethanol : water (60 mL) was added 7.0 g of hydrazine hydrate. The resulting reaction mixture was refluxed under nitrogen atmosphere for 18 hr. After cooling down to room temperature, the reaction mixture was filtered. The resulting polymeric solid was treated with 200 mL of deionized water and heated to 60°C for 1 hr. It was filtered and was washed with 3 × 100 mL of deionized water. The polymer suspension was filtered and was dried at 60°C under reduced pressure, yielding 2.7 g of an off-white solid.

Synthesis of Hydrogels Bearing Guanidinium Groups

Appropriate polymeric guanidinium salts were prepared by guanylation of different polymeric amine gels using 1-H-pyrazole-1-carboxamidine as the guanylating agent. The

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detailed synthetic procedure is given for the synthesis of poly(allylguanidinium)chloride gel, which serves as a representative example.

At first, the partially protonated polyallylamine gel was converted to its free base form. To 25.0 g of the partially protonated, epichlorohydrin crosslinked polyallylamine (see above) was added 500 mL of deionized water. While stirring, aqueous NaOH solution (50%, w/w) was added dropwise to the polymer suspension and the pH was monitored regularly. The addition of NaOH was discontinued after the pH of the suspension reached ~12.0. The stirring continued for additional 1 hr. The polymer gel was filtered and washed thoroughly with deionized water until the conductivity of the polymer suspension was less than 0.5 mS/cm. The polymer was filtered and dried under vacuum yielding 17.0 g of an off-white solid.

In a 500 mL, round-bottomed, three-necked flask were placed 5 g of the above polyallylamine gel (free base) and 100 mL of deionized water. A solution of 14 g of 1-H-pyrazole-1-carboxamidine · HCl and 9.3 g of sodium carbonate dissolved in 100 mL of deionized water was added to this polymer suspension. The resulting reaction mixture was stirred at room temperature under nitrogen for 24 hr. The polymer suspension was filtered, dispersed in 500 mL of deionized water, stirred for 40 min, and filtered. This process was repeated four more times, at which point the conductivity of the polymer suspension was 0.3 mS/cm. Finally, the polymer was dispersed in 100 mL of deionized water and 4 mL of conc. HCl was added. After stirring for 30 min, the polymer was filtered and dried at 60°C under reduced pressure yielding 8 g of the poly(allylguanidinium) hydrochloride as an off-white solid.

Evaluation of In Vivo Bile Acid Sequestration Properties

After a week of acclimation to the facility, hamsters were transferred to metabolic cages to separate urine and feces. Animals were presented a casein-based purified feed with 10% fat added, plus a predetermined amount of the drug. The control group was given no drug. The food was presented for 3 days and fecal material was collected on the final 2 days of drug treatment. The fecal material was freeze-dried and ground in an amalgamator to a uniform powder. The powdered material (1 g) was placed in the extraction cell and a solution of 100 mM NaOH in 80% aqueous methanol was used for extraction. This solvent system was found to be appropriate to dissolve most of bile acids sufficiently and it is basic enough to hydrolyze bile acid esters. The esters commonly occur in the feces and become difficult to extract if not hydrolyzed. The extraction process was accelerated by keeping the sample and solvent at 100°C under a pressure of 1500 psi. A portion (0.25 mL) of the extract was evaporated and reconstituted in bovine calf serum. The sample was then analyzed enzymatically for bile acid concentration with a colorimetric assay.[20]

RESULTS AND DISCUSSION

The desired structural features that would make a cationic hydrogel potent BAS include: (i) strong electrostatic interaction with bile acids over the pH range of the GI tract, (ii) adequate swelling characteristics under physiological conditions; (iii) optimum cationic charge density; and (iv) incorporation of additional binding features to enhance

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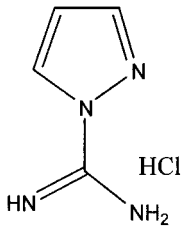
the interactions between bile acids and the polymer resin. All these features are expected to enhance the capacity of the sequestrants and protect the polymer–bile acid complexes from the desorbing forces in the GI tract. Cholestyramine is one of the FDA approved commercial BAS, which has been used as a pharmaceutical agent for the reduction of elevated levels of plasma cholesterol. This polymeric resin contains benzyl trimethyl ammonium groups along the polymer backbone, is less hydrophilic, and is a weak sequestrant in vivo. Therefore, the design and development of cationic polymer gels that possess functional groups exhibiting stronger affinity towards anionic bile acids and higher binding capacity would constitute novel classes of BAS. Guanidinium group-containing hydrogels are expected to provide strong binding towards bile acids by presenting two parallel hydrogen bonds in addition to electrostatic attraction. This kind of binding motif is known in the nature during the complexation of arginine containing proteins with different oxyanionic substrates of biological significance.[21]

Synthesis and Characterization Polymeric Amines and Poly(Guanidinium) Hydrogels

The importance of guanidinium groups as key structural elements in a number of areas ranging from biomimetic chemistry to medicinal chemistry has led to development of a wide variety of guanylation reactions.[22] The typical procedure for the synthesis of guanidinium derivatives involves the reaction of a guanyl transfer agent with a primary or secondary amine. The key to the success of these guanylation reactions is the enhanced electrophilicity of the guanyl transfer reagent. A variety of guanylating reagents have been developed over decades that produce guanidinium compounds with moderate to high yields.[23,24] Among various reagents developed, 1-H-pyrazole-1-carboxamidine (**1**) has been found to be a promising reagent. This reagent introduces guanidinium functionality directly without involving any additional deprotection steps.[25]



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Although a large body of literature is available dealing with syntheses of low molecular guanidinium compounds, reports pertaining to syntheses of polymeric guanidinium salts are relatively scant.[26] We decided to use 1-H-pyrazole-1-carboxamidine as the guanylating reagent to prepare various polymeric guanidinium compounds. Since the polymeric amine precursors are crosslinked gels and swell in water, this water-soluble guanylating reagent appeared to be particularly attractive. This reagent

is commercially available and is known to react with both primary and secondary amines, although with more ease with the former.[25]

Different amine containing polymers were used as precursors to prepare these polymeric guanidinium compounds. Both primary and secondary amine groups were used. The precursor polymeric amines were used in the form of lightly crosslinked hydrogel and were prepared by reacting linear polymeric amines with appropriate amounts of epichlorohydrin. Use of the crosslinked gels for chemical modification has made the purification and isolation of guanylated polymer much easier without compromising the reactivity of polymer bound amine groups towards the guanylating reagent.

The structures of different polyamines used in this study are shown in Fig. 1. While polyvinylamine (**P-1**) is commercially available, polyallylamine (**P-2**) and polydiallylamine (**P-3**) were synthesized readily by the free radical polymerization of the hydrochloride salts of corresponding amine monomers. Polymers containing pendant aminoalkyl substitutents were prepared by a polymer modification method. For this purpose polydiallylamine was used as the precursor polymer. Reaction of this polymer with different ω-phthalimido alkyl bromide produced poly{*N*-(ω-phthalimido)alkyl diallylamine}. Deprotection of pthalimido groups to generate polymer gels bearing pendant aminoalkyl substituents was accomplished by treating these polymers with hydrazine hydrate. This two-step polymer modification reaction for polymeric amine synthesis is illustrated in Sch. 2. By choosing the appropriate ω-phthalimido alkylbromide reagents, polymers containing primary amine groups located at varying distances from the backbone were prepared (Structure **P-4**–**P-7**). The extent of incorporation of aminoalkyl groups to polydiallylamine backbones was quite satisfactory, as is evident from their elemental analysis data (Table 1).

The general method for the synthesis of polyguanidinium salts using the amine containing polymers as precursors is outlined in Sch. 3. Aqueous suspensions of polymeric amine gels in the form of their free bases were treated with 1-H-pyrazole-1-carboxamidine. The reaction was quite facile with polymers containing primary amine groups. For these polymeric amines, the amine groups were significantly transformed to polyguanidinium salts within 24 hr at room temperature. However, for the polymer containing secondary amine, such as polydiallylamine, the reaction mixture needed to be heated at 60°C. Purified polymeric guanidinium salts were isolated by repeated washing of the modified gels with water, which removed all unreacted guanylating agents as well as pyrazole byproducts. Transformation of these polymer bound amine groups to guanidinium groups was established from their elemental analysis data (Table 2). By using three-fold excess of the guanylating agent, it became possible to transform over 90% of the amine groups to guanidinium groups.

Evaluation of In Vivo Bile Acid Sequestration Properties

The bile acid sequestration properties of these polyguanidinium hydrogels were evaluated in vivo using hamsters as the animal model. Published literature on the development of novel BAS has predominantly relied on in vitro experiments. According to some of these publications since the bile acid binding measurements were carried out under conditions that are likely to be encountered in vivo,[27] the results may predict a true in vivo situation. However, in addition to the presence of a gradient of pH and ionic

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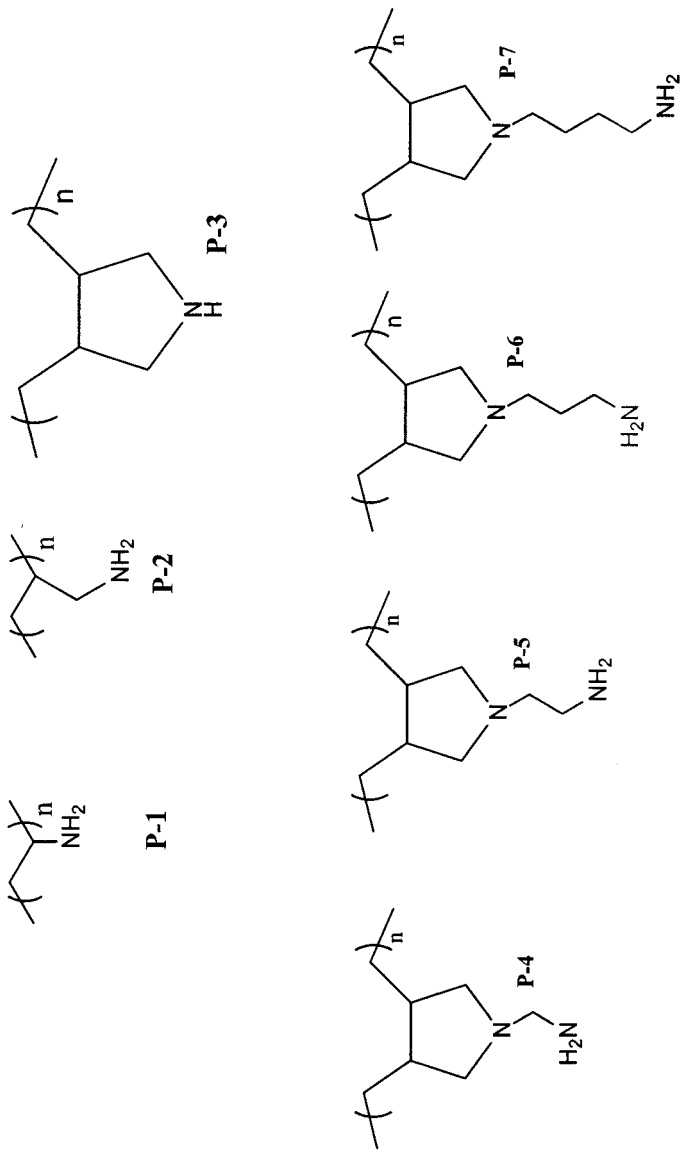

Figure 1. Structures of various polymeric amine used as precursors to introduce guanidinium groups.

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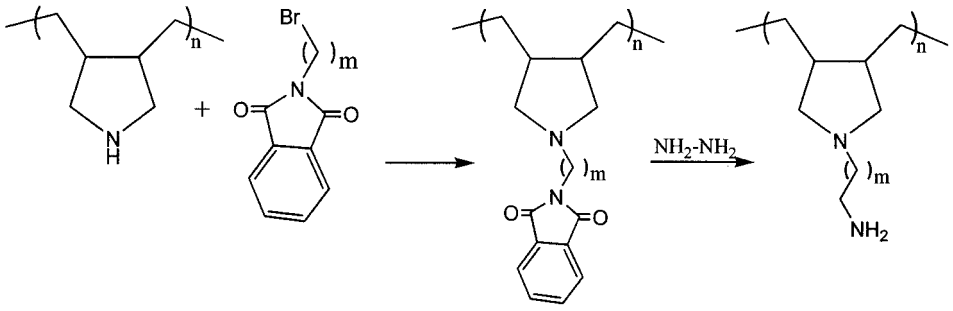

Scheme 2. Synthesis of polymeric amines with pendant primary amine groups.

strengths in the GI tract, transporter-mediated active reuptake of bile acids from the gut have also been reported.[28] These in vitro experiments might have underestimated the role of the transporter-mediated desorbing forces, and thereby possibly overestimated the in vivo potencies of different sequestrants. In order to obtain a more reasonable picture of the potencies of these guanidinium group-containing BAS, we have decided to forego in vitro binding experiments and evaluate the bile acid binding properties of these sequestrants under true biological conditions.

The in vivo bile acid sequestration experiments were carried out by feeding hamsters with polyguanidinium hydrogels as components of their daily diet and measuring the bile acid contents in their feces. An increase in bile acid content of the feces of treated animals (in comparison to control animals) provides a direct measurement of the sequestering abilities of these polymers under physiological conditions. Thus, it becomes possible to estimate to what extent these polymers could bind and effectively remove bile acids from the GI tract. The results on in vivo bile acid sequestration properties of these polymers are summarized in Table 3.

Analysis of in vivo results reveals that in general, all guanidinium functional polymers sequestered bile acids to some extent. Furthermore, most of these polymers were found to be somewhat more potent than the commercially available BAS, cholestyramine. However, no clear structure–activity relationship was observed for polymers of different backbones or role of the spacer arms between the polymer backbone and the

Table 1. Synthesis of poly(*N*(*ω*-amino)alkyl diallylamine by chemical modification reaction of poly(diallylamine).

Polymer ID	Type of (*ω*-phthalimido)-alkylbromide used	Elemental composition (C/N)	
		Before NH_2NH_2 treatment	After NH_2NH_2 treatment
P-4	1-Pthalimidomethyl	6.69	6.02
P-5	2-Pthalimidoethyl	5.11	4.51
P-6	3-Pthalimidopropyl	7.35	5.34
P-7	4-Pthalimidobutyl	7.39	5.41

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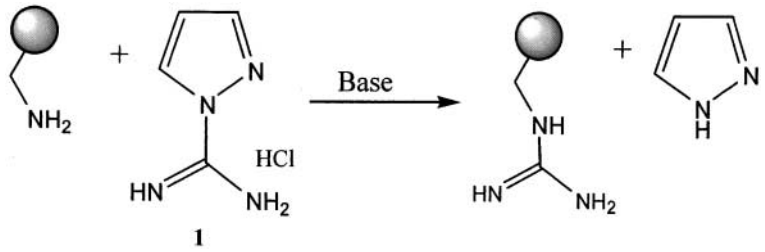

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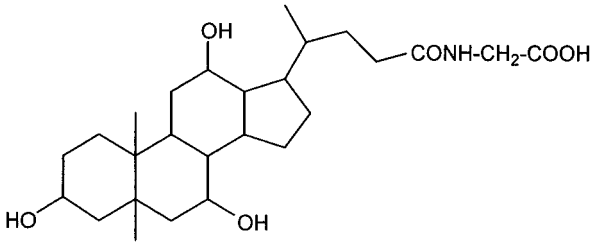
Scheme 3. Reaction of polymeric amine with 1-H-pyrazole-1-carboxamidine to prepare polymeric guanidinium salts.

guanidinium groups. Moreover, the precursor amine polymers showed equal or better bile acid binding properties compared to their guanidinium counterparts. Since guanidinium groups are known to interact with carboxylate anions through electrostatic as well as hydrogen bonding interactions, they should lead to stronger interactions than amines. In the light of this background, the observed in vivo data is somewhat puzzling. A careful interpretation of the anion binding properties of guanidinium groups and nature of bile acids in the gut may provide some insight on these observed results. A possible explanation is thus given as follows. The bile acids in the GI tract are present as conjugates with taurine (sulfonic acid residue) and glycine (carboxylic acid residue).[29] Thus, taurocholic (**2**) and glycocholic acids (**3**) are two typical bile acids present in the gut.



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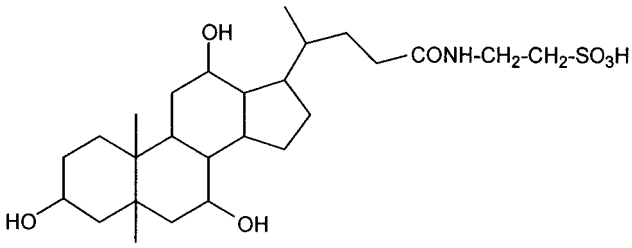

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Sulfonic acid is a stronger acid than carboxylic acid. Furthermore, for a given anionic species, the strongest electrostatic interaction between the anionic species and cationic species occurs when the cationic species has the highest localized charge density. In other words, an increase in the positive charge density of the cationic ligand

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Table 2. Synthesis of polyguanidinium salts using various polymeric amine hydrogels.

Polymer ID	Type of precursor polymeric amine used[a]	Elemental composition (C/N)	
		Precursor polymer	Polyguanidinium salt
P-8	**P-1**	2.24	1.95
P-9	**P-2**	3.31	1.63
P-10	**P-3**	6.26	2.69
P-11	**P-5**	4.51	2.86
P-12	**P-6**	5.34	3.08

[a]For structures of these polymeric amines, see text.

would increase the stability constant of its complex with the anion. Since the charge on the guanidinium group is more delocalized than on the ammonium group, electrostatic interaction is expected to be stronger with the latter.[30] However, the guanidinium groups present a more favorable geometry to form two linear hydrogen bonding interaction sites with various anionic groups. In general anion complexation properties of guanidinium groups are independent of pH. However, anion binding properties of amine are limited to the pH range, where both protonated amines and anions of interest can coexist. Relative acidity of the GI tract (pH range of ~2–5 in the stomach and 5–6.5 in the small intestine) would render most of the polymeric amine groups to be present predominately in protonated forms. Due to their low p*K*a values, sulfonic acid residues of taurine conjugates are likely to be present in the form of dissociated anions in the gut. As a result, electrostatic interaction may be the dominant binding force between the cationic polymer gels and bile acids. On the contrary, acid groups of glycine-conjugated bile acids may be partially present in the protonated acid form. While protonated carboxylic acid may not interact with ammonium groups through electrostatic interaction, they could interact with guanidinium groups through hydrogen bonding interaction. It has been reported that

Table 3. Results on in vivo bile acid sequestration properties of polyammonium and polyguanidinium group containing hydrogels.

Polymer ID[a]	Dose in feed (w/w in %)	Percentage above control
P-1	0.2	35
P-2	0.2	43
P-3	0.2	134
P-4	0.2	103
P-5	0.2	164
P-6	0.2	115
P-7	0.2	144
P-8	0.2	30
P-10	0.2	84

[a]For description of these polymers, see text and Table 2.

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taurine conjugated bile acids are the major de novo constituents the of bile conjugates are likely to be present in the form of dissociated anions in the gut. As a result, electrostatic interaction may be the dominant binding force between the cationic polymer gels and bile acids. On the contrary, acid groups of glycine-conjugated bile acids may be partially present in the protonated acid form. While protonated carboxylic acid may not intereact with ammonium groups through electrostatic interaction, they could interact with guanidinium groups through hydrogen bonding interaction. It has been reported that taurine conjugated bile acids are the major de novo constituents the of bile acid pool of hamsters during depletion studies, where the bile duct has been diverted.[31] As mentioned above, although guanidinium groups present more favorable hydrogen bonding interaction sites to anionic groups, stronger electrostatic interaction between taurine conjugated bile acids and polymeric ammonium salts are expected. We tentatively attribute this to be the possible reason behind the observed low in vivo efficacy of guanidinium functional polymer compared to the corresponding polymeric amine sequestrants. However, the complexity of biological system precludes us from drawing any definitive conclusion.

CONCLUSION

A facile synthetic method has been developed to prepare novel polymer structures containing novel amine and guanidinium groups. These cationic ligands have been evaluated as BAS, and hence as cholesterol lowering agents. The bile acid sequestration properties of these guanidinium-functionalized polymers were compared with those of the corresponding polymeric amine precursors. Although the guanidium group is known to be a better anion chelator over a wider pH range, under the physiological conditions the polyammonium compounds exhibited better bile acid sequestration properties than the polymeric guanidinium salts.

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