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The Effects of Cross-Linking on the Binding of Bile Acids by Poly(acrylamide) Resins

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements of the degree of
Master of Science

Department of Chemistry
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Abstract

The primary goal of this study was to investigate the effect of the density of positively charged groups on the binding of bile acids by poly(acrylamide) resins with amine-containing pendent groups. For this purpose, lightly cross-linked poly(methyl acrylate) beads were functionalized by the reactions with diamines using two different methods, one of which eliminated additional cross-linking during the functionalization procedure. A comparison was made between the swelling characteristics, the isotherms for the binding of cholate anions, and the degree of cooperativity of these resins. Interestingly, the elimination of additional cross-linking not only improved the capacity of the resins to bind cholate anions but it also increased the selectivity for bile acid anions as well as their degree of positive cooperativity. The high degree of cooperativity of minimally cross-linked poly(acrylamide) in binding bile acid anions probably indicates the existence of bile acid micelles or mixed micelles within the beads cavities.

To rationalize the effect of cross-linking density on the *in vitro* sorption efficiencies, a microstructure model for three dimensionally cross-linked beads was introduced. This model was developed according to the local degree of cross-linking and is based on the size of functional units and bile acids.

Furthermore, for the first time the effect of various organic counter-ions, such as propionate, acetate, benzoate, and salicylate on the *in vitro* sorption of cholate by poly(acrylamide) resins was studied. It was determined that the sorption efficiency can be improved significantly by replacing the chloride counter-ions with organic counter-ions of low relative affinity. It was demonstrated that functionalized poly(acrylamide) resins having acetate and propionate counter-ions possess a relatively better binding behavior for cholate anions than those resins with Cl^- or I^- counter-ions.

Résumé

L'objecif principal de cette étude a été d'éxaminer l'effet de la densité des groupes chargés positivement sur l'adsorption des acides biliaires par des résines polyacrylamides contenant des groupes pendant qui contiennent des amines. Dans ce but, des billes de polyacrylate de méthyle à liaison croisée ont été fonctionalisés par des réactions des di-amines utilisant deux méthodes différentes; une d'elles élimine la formation de liaisons croisées additionelles durant le procéssus de fonctionalisation. Une comparaison a été faite entre les caractérisques de boursouflement, les isothermes pour l'adsorption des anions cholate et le degré de coopérativité de ces résines. L'élimination des liaisons croisées additionelles augmente non seulement la capacité des résines pour l'adsorption des anions cholates mais aussi, la sélectivité des résines pour les anions des acides billiares et le degré de la cooperativité. Le haut degré de cooperativité dans les résines polyacrylamides à base de liaisons croisées dans le processus d'adsorption avec les anions d'acides biliaires démontre probablement l'existence de micelles d'acides biliaires ou de micelles mixtes dans les cavités des billes.

Pour comprendre l'effet de la densité des liaisons croisées sur l'éfficacité de la sorption *in vitro*, un modèl basé sur la microstructure des billes aux liasons croisées en trois dimensions a été étudié. Ce model a été développé en accord avec le degré local de liaisons croisées et est basé sur la grandeur des unités fonctionnelles et des acides biliaires.

Pour la première fois, l'effet de plusieurs contre-ions organiques comme le propionate, l'acetate, le benzoate, et le salicylate sur la sorption *in vitro* des anions cholates par la résine polyacrylamide a été étudié. Il a été démontré que l'éfficacité de sorption de la résine polyacrylamide pour les acides biliaires peut être amélioré en ramplaçant le contre-ion Cl^- avec un contre-ion organique d'affinité relativement faible. Il a été démontré que les résines de polyacrylamides qui ont été fonctionalisées et qui ont comme contre-ion l'acetate ou le proprionate possèdent un mode d'adsorption relativement supérieur pour les anions cholate par rapport aux résines utilisant Cl^- ou I^- comme contre-ion.

Acknowledgment

I would first like to express my sincerest gratitude to my research director, Professor G. R. Brown, whose guidance, support, and friendship made my stay at McGill a valuable and memorable experience.

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Chapter 1

PC : phosphorylcholine;
Apo E : apolipoprotein E;
LP : lipase lipoprotein;
HDL : high density lipoprotein;
LDL : low density lipoprotein;
VLDL : very low density lipoprotein;
CETP : cholesteryl ester transfer protein;
BA : bile acid;
Chol : cholesterol;
FA : fatty acid;
MG : monoglycerides;
TG : triacylglycerols;
CE : cholesteryl esters;

Figure 1.9 A schematic diagram of the areas of the digestive tract where the passive and active transport processes occur. Even though the passive transport process can occur in all parts of the small intestine and colon, the major transport process for re-absorption of bile acids is through the active transport system in the ileum (Reference 18).

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Figure 1.19 Schematic diagram of the structure proposed for micelles: (a) bile acid (cholate) (b) saponin, and (c) saponin-bile acid mixed micelle (Reference 88).

Figure 1.20 Chemical structure of a bile acid dimer (Reference 18).

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List of Symbols

Symbols	Definition
Ala	alanine
Apo E	apolipoprotein E
BA	bile acid
Boc	di-*tert*-butyl bicarbonate
C^-	counter-ion
CA^-	cholate anion
CE	cholesteryl ester
C_{eq}	concentration at equilibrium
CETP	cholesteryl ester transfer protein
CHD	coronary hearth disease
Chol	cholesterol
Ci	initial concentration of surfactant
CK	creatine kinase
CMC	critical micelle concentration
Cp	amount of functional groups on the polymer
$(C_s)_{0.5}$	equilibrium concentration at half-bound point
DCM	dichloromethane
DD	two neighboring occupied site on the polymer matrix
DG	diglycerides
DIEA	diisopropyl ethylamine
dL	deciliter
DMF	dimethylformamide
FA	fatty acid
FT-IR	fourier transform infrared
HDL	high density lipoprotein
HMG-CoA	3-hydroxyl-3-methylglutaryl coenzyme A
HPLC	high performance liquid chromatography
K	binding constant (equilibrium constant)
K'	selectivity of exchanger phase
$K_{a/b}$	selectivity coefficient (or relative affinity coefficient)

K_o	binding constant of surfactant molecule bound to an isolated binding site
K_{th}	thermodynamic equilibrium constant
LDL	low density lipoprotein
LP	lipase lipoprotein
Lys	lysine
MG	monoglycerides
NaCA	sodium cholate
NaGCA	sodium glycocholate
NMR	nuclear magnetic resonance
nn	interactions between the non-polar part of the ion and resin
ns	interactions between the non-polar part of the ion and solution molecule
OO	two neighboring free surfactant sites
P	osmotic pressure
PC	phosphorylcholine
PMA	poly(methyl acrylate)
ppm	part per million (units for NMR chemical shift)
R	gas constant (8.3143 J/mol K)
TFA	trifluoroacetic acid
TG	triacylglycerols
Tris	tris(hydroxymethyl)-aminomethane
u	cooperativity parameter
V''	partial molar volume
V^*	hydrated volume of counter-ions
VLDL	very low density lipoprotein
V_o	volume of dry resin
V_p	volume of swollen resin
X	fraction of counter-ions in the solution phase
X^*	fraction of counter-ions in the exchanger phase
α	activity
β	degree of binding (molar ratio of bound surfactant to total binding site)
γ''	activity coefficient of ions in resin phase
γ'	activity coefficient of ions in solution phase
Π	swelling pressure

CHAPTER ONE

INTRODUCTION

1.1 Hypercholesterolemia and Coronary Heart Disease

1.1.1 Cholesterol and Coronary Heart Disease

Coronary heart disease accounts for approximately 1 of every 3 deaths, or nearly 600,000 deaths in the United States each year (1). Not only is cardiovascular disease the leading cause of deaths but it is also responsible for a significant number of illnesses which contribute considerably to health care costs (2,3). It is generally known that the major contributing factor is a high cholesterol level. According to the results of the Lipid Research Clinics Coronary Primary Prevention Trial, there is a direct relationship between high cholesterol levels and the incidence of coronary heart disease (CHD) (4). A wide range of studies, such as pathologic studies of the atherosclerotic plaque, metabolic studies, and genetic studies have provided strong support for the hypothesis that decreasing cholesterol levels reduces the risk of CHD (5-7). Since most of the cholesterol in plasma is transported in the low density lipoprotein (LDL) form, the presence of this lipoprotein is of major importance in the link between the plasma cholesterol level and CHD.

One of the major causes for coronary heart disease is the development of atherosclerosis, which is a thickening of the arterial wall due to the accumulation of lipids, primarily cholesteryl esters. Over many years, atherosclerotic plaque eventually causes arterial occlusion. This results in a decrease of blood flow to the heart which eventually leads to a heart attack and/or stroke. Even though other factors, such as smoking, hypertension, and diabetes, contribute to the development of the disease, a high blood cholesterol level is the major factor for CHD (8).

1.1.2 What is Hypercholesterolemia?

Hypercholesterolemia is defined as a cholesterol concentration associated with significantly increased risk of CHD. This definition brings up the problem of defining what is a "significant increase in risk". According to the 1985 Consensus Conference, a moderate risk for middle-aged adults was defined as a plasma cholesterol level of 240

mg/dL (6.21 mmol/L) and high risk was placed at 265 mg/dL (6.85 mmol/L) (9). Furthermore, it was indicated that a level between 200-240 mg/dL (5.17 to 6.21 mmol/L) carries at least mild increase in risk. In other words, in a middle aged person hypercholesterolemia would be defined as a plasma cholesterol level of over 240 mg/dL (6.21 mmol/L) which actually doubles the risk for CHD compared with a level of 200 mg/dL (10).

1.1.4 The Ideal Plasma Cholesterol Level

Because of the positive correlation between the plasma cholesterol level and the risk of CHD, many investigators believe that the average cholesterol level for the whole population should be as low as possible (10). The ideal cholesterol range may be in the range of 130-190 mg/dL (3.36 to 4.91 mmol/l). If this goal were achieved, the rate of CHD would be reduced by 30% to 50% (11).

1.1.3 The Cause of Hypercholesterolemia

One of the causes of hypercholesterolemia is abnormalities in the gene encoding for LDL receptors in the liver. However, this condition applies to only 2% of the cases of hypercholesterolemic patients. The other causes of hypercholesterolemia are improper diet and defective genetics, or a combination of the two (12). Over production of very low density lipoprotein (VLDL), the precursor of LDL, is one of the genetic causes of hypercholesterolemia. Certainly, diet can also affect the plasma cholesterol level; some people are sensitive to excess dietary cholesterol, saturated fatty acid, or total calories and respond with an unusually marked increase in their LDL level (10).

1.2 Bile Acids And Their Properties

1.2.1 Chemical Structures of Bile Acids

Bile acids are acidic steroids synthesized from cholesterol in the liver. They are mainly carboxylic acids containing 22 to 28 carbon atoms. Their skeleton, which resembles that of cholesterol, is derivative of cyclopentanophenantrene (13). In nature,

the common bile acids are saturated C24 acids derived from 5β-cholanic acid (Figure 1.1). The naturally synthesized bile acids have 1, 2 or 3 hydroxyl groups in equatorial positions and a short aliphatic side chain at one end which has a terminal carboxyl group. The primary bile acids, cholic acid (with 3 OH groups) and chenodeoxycholic acid (with 2 OH groups), are synthesized in the liver and conjugated with one of two amino acids, glycine or taurine (14). All of the bile acids leaving the liver are in the conjugated form.

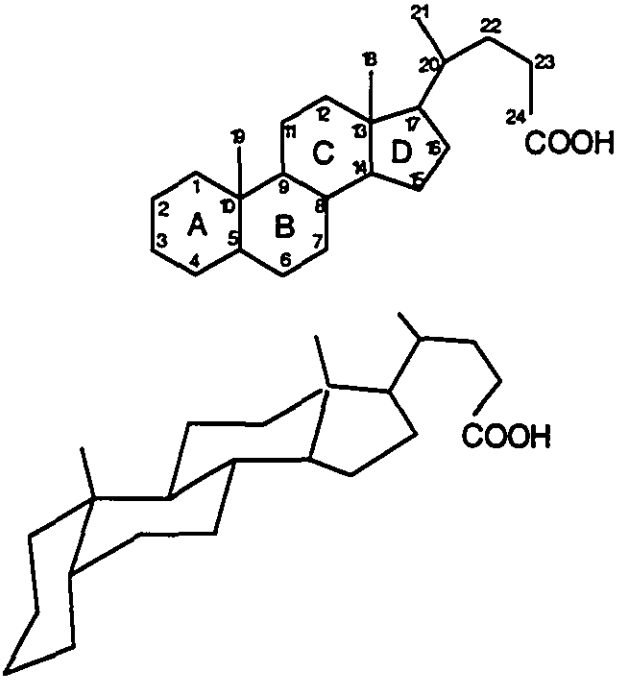
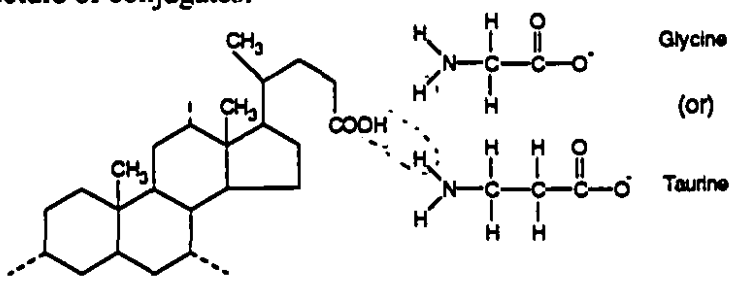


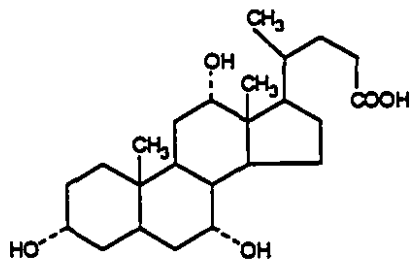
Figure 1.1 Chemical structure of 5β-cholanic acid, the precursor of naturally occurring bile acids.

Conjugation is through a peptide linkage between the amino group of taurine or glycine and the carboxyl group of bile acids (Figure 1.2). Primary bile acids are modified to secondary bile acids by the action of the 7α-dehydroxylating enzyme of bacteria in the

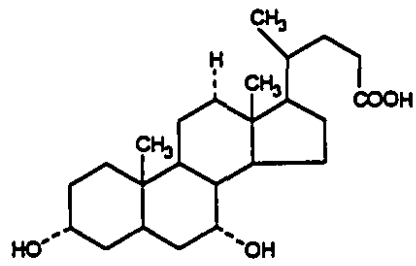
A) General structure of conjugates:



B) Primary bile acids:

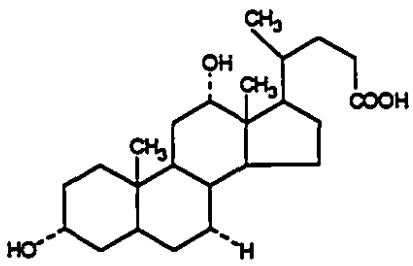


Cholic

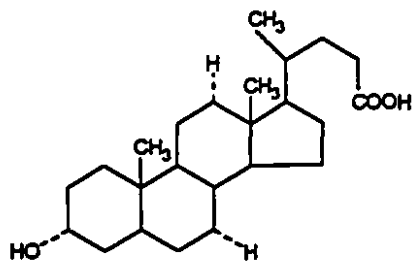


Chenodeoxycholic

C) Secondary bile acids:



Deoxycholic



Lithocholic

Figure 1.2 Structures of major bile acids (Reference 15).

lower small intestine. The secondary bile acids, deoxycholic acid and lithocholic acid, are formed by the dehydroxylation of cholic and chenodeoxycholic acid, respectively (14). Both primary and secondary bile acids are also deconjugated by the action of bacterial enzymes. After re-absorption, the deconjugated bile acids are reconjugated in the hepatocytes. Figure 1.2 shows the structures of primary and secondary bile acids and the conjugated forms. Conjugation of bile acids by amino acids under physiological conditions has a couple of functional consequences (16,17). First, it makes the bile acids more resistant to precipitation by precipitants such as calcium ions. Second, it reduces the passive transport adsorption in the jejunum and ileum and as a consequence more bile acids are available for digestion of fats. The mean ratio of taurine to glycine is approximately 1:3 (mole:mole), and all of the bile acids are conjugated to approximately the same ratio (17).

The hydroxyl groups in the bile acid molecule are all in equatorial positions on the side opposite to the methyl groups. Therefore, the bile acid molecules consist of two distinct hydrophobic and hydrophilic areas. Figure 1.3 shows the structure of cholic acid indicating the hydrophobic and hydrophilic portions. This characteristic of bile acid molecules makes them act as detergents and plays an important role in the absorption of lipids in the intestine. The degree of lyophilicity of each bile acid depends on the number of hydroxyl groups on the rings (18).

1.2.2 Micelle Formation of Bile Acids

The amphiphilic character of bile acids enables them to associate into micelles in aqueous solution above the critical micelle concentration (CMC). In aqueous solution, the self-aggregation decreases the contact of the hydrophobic portion of the molecules with water. The hydrophobic section is directed inward and the hydrophilic portions (carboxyl and hydroxyl groups) point outward. The hydroxyl groups further increase the aggregation number by the formation of hydrogen bonds. Figure 1.4 shows the structures of primary and secondary micelles.

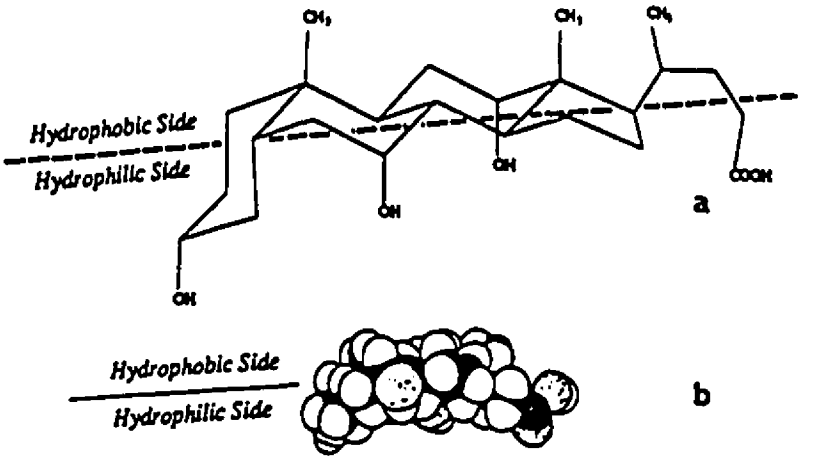


Figure 1.3 The conformation of cholic acid, indicating the hydrophobic and hydrophilic parts of the molecule: (a) perspective structure; (b) Stuart-Briegleb space filling model (Reference 19).

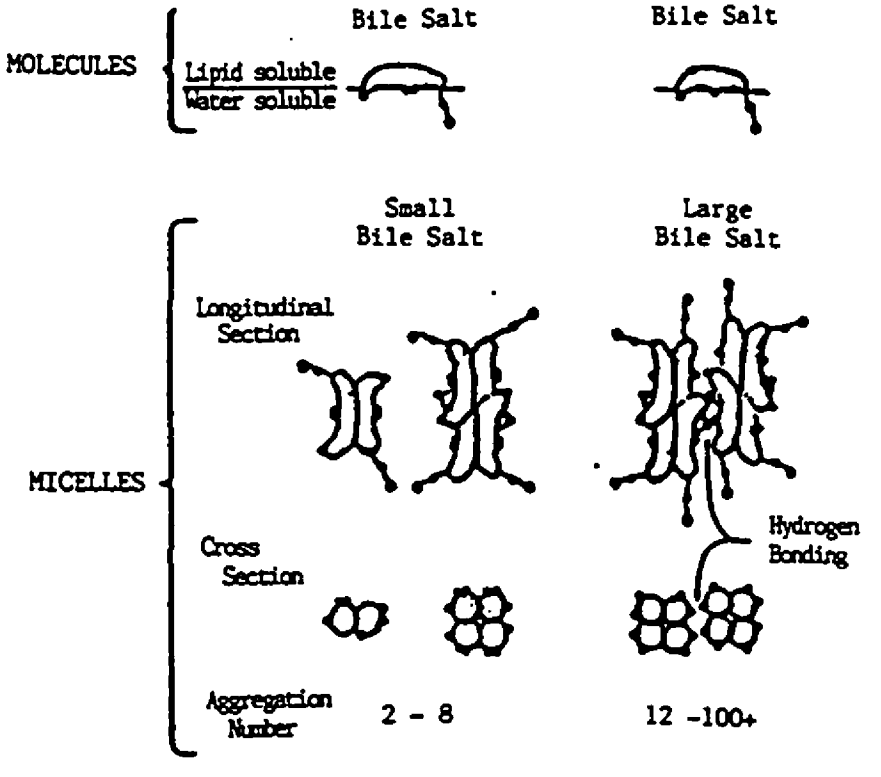


Figure 1.4 Structure of small micelles (aggregation occurs via hydrophobic interaction) and large micelles (aggregation occurs via hydrogen bonding as well as hydrophobic interaction) (Reference 19).

One of the advantages of micelle formation is that hydrophobic lipid materials are included into the bile acid micelles to create "mixed micelles". Figure 1.5 shows the structure of such mixed micelles. The creation of mixed micelles leads to an increase in the solubility of lipid-like molecules. The (CMC) for naturally occurring bile acids is in the range of 5-15 mM in distilled water and about 5-15 μM in the presence of physiological saline (20,21).

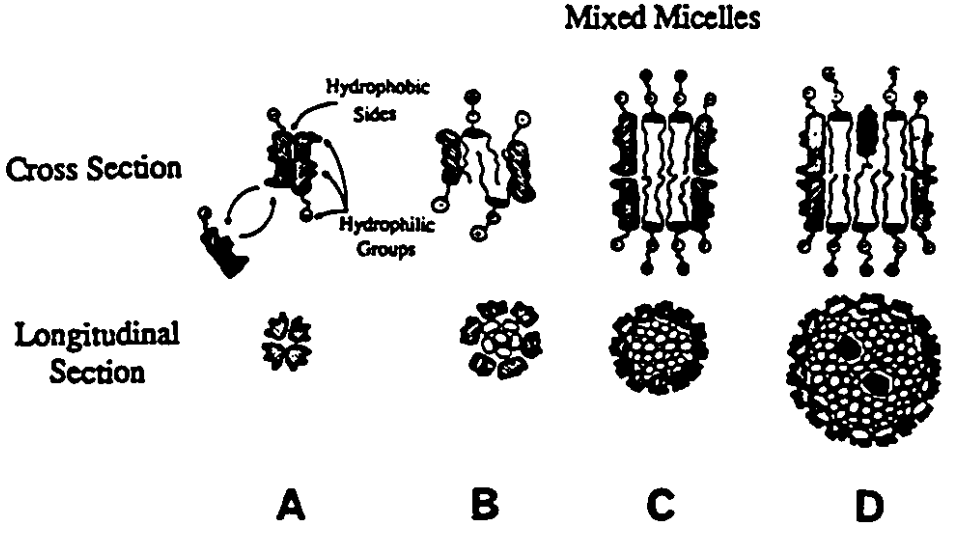


Figure 1.5 The structure of bile salt micelles: A: a simple micelle structure of bile salts; B, C, and D indicating the structure of mixed micelles with lecithin and cholesterol (Reference 19).

In general, human bile has a composition of about 72% bile acids, 21% lecithin, 7% cholesterol (22), and the bile acids which make up the majority of the bile are about 40% cholic acid, 40% chenodeoxycholic, and 20% deoxycholic (23). The role of bile acids in the intestine is to serve as emulsifying agents that assist in the digestion of fats. The bilary cholesterol is probably required for the proper assembly of mixed micellar aggregates and efficiency of digestion of fats (10).

1.2.3 The Physiology of Bile Acids

Generally, primary bile acids are synthesized from cholesterol in the liver; then they are conjugated before they are transported into the bilary canaliculi. They are stored and concentrated in the gallbladder and secreted into the duodenum (part of the intestine) at the time of ingestion of a meal. After facilitating the absorption of lipids, they are re-absorbed by small intestine. For the digestion and absorption of lipid nutrients, the bile salts functions are as follows (23,24): 1) They emulsify the fat constituents of the meal. The emulsification increases the surface area of the lipids and allows greater activity of pancreatic lipase that breaks down the triacylglycerides. 2) They prevent the denaturation of pancreatic lipase. 3) They create mixed micelles with cholesterol, free fatty acids, triglycerides, phospholipids, and fat soluble vitamins for solubilization of these molecules. As a result, the role of bile acids (as they progress down the digestive tract) changes from stabilizing to solubilizing and/or transport (15).

1.3 Enterohepatic Circulation

The cyclical movement of bile acids from the liver into the intestine, back to the liver and back into the intestine is called the "enterohepatic circulation". Figure 1.6 presents the enterohepatic circulation. After ingestion of a meal, the conjugated bile acids are expelled from gallbladder into the duodenum. Bile acids facilitate the

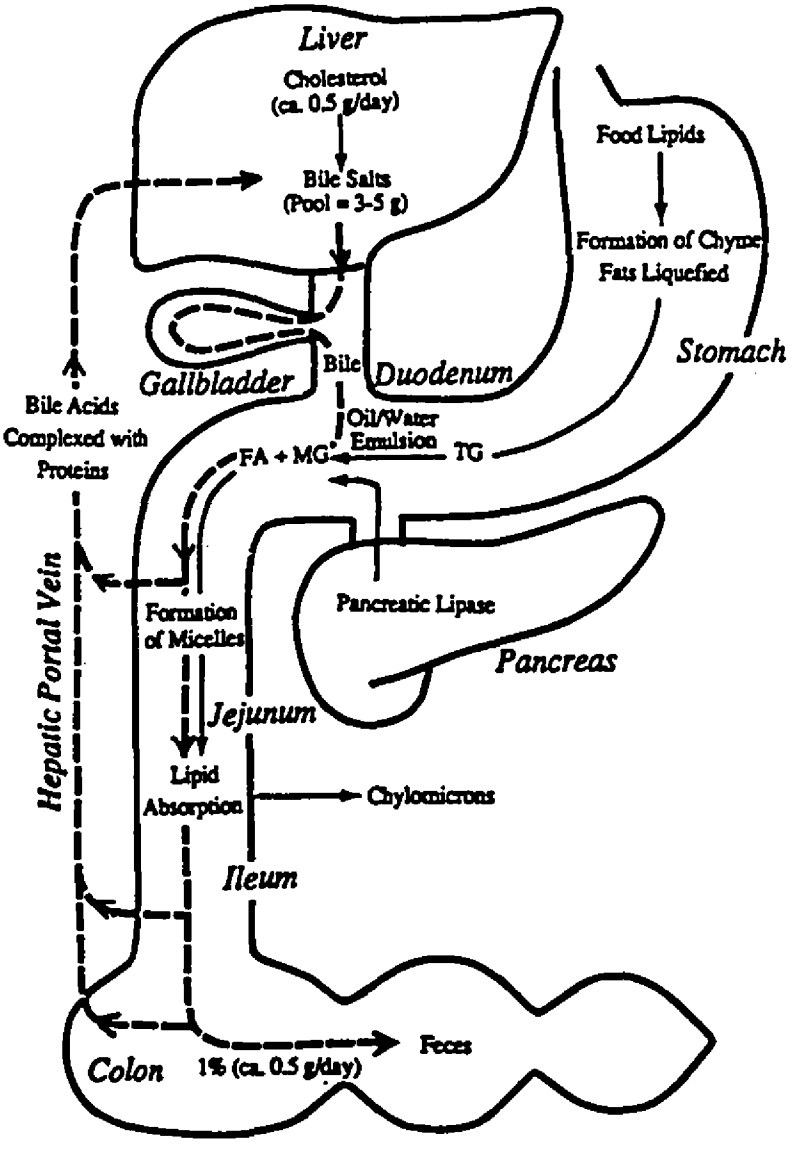


Figure 1.6 The "enterohepatic circulation" (Reference 25) indicating the 99% resorption of bile salts from intestine. (TG:triacylglycerol, FA:long-chain fatty acids, MG:monoglycerol, DG:diglycerides)

absorption of dietary lipids in the jejunum and ileum. Eventually they are re-absorbed, mainly by the active transport system in the terminal ileum. They are returned to the liver by the portal vain.

In total, the bile in humans, which contains 3-5 grams of bile and about 0.25 grams of cholesterol, circulates 6-10 times per day. Thus, 18-50 g of bile acids and 2.5 g cholesterol enter the duodenum per day. The absorbed bile salts exert negative feedback on their own synthesis, and hence on cholesterol breakdown. However, during each cycle about 1% of the bile acids are lost; therefore, in order to maintain the bile pool the liver synthesizes more bile acids from cholesterol to replace the fraction that is lost (18). This loss seems to be the major route for the elimination of cholesterol from body. Thus, if the re-absorption of the bile salt could be impeded, more cholesterol would be consumed by liver, which would lead to a reduction of the plasma cholesterol level. This idea has been the basis of many studies for the development of the lipid lowering drugs.

1.3.1 Physical Chemistry of the Enterohepatic Circulation

A total of 4 membranes and at least 7 transport proteins participate in the enterohepatic circulation (Figure 1.7) (18): 1) The transport of bile acids at the brush border membrane is an active co-transport system which depends on the concentration gradient of Na^+ ions into the enterocytes. The bile acids are transported through both ileal and hepatic cytosols by Z-proteins. 2) Bile acids are exported from the enterocytes into the portal circulation via active transport which may obtain its energy from the intra- to extra-cellular electrical potential of the enterocytes. At this point, bile acids are transported by albumin and possibly LDL. 3) Bile acids enter the sinusoidal membrane of the hepatocytes via an active co-transport system, which again depends on the gradient of sodium ions into the hepatocyte. 4) Finally, bile acids leave the hepatic cytosol at the canalicular membrane via an active transport process which is directly coupled to a Na^+-K^+-ATPase. Also, several different hepatic proteins appear to participate in the cytosolic transport of bile acids (18).

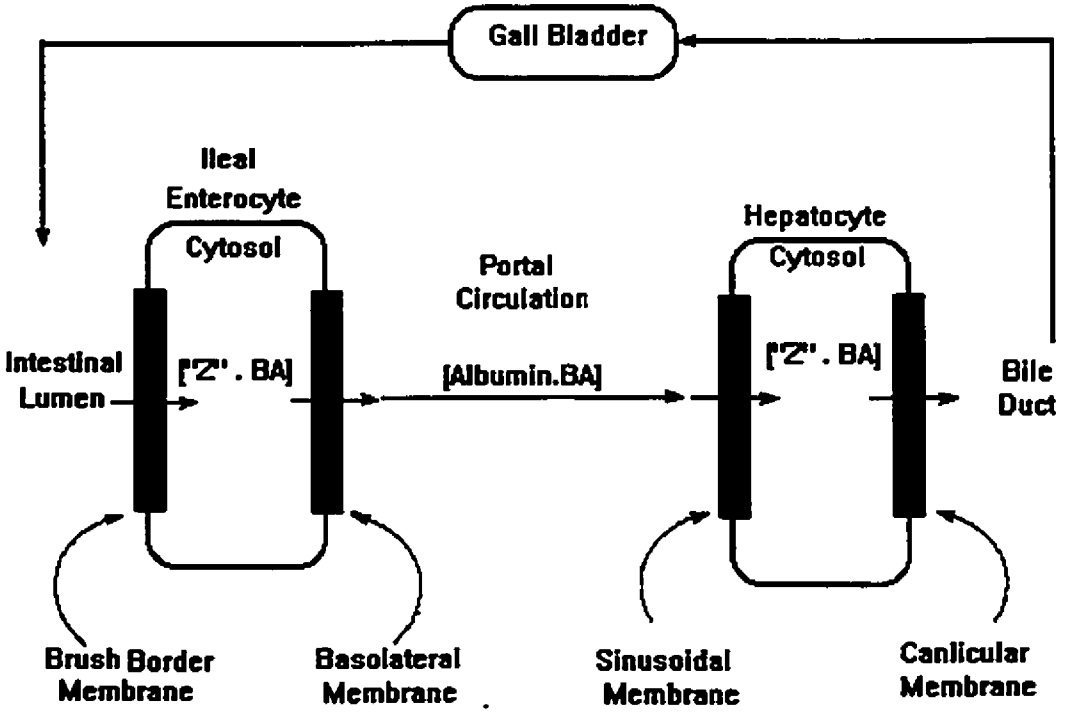


Figure 1.7 Physical chemistry of the enterohepatic circulation of bile salts. The bile salts in enterohepatic circulation travel through at least four cellular membranes (BA:bile acids; "Z":Z-proteins) (Reference 18).

1.3.2 Biochemistry of Cholesterol and Bile Acids

The study of the biochemistry of bile acids and cholesterol reveals that the excretion of bile acids is equivalent to the excretion of cholesterol with respect to its effect on the concentration of cholesterol in plasma. Figure 1.8 shows the physical inter-relationship between the various proteins, cholesterol, and bile acids which is hypothesized to occur in the plasma (18): 1) Cholesterol is removed from extra-hepatic tissues, macrophages, fibroblasts, and other cells within the artery. Cellular cholesterol first appears in the blood stream as pre-β-high density lipoprotein (pre-β-HDL) particles. The pre-β-HDL particles have a $t_{1/2} < 10$ minutes and act as mediators for the transfer of cholesterol (26). 2) Then cholesterol is esterified with fatty acids and the cholesteryl ester is bound to HDL. 3) Cholesteryl esters are then delivered to hepatocytes by the HDL particles. This delivery may occur via several paths: a) Cholesteryl esters and triacylglycerols may be equilibrated between HDL, LDL, VLDL via the action of the cholesteryl ester transfer protein (CETP). b) HDL particles may accumulate apolipoprotein E and be taken up by the hepatic apolipoprotein E receptors. c) Cholesteryl esters may be selectively taken directly from HDL by the liver with return of HDL to circulation. Also, the increased activity of LDL receptors on the hepatic surface favors reverse transport of cholesterol via HDL to LDL route (27).

1.4 Intestinal Physical Chemistry

1.4.1 Passive Transport

Passive transport is a transport process which can occur spontaneously and results in a decrease in free energy. In general, bile acids can be absorbed at all levels of the gastrointestinal tract via passive transport if the pH is sufficiently low to protonate the bile salt anions (Figure 1.9). The passive transport of bile acids across a microvillus

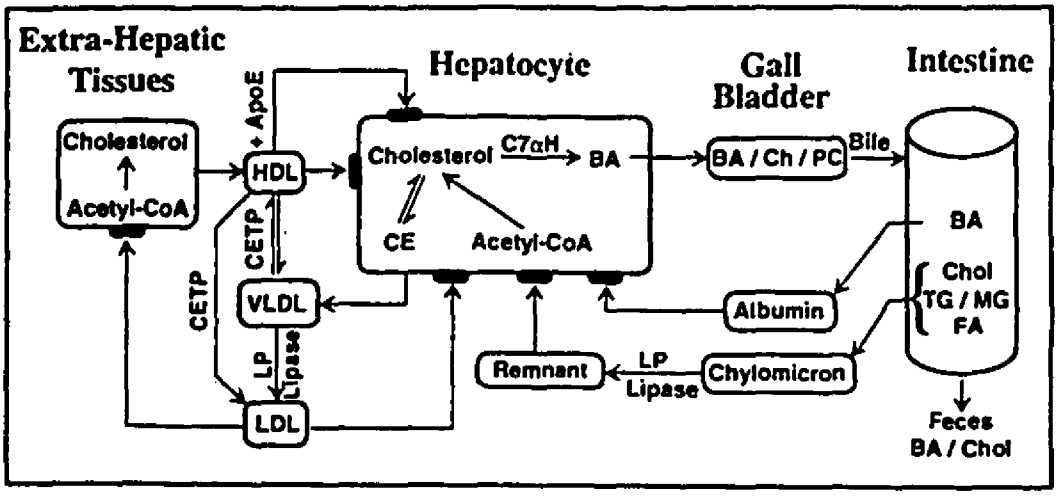


Figure 1.8 Interactions between fecal bile acid, cholesterol, LDL, VLDL, and HDL. The bile acids excreted through feces are related to the serum concentration of cholesterol through a complex series of interactions (Reference 18).

PC : phosphorylcholine;
Apo E : apolipoprotein E;
LP : lipase lipoprotein;
HDL : high density lipoprotein;
LDL : low density lipoprotein;
VLDL : very low density lipoprotein;
CETP : cholesteryl ester transfer protein;
BA : bile acid;
Chol : cholesterol;
FA : fatty acid;
MG : monoglycerides;
TG : triacylglycerols;
CE : cholesteryl esters;

membrane shows the following characteristics (18): 1) It occurs only down an existing concentration gradient; 2) It has a linear relationship to the concentration of bile acids, 3) It is not affected by competitive phenomena, and 4) It is uninfluenced by anaerobiosis or metabolic inhibition.

It should be noted that the passive transport is not the only absorption process for bile acids in the gastrointestinal tract. One of the important aspects in the transport process of bile acids is that their concentration decreases as the bile progresses down the intestinal tract. Another fact is the existence of bile acid micelles. The mixed micelles of bile acids serve to limit the concentration of un-associated bile acids in the intestinal tract. Moreover, as the bile acids progress down the intestine de-conjugation occurs, which causes the pK_a of the bile acids to increase. At the same time, the pH of the intestinal contents increases. As a result, the pH and pK_a's are always mismatched in a way that limits the protonation of bile acid anions. This implies that, even though the passive transport of bile acids can occur throughout the digestive tract, the passive transport mechanism does not occur significantly during normal digestion (18). Therefore, there must be another transport process which accounts for the highly efficient re-absorption of bile acids.

1.4.2 Active Transport

The uptake of bile acids can also occur via active transport in the ileum (28). Active transport is a transport process which requires a coupled input of energy. It is operative mainly on the ileal brush border membrane on the ileum, and it is accompanied by the following phenomena (29): 1) Bile acids move against their concentration gradient. 2) It manifests competitive inhibition of uptake of one bile acid by other structurally related bile acids. 3) It can be inhibited by anaerobiosis or metabolic inhibition.

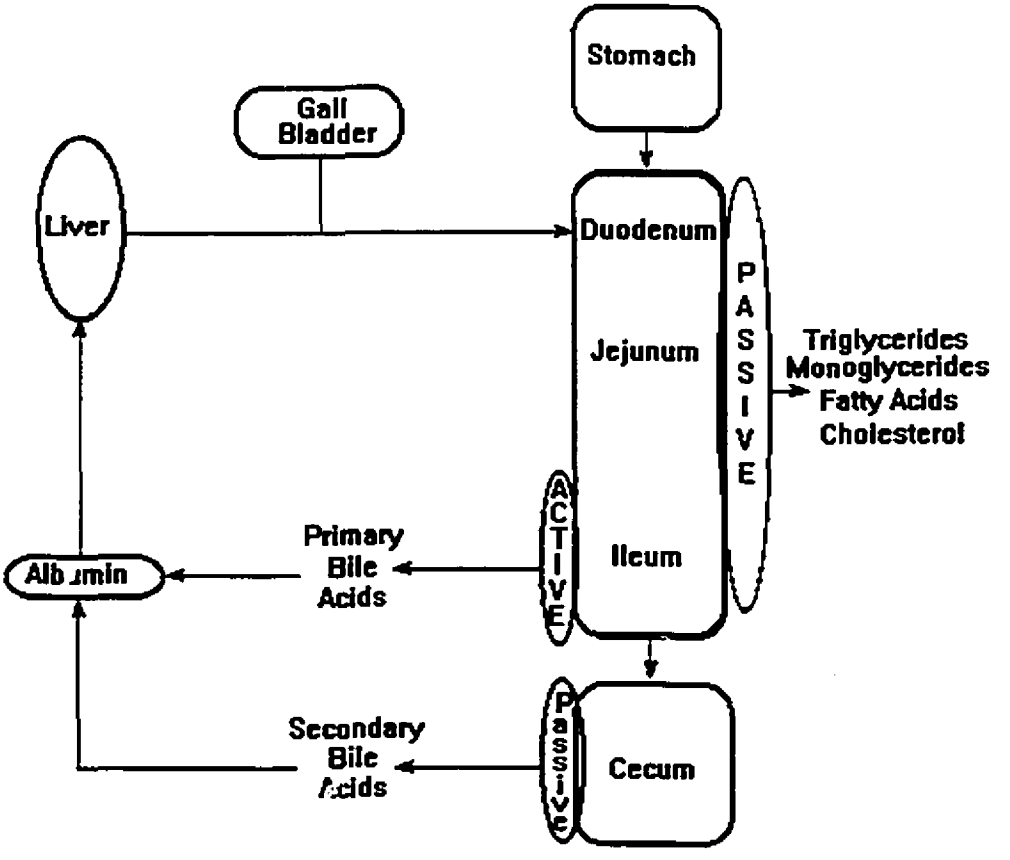


Figure 1.9 A schematic diagram of the areas of the digestive tract where the passive and active transport processes occur. Even though the passive transport process can occur in all parts of the small intestine and colon, the major transport process for re-absorption of bile acids is through the active transport system in the ileum (Reference 18).

In general, the energy that drives the active transport process for bile acids is derived from the gradient of sodium ions which exists across the brush-border membrane. It also depends on the activity of Na^+-K^+-ATPase, which is probably located on the basolateral membrane of the enterocytes (30). The transport process of bile acids across the brush border membrane by Na^+ is due to flux electrical coupling to satisfy overall electrical neutrality (18). The Gibbs free energy change is great enough to compensate for the free energy required to move bile anions into the cytosol, which is anti-parallel to its concentration gradient. The active transport process is so efficient that the full transport velocity continues even when the interluminal concentration of bile acids has dropped to a level much below 1 mM (31). The active transport occurs not only with high velocity but also with very high efficiency.

1.5 Treatment of Hypercholesterolemia

During the past 30 years, considerable efforts have been made to find new methods to reduce cholesterol levels. These include changes in diet, exercise, drugs (both systemic and non-systemic), and finally the most drastic approaches such as by-pass surgery. In some cases a combination of these methods of treatment has been used (32).

It is obvious that appropriate diet and exercise reduce the blood plasma cholesterol level. However, in some cases of hypercholesterolemia, diet alteration and exercise are not enough. It has been accepted that the dietary modification is not as potent as lipid lowering drugs. Therefore, the patients with a high risk of CHD require lipid lowering drugs. However, one of the main concerns of using drugs is the side effects. On the other hand, one of the factors which should be considered is the determination of benefit/risk ratio (10). Therefore, it is reasonable to look for a drug with the least side effects or, in other words, with high benefit/risk ratio.

Lately, advances in biochemistry have led to new drug strategies. One of the most successful groups of lipid lowering drugs is bile acid sequestrants. They are non-absorbable ion-exchange resins which cause less complications and fewer side effects. The most effective and safe cholesterol lowering drugs are the bile acid sequestrants which have been used since 1959. The two available sequestrants are cholestyramine (Questran®) and colestipol hydrochloride which are orally administered, and are not absorbable in the gastrointestinal tract. These function in the gastrointestinal tract through physical interactions. They are ion-exchange resins which act by binding the bile salt anions (the major end-product of cholesterol metabolism).

In general, there are 2 classes of lipid lowering drugs: (1) systemic (absorbable) and (2) non-systemic (non-absorbable). The main mode of action of the systemic drugs is through absorption from digestive tract, after which they decrease the rate of biosynthesis of cholesterol in liver. On the other hand, non-systemic lipid lowering agents are not absorbed from gastrointestinal tract, and they function in the human intestine through physical interactions with bile acid anions. In this thesis, the main emphasis is on the non-absorbable lipid lowering agents. The various non-systemic lipid lowering agents will be categorized into 4 major groups according to the mode of action, and these groups will be divided further into subgroups according to their chemical structures and properties. Several examples of each group will be presented, and the main interactions involving the sequestering of bile acids by these compounds will be discussed.

1.5.1 Systemic (Absorbable) Lipid Lowering Drugs

The exact mechanism of the hypolipidemic action of absorbable agents such as Clofibrate®, nicotinic acid, and Probucol® is less clear than that of their non-absorbable counterparts. Some of these systemic drugs inhibit hepatic VLDL synthesis and/or release, while others stimulate the removal of lipoproteins from the blood or interfere directly with cholesterol synthesis (33).

One of the main groups of systemic drugs is the hydroxymethyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors, such as Lovastatin® and its analogue, Compactin®. The HMG-CoA reductase inhibitors block the action of the enzyme which catalyzes the reduction of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate which is in the synthesis sequence of cholesterol from acetyl coenzyme A (34,35). Mevalonate is metabolized along several possible routes, one of which leads to cholesterol formation (36) (see Figure 1.10).

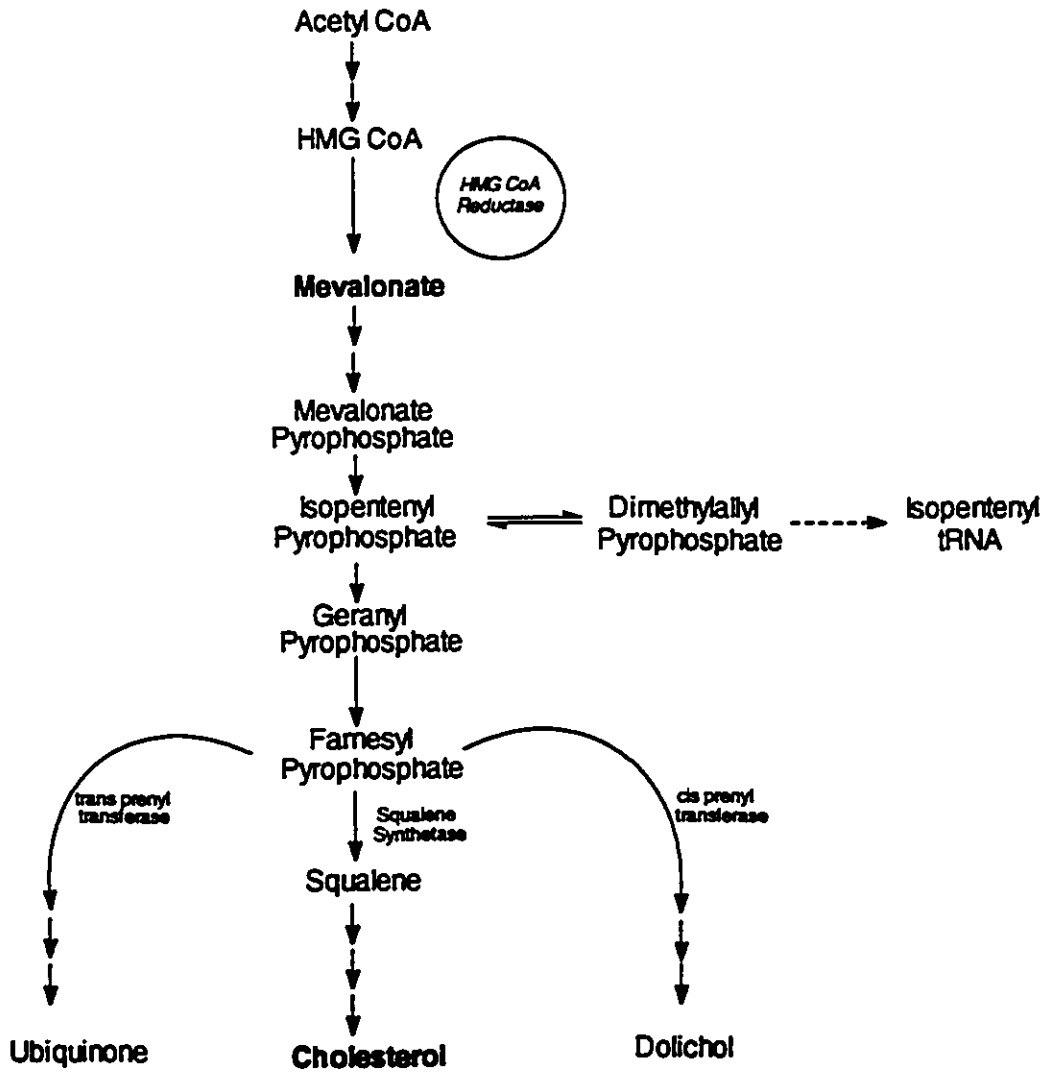
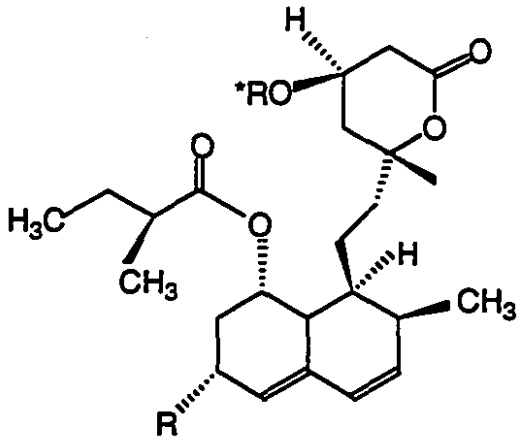


Figure 1.10 A schematic representation of pathways of mevalonate metabolism in cholesterol synthesis sequence (Reference 36).

The inhibition of cholesterol biosynthesis by the absorbable drugs forces the cells to rely more on exogenous sources of cholesterol which it does by synthesizing more LDL receptors; therefore, they take up an increased amount of cholesterol from circulation and this eventually decreases the concentration of LDL cholesterol in the blood stream (37). Figure 1.11 shows the chemical structure of Lovastatin®, also referred to as Mevinolin®, an example of absorbable lipid lowering agents.



a) R = CH_3 , *R = H

b) R = *R = H

Figure 1.11 Chemical structures of a) Mevinolin® and b) Compactin®, the absorbable drugs used as lipid lowering agents.

Inhibitors of HMG Co A reductase may be the most potent cholesterol lowering agents; however, the administration of systemic lipid lowering drugs can result in drastic side effects. For example, the administration of Lovastatin® can cause myalgia, elevation in creatine kinase levels (CK) and myositis, muscle pain (which may accompanied by fever),

rhabdomyolysis, increased incidence of hepatocellular carcinomas, and increased incidence of skeletal malformation in fetuses (38).

1.5.2 Non-systemic Lipid Lowering Drugs

The non-systemic lipid lowering drugs act within the gastrointestinal lumen. Their main mode of action is to interrupt the re-circulation of the bile acids or to reduce the re-absorption of cholesterol from the intestines. In this way the feedback inhibition of bile acid synthesis is diminished, and conversion of cholesterol to bile acids is stimulated. Thus, the decrease in the hepatic content of cholesterol causes the liver to replenish its cholesterol pool by stimulating endogenous cholesterol biosynthesis and/or by synthesizing additional LDL receptors on the surface of the cells. Since the site of action of non-systemic lipid lowering agents is restricted to the digestive tract, they have fewer side effects. Those which have been reported are considered to be minor; for example, administration of cholestyramine causes heartburn, bloating, and constipation, which are milder than the side effects of the absorbable counterparts (36). Because of limited side effects, the long term clinical study, familiarity, and understanding of the interactions involved in the sequestering of bile acids, much more attention has been paid to non-absorbable drugs recently.

Non-absorbable lipid lowering agents can be classified into 4 major groups according to their specific function: 1) Inhibitors of diffusion through the microvillus membrane; 2) Agents which enhance the excretion of cholesterol, which can be divided in to two groups: (a) inhibitors of passive transport of cholesterol, and (b) sequestrants and precipitants of cholesterol; 3) Agents for enhanced excretion of bile acids, which can be characterized into 5 groups: (a) soluble organic, (b) insoluble organic, (c) inorganic, (d) hydrophobic, and (e) dietary saponins; and 4) Inhibitors of active transport. Figure 1.12 represents the schematic classification of lipid lowering agents, focusing mainly on different types of non-systemic agents.

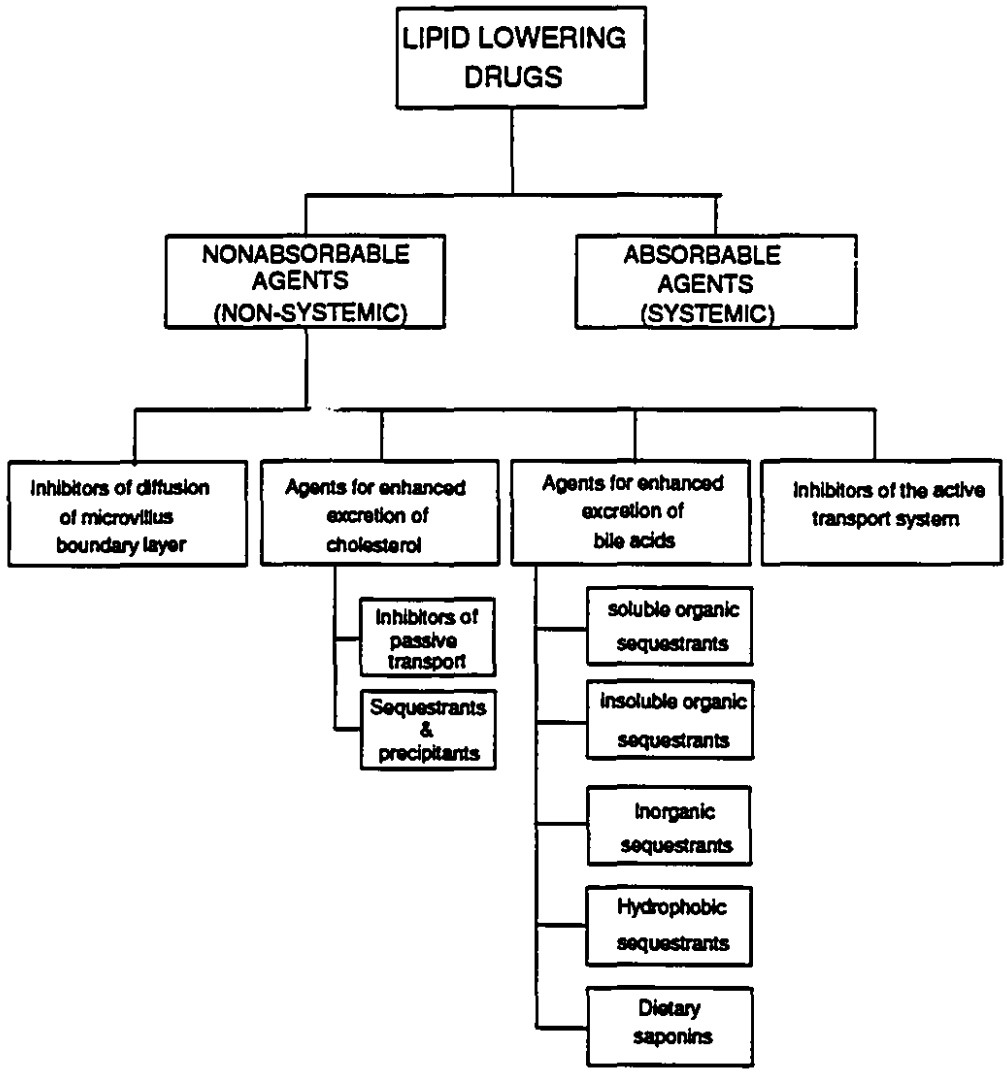


Figure 1.12 Schematic classification of non-systemic cholesterol lowering drugs, according to their specific functions.

1.5.2.1 Inhibitors of Diffusion Through Microvillus

The inhibitors of diffusion through microvillus are simply water-soluble dietary fibers which are poorly digestible polysaccharides. Their main mode of action is to increase the solution viscosity of the contents of the intestine, which leads to decreased translational diffusion of all of the species in the small intestine. The reduction of translational diffusion through the microvillus membrane has a great effect on efficiency of adsorption and re-absorption of cholesterol and bile acids, respectively. As a result, dietary fibers probably inhibit both passive and active absorption of the bile acids and sterols (39). However, there is no selectivity toward bile acids over phospholipids or cholesterol. Table 1.1 shows a few examples of the reported decrease in the serum cholesterol levels by dietary fibers.

Table 1.1 The percent decrease in serum cholesterol when subjects were treated with different dietary fibers for specific periods of time. The data on each dietary fiber were collected from different studies in the literature. The subjects of the experiments were different in each study; some of the studies were applied on rats, some on humans, and some on hamsters; so that, direct comparison cannot be made (References 39-44).

compounds	dosage/day	duration	% reduction
1) Plantago ovata mucilloid (40)	15 g/day	2 weeks	<5%
2) Guar gum (39)	"	"	5-10%
3) Pectin (41)	"	"	5-10%
4) Ceratonia siliqua gum (42)	8-30g/day	8 weeks	17%
5) Sterculia urens Roxb. gum (43)	24g/day	4 weeks	10%
6) Acacia verek gum (44)	25-30g/day	3 weeks	small amn.

1.5.2.2 Agents That Enhance The Excretion of Cholesterol

This class of lipid lowering agents has two main modes of action: (1) Inhibition of passive transport of cholesterol and (2) Precipitation of cholesterol and sequestering of cholesterol.

1.5.2.2.1 Inhibitors of the Passive Transport of Cholesterol

In the literature there are numerous examples of inhibitors of passive transport of cholesterol, of which only some representative examples are presented here (see Figure 1.13):

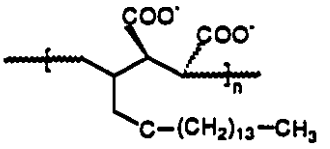
A) *Surfomer (α-OMA)*: Surfomer is a hydrolyzed form of a synthetic alternating copolymer of 1-octadecane and maleic anhydride (45). According to Turley (46), surfomer (α-OMA) decreases the hydrophobicity of the microvillus membrane. This is reflected by an increase in the free energy associated with uptake of cholesterol.

B) *Stigmastanyl phosphorylcholine*: It belongs to the family of steroidal betaines and is a steroidally substituted derivative of choline hydroxide (47). It mainly inhibits the re-absorption of cholesterol from the intestine, and it is useful for treatment of hypercholesterolemia when high dosages are administered (47). It also creates mixed micelles with cholesterol, phosphoryl choline and glycocholate.

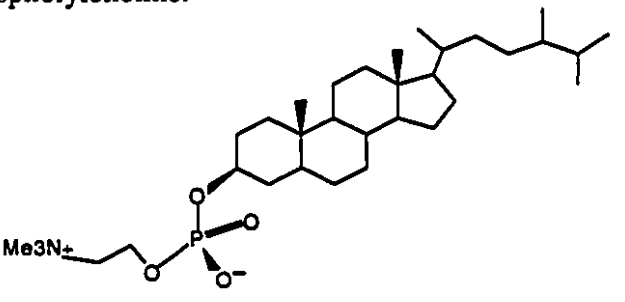
C) *Neomycin*: Neomycin acts as an *in vitro* bile acid precipitant but it does not increase the fecal excretion of bile acids. Neomycin increases the excretion of cholesterol by interfering with the absorption of cholesterol in the jejunum (48-51). It is thought that neomycin also functions by the inhibition of the bacterial fermentation of dietary fiber within the intestinal tract (18). With a daily dosage of 1-2 grams, neomycin can decrease LDL cholesterol levels by 20-25%.

D) *Sucrose polyester (SPE)*: Sucrose polyester (*Olestra*®) is a mixture of hexa-, hepta-, and octa-fatty acid esters of sucrose. It is not absorbed from the digestive tract because it is not hydrolyzed by the normal lipases that are present in the digestive tract (18).

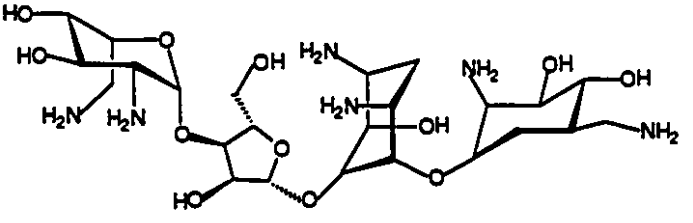
a) Surfomer:



b) Stigmastanyl phosphorylcholine:



c) Neomycin:



d) Sucrose polyester:

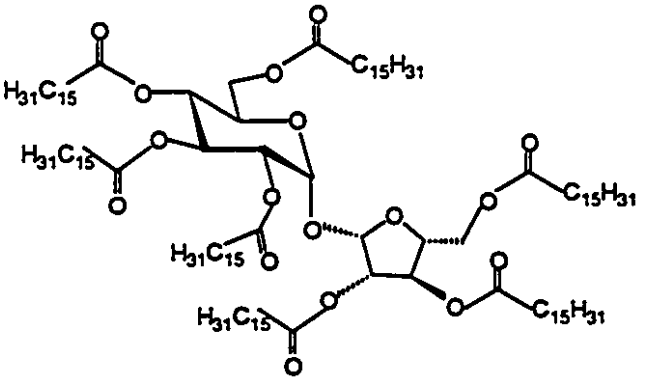


Figure 1.13 The chemical structures of a) surfomer, b) stigmastanyl phosphorylcholine, c) neomycin, and d) sucrose polyester; compounds which inhibit the passive transport of cholesterol (Reference 18).

The interesting aspect about this compound is that it reduces the absorption of exogenous dietary cholesterol by solubilizing cholesterol into a large non-absorbable oil phase (52). However, it does not significantly increase the fecal excretion of bile acids (53).

1.5.2.2.2 Precipitants And Sequestrants of Cholesterol

Some examples of lipid lowering agents that act as precipitants of cholesterol include neutral sterols, γ-oryzanol, and Pluronic L-81 (see Figure 1.14).

A) *Plant-derivative neutral sterols*: β-sitosterol, fucosterol and stigmasterol are examples of plant derivative neutral sterols which have very similar structures (Figure 1.14A). Their main mode of action is to create mixed micelles with cholesterol and to increase the fecal output of cholesterol (54,55). Specifically, they displace cholesterol from taurocholate micelles which causes the cholesterol to precipitate in the lumen of the small intestine (56). It is also noteworthy that even though the plant-derivative neutral sterols are not macromolecules, they are poorly absorbed in humans (57).

2) *γ-Oryzanol*: γ-Oryzanol is the ferulic acid ester of cyclo-octanol (Figure 1.14B). Rice bran oil is the richest available source of dietary γ-oryzanol. According to previous studies (18,58,59), administration of rice bran oil decreases the level of serum triacyl glycerols, LDL cholesterol, and VLDL cholesterol while HDL cholesterol remains unchanged. The exact mode of action of γ-oryzanol is unclear. According to Sharma (58), rice bran oil may create an environment unsuitable for micelle formation, or it may have a negative effect on the re-absorption of cholesterol. It is also suggested that γ-oryzanol may even have systemic effects, such as inhibition of cholesteryl esterases and HMG-CoA reductase (60).

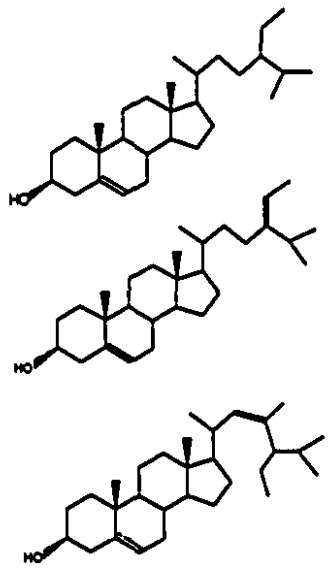
3) *Pluronic L-81*: This compound is a non-ionic hydrophobic surfactant consisting of a (A-B-A) triblock copolymer of ethylene oxide and propylene oxide with molecular weight of about 2700 (Figure 1.14C) (61). It increases the fecal excretion of neutral

A) Plant derived neutral sterols

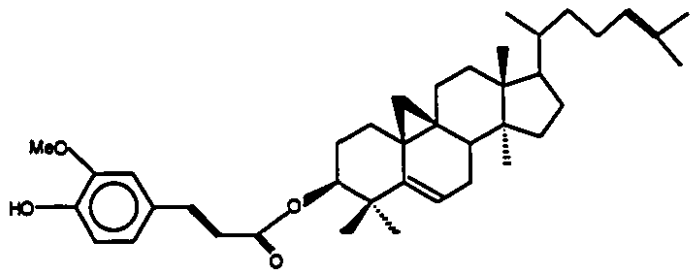
a) β-Sitosterols

b) Fucosterols

c) Stigmasterols



B) γ-Oryzanol



Ferulic Acid + Cycloartenol

C) Pluronic L-81

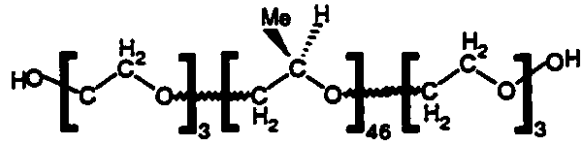


Figure 1.14 The chemical structures of (A) β-sitosterol, fucosterol, and stigmasterol (which are the members of plant-derivative neutral sterols family), (B) γ-oryzanol, and (C) Pluronic L-81; these compounds tend to lower the serum cholesterol by precipitation and/or sequestering of cholesterol (Reference 18).

sterols. The exact mode of action of Pluronic L-81 as a lipid lowering agents is not yet known. However, it is well known that the tri- or di-block copolymers consisting of hydrophobic and hydrophilic blocks create polymeric micelles. Thus, it is possible that Pluronic L81 creates mixed micelles with bile acids, cholesterol and neutral sterols.

1.5.2.3 Agents Which Enhance the Excretion of Bile Acids

This class of lipid lowering agents, which increase the fecal excretion of bile acids, includes the most effective non-systemic agents. In general, the agents for enhanced excretion of bile acids can be divided in to 5 groups according to their chemical structure: (1) soluble organic, (2) insoluble organic, (3) inorganic, (4) hydrophobic, and (5) dietary saponins.

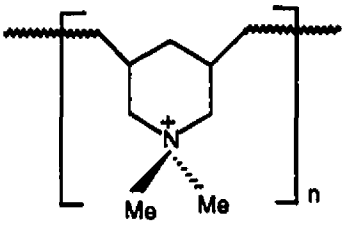
1.5.2.3.1 Soluble Organic Sequestrants

Among the non-systemic lipid lowering agents, soluble organic sequestrants show greater *in vivo* affinity for bile acids than cholestyramine (up to 10 times greater efficiency). However the efficiency comes at the cost of more serious side effects and toxicity (18). Some of the examples of soluble organic lipid lowering agents include:

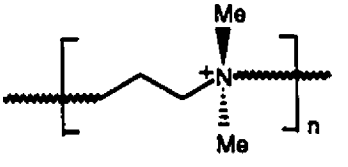
A) CAT-FLOC®: CAT-FLOC is poly[diallyldimethyl ammonium chloride] which is a water soluble linear polymer with a molecular weight of about 200 kDa (Figure 1.15A). It has been reported that *in vitro* it binds 1.8-fold the bile salt adsorbed by cholestyramine; on the other hand *in vivo*, 1.8 g/day CAT-FLOC sequester as much as bile salt as cholestyramine administered at 12 g/day (18,62).

B) 3,3-Ionene: It is poly(dimethyliminotrimethylene chloride) which is a water soluble linear polymer (Figure 1.15B). *In vitro*, 3,3-ionene binds 2.5-fold the amounts of bile salt anions that are adsorbed by cholestyramine. It was found that *in vivo* (in a dog model), 1.2 g/day 3,3-ionene sequesters as much bile salt anion as does cholestyramine at 12 g/day (18,62).

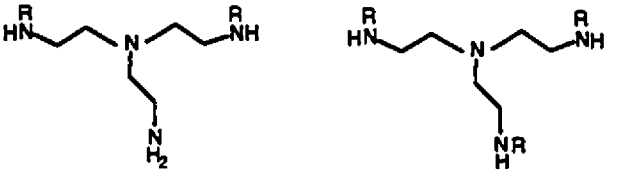
A) CAT-FLOC



B) Ionene



C) N-(cycloalkyl)alkylamine



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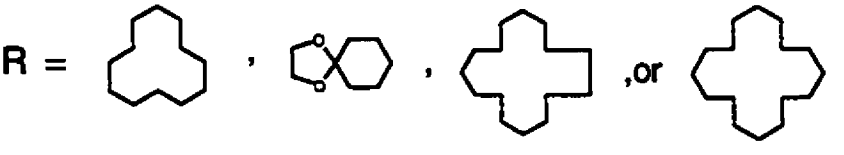


Figure 1.15 Chemical structures of a few organic soluble sequestrants: A) CAT-FLOC, B) ionene and quaternized ionene, C) N-(cycloalkyl amine) (References 18,63).

Surprisingly, both CAT-FLOC and 3,3-ionene sequester more bile salt anions *in vivo* than *in vitro*. Several explanations can be given; for example, (a) there may be an

interaction between the linear polymer and transport proteins, or/and (b) these polymers may increase the solution viscosity of the contents of the digestive tract. Understanding the exact mode of action of these sequestrants requires further experiments.

C) N-(cycloalkyl amines): Thomas and his coworker in the UpJohn laboratory (63) synthesized a series of novel lipophilic polyamines by the sodium cyanoborohydride-mediated reductive amination of various ketones and aldehydes using the polyamine tris(2-aminoethyl)amine. The most potent compounds which they synthesized were di- and tri-cyclododecyl substituted amines (Figure 1.15C). It was shown that in terms of drug potency, one of the important factors is the hydrophobicity of the appended cycloalkyl groups. For example, molecules with smaller ring size, such as cyclohexane, were found to be almost inactive while those with larger rings exhibited increased potencies. According to the authors (63), the most important interactions are hydrophobic interactions and acid-base interactions with bile acids. The study using the rat model indicated that some of these compounds are up to 29 times more potent than colestipol hydrochloride.

D) Encapsulated soluble anion-exchanger: Encapsulation of soluble organic agents is one of the most interesting ideas for sequestering of bile acids. It is thought that there are some substances in the digestive tract which interfere with precipitation or sequestering of bile acids. By encapsulation the sequestrant can be encased into coacervate vesicles which can be prepared from a variety of non-toxic polymeric materials. These form a flexible, semi-permeable barrier structure which is permeable to bile acids but excludes interfering substances. It is expected that in this way the bile acid would be precipitated or sequestered by the soluble organic agents inside the vesicles, so that the bile acids are trapped inside the vesicles. One of the examples is provided by the experimental work of Cerami (18). He prepared the vesicles using a copolymer of polystyrene sulfonate and vinylbenzyl trimethylammonium chloride. The pore size of the vesicles was estimated to be about 20 Å, sufficiently large to permit passage of bile

acids but small enough to block the passage of molecules having molecular weights >800. Another example of encapsulation is Diaion HPA25®, by Shuhei (64), in which an anion-exchange resin was encapsulated in poly(hydroxyethyl methacrylate) to prevent absorption of albumin, while it allowed the passage of bilirubin and bile acids.

For the purposes of this general review, only the most important organic soluble agents have been discussed. However, there are other examples of soluble organic lipid lowering agents, such as quaternized poly(aspartate) (18), quaternary ammonium salts of natural polysaccharides (65,66), and antiatherosclerotic silanes such as ethyl-4-[14-(trimethylsilyl)tetradecanamido]benzoate (67), which have been reported to be more potent than cholestyramine and colestipol. It should be kept in mind that although organic soluble lipid lowering agents are among the most potent sequestrants for bile acids, the toxicity of this group of compounds is their biggest disadvantage (18).

1.5.2.3.2 Insoluble Organic Sequestrants

It is thought that the insoluble anion-exchange resins would cause fewer harmful side effects than any other classes of lipid lowering agents. The mode of action of anion-exchange resins is the best understood mechanism for the sequestering of bile acids. However, the main disadvantage of anion-exchange resins as lipid lowering agents is the fact that relatively large quantities are needed.

Lately, major research efforts have been focused on the synthesis of anion-exchange resins with increased binding efficiency for bile acids. There are mainly four factors that contribute to these efforts (18): 1) The clinical success of cholestyramine and colestipol (long term clinical trials and familiarity with the effect of the resins *in vivo*). 2) The mode of action (physical chemistry) of anion-exchange resins is well understood. 3) It is relatively easier to commercialize and synthesize them in large quantities. 4) These resins do not have serious side effects, and they can probably be categorized under the safest lipid lowering drugs. Here, some of the well known insoluble organic sequestrants are discussed briefly.

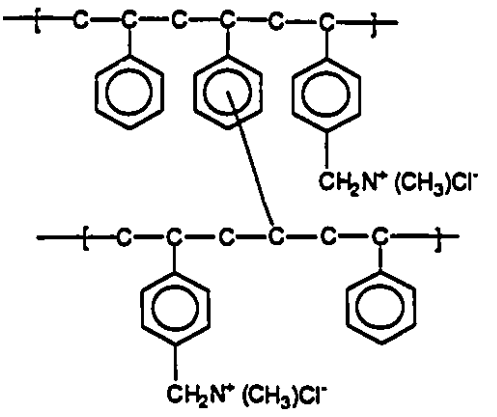
A) Cholestyramine (DOWEX 1-X2)

Cholestyramine was first introduced by Bergen in 1959 (68). It consists of a poly(chloromethyl styrene) matrix which is cross-linked with divinylbenzene and quaternized with trimethyl amine (Figure 1.16A). The main interactions of this resin with bile acids are electrostatic interactions, involving the quaternary amines, and hydrophobic interactions involving the phenyl rings. Even though cholestyramine has had good performance through the years, it has a low efficiency *in vivo*, and as a result large dosages are required for the treatment of hypercholesterolemia. For example, *in vivo*, the binding efficiency is about 2 to 3 % of the theoretical capacity, based on the member of quaternary ammonium sites within the resin (18).

Since the main interactions in the sequestering of bile acids by cholestyramine are hydrophobic and electrostatic interactions, recent studies aimed to increase one or both of these interactions which basically means to increase the Langmuir affinity. To increase the electrostatic interactions, attempts have been made to increase the density of quaternary amines (19,69,70). On the other hand, by increasing the length of hydrophobic pendent groups, the hydrophobic interactions can be enhanced (69,70).

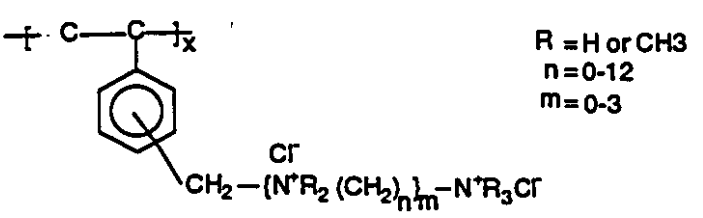
Within the last 20 years, scientists have been trying to modify cholestyramine to increase its *in vivo* binding capacity. The best examples are the polystyrene-based resins which were synthesized by Smith, Kline, and French (18), in which the structure of the functional group was varied and different lengths were used for the pendent groups. Another related resin which has better performance than cholestyramine is cholestyramine with quaternized imidazole. This resin is 1.5-fold more potent than cholestyramine *in vivo* (71). Also, Wu (70) and Whittaker (19) synthesized a number of cholestyramine-related resins, and they studied the effects of changes in the density of

A) Cholestyramine



B) Styrene-based resins

a)



b)

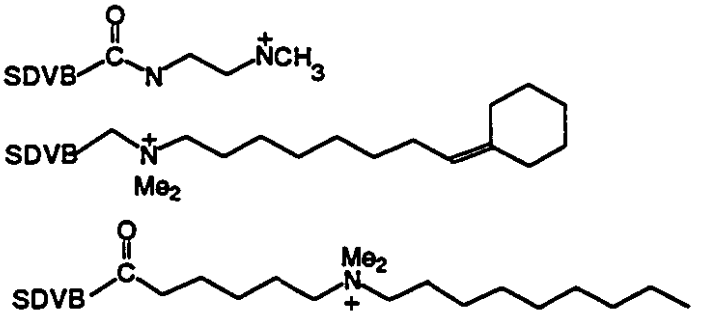


Figure 1.16 Chemical structure of A) cholestyramine and B) cholestyramine related compounds having a polymeric matrix of styrene divinylbenzene, a) polymeric resins synthesized by Wu and Whittaker and b) a few examples of polystyrene based resins synthesized by Smith, Kline, and French (References 18,19,70).

positively charged groups and the length of the hydrophobic spacer in the pendent groups in detail. Figure 1.16B shows some examples of a polystyrene matrix with different pendent groups.

B) Colestipol®

Colestipol® is another ion-exchange resin which has performed well in the market. It is a cross-linked polymer which is synthesized as a copolymer of tetra ethylene-pentamine and epichlorohydrin (72). Clas reported that the binding capacity of colestipol can be increased significantly by quaternization of the amino groups (73). Unlike cholestyramine, colestipol has minimal hydrophobic interactions with bile acid anions; only those involving the small hydrophobic moiety of methylene groups on the quaternary amine groups. However, the main interaction of cholestipol is electrostatic.

The main advantage of colestipol result from the existence of quaternary amine groups directly in the main chain of the resin. In other words, the number of positive charges in the resin does not depend on the number of pendent groups.

C) Poly(acrylamide) resins

Recently attention has been focused on finding a replacement for cholestyramine which has better water-swellability. As a result, poly(acrylamide) resins have been chosen to be the alternative polymer support for bile acid sorbents (19,25,69,70,74,75). One of the main advantages of poly(acrylamide) resins is the high swellability in aqueous solution; unlike polystyrene, the poly(acrylamide) backbone has a hydrophilic character. The high swellability allows the bile acid anions to penetrate the beads, and as a result a greater number of functional sites is available for exchange process.

As an example, Zhu, et al. (25,74) prepared novel polymeric sorbents for bile acids by attaching the peptide sequence (a) Lys-Ala_3-, (b) Lys_3-Ala_3-, and (c) Lys_5-Ala_3- onto a water-swellable polyamide resin, where Lys and Ala represent lysine and alanine residues, respectively. The binding capacity increased as the oligopeptide sequence length increased. They concluded that the longer sequence length plays an important

role; in which the pendent groups can adopt a specific conformation. Even though a combination of ionic and hydrophobic interactions can account for the binding of bile acids, the interaction with bile salt anions may be facilitated by conformational changes (74).

As a further extension, Wu, et al. (69,70,75) synthesized a series of poly(acrylamide) resins having di-, tri-, or tetramine pendent groups with carbon spacers of various sizes. They studied the sorption of bilirubin (75) and bile acids (69,70) by these resins. In these studies, the electrostatic interactions between the positively charged amine groups and bile acid anions were considered to be the main driving force for binding. It was suggested that in addition the hydrophobic interactions between the hydrophobic spacer of the polymer and hydrophobic moiety of bile salt anions reinforce the binding. Since polyacrylamide is not as hydrophobic as cholestyramine, increasing the hydrophobicity of the pendent group increases the binding efficiency of the resin. Furthermore, they found that there is a positive cooperativity in the binding, which was attributed to hydrophobic interactions and H-bonding among bile acids bound at adjacent positions within the resin beads (69,70).

D) Poly(methylimidazol)

This insoluble organic lipid lowering agent was synthesized by copolymerization of 2-methylimidazol and epichlorohydrin by the Mitsubishi Yuka Company (76); Figure 1.17 shows its chemical structure. The binding capacity of this resin for bile acids in a rabbit model was reported to be 4.3-fold that cholestyramine. The theoretical ion-exchange capacity of poly(methylimidazol) is about 40% higher than that of cholestyramine. One of the interesting aspects of this resin is that the functional quaternary amines are part of the resin matrix; it is a member of the imidazole cyclic structure in which the positive charge is shared by 2 nitrogen atoms. Furthermore, the swellability of poly(methylimidazol) is almost twice that of cholestyramine (76). Table

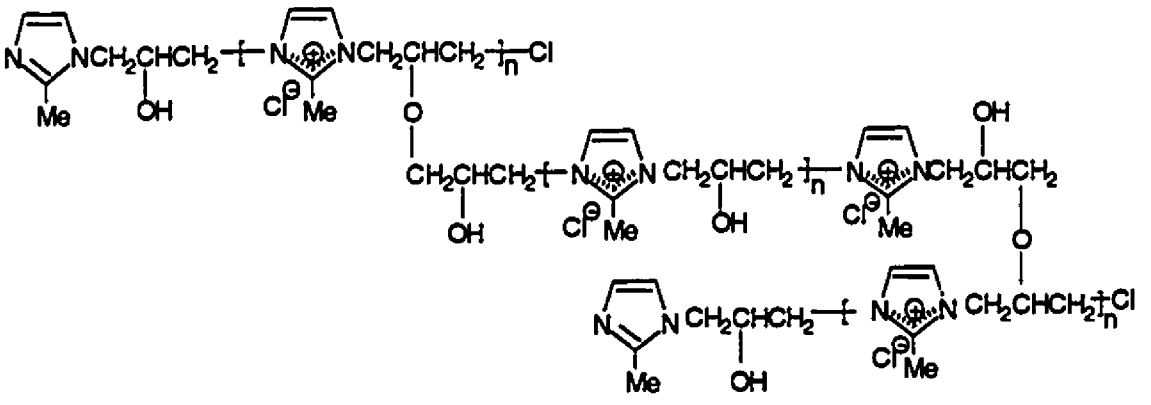


Figure 1.17 Chemical structure of poly(methylimidazol) (Reference 76).

Table 1.2 Chemical properties of poly(methylimidazol) and cholestyramine (Reference 76).

Parameter	Poly(methylimidazol)	Cholestyramine
Polymer skeleton	Epoxide	Styrene/divinylbenzene
Functional group	Imidazolium salt	Trimethyl ammonium salt
Ion exchange capacity, meq/g	4.9	3.0
Apparent density, g/cm^3	0.71	0.68
Swelling, mL/g	12 ~ 15	6 ~ 8

1.2 compares some of the chemical properties of cholestyramine and poly(methylimidazol). It has been reported that the ion exchange capacity of poly(methylimidazol) is about 1.6 times higher than that of cholestyramine. The apparent density of this polymeric resin is almost the same as that of cholestyramine, and the swelling ratio of poly(methylimidazol) in water is about twice that of cholestyramine (76).

E) Cyclodextrins

Cyclodextrins are macrocyclic oligosaccharides that can act as serum cholesterol level depressants (77). The interactions between bile salt anions and cyclodextrins in aqueous solution have been studied in detail by Tan, Zhu and Brown (78). Specifically, using NMR titration methods they studied the interaction of bile salt anions with β-and γ-cyclodextrins, which consist of seven and eight glucosidic units, respectively. They concluded that the main driving force for the binding of bile salt anions with cyclodextrins is hydrophobic interactions. Interestingly, the complex with β- and γ-cyclodextrins involves the penetration of the anions into the cyclodextrin cavities, which are comparable in the size with bile salt anions (78).

1.5.2.3.3 Inorganic Sequestrants

Antacids that contain various metallic hydroxides or salts, such as $Al(OH)_3$, $Mg(OH)_2$, $CaCO_3$, $AlPO_4$, and $Ca_3(PO_4)_2$ have been found to bind large amounts of bile salts (70,79-85). In general, the interaction of the majority of inorganic sequestrants involves simple a salt formation or precipitation.

A) Calcium Carbonate and Aluminum Hydroxide

Saunders and coworkers (82) studied the effects of administrating 6 grams calcium carbonate or 7.2 grams aluminum hydroxide per day to human subjects over a 3 week period. They concluded that a daily therapeutic dosage increases the fecal bulk and the fecal excretion of bile acids by 87% and 79%, respectively. It was reasoned that since the oral intake of Ca^{2+} increases the fecal excretion of fat, the Ca^{2+} precipitates

fatty acids and presumably bile acids (82). According to these authors, calcium and aluminum ions can alter the interaction between the colonic epithelium and its contents by precipitating ionized bile acid anions as insoluble calcium (or aluminum) compounds. Furthermore, it has been reported that calcium ions limit the solubility of bile acid anions, especially those of the unconjugated bile acids (83).

B) Pepto-Bismol®

Pepto-Bismol® consists of veegum and bismuth subsalicylate (2-hydroxybenzoic acid bismuth (3^+) salt) both of which can individually sequester bile acids (84). Veegum actually is processed montmorillonite clay which is composed entirely of aluminum-magnesium silicate ($Al_2MgO_8Si_2$); it forms a colloidal dispersion of high viscosity (84). *In vitro*, the effect of Pepto-Bismol on the bile acid concentration is comparable to cholestyramine; however, extensive *in vivo* studies have not been performed yet. In general, the mode of action of Pepto-Bismol as a bile acid sequestrant is as a precipitation of bile acids (84).

One of the disadvantages of inorganic sequestrants is that they tend to create complications in biological systems, e.g. constipation (82) and gallstone formation (85). In some cases the side effects are so dramatic that it is considered to be too toxic because normally large doses are required. Furthermore, they must be administered over a long term (80,81).

1.5.2.3.4 Hydrophobic Lipid Lowering Agents

These agents absorb bile acids only via hydrophobic interaction and hydrogen bonding. They contain no anion exchange sites. Because of the limited number of interactions, this class of non-systemic lipid lowering agents has not been very successful. Some of the examples of hydrophobic sequestrants reported in the literature are polyurethanes (18) and charcoal beads (86,87).

1.5.2.3.5 Dietary Saponins

Saponins are tri-terpene or steroid glycosides which are linked to one or more sugar chains (Figure 1.18) (88). Saponins are found in many plants. They act as a resistance factor against fungal infection. They function mainly by means of their ability to sequester neutral sterols and disrupting the cell membrane (88). One of the advantages of the use of saponins related to non-systemic agents is that they do not absorb through the gut wall easily.

To be more specific, saponins have 4 direct effects on the excretion of bile acids and cholesterol: (1) direct precipitation of cholesterol, (2) loosening of the intestinal mucosa which causes the loss of endogenous cholesterol, (3) complexation with bile acids through micellar aggregates, and (4) inhibition of all transport phenomena by high molecular weight aggregates (88). These effects are present in all saponins to some extent. In general, saponins as non-systemic lipid lowering agents can be divided into two different classes according to their function: (1) precipitants of cholesterol, and (2) sequestrants of bile acids. Figure 1.18 shows the chemical structure of soy bean saponin (glycine max saponin).

1.5.2.3.5.1 Saponins as Precipitants of Cholesterol

Some of examples of saponins which precipitate cholesterol include: 1) Digitonin (Fox glove), which forms the digitonide and creates a non-covalent complex with cholesterol (89). 2) *Medicago sativa* saponin (*alflafa*) which increases the fecal excretion of cholesterol. It has been suggested that complexation and precipitation of cholesterol in the lumen may be the cause (90). 3) *Avena sativa* (Oat), oat bran is one of the sources of Avena sativa saponin. *Avena sativa* reduces the total plasma cholesterol and increases the HDL (91).

1.5.2.3.5.2 Saponins as Bile Acid Sequestrants

The main mode of action of these saponins is the creation of mixed micelles with

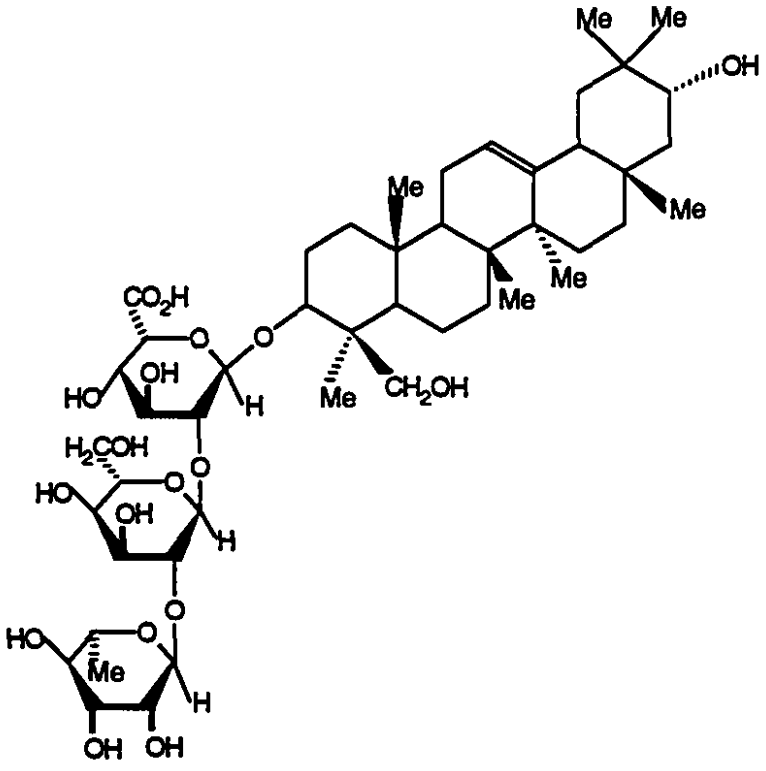


Figure 1.18 Chemical structure of one of the saponins from soya beans (glycine max saponin) (Reference 88).

bile acids. Saponins themselves aggregate by hydrophobic interactions involving their steroid rings leaving the sugar moieties exposed to water. The saponin micelles would appear to be restricted in size by steric repulsion of the bulky sugar groups. On the other hand, the combination of saponins with bile acid anions increases the separation of the sugar residues and the charged groups; as a result they reduce both electrostatic and steric repulsion (88). Thus, the saponins create large mixed micelles with bile acids, with a relative size that depends on the chemical structure of saponins. These mixed micelles usually have a loose internal structure and are highly hydrated. Furthermore, electron micrographs (88) indicate that the stacking of tri-terpene and steroid rings is in the form of helices. Figure 1.19 shows the proposed structure of mixed micelle of saponins and bile acids.

The examples of this group of saponins are (a) *quillaia saponaria*, (b) *saponaria officinalis*, and (c) glycine max (soy bean).

1) *Quillaia saponaria* saponin: With cholate it creates mixed micelles with molecular weights of up to 100 kDa at a ratio of 40:1 (saponin:cholate) (92). As a result, *quillaia* saponin reduces the concentration of free bile acids, and therefore it effects both passive and active transport.

2) *Saponaria officinalis* (saponin white): With cholate it forms mixed micelles with molecular weights of up to 1000 kDa at a ratio of 40:1 (saponin:cholate). *Saponaria officinalis* not only increases the fecal excretion of bile acids, but it also causes interruption of both the active and passive transport due to a reduction of the concentration of free bile acids.

3) Glycine max saponin (soya bean saponin): Glycine max saponin is contained in most kinds of beans; such as soy, kidney, butter and field beans. With cholate it creates mixed micelles with molecular weights of up to 2000 kDa with a ratio of 8:1 (saponin:cholate) (92).

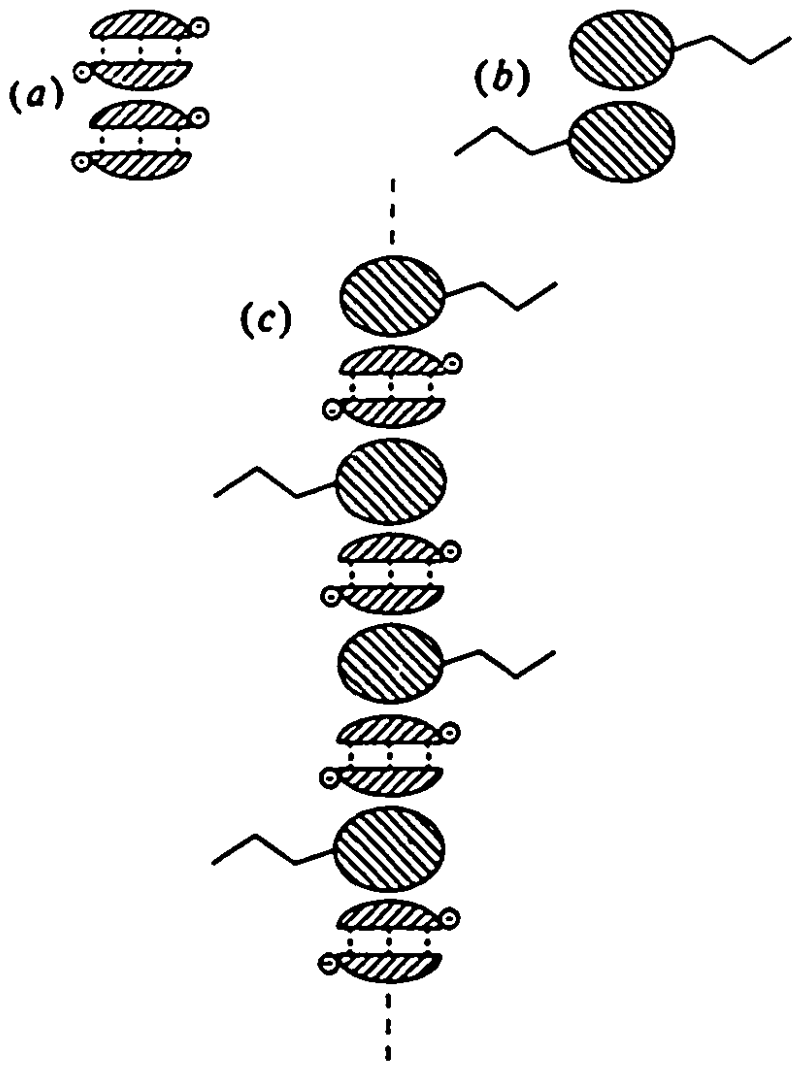


Figure 1.19 Schematic diagram of the structure proposed for micelles: (a) bile acid (cholate) (b) saponin, and (c) saponin-bile acid mixed micelle (Reference 88).

The reason that soya saponin creates much larger mixed micelles may be that its steroid rings do not have any carboxylic acid or charged groups, while quillaia and officinalis saponins do. Therefore, the mixed micelles created by soya saponins are more uniformly hydrophobic and there are fewer constraints to the way in which these molecules can pack with the steroid rings of bile acids.

1.5.2.4 Inhibitors of Active Transport

Another interesting approach to increasing the fecal excretion of bile acids is the inhibition of the active transport system. Since most of the bile acids re-absorb from digestive tract via active transport, this would probably be an effective method to reduce the re-absorption of bile acids. Some of the ways that the active transport system in the digestive tract can be retarded include: (1) inhibition of the Na^+ ion gradient and (1) competitive binding to bile acid transport protein.

1.5.2.4.1 Inhibitors of the Na^+ Ion Gradient

Active transport of bile acids from the intestine to the enterocytes requires the inward gradient of Na^+ ions across the cell membrane. Theoretically, the active transport can be retarded by depolarizing the gradient of Na^+ concentration. However, depolarization is a great assault on the biochemistry of intestinal enterocytes. Therefore, this is not clinically useful simply because of the side effects. One of the examples of inhibitors of the Na^+ gradient in the literature is auranofin, [(1-thio-β-D-glucopyranosato) (triethylphosphine) gold 2,3,4,6-tetra-acetate] (93). Actually, auranofin is an orally active and effective drug in the treatment of rheumatoid arthritis. Auranofin inhibits the absorption of nutrients, bile acids, sodium, and even fluids in the small intestine and colon which cause disturbances of bowel function (93). It has been suggested that the inhibition of sodium linked absorption may be due to the reduction in Na^+-K^+ ATPase (pumps Na^+ ions out of enterocytes) activity (94). According to the mode of action of inhibitors of active transport, it might be considered as a systemic lipid lowering agent as well.

1.5.2.4.2 Competitive Binding to Transport Protein

In this approach the lipid lowering agent competitively binds to transport proteins, which eventually reduces the capacity of the protein to transport bile acids. One of the main requirements for this kind of agents is that they must have a greater affinity constant than bile acids for binding by the protein. Among the best candidates are the bile acid dimers (Figure 1.20). First of all, the bile acid dimers have a high affinity for transport protein. Secondly, it has been reported that the bile acid dimers are not absorbed through the digestive tract, and do not enter into the blood circulation (18).

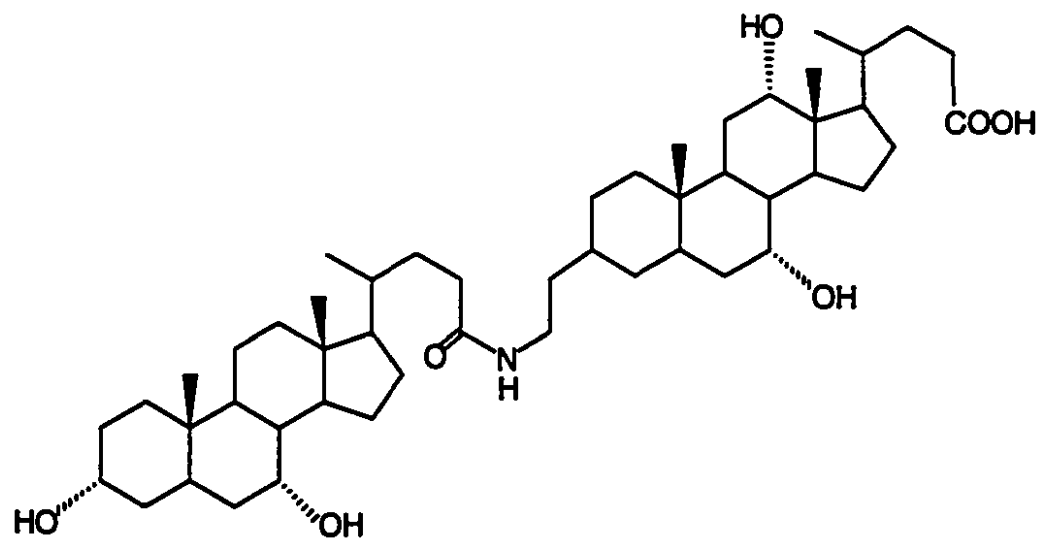


Figure 1.20 Chemical structure of a bile acid dimer (Reference 18).

1.6 Summary

Past studies and research have shown that an elevated serum cholesterol level in healthy people is a prediction of the future development of CHD. Furthermore, data from clinical intervention trials have shown reductions in mortality due to CHD when the serum cholesterol levels are reduced (3). Other studies also support the generally accepted hypothesis that lowering elevated blood cholesterol levels, specifically the blood level of low density lipoprotein (LDL) cholesterol, reduces the risk of heart attacks due to coronary heart disease (5-7).

The direct relation between serum cholesterol and bile acid has been well documented. So far, the most effective and safe method of decreasing the serum cholesterol level is to increase the fecal excretion of bile acids (by bile acid sequestrants). Thus, it is very important to understand the physical, chemical, and physiological properties of bile acids; also it is essential to recognize the mechanism of re-absorption as well as the physical chemistry of the intestine. According to the literature, the major transport process for the re-absorption of bile acids from the intestines is the active transport mechanism. The active transport system is so efficient that it practically strips bile acids off of the sequestrants. Bile acid sequestrants such as cholestyramine and cholestipol have been around for as long as 30 years. However, their main drawback, their high dosage, limits their potential usefulness. Recently, researchers in the field of bile acid sequestrants have focused their attention on increasing the capacity and selectivity of these sequestrants for bile acids.

In this chapter the various kinds of lipids lowering agents were presented; specifically a number of bile acid sequestrants have been described. It is important to review the main interactions involved in the sequestering of bile acids, not only to develop a better understanding of overall process, but also to develop a strategy for the improvement of future lipid lowering agents. According to the mode of action of a

variety of lipid lowering agents introduced in this chapter, the list of important factors which contribute to sequestering of bile acids include (in no specific order):

1) Increase of the solution viscosity of the contents of the intestines;

2) Inhibition of bacterial fermentation;

3) Decrease in the hydrophobicity of the microvillus membrane;

4) Formation of mixed micelles with cholesterol and bile acids;

5) Precipitation of cholesterol and bile acids;

6) Trapping of the cholesterol in large non-absorbable oil phase;

7) Ionic interactions;

8) High swelling characteristics in water;

9) Increase in the number of functional groups;

10) Exclusion of the interference material by encapsulation;

11) Hydrophobic interactions;

12) Inhibition of the Na^+ ion gradient;

13) Reduction of the capacity of transport protein for bile acids;

Some of the sequestrants exhibit more than one of these factors for the sequestering of bile acids and cholesterol.

The overall fundamental characteristic of all of the insoluble organic agents is that they embody equilibrium processes which can be described by a Langmuir affinity constant. Also, they embody reversible sorption and desorption processes. At the same time, the active transport system of bile acids has remarkable characteristics: (1) full transport velocities continue even at intraluminal concentrations of bile acid of about 1 μM, and (2) they proceed with very high efficiency. In other words, the active transport mechanism in the ileum is so efficient that it tends to strip bile acid off of the resin. An

important point which is most often forgotten is that loading and unloading of resin with bile acids is not symmetrical. Loading happens mainly in the duodenum, and unloading happens in the ileum under very different conditions and pH. Therefore, it is very important to conduct further studies on the desorption of bile acids from the resins.

Within the last 20 years, a major emphasis has been placed on the non-systemic lipid lowering agents such as ion-exchange resins. The insoluble organic anion-exchange resins have become a primary area of lipid lowering research. One of the well known non-systemic lipid lowering drugs is cholestyramine; however, the disadvantage of cholestyramine is its low binding capacity for bile acids *in vivo*. The binding capacity of cholestyramine *in vivo* is about 50-fold less than its theoretical capacity, and as a consequence high dosages are needed (18).

Recently a major effort has been made to overcome the problem of low capacity of anion-exchange resins *in vivo*. One of the major approaches to overcome this problem is to increase the affinity of the sequestrant for bile acids, which is the most direct attack to the problem. Basically increasing the Langmuir binding constants is the goal of this method.

It is well accepted that one of the most important interactions involved in the sequestering of bile acids by anion-exchange resins involves electrostatic interactions. Therefore, increasing the electrostatic interactions should increase the affinity of anion exchange resins for bile acids. This can be achieved by increasing the density of cationic sites (functional groups) on the resin, which can be done by 2 different methods. The first approach is to incorporate the cationic sites directly into the polymeric matrix rather than in the pendent groups. This approach is limited to the chemical structure of polymer. One example of this kind of anion-exchange resin is poly(methylimidazol), in which the cross-linking does not affect the number of functional sites. The second, and more practical approach, is to increase the number of quaternary ammonium groups by decreasing the overall cross-linking of polymeric matrix, so that more free functional

units are available for interaction with bile acids (95). Furthermore, a lower degree of cross-linking accounts for the higher diffusion rates of bile acids inside the resins.

1.7 The Present Work

The focus of this work is modify the chemical structures so as to increase the binding capacity of poly(acrylamide) resins for bile acids, and to study the selectivity of these resins. The basic idea is to improve the electrostatic interactions by increasing the density of positive charge on the resin. One of the major and practical methods to increase the density of positive charge is to increase the density of pendent groups (functional groups) on the resin. It is suggested that a higher functionality can be achieved by decreasing the cross-linking of the polymeric matrix.

In this work, attempts were made to eliminate or minimize the additional cross-linking of poly(methyl acrylate) that occurs during the functionalization process. This should result in a higher number of available functional groups on the resin; thus, increase the ionic interaction of the resins. Furthermore, a lower degree of cross-linking should result in a high degree of swelling of the resins in aqueous solution, and a more highly swollen resin facilitates the diffusion of bile acids into the resin matrix. The next chapter presents the experimental method for functionalization of poly(methacrylate) resins using mono-protected diamine functional units.

Chapter 2 describe the synthesis, characterization, and bile salt sorption characteristics of ammonium-containing poly(acrylamide) resins. A new strategy for the functionalization of poly(methyl acrylate) is proposed which is similar to the solid phase peptide synthesis. Cross-linked poly(methyl acrylate) is functionalized according to both previous and new methods, and a comparison is made between the sorption characteristics of the resulting beads. Based on the idea of local degree of cross-linking, a micro-structure model for three dimensionally cross-linked beads is introduced.

Furthermore, the swelling characteristics of the synthesized beads are presented, and the effect of temperature on the degree of swelling is evaluated.

By evaluating the sorption isotherms, the importance of cross-linking on the binding efficiency of bile salt anions is discussed in detail. Also, in Chapter 2, a comparison is made between the resins having the same length of hydrocarbon pendant but a different number of ammonium groups in their pendent group. The result of this comparison indicates the importance of hydrophobic interactions along with ionic interactions involved in the sorption of bile acids by the poly(acrylamide) resins.

The binding of bile acids by poly(acrylamide) resins has been reported to be a highly cooperative process (69,70). This cooperativity is most often attributed to the contribution of hydrophobic interactions and hydrogen bonding between bile acids found at sites adjacent to each other (69,70). The cooperativity parameter is calculated, and comparison is made between the degree of cooperativity of synthesized resins.

Chapter 3 provides a study of the effect of replacing the chloride with organic counter-ions on the selectivity of poly(acrylamide) resins for bile salts. The organic counter-ions that were studied include benzoate, salicylate, acetate, and propionate. Furthermore, a background review on the selectivity of the ion-exchangers for large organic ions is presented. The major factors that determine the course of the exchange process of organic ions is discussed. The isotherms for the sorption of cholate (CA^-) by poly(acrylamide) resins having various organic counter-ions are determined and compared.

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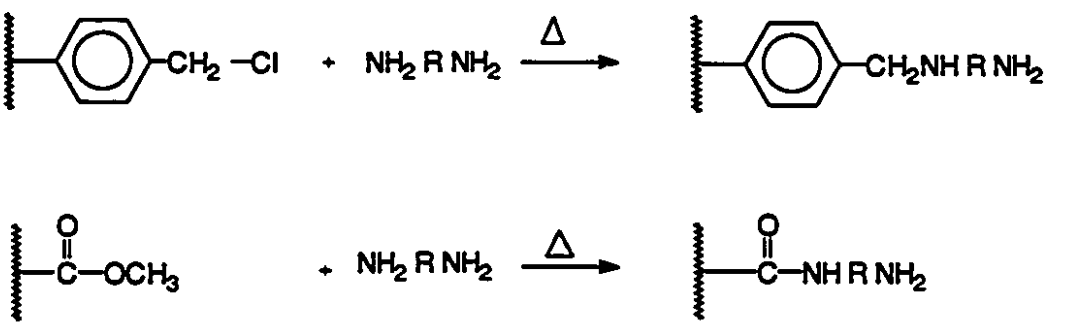
CHAPTER TWO

FUNCTIONALIZATION OF CROSS-LINKED POLY(METHYL ACRYLATE) RESIN

2.1 Introduction

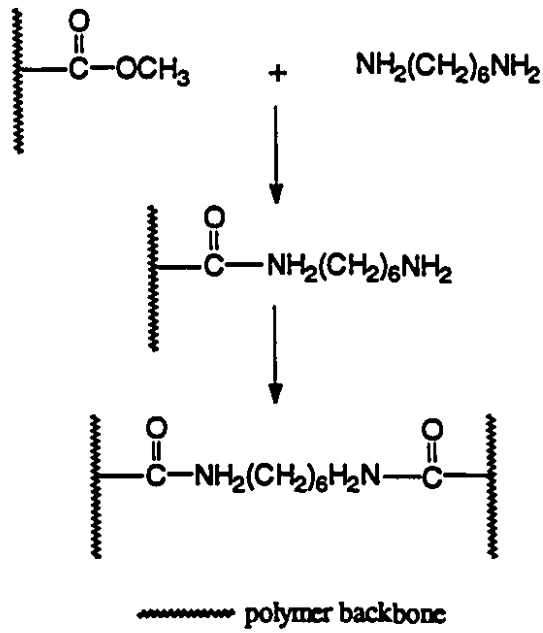
As described in previous Chapter, among the various bile salt binding agents, those that are most promising almost always belong to the category of ammonium-containing anion exchange resins, e.g., cholestyramine, poly(2-methylimidazol), and poly(acrylamides). For these resins the electrostatic interactions of the positively charged amine with the bile acid anion is the most important interaction involved in the sequestering of bile acids (1). As a result, the binding capacity of the resin depends largely on the number of available functional groups.

In general, the procedures used in this laboratory for functionalization of poly(methyl acrylate) (1) and polystyrene resins (2) by reaction with multifunctional amines were successful. The general mechanism is a simple nucleophilic substitution, which proceeds according to the following chemical equations:



However, the process has a disadvantage. In this process, the amine functional units were simply introduced to the polymer backbone at high a temperature, which led to the formation of additional cross-links (3). Since the additional cross-linking decreases the percentage of functional groups available, it decreases the capacity of the resins to bind bile acids.

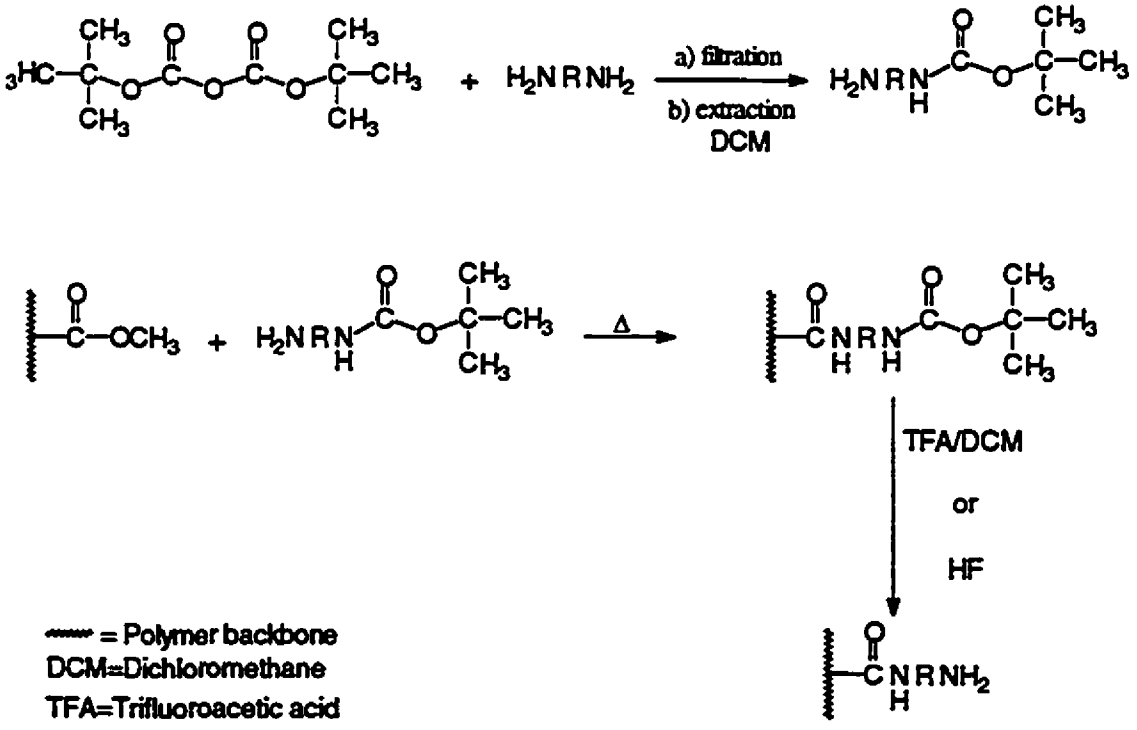
The functionalization of cross-linked poly(methyl acrylate) beads by the reaction with multifunctional amines was studied extensively by Yu, et al. (3-6). Yu studied the kinetics and mechanism of the functionalization reaction by high resolution ^{13}C CP/MAS solid state NMR and demonstrated that it results in the formation of additional cross-linking (3). The additional cross-linking is summarized by the following equations (3):



He pointed out that the formation of the additional cross-links hampers the functionalization reaction rate by affecting the diffusion of amines into the polymeric beads. He suggested that in order to avoid additional cross-linking the functionalization of PMA should be done in the presence of a large excess of the multifunctional amine (3).

It has also been pointed out (4) that the mobility of the N-alkyl side chain groups plays an important role in the binding process. It is thought that an increase in flexibility of polymeric chains should facilitate the accessibility of sorbate (bile acid anions) to the functional groups. However, a high degree of cross-linking decreases the flexibility of the beads, which in turn decreases the extent of binding of bile acid anions.

The interest of the present work is to introduce an alternative functionalization method that eliminates, or minimizes, the additional cross-linking. One of the ways by which this can be done is by using diamines which have a protecting group, e.g., Boc group, on one of the amines of diamine molecules. This is a similar approach to that used in solid phase peptide synthesis. The mono-protected diamine can be attached to the polymer backbone, and the protecting group can be removed later under acidic conditions. By this new method, additional cross-linking will be minimized so that the density of functional groups on the resins is increased, and as a result the binding capacity of the resins will be increased. The following equations summarize the reactions used in the new method of functionalization of poly(methyl acrylate), PMA, resin:



This chapter presents the synthesis and characterization of cross-linked poly(methyl acrylate) beads and describes the new method of functionalization. Cross-linked poly(methyl acrylate) beads are functionalized by the reaction with 1,6-hexanediamine using both the new and previous methods. Furthermore, some of the PMA beads are functionalized by reaction with 1,12-dodecanediamine and bis-(hexamethylene) triamine using methods previously developed by Wu (1). The functionalized beads are quaternized, and a comparison is made between these two types of functionalization procedures on the basis of the ion-exchange capacity of the resins.

Subsequently, the swelling behavior of synthesized resins is presented. The effect of temperature on the degree of swelling is evaluated. Since the extent of bead shrinkage has a direct effect on the extent of binding of bile acids, the relative degree of shrinkage of poly(acrylamide) beads is evaluated by the introduction of bile salt solution to swollen beads.

Finally, the sorption characteristics of these functionalized resins for bile salts are presented. A comparison is made between the beads prepared here and those which were prepared previously by Wu, and the relationship between their water swellability and sorption capacity is investigated. The binding of bile acids by the poly(acrylamide) resins is determined to be a cooperative process, the degree of cooperativity of synthesized resins is calculated.

2.2 Experimental

2.2.1 Synthesis of Poly(Methyl Acrylate) Beads

Cross-linked poly(methyl acrylate) beads were synthesized via suspension polymerization according to methods developed in this laboratory (1). The cross-linking agents used in this reaction were divinyl benzene and triallyl-1,3,5-triazine-2,4,6-(1H,3H,5H) trione. Benzyl peroxide, butyl ether, and poly(vinyl alcohol) (with molecular weight of 124,000-186,000) were used as the initiator, pore-forming agent,

and surfactant, respectively. The chemicals used for this synthesis were purchased from Aldrich Chemical Co., and they were used as received.

The solution of poly(vinyl alcohol) (15 grams in 2500 mL distilled water) and the solution of NaCl (80 grams in 200 mL distilled water) were made separately. A 1000 mL aliquot of the poly(vinyl alcohol) solution was added to the NaCl solution, and the solution was filtered through a cloth. From the filtrate, 1000 mL was taken and placed into a 2 liter 3 neck flask, previously set up in a water bath at 50 °C. Methyl acrylate (192 grams), divinyl benzene (5.1 grams), triallyl-1,3,5-triazine-2,4,6-(1H,3H,5H)-trione (4.4 grams), benzyl peroxide (3 grams), and butyl ether (50 grams) were added to the flask containing the solution of poly(vinyl alcohol) and NaCl. The mixture was stirred with mechanical stirrer at a high speed (approximately 200 rpm). The temperature was slowly increased from 50 °C to 68 °C over a 3 hour period, and the reaction was allowed to proceed for 16 hours. The temperature was then increased further, to 85 °C, and maintained for another 24 hours. In the final step the temperature was raised to 95 °C and maintained for 60 hours.

The product (cross-linked PMA beads) was filtered and washed with hot distilled water several times. The beads were then added to a large amount of 95 % ethanol and allowed to stir over night. They were extracted with ethanol in a Soxhlet extractor for 5 days. Finally, the beads were dried in a vacuum oven (50 °C) and stored in a desiccator.

The solid state ^{13}C NMR spectrum of synthesized PMA is presented in (Figure 2.1A). The peaks at 175 ppm, 52 ppm, and 40 ppm correspond to (C=O), (OCH_3), and (CH_2 and CH), respectively. Figure 2.1B presents the FT-IR spectrum of PMA beads, in which the band at 1770 cm^{-1} corresponds to (C=O), and the small bands at 3000 cm^{-1} represent the (C-H) stretching.

2.2.2 Synthesis of N-*tert*-Butoxycarbonyl-1,6-Hexanediamine

The N-*tert*-butoxycarbonyl-1,6-hexanediamine was synthesized by the reaction of

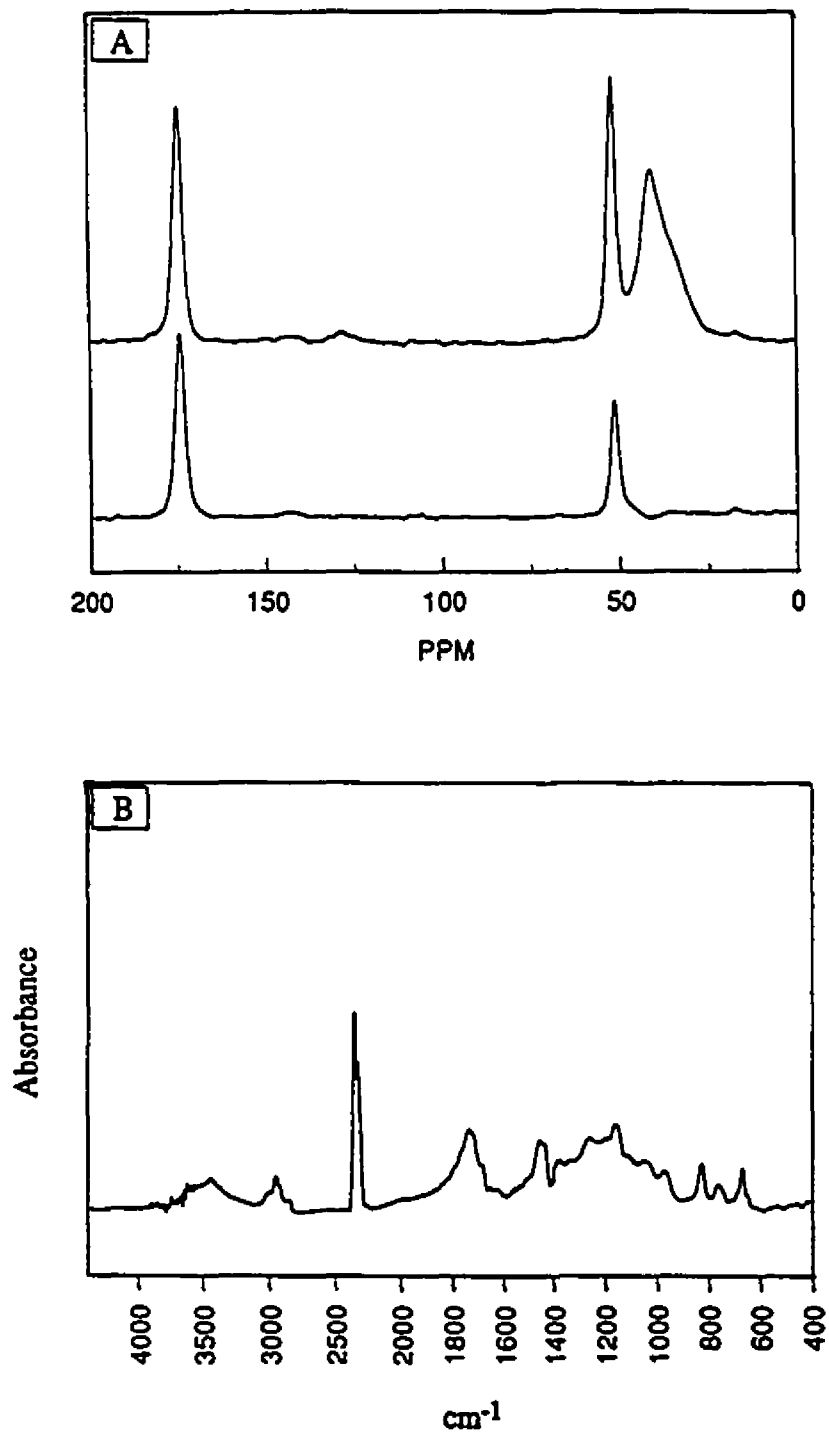


Figure 2.1 (A) The solid state ^{13}C CP/MAS NMR spectrum of PMA beads. The upper portion is the complete spectrum, and the lower is the spectrum without (CH and CH_2) peaks. (B) The FT-IR spectra of KBr pellet containing ground PMA beads.

1,6-hexanediamine with di-*tert*-butyl bicarbonate in dioxane (as the solvent) (7,8,9). To obtain a minimum amount of bis-substituted product, a molar ratio of 5:1 excess of 1,6-hexanediamine to di-*tert*-butyl bicarbonate was used. The bis-substituted product was easily removed by taking advantage of its insolubility in water. After removing the dioxane, water was added to the reaction flask, from which the bis-product could be removed by filtration; the mono-protected diamine was then extracted from the aqueous phase with dichloromethane (DCM).

The detailed reaction procedure is as follows: A solution of di-tert-butyl bicarbonate (21.23 g, 0.097 mole) in dioxane (300 mL) was prepared and added over a 6 hour period to a solution of 1,6-hexanediamine (59.78 g, 0.52 mole) in dioxane (300 mL). The mixture was allowed to stir for 24 hours, and the dioxane was removed using a rotary evaporator. Distilled water (600 mL) was then added to the residue and mixed well. The insoluble bis-substituted product (which appeared as white solid, 4.3 g) was separated by the filtration. The filtrate was extracted with DCM (3 × 250 mL). At this point, the DCM was back-washed with distilled water to remove any excess diamine. Finally, the DCM was evaporated using a rotary evaporator, and the mono-protected diamine appeared in the form of an oil (27.33 g) which solidified over a 20 day period.

To verify that the desired product had been obtained, analyses were performed by FT-IR and NMR spectroscopy. The FT-IR spectrum of the product (N-tert-butoxycarbonyl-1,6-hexanediamine) is presented in (Figure 2.2). Since the product was in the form of oil, it was directly applied onto a KBr pellet as a thin film. In the FT-IR spectrum, the band at 3200-3400 cm^{-1} corresponds to NH and NH_2 stretching, the band at about 1670 cm^{-1} corresponds to C=O stretching, and the band at the frequency range of 1520-1550 cm^{-1} indicates the amide II band (combination of NH deformation and C-N stretching). Figure 2.3 presents the NMR spectrum indicating the structure of N-tert-butoxycarbonyl-1,6-hexanediamine: 1.4 ppm (singlet, 9H, **BOC**); 1.3-1.6 ppm

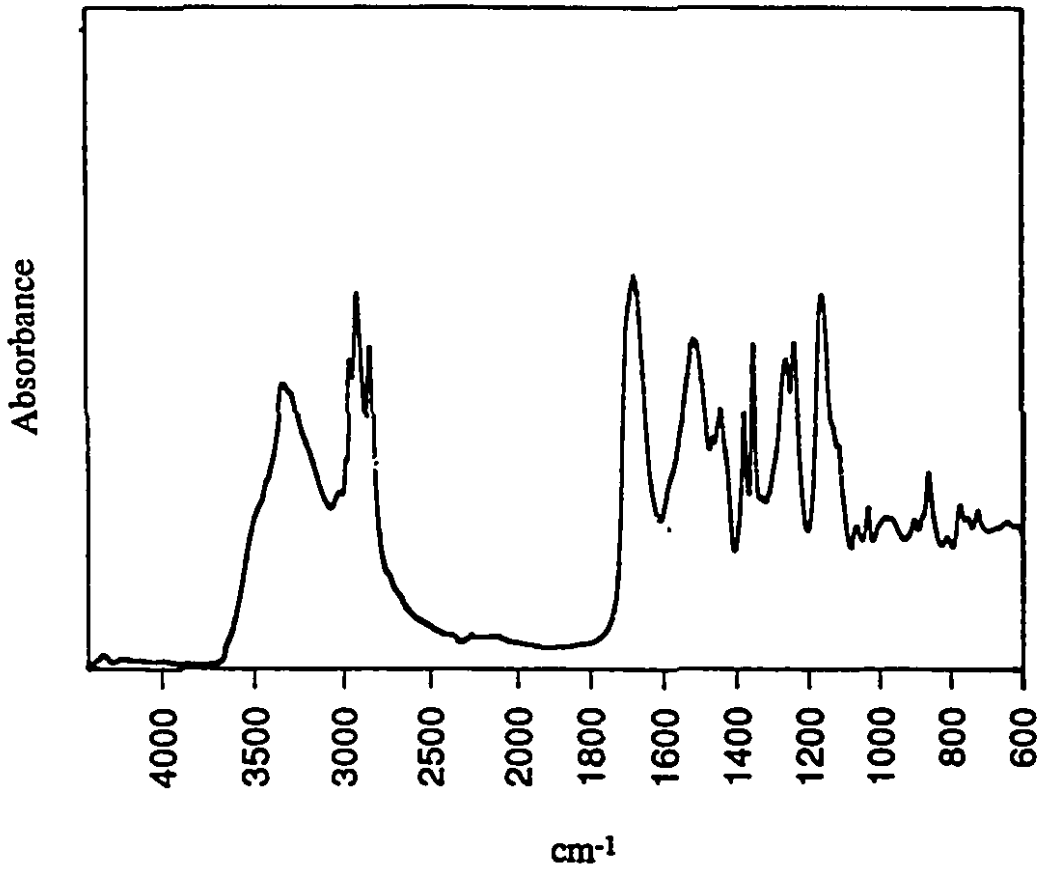


Figure 2.2 FT-IR spectra of N-*tert*-butoxycarbonyl-1,6-hexanediamine indicating the NH and NH_2 stretching, C=O stretching, and combination of NH deformation with C-N stretching at about 3200-3400, 1670, and 1520-1550 cm^{-1}, respectively.

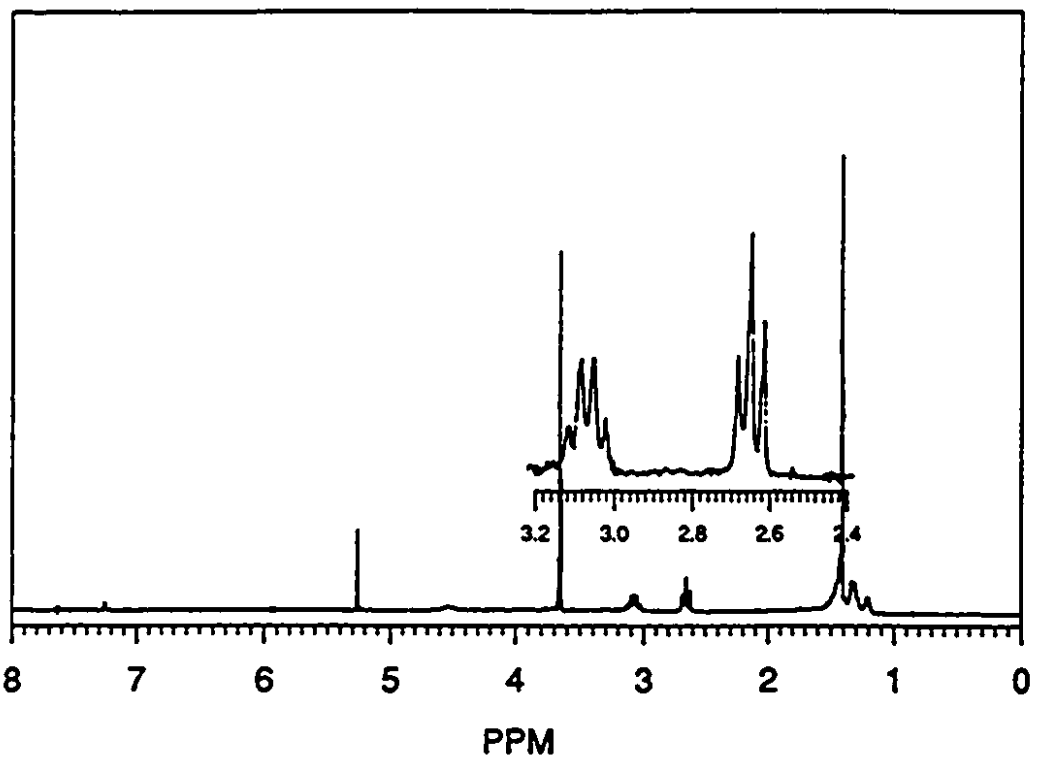


Figure 2.3 The proton NMR spectrum of N-*tert*-butoxycarbonyl-1,6-hexanediamine.

(multiplet, 8H, $CH_2CH_2CH_2CH_2$); 1.2 ppm (singlet, 2H); 2.65 ppm (triplet, 2H, CH_2NHBOC); 3.0-3.15 ppm (multiplet, 2H, CH_2NH_2); and 5.3 ppm (singlet, 1H, NH).

2.2.3 Functionalization of Cross-linked Poly(methyl acrylate) Beads Using the New Method

As a preliminary quick check, a linear poly(methyl acrylate) (with molecular weight of 30700) was reacted with N-tert-butoxycarbonyl-1,6-hexanediamine to determine whether the functionalization is still accompanied by cross-linking. The reaction was set up under a nitrogen atmosphere, using a molar ratio of 2:1 excess mono-protected diamine. A reaction temperature of 110 °C was used. To follow the reaction, ^{13}C NMR spectra were taken using the peak at 174 ppm which indicates the presence of (N-C=O). According to Yu (3), the functionalization of linear PMA with multifunctional amines, such as (tris-(hydroxymethyl)amino)methane, resulted in gel formation, as indicated by the creation of a viscous solution. However, the reaction of linear PMA with mono-protected diamine gave no evidence of any gel formation. As a result, it was concluded that the reaction with mono-protected diamine is not accompanied by formation of additional cross-links.

2.2.3.1 Reaction of N-*tert*-Butoxycarbonyl-1,6-Hexanediamine With PMA Beads

To develop an appropriate procedure for the functionalization of cross-linked poly(methyl acrylate) beads by the reaction with the mono-protected diamine, four reactions were set up under a nitrogen atmosphere. In these reactions, conditions such as the mole ratio of mono-protected diamine to PMA, solvent, and time of reaction were varied. For the first reaction (I) DMF and toluene (50:50) were used as the solvent. The second reaction (II) was done using only DMF as the solvent, having an amine concentration of 1.1 M (on the basis of mono-protected diamine). The third reaction (III) was done using DMF as the solvent, but in this case, a high concentration based on N-

tert-butoxycarbonyl-1,6-hexanediamine was used. In the fourth reaction (IV), the mono-protected diamine was introduced to PMA beads without any solvent. All of these reactions were heated under reflux for a certain period of time. Table 2.1 presents the conditions for the above reactions.

Table 2.1 Summary of reaction conditions applied to functionalize the PMA beads.

Rxn #	polymer backbone	functional units	solvent	conc. (M)	ratio of PMA to functional units	temp.°C	time per day	[a]extent of reaction
I	cross linked PMA	mono-prot. 1,6-diamine	DMF/Toluene 50:50	0.77	1:1.9	110	6	slightly <7%
II	"	"	DMF	1.1	1:2.4	145-150	6	60-68%
III	"	"	"	6.3	1:3.9	140-150	6	90-97%
IV	"	"	NO solvent	-	1:4.7	120-130	8	100%

(a) Estimated from the relative areas of FT-IR bands at 1660 cm^{-1} to 1770 cm^{-1}.

FT-IR spectroscopy revealed that reactions I and II were unsuccessful; however, reactions III and IV were determined to be successfully completed. It was concluded that the functionalization of PMA beads requires a concentrated solution of mono-protected diamine in DMF as the solvent, or no solvent at all.

The procedure used for reaction IV was as follows: Well dried poly(methyl acrylate) beads (1.9 g, 0.034 mole) prepared as described above were added to a reaction flask containing N-tert-butoxycarbonyl-1,6-hexanediamine (34.74 g. 0.161 mole). The temperature was raised slowly to about 125-130 °C during a 5 hour period. The reaction

mixture was allowed to reflux for 8 days under a nitrogen atmosphere. Following the reaction, the functionalized PMA beads were washed with warm DMF followed by several washes with 95% ethanol. They were then extracted with ethanol in a Soxhlet extractor for 24 hours. Finally the beads were dried in a vacuum oven (60 °C) for 24 hours.

Figure 2.4 presents the FT-IR spectra of cross-linked PMA beads, before and after functionalization by the reaction with N-tert-butoxycarbonyl-1,6-hexanediamine. The most obvious changes in the spectrum are the appearance of the band at 1670 cm^{1}, showing the stretching of C=O in the presence of C-N, and the disappearance of the band at 1770 cm^{-1} which corresponds to the reaction of C=O. Furthermore, the appearance of a new band at 1560 cm^{-1}, which is the amide II band (combination of NH deformation and C-N stretching), indicates that the functionalization process was successful.

To develop a better understanding of the reaction rate, some beads were taken out every 24 hours and analyzed by the FT-IR spectroscopy. Figure 2.5 presents the FT-IR spectra of reacted beads at different times of reaction. In general, it is observed that for a period of up to 40 hours the extent of reaction increases regularly, and after 40 hours the reaction rate decreases.

2.2.3.2 Deprotection of Functionalized PMA Beads

A number of methods have been described in the literature for the removal of the t-butoxycarbonyl groups (10-14). One of the most widely used reagents for removing the amine protecting groups, such as t-butoxycarbonyl, is trifluoroacetic acid, TFA. According to Karlsson, et al. (10), trifluoroacetic acid-methylene chloride 1:1 (v/v) is the fastest and most reliable de-protecting medium. He reported that 50% TFA in DCM readily removes the Boc groups in less than 60 minutes. However, the deprotection of functionalized PMA beads, by the application of 50% TFA in DCM, did not occur in satisfactory manner. Even after a period of 20 hours the value determined for the amine

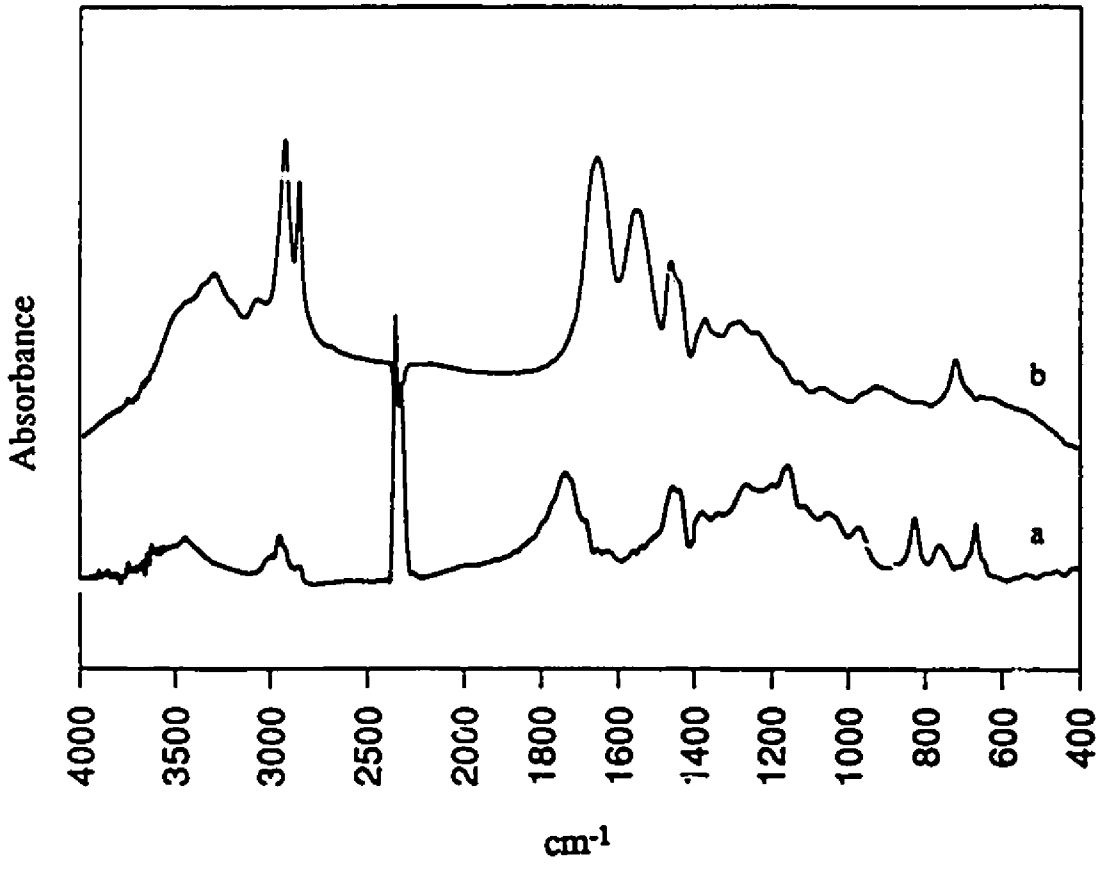


Figure 2.4 The FT-IR spectra of: (a) cross-linked PMA and (b) functionalized PMA with N-*tert*-butoxycarbonyl-1,6-hexanediamine, comparing the unfunctionalized PMA with functionalized PMA. (For clarity, these spectra are normalized in such a way as to prevent overlapping.)

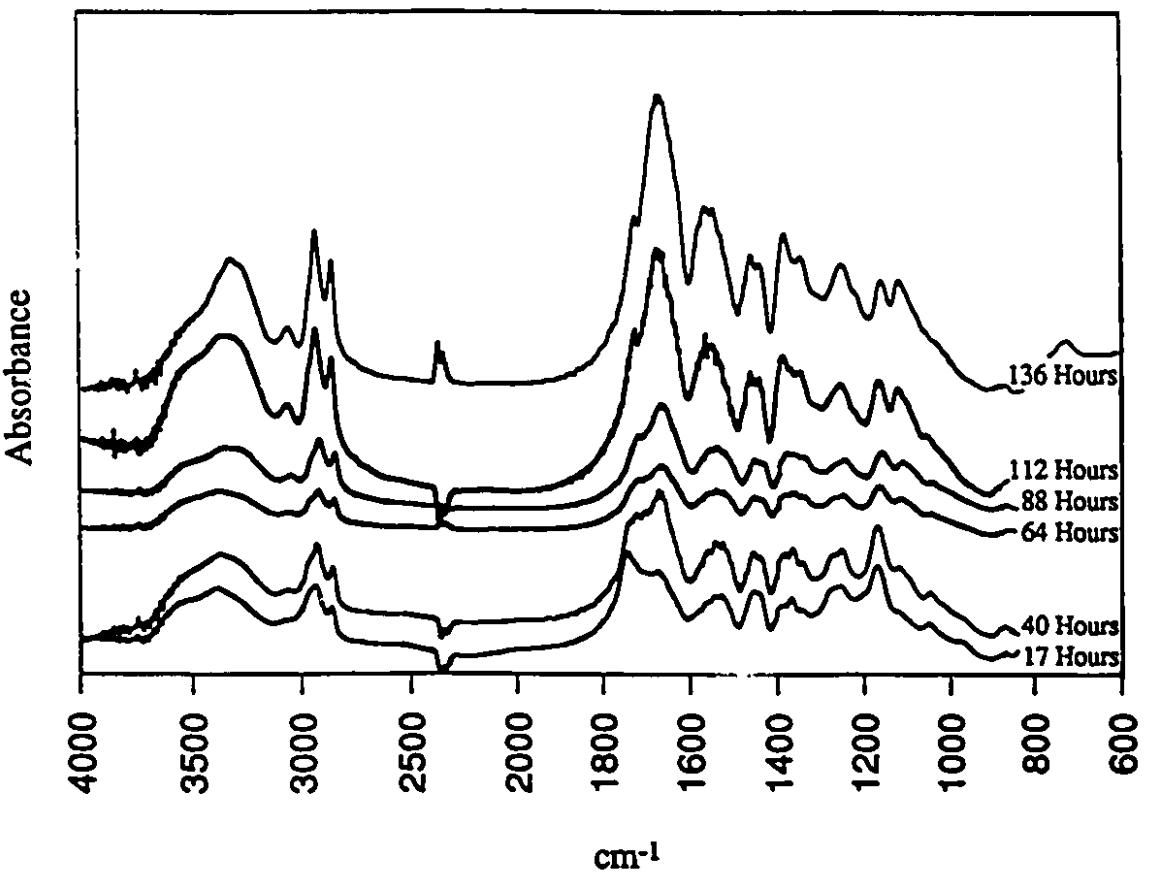


Figure 2.5 FT-IR spectra of functionalized PMA with N-*tert*-butoxycarbonyl-1,6-hexanediamine at 0, 17, 40, 64, 88, 112, and 136 hours reaction time indicating that the reaction proceeds by the strong appearance of N-C=O band at 1670 cm^{-1} and disappearance of C=O band at 1770 cm^{-1}. (For clarity, these spectra are normalized in such a way as to prevent overlapping.)

functionality of the polymer was very low. In the PMA system the application of TFA appeared to create some complications, possibly, TFA salt formation. The formation of TFA salt with protonated amine on the matrix is expected to decrease the functionality value. On the other hand, the failure to remove the Boc groups by TFA in PMA system may also be related to the swelling characteristics of this polymer. The most widely used polymeric resin for solid phase peptide synthesis is styrene-divinylbenzene, which swells easily in the TFA/DCM solution. On the other hand, as was demonstrated in this experiment, PMA beads do not swell significantly in TFA/DCM.

Since the solution of TFA did not remove the Boc groups effectively in the PMA system, an alternative reagent, hydrogen fluoride (HF), was used. Hydrogen fluoride is strongly acidic, which makes it an excellent solvent and protonating agent for the acidolysis of various protective groups (13). One difficulty in the use of HF is the caustic action of this reagent on glass. As described in the literature (13,15), the reaction was carried out in a cylindrical reaction vessel made of synthetic resins (Teflon) which was connected to a vacuum line.

To free the Boc groups from the functionalized PMA beads, well dried beads were placed in the reaction vessel and reacted with HF for about 45 minutes, at 0 °C. Excess HF was removed with the vacuum aspirator. The beads were then carefully added to diethyl ether and washed repeatedly with DCM, DMF, water, and ethanol. After a proper wash, the beads were neutralized with 10% diisopropyl ethyl amine (DIEA) in DCM, and washed again with DCM and ethanol. Finally the de-protected beads were extracted with ethanol in a Soxhlet extractor for 24 hours and dried in a vacuum oven (60 °C) for 24 hours. Figure 2.6 presents the FT-IR spectra of PMA before and after deprotection. Successful removal of Boc groups by HF is indicated by the disappearance of the bands at 1150-1200 cm^{-1} corresponding to the t-butyl group. Furthermore, the splitting of the band at about 1650-1670 cm^{-1} also disappeared.

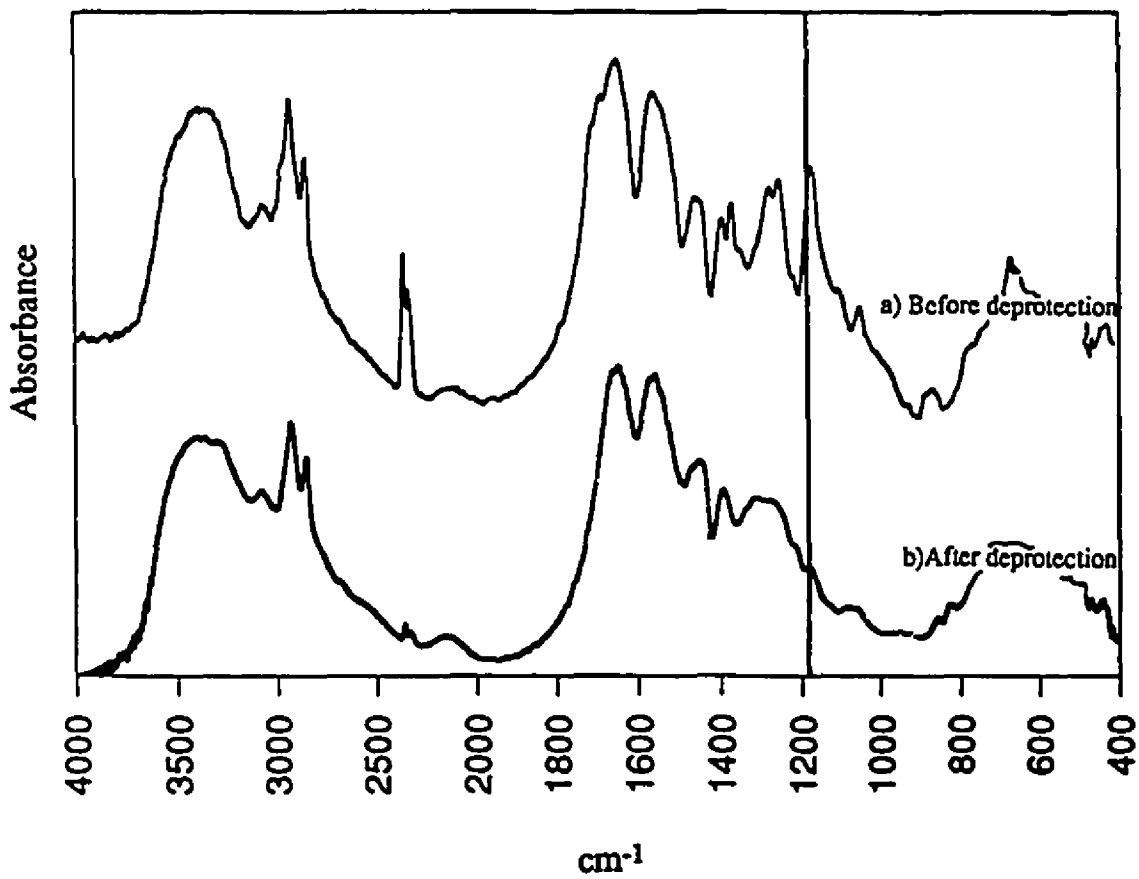


Figure 2.6 FT-IR spectra of functionalized PMA with N-*tert*-butoxycarbonyl-1,6-hexanediamine a) before deprotection and b) after deprotection; the disappearance of *t*-butyl group band at 1150-1200 cm^{-1} indicates the successful removal of Boc groups. (For clarity, these spectra are normalized in such a way as to prevent overlapping.)

2.2.4 Functionalization of PMA Beads Using the Previous Method

The PMA beads were functionalized by the reactions with 1,6-hexanediamine, 1,12-dodecanediamine, and bis (hexamethylene) triamines using the method developed by Wu (1). In all cases, well-dried PMA beads were added to a large excess of the amine at room temperature. The beads were allowed to swell in the multifunctional amine for several hours. The reaction temperature was raised slowly to about 160-205 °C, depending on the boiling point of the amines. The reaction mixture was allowed to reflux for 8 days. The functionalized beads were washed with DMF and 95% ethanol, and extracted in a Soxhlet extractor with ethanol for 3 days. Finally they were rinsed with ethanol and placed in a vacuum oven (60 °C) for 24 hours. The functionalized PMA beads were stored in a desiccator until the quaternization process was performed.

To verify that the desired resins had been obtained, FT-IR spectroscopy was performed. Figure 2.7 presents the FT-IR spectra of PMA and various functionalized PMA beads. These are identical to those reported previously in the literature (1,16). The FT-IR spectrum of the PMA beads, Figure 2.7, indicates the characteristic of C=O stretching vibration at 1800 cm^{-1}. Furthermore, the bands 1200 cm^{-1}, 1450 cm^{-1}, 2950 cm^{-1} correspond to C-O stretching, C-H deformation, and C-H stretching, respectively. The main characteristics of these spectra are the strong N-H stretching bands at 3300 cm^{-1}, the appearance of the C=O amide stretching band at 1670 cm^{-1}, and the appearance of a new band at 1550 cm^{-1} corresponding to the amide II band (combination of NH deformation and C-N stretching). Moreover, the disappearance of the (C-O) stretching band at 1200 cm^{-1} along with (C=O) stretching at 1770 cm^{-1} indicate the completion of the functionalization reaction. The relative size of C-H stretching bands at about 2900-3000 cm^{-1} increases with increased in the length of the CH_2 of pendent group (1).

2.2.5 Quaternization of the Poly(acrylamide) Beads

The functionalized PMA beads were quaternized by the reaction with methyl

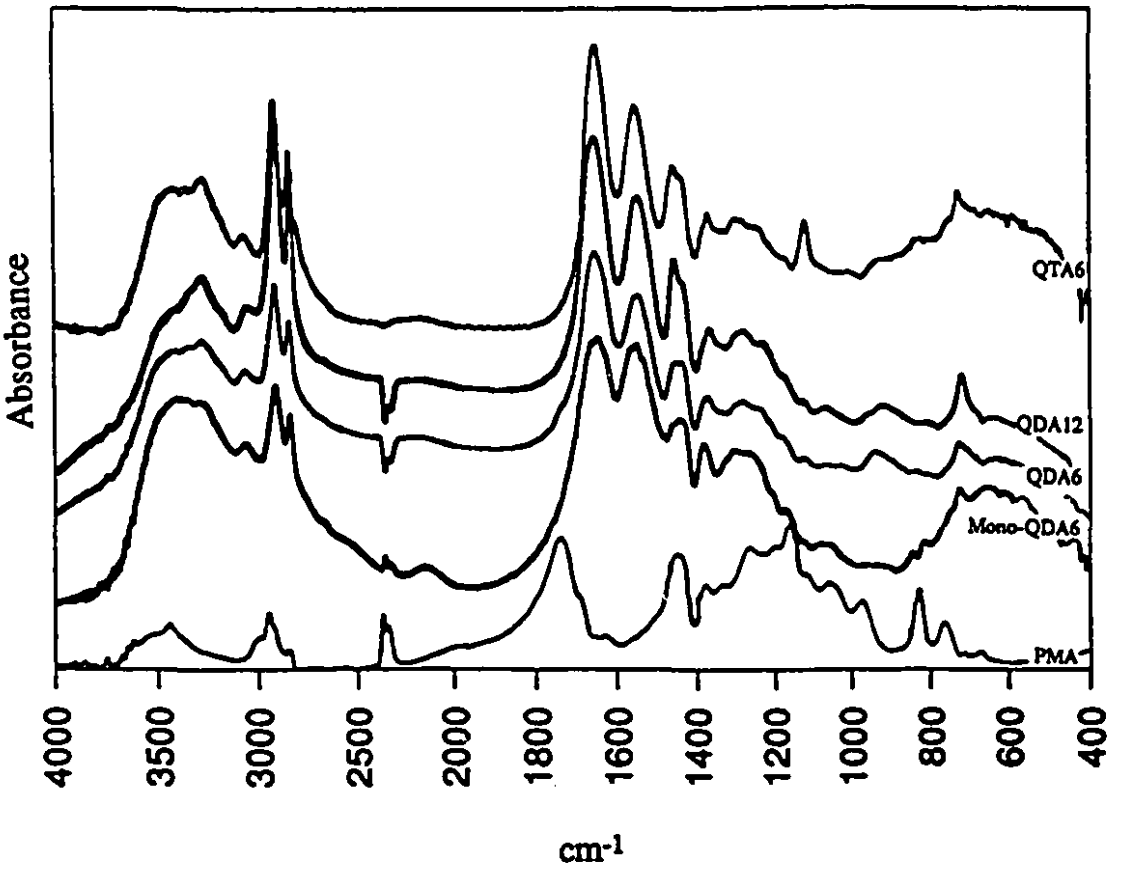


Figure 2.7 The FT-IR spectra of KBr pellets containing ground PMA resins functionalized with various functional units. (For clarity, these spectra are normalized in such a way as to prevent overlapping.)

iodide and potassium bicarbonate in methanol (17). For each millimole of PMA beads, one mL CH_3I, 1 g $KHCO_3$, and 20 mL of CH_3OH were added; in other words, on a molar basis a 16-fold excess of CH_3I and 10-fold excess of $KHCO_3$ was used (18). In this process, the PMA beads were added to methanol and allowed to stir for 1 hour; then the $KHCO_3$ was added to the flask and stirred for 1 hour. Finally CH_3I was added and the reaction mixture was stirred for 10 days. After the reaction, the beads were added to a large amount of 95% ethanol and were washed several times with 95% ethanol and distilled water. Next, the quaternized beads were extracted with 95% ethanol in a Soxhlet extractor for 5 days.

To replace the I^- counter-ions with Cl^-, well dried beads were treated with aqueous NaCl (1.7 M) solution for 3 days. The beads were washed with hot distilled water several times and subsequently placed in the column and flushed with distilled water for 2 days. Finally, the beads were placed in a Soxhlet extractor once more and extracted with 95% ethanol for 24 hours. After drying the beads in a vacuum oven (at 60 ^oC) overnight, they were stored in a desiccator. Figure 2.8 presents the solid state ^{13}C NMR spectra of PMA and quaternized poly(acrylamide) resins with various functional units. The peak at about 26 ppm corresponds to carbons on the pendent groups, that at 40 ppm corresponds to carbons of polymeric backbone, that at 52 ppm corresponds to methyl groups, that at 68 ppm corresponds to carbons next to the quaternary amines, and at 175 ppm corresponds to carbonyl groups.

2.2.6 Determination of the Amine Functionality

The amine functionality of protonated beads was determined by acid-base back titration. To perform the titration, 50 mL of HCl (0.09741 N) was added to (200-300 mg) of resins. The flasks were agitated for 12 hours with a mechanical shaker. The solution was filtered and the excess HCl was titrated with NaOH (0.0835 N). The amine functionality values, chemical structures, and symbols of functionalized PMA before and

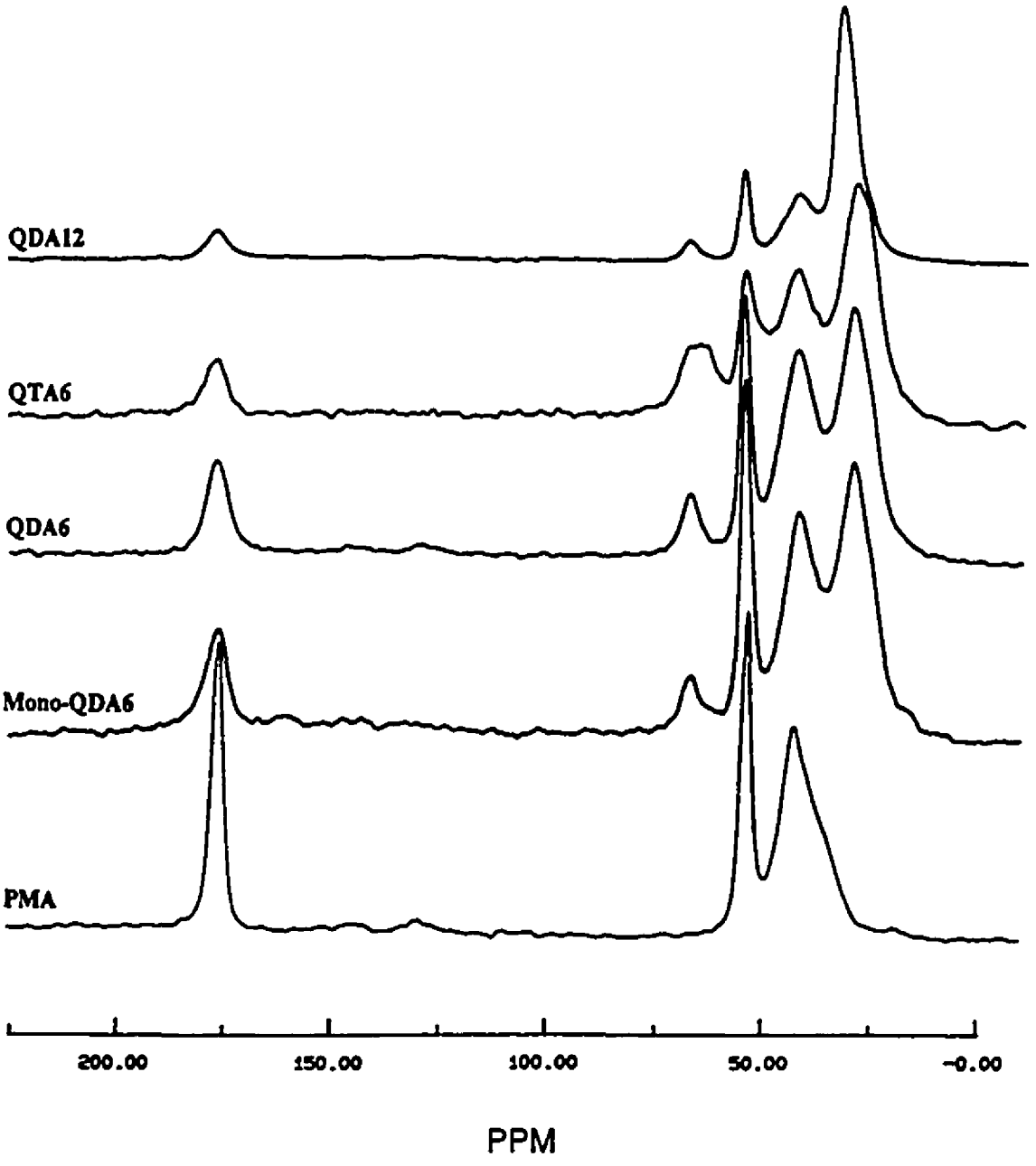


Figure 2.8 Solid state ^{13}C CP/MAS NMR spectra of quaternized PMA resins with various functional units. The peaks at 26, 52, 68, 80, and 175 ppm correspond to carbons on the pendant groups, (CH_3), carbons next to the quaternary amines, carbons on the backbone, and (C=O), respectively.

after quaternization are presented in (Table 2.2). The increase in mass after quaternization results in a small decrease in the number of functional groups per unit mass of the resins.

The functionality values of the quaternized poly(acrylamide) beads were determined by potentiometric titration using a Mettler DL21 automatic titrator. The instrument was set up to add an appropriate amount of titrant corresponding to a signal increase of 4 mV every 20 seconds. The reference electrode was a silver-silver chloride,

Table 2.2 The chemical structure, symbols, and functionality values of the pendent group. The symbol (P) represents the cross-linked poly(acrylamide), common to all resins, and (Q) represents the quaternized resin. Ref.QDA12 resin which was synthesized before was used as a reference.

Chemical structure of pendent groups	Symbols	functionality values (pendent groups mmole/g resin)
P-$(CH_2)_{12}$-NH_2	DA12	2.34
P-$(CH_2)_{12}$-$N^+(CH_3)_3$ Cl^-	QDA12	2.05
P-$(CH_2)_6$ -NH_2 $(CH_2)_6$-NH_2	TA6	5.84
P-$(CH_2)_6$ -$N^+(CH_3)_3$ $Cl^-(CH_2)_6$-$N^+(CH_3)_3$ Cl^-	QTA6	3.63
P-$(CH_2)_6$ -NH_2	DA6	3.95
P-$(CH_2)_6$ -N+$(CH_3)_3$ Cl^-	QDA6	2.80
P-$(CH_2)_6$ -NH_2	Mono-DA6	3.42
P-$(CH_2)_6$ -$N^+(CH_3)_3$ Cl^-	Mono-QDA6	2.56
P-$(CH_2)_{12}$-$N^+(CH_3)_3$ Cl^-	Ref.QDA12	2.14

and the other electrode was a silver wire. The solution of silver nitrate ($AgNO_3$) was used as the titrant, and potassium nitrate (KNO_3) was used as the solution to "drive out" the counter-ion.

To perform the titration, 30 mL of KNO_3 (0.5 M) solution was added to (100-150 mg) quaternized beads, and the mixture was agitated for 12 hours with a mechanical shaker. The content of the bottles were titrated directly with $AgNO_3$ (0.0998 M). As an example, Figure 2.9 shows a titration curve obtained for the potentiometric titration of resin QDA12.

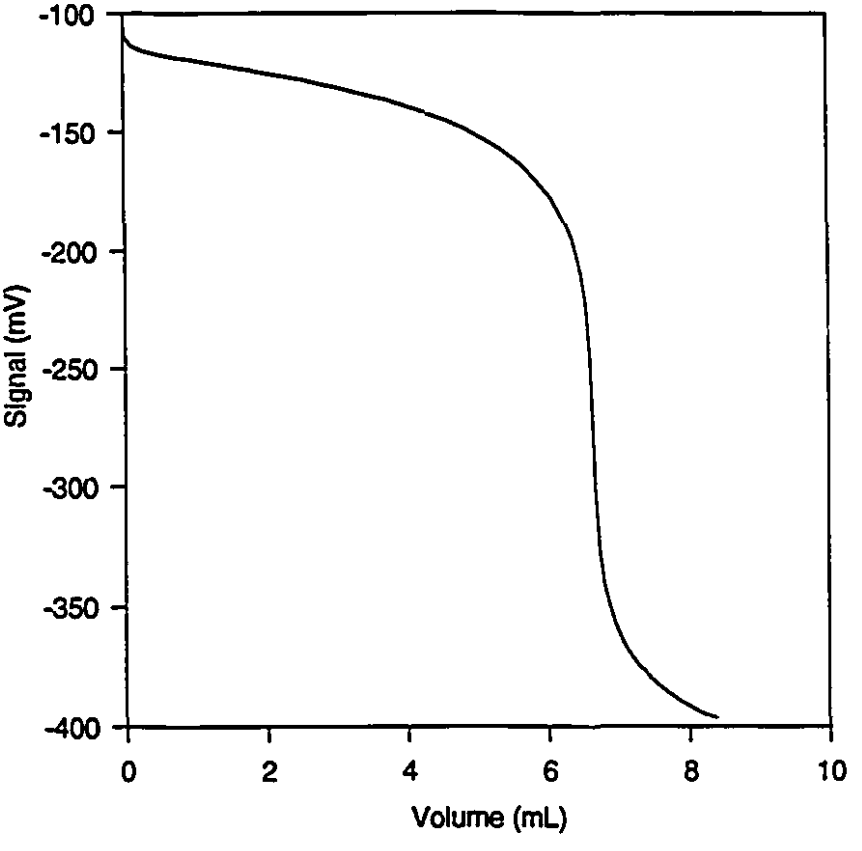


Figure 2.9 Potentiometric titration curve for quaternized resin QDA12. The X-axis indicates the volume of silver chloride added in mL, and the Y-axis indicates the signal observed by Mettler titrator in mV.

During the titration experiments, it was noticed that the titration curve of some of the resins appeared to have 2 end points (Figure 2.10). This was attributed to the presence of 2 counter-ions with different electronic properties. It was concluded that within the resin matrix both counter-ions of (I^- and Cl^-) existed, and the Cl^- wash did not replace all of the I^- counter-ions. As a result, these beads were treated with the solution of NaCl again. After this procedure, the titration curve had only one end point. The titration method may be used in the future to determine the proper replacement of counter-ions.

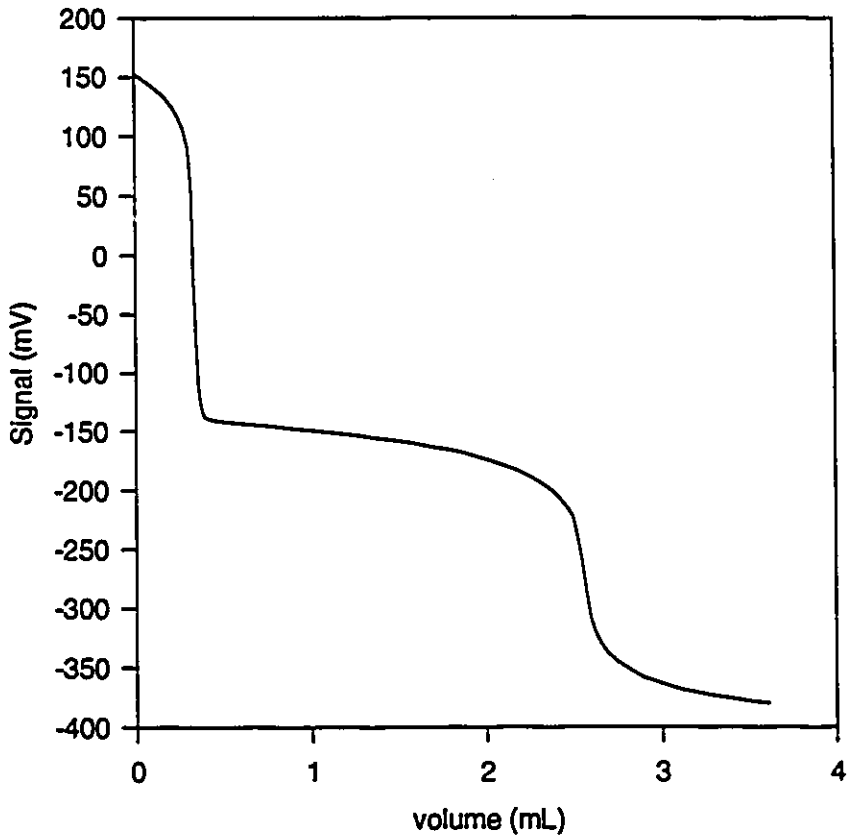


Figure 2.10 The potentiometric titration curve for quaternized resin QDA6, showing two end points, which indicates the existence of I^- counter-ions within the polymeric matrix.

2.2.7 Physical Appearance of Poly(acrylamide) Beads

The physical appearance of the beads was studied using photomicrographs of the beads taken with a 35 mm Nikon camera mounted on a Nikon Optiphotpolarized light microscope. Figure 2.11 presents the photomicrographs of the QDA12 and QDA6 beads (which were synthesized here) and the reference QDA12 beads (which were synthesized previously by Professor D. Gravel of the University of Montreal). Moreover, Figure 2.12A shows the photomicrographs of the dry Mono-QDA6 beads, and Figure 2.12B shows the same beads dispersed in water. The physical appearance of the final quaternized poly(acrylamide) beads in this study differs from the resins synthesized previously, in both size and shape. The size of these beads is less, with a diameter in the range of 10-100 microns; whereas the poly(acrylamide) beads prepared by Dr. Gravel had diameters in the range of 300-600 microns. In addition to the size, the shape of these beads is also different. The previously synthesized beads were spherical, while the beads that were prepared here are of an irregular shape.

Before functionalization, the PMA beads that were synthesized in this study were almost identical in shape and size to the previously synthesized beads. However, when these beads were quaternized and the I^- counter-ions were replaced by Cl^-, they lost their original shape. It seemed that the disintegrated, or exploded, into small pieces. It is possible that the lower degree of cross-linking may account for this phenomenon. In general, the swelling is produced by osmotic pressure in the interior of the ion-exchange resin. For example, if the beads are lightly cross-linked, a high swellability is expected. If the osmotic pressure inside the beads is much greater than the external pressure, and the polymeric network is not strongly cross-linked, the polymeric matrix can break apart before the system reaches an equilibrium. Therefore, very small pieces result from each bead. In the next section, the swelling characteristics of these resins in water will be determined.

Figure 2.11 The photomicrographs of: (A) the reference QDA12 beads, (B) the QDA12 beads, and (C) the QDA6 beads.

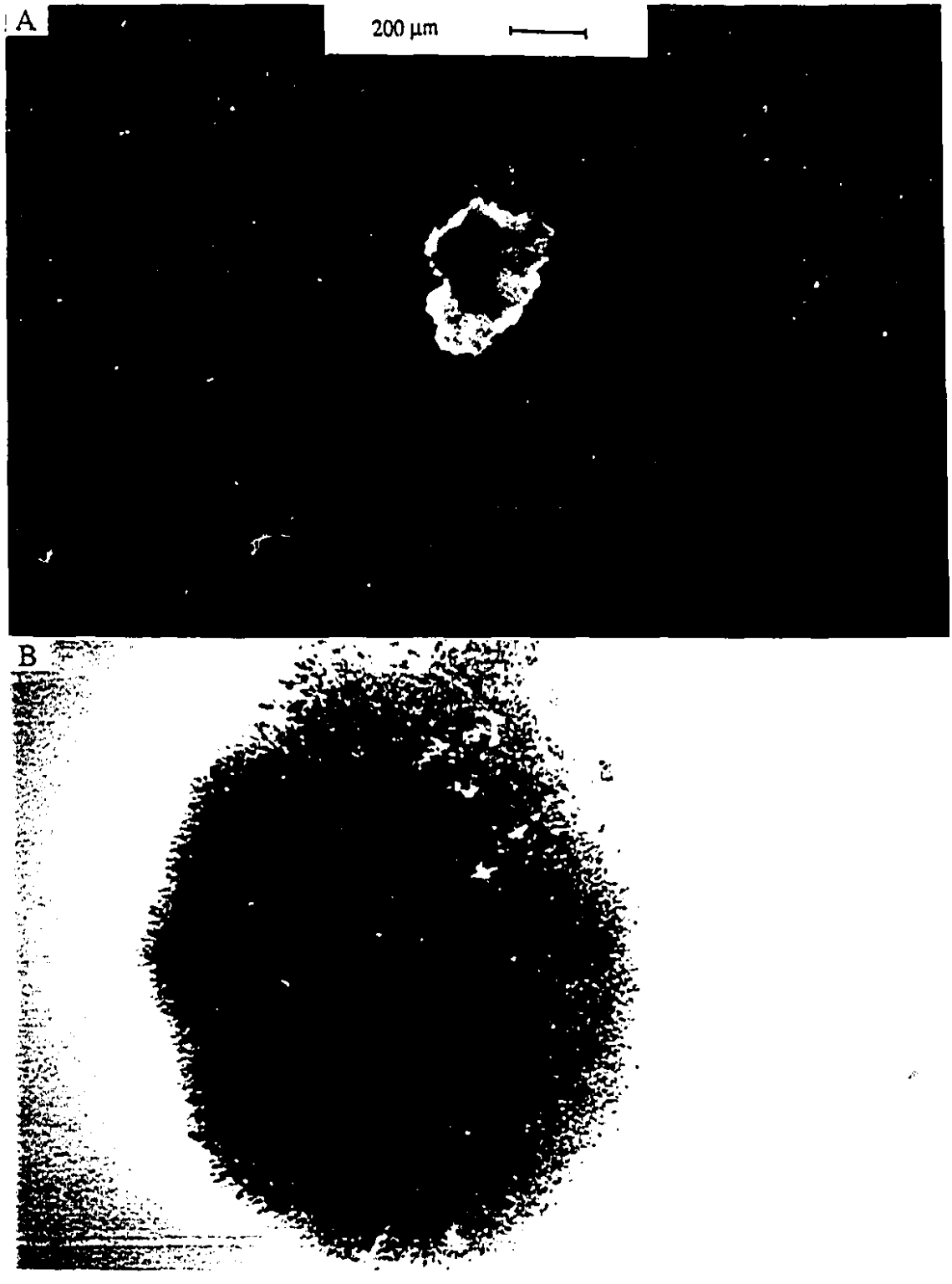


Figure 2.12 The photomicrographs of: (A) a dry Mono-QDA6 bead and (B) the same beads in water.

2.2.8 Swelling Characteristics of the Poly(acrylamide) Beads

When a dry ion-exchange resin is placed in an aqueous solution, it swells to a degree that depends on the hydrophobicity. Water enters the resin matrix to solvate both the fixed ions on the resin and the mobile counter-ions. The swelling phenomenon can be considered as a result of the osmotic pressure difference between the two phases, the dilution of the concentrated resin phase, or as an electrostatic repulsion of the resin fixed ionic groups. The swelling process is opposed by the rigidity of the resin matrix. The extension of the polymer chains due to absorption of the solvent stretches the cross-links; in turn, they exert an elastic counter pressure which opposes the swelling. Equilibrium is reached when the elastic counter pressure is equal to the osmotic pressure (19). The degree of swelling depends on the degree of cross-linking of the resins, the number of exchange sites on the resin, and the nature of the fixed charge groups as well as the counter-ions. In any case, the amount of swelling obviously depends upon the degree of cross-linking in the resin.

In a general sense, if the phase boundary is permeable to the solvent, a concentrated salt solution cannot be in equilibrium with one that is more dilute at the same pressure as osmosis will take place. Conversely, if the concentrated solution is contained in a semipermeable membrane, its osmotic pressure will build up until the equilibrium is reached. The absorption of water by resin causes the polymer network to stretch and set up an internal "swelling pressure (tension)" (20).

2.2.8.1 Water Swellability

An attempt was made to study the swelling characteristics of poly(acrylamide) beads. The percent swellability of the beads was determined. The effect of temperature on the swelling behavior of beads in water and in sodium cholate solution was studied. Furthermore, the degree of swelling and shrinking was evaluated by changing the solution covering the beads.

The change in the volume of the beads was followed using a video camera mounted in front of a water bath. It transmitted images to a computer and to a secondary monitor (Electrohome RGB) for display. The images were recorded on videotape using a Mitsubishi U80 video cassette recorder for later analysis. A video analysis software program (JAVA, Jandel scientific) was used to measure the level of beads inside the capillary tubes. The length of the beads inside the capillary tubes was taken as the average of 6-10 measurements. The degree of swelling was determined according to the equation (1):

$$\text{Swellability} = (V_P - V_o) / V_o \times 100$$

where V_o is the volume of dry resins, and V_P is the volume of wet resin.

Since the beads were very small and irregular in shape, the volume change was performed in capillary tubes. A few milligrams of each resin were placed in a series of capillary tubes. The capillary tubes were spun in a centrifuge for 5 minutes to achieve a proper packing. The tubes were placed vertically in front of a video camera, and the level of the beads inside the capillary tubes was recorded. The capillary tubes were then filled with distilled water by microsyringe and centrifuged for 5 minutes. After 4 hours, the level of the swollen beads was recorded by video camera. For each resin, the swellability determination was performed in 4 different capillaries, and the average values are reported in Table 2.3. It is of note that the water swellability of resin QDA6 prepared in this study is about twice that of the one prepared by Wu (1). Furthermore, the swellability of Mono-QDA6 resin is even greater than that of the QDA6 resin prepared for this study. Clearly, this confirms that the previous method of functionalization results in additional cross-linking.

Table 2.3. The water swellability of poly(acrylamide) resins at 25 °C, comparing the water swellability with resins prepared previously in this laboratory.

Resin	H_2O swellability
PMA	30%
Mono-QDA6	158%
QDA6	117%
QDA12	69%
PMA[a]	16%
Q-PDA6[a]	67%

(a) The data obtained form reference (1).

2.2.8.2 Effect of Temperature on Swellability

To study the effect of temperature on the volume of the beads, a water bath was set up, and the capillary tubes containing the beads were placed vertically inside the water bath. The temperature of the water bath was increased from 0 to 75 °C. At 5 °C intervals, the beads were allowed to equilibrate for 30 minutes and the level of the beads was recorded with a video camera.

Figure 2.13 shows the effect of temperature on the percent swellability of Mono-QDA6, QDA6, and QDA12 resins in water and in a solution of sodium cholate (NaCA, 50 mg/dL). It appears that temperature does not have a significant effect on the extent of swellability of the resins QDA12 and QDA6. However, the percent swellability of resin Mono-QDA6 behaves somewhat differently and increases as the temperature increases. The effect of temperature on the volume of swollen beads is less obvious in NaCA solution. The percent swellability of the resins QDA6 and QDA12 are unaffected by temperature; however, the volume of the swollen Mono-QDA6 still increases as the temperature increases. Furthermore, the degree of swellability of the resins in water is higher than in the solution of sodium cholate (Figure 2.13).

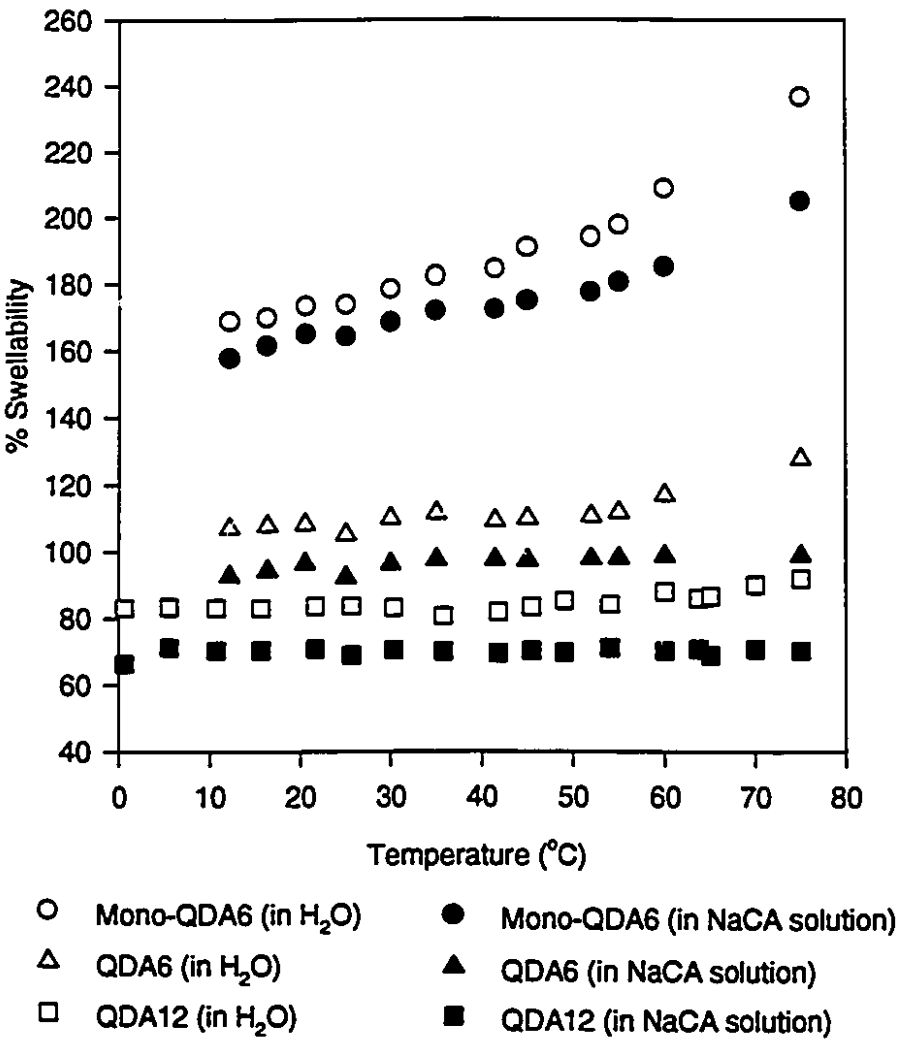


Figure 2.13 Effect of temperature on the degree of swelling of the resins Mono-QDA6, QDA6, and QDA12 in water and in NaCA solution (of about 50 mg/dL).

2.2.8.3 Effect of Change in the Solution Phase on Swellability

Further studies were made to evaluate the effect of change in the solution on the swelling behavior. The swelling procedure and the method of recording volumes were as described above. The effect of solution composition was studied by changing the solution phase in the capillary tubes. In this experiment, a few milligrams of each resin were placed into 2 sets of capillary tubes. The volume of dry resin was recorded with the video camera. Water was added to the first set of tubes and a the solution of sodium cholate (50 mg/dL) was added to the second set. The tubes were placed in a water bath and the temperature was increased from 0 to 60 °C. At 10 °C intervals, the beads were allowed to equilibrate for 30 minutes and then the volume of the beads was recorded. After cooling the beads, the water in the first set of tubes was replaced by aqueous NaCA solution (50 mg/dL) and the solution of the second set of tubes (NaCA solution) was replaced by water. It should be noted that when changing the solutions inside the tubes, the beads were washed several times with the new solution. For each washing process, the beads were allowed to equilibrate for several hours. After changing the solutions, the temperature of the water bath was increased to 60 °C and then decreased to 10 °C, during which the level of the beads was recorded every 10 degrees.

For the first set of tubes, the effects of the solution phase and temperature for the resins QDA12, QDA6, and Mono-QDA6 are presented in (Figure 2.14). When the water is replaced by a sodium cholate solution, the resins Mono-QDA6 and QDA12 shrink; however, in the case of the resin QDA6 the volume change is very small.

The effects of solution phase and temperature for the resins QDA12, QDA6, and Mono-QDA6 in a second set of tubes are presented in Figure 2.15. As expected, when the sodium cholate was replaced by water, the swelling of resins Mono-QDA6 and QDA12 increased significantly. The increase in volume indicates the desorption of bile acid from the resins. In the case of QDA6 resin, an insignificant volume change resulted

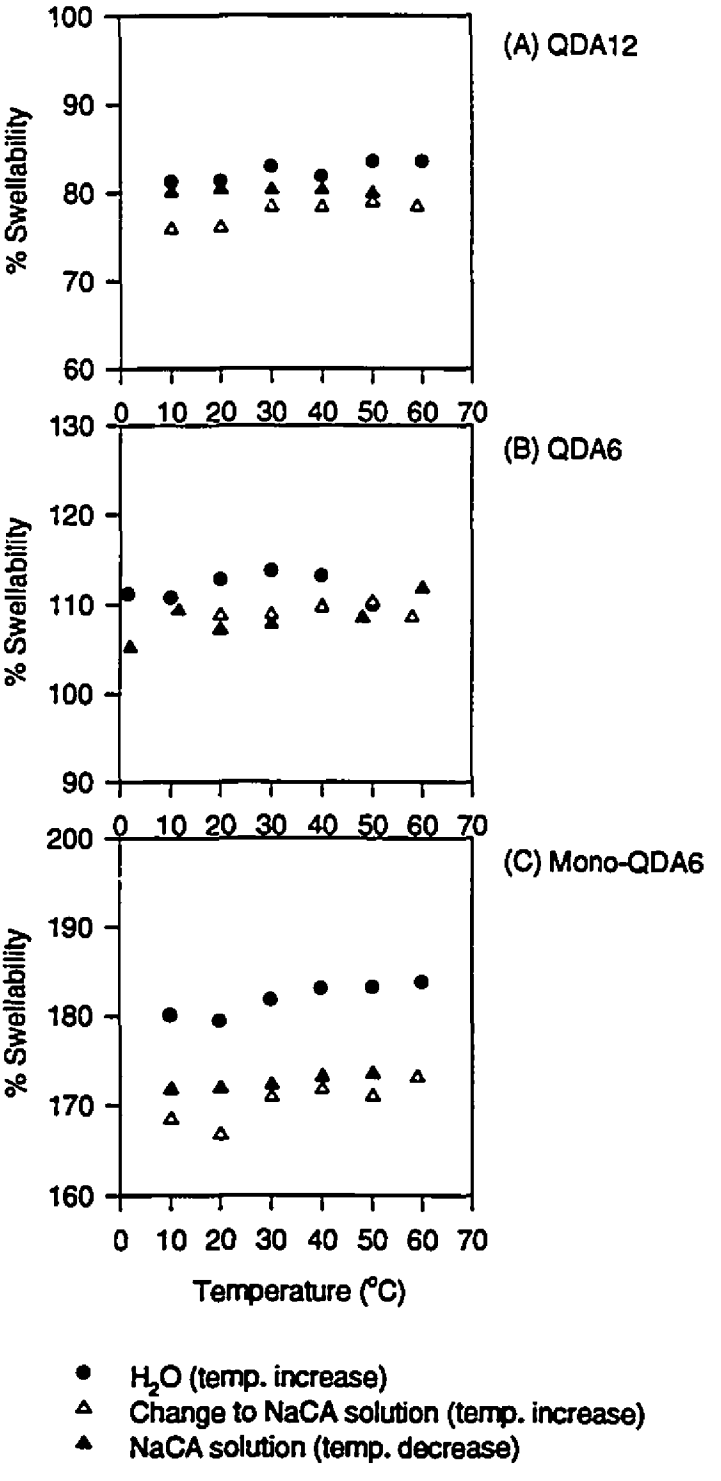


Figure 2.14 Effect of replacement of water with a solution of NaCA on the temperature change of the degree of swelling of resins QDA12, QDA6 and Mono-QDA6.

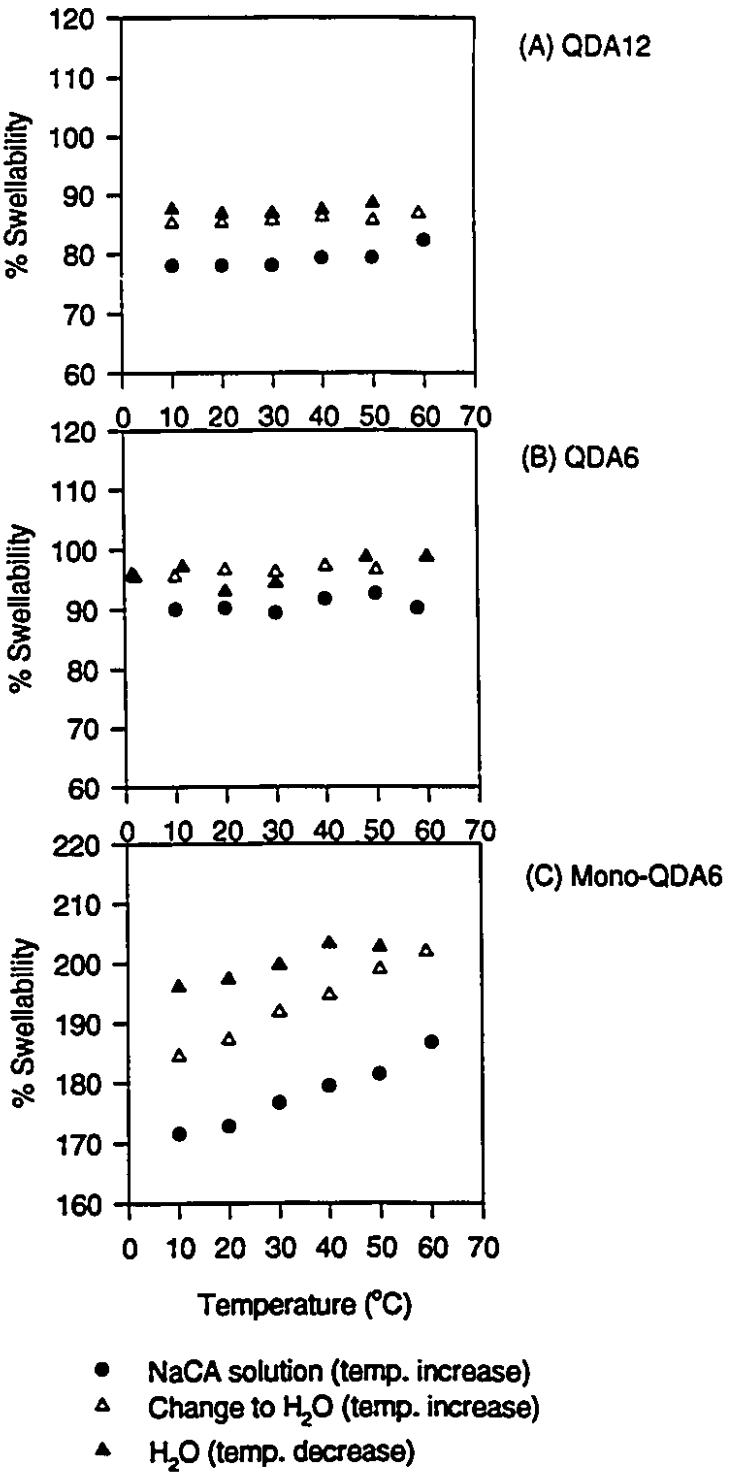


Figure 2.15 Effect of replacing the NaCA solution with water on the temperature change of the degree of swelling of the resins QDA12, QDA6, and Mono-QDA6.

upon changing the solution phase (Figure 2.14.B and 2.15.B). The reason might be that (a) resin QDA6 has a higher degree of cross-linking than the Mono-QDA6 resin, and (b) it is possible that more time is required for the QDA6 beads to reach an equilibrium. As shown in Figure 2.15, the percent swellability of resins during the decrease in temperature is slightly higher than that during the increase in temperature. The reason for this might be the insufficient equilibration time between the measurements

2.2.9 Sorption Experiments

Attempts were made to follow the established sorption procedure developed in this laboratory (1,18,21). Since the prime objective of these experiments was to evaluate and compare the sorption characteristics of these resins, the sorption capacities of the resins for sodium cholate alone were determined. The sorption experiments were performed using aqueous bile acid solutions, both buffered and non-buffered. Tris-HCl (tris(hydroxymethyl) amino ethane, Aldrich) was used as the buffer. The tris-HCl buffer has a pH range of 7-9 corresponding to the pH range of the gastrointestinal tract, which has a pH of 7-8 (22). In these experiments, a tris-HCl buffer (2.5 mM) at pH 7.2 was used.

Stock solutions of cholic acid sodium salt (50 mg/dL) were prepared, both in water and in an aqueous buffer. The quaternized beads (~ 10 mg) were weighed into a series of glass bottles of different sizes. To achieve a range of equilibrium concentrations of the bile salt, the volume of the stock solution added to the bottles was changed (ranging from 6 to 32 mL). The bottles were agitated with a mechanical shaker at 120 oscillations per minute for 12 hours. Aliquots were withdrawn and were filtered to obtain a clear solution for HPLC analysis. The QDA12 resin synthesized previously by Professor D. Gravel of the University of Montreal was used as the reference material for this study.

2.2.10 Analysis of Cholic Acid

The bile acid concentrations were determined by the high performance liquid chromatography (HPLC) technique using a C-6 (5 microns) siloxane hexyl reverse phase column (CSC) which was developed in this laboratory by Zhu, et al. (23). The detector used in these experiments was a differential refractometer (Waters 410) detector. The mobile phase was a solution of HPLC grade methanol and 0.1 M aqueous acetic acid, at a volume ratio of 80:20. The calibration with standard solutions presented a linear relationship between the peak areas and the concentration of sodium cholate (Figure 2.16). The standard sodium cholate solution was run before, during, and after each set of sorption experiment by HPLC.

2.3 Results And Discussion

2.3.1 Effect of Additional Cross-Linking on the Sorption of Cholate

The isotherms for the sorption of cholate from sodium cholate solution, at room temperature, by the QDA6 resin (i.e., the one functionalized by the previous method) and the Mono-QDA6 resin (which was functionalized by new method) are presented in Figure 2.17 (water) and in Figure 2.18 (Tris-HCl buffer solution). For both media the Mono-QDA6 resin exhibits binding properties that are significantly superior to that of QDA6 resin. These results demonstrate the importance of the additional cross-linking during the functionalization process. The difference in the binding capacities of these two resins is more pronounced for the tris buffer solution, especially at low equilibrium concentrations (Ceq) (Figure 2.18). For the tris buffer solution the isotherms for both resins have S-shaped isotherms, indicating a positive cooperativity in the binding process, which is thought to be due to hydrophobic interactions and hydrogen bonding involving bile salt anions bound at adjacent sites inside the resin beads (1,26). Numerical values of the cooperativity parameter of these beads is presented in Section 2.3.7.

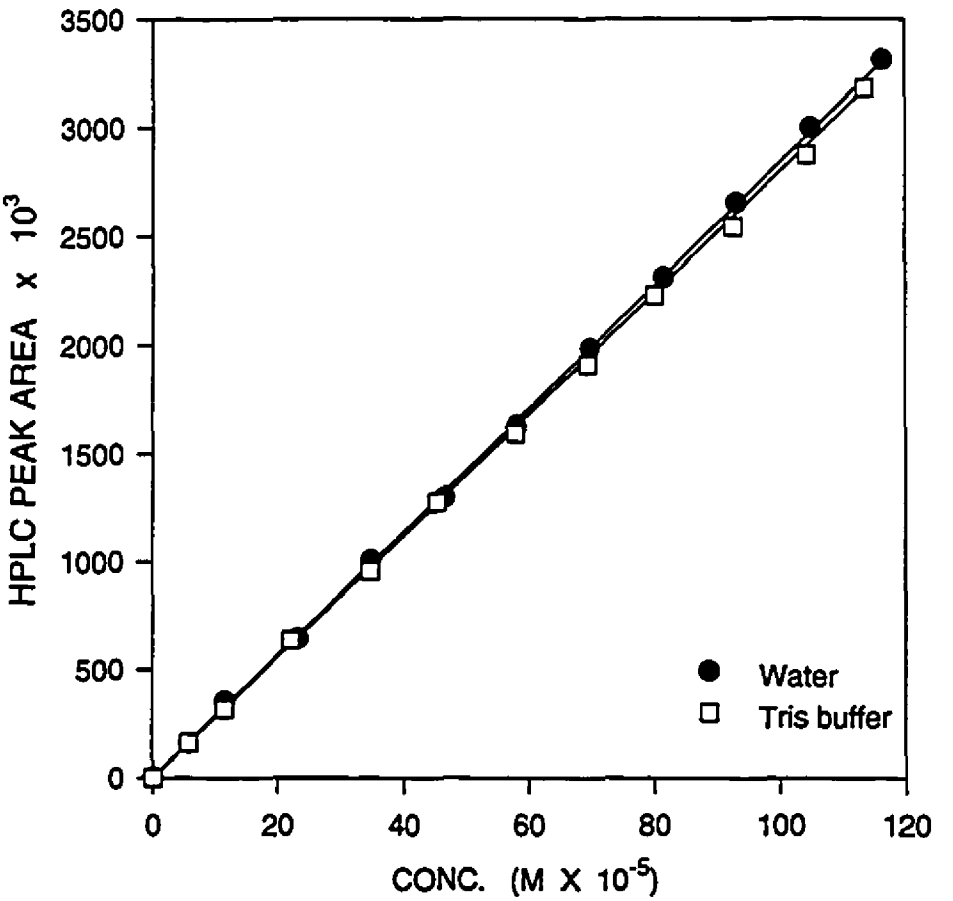


Figure 2.16 The relationship between the peak area of HPLC chromatogram and the concentration of NaCA in water and in 0.0025 M tris buffer (pH 7.2).

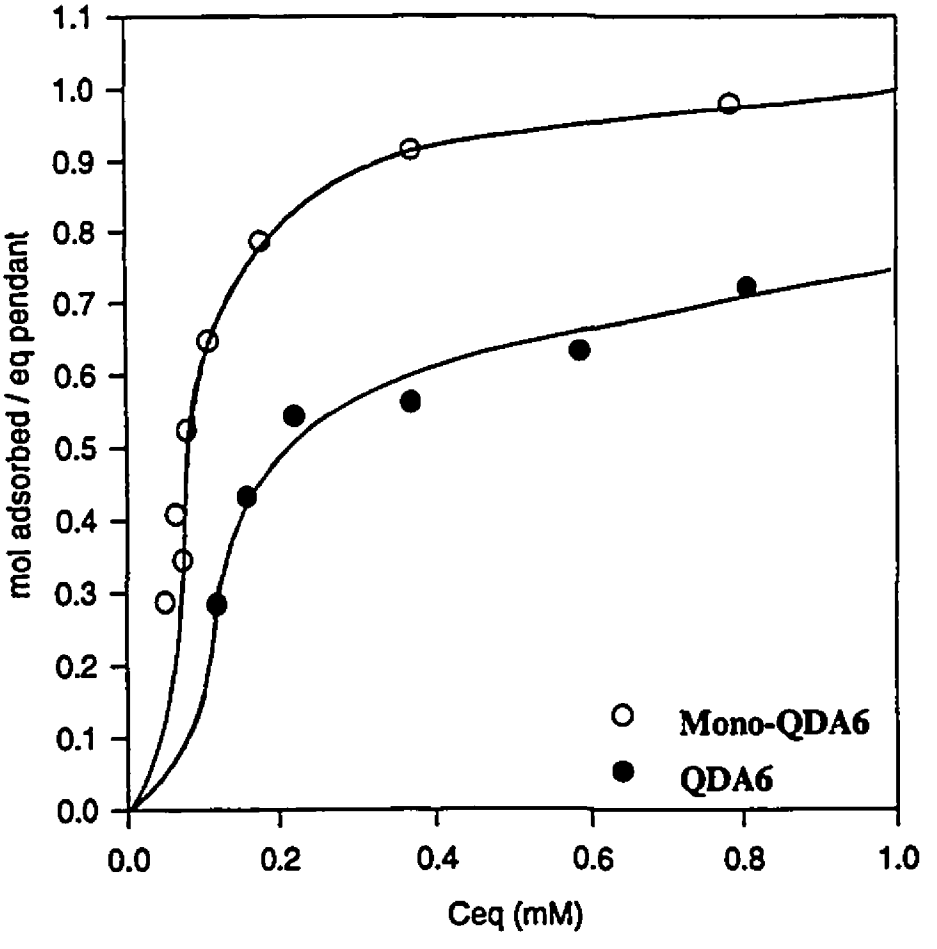


Figure 2.17 The isotherms for the sorption of cholate by the quaternized resins in water at 20 °C; showing the superiority of the new method of functionalization.

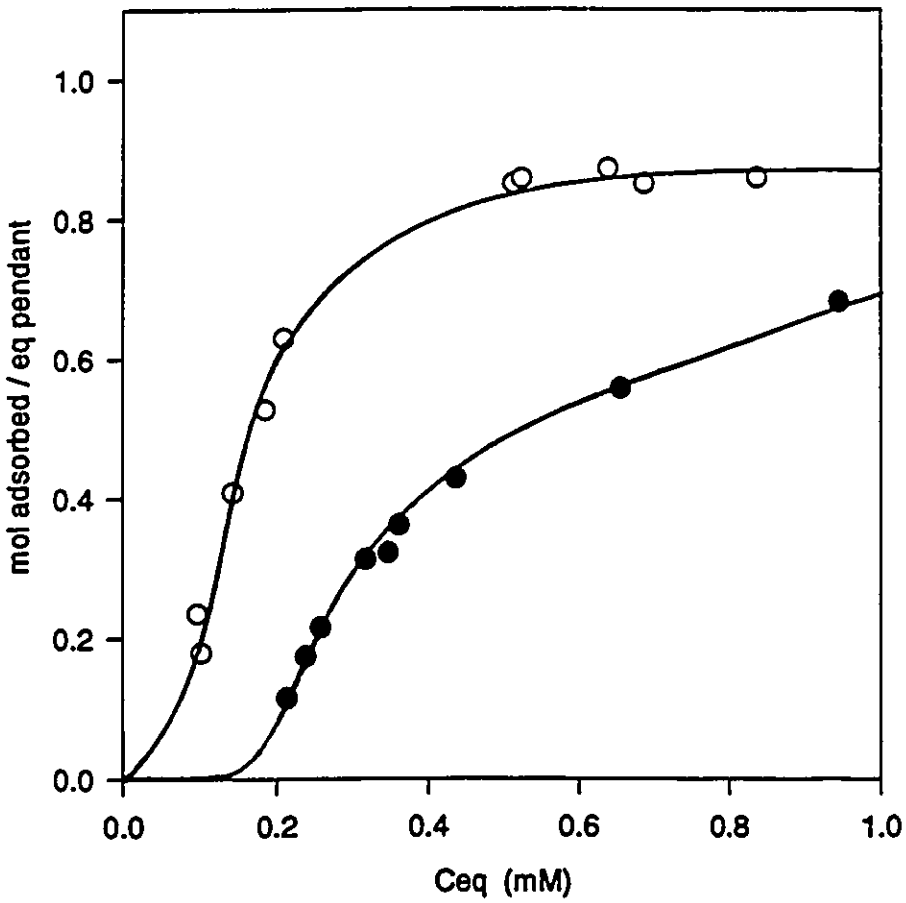


Figure 2.18 The isotherms for the sorption of cholate by the quaternized resins in 0.0025 M tris buffer (pH 7.2) at 20 °C; showing the greater sorption capacity of Mono-QDA6 resin.

It may be recalled that, as indicated in Table 2.2, the ion-exchange capacity of Mono-QDA6 is less than that of the resin QDA6 (2.6 as compared with 2.8 mmol/g resin). However, the Mono-QDA6 resin shows a greater binding efficiency than resin QDA6 for cholate anion, approaching full replacement of Cl^- anions. These results are a strong indication of the successful diffusion of bile salt anions within the resin having the lower degree of cross-linking, resin Mono-QDA6.

2.3.2 Comparison Between Mono-QDA6 And QDA6 Resins

Evaluation of the functionality values and the sorption isotherms shows that the binding process does not depend solely on the overall value of ion-exchange capacity of the resins. It seems that the diffusion of bile salt anions into the resins is also an important aspect.

To evaluate and make comparisons between these two resins synthesized in different ways, several important questions should be addressed. First of all, why does the Mono-QDA6 resin have a lower functionality than QDA6? As was discussed earlier, one of the original goals of developing an alternative method of functionalization was to create resins with a higher ion-exchange capacity by elimination of additional cross-linking. However, the potentiometric titration results in Table 2.2 indicate a lower functionality value for resin Mono-QDA6 than for resin QDA6. Secondly, why does the Mono-QDA6 resin, with a lower ion-exchange capacity, bind $cholate^-$ more efficiently than the QDA6 resin? It is well recognized that the primary interaction involved in the sorption of bile salt anions by ion-exchange resins involves electrostatic interaction (1,26). Therefore, it is expected that the resin with higher ion-exchange capacity (the QDA6 resin) should bind cholate more efficiently than the resin with the lower amine functionality (the Mono-QDA6 resin). Despite these facts and expectations, there are other aspects, such as the diffusion process, which are important to the overall sorption process.

2.3.2.1 Evaluation of Functionality Values For Mono-QDA6 And QDA6 Resins

To develop an explanation for the first question, an assumption is made. In 1955, in a study of selectivity of 5, 10, 15, and 25 percent cross-linked polystyrene-divinyl benzene resins for hydrogen, sodium, and potassium ions, Reichenberg and McCauley (19), concluded that the ion-exchange sites in a resin are not all identical, and they suggested that the most possible cause of the variation is the extent of cross-linking in different parts of the same beads. Here, in a similar manner, it is assumed that the degree of cross-linking is not uniform throughout the poly(acrylamide) beads; for example, there are various regions within the resin beads which have high density of cross-linking and some areas with lower cross-linking. Thus, the ion-exchange resin beads can be divided into several distinct regions according to the degree of local cross-linking (27). To simplify this qualitative discussion, the various regions in the beads can be placed into three main categories: (1) high, (2) medium, and (3) low density of local cross-linking. Figure 2.19 presents a schematic representation of cross-linked beads which indicates the three distinct regions.

Based on the idea of differences in the degree of local cross-linking, it is assumed that there are areas within the beads which are accessible to the 1,6-hexanediamine molecule but inaccessible to N-tert-butoxycarbonyl-1,6-hexanediamine molecules. It is clear that the size of these two molecules is quite different since the 1,6-hexanediamine has a molecular weight of 116 while N-tert-butoxycarbonyl-1,6-hexanediamine has a molecular weight of 216, i.e., about 86% heavier. Furthermore, the bulky tertiary butyl group of the Boc protecting group inhibits the movement of the molecules inside the beads. Thus, it can be concluded that there are areas within the PMA beads that are accessible to the diamine molecules, so that grafting to the polymer chain can occur, but are inaccessible to the mono-protected diamine. In this discussion, these areas within the PMA beads are referred to as region I (areas of high degree of cross-linking). It is clear

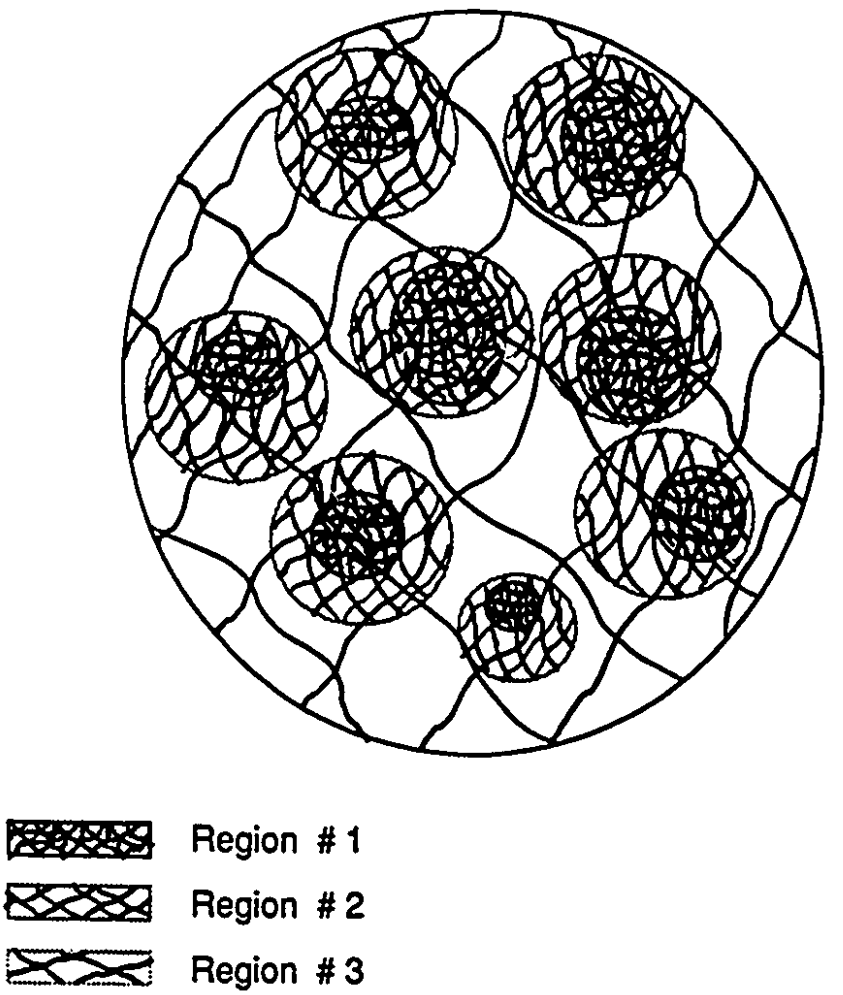


Figure 2.19 A schematic representation of the proposed interior microstructure of three dimensionally cross-linked resin beads, indicating the various areas within the bead based on the local degree of cross-linking. In relation to this study, the areas within the beads in which bile salt anions can successfully diffuse are indicated as region 3.

that inside region I functionalization by the reaction with mono-protected diamine cannot occur whereas the reaction with unprotected diamine can occur readily. Also, it should be pointed out that in this region the probability of forming additional cross-linking is relatively high. Thus, for the beads that were reacted with mono-protected diamine by the new method the functionality value of region I would be zero, but there is some finite value in region I for the beads that were prepared by the previous method.

There are also other areas in the PMA beads that are readily accessible to both diamine and mono-protected diamine but are inaccessible to the bile salt anions. These areas of the PMA beads are referred to as region II, areas with a medium degree of cross-linking. Since this region will be functionalized by both reactions, it contributes to the ion-exchange capacity values for both method of functionalization. However, as in region I, it is not useful in the sense that bile salt anions cannot be bound, simply because they cannot reach the active sites.

Finally there are areas within the PMA beads that are accessible to the diamine molecules, mono-protected diamine molecules, as well as bile salt anions. These areas in PMA beads are called region III, and are areas of a low degree of cross-linking. This region is the most important region within the PMA beads because the actual sorption of bile salt anions occurs here.

The presence of positively charged ammonium groups in region I of the resin QDA6 contributes to a higher overall functionality value of this resin, relative to Mono-QDA6 resin. It is of note that the difference is relatively small (< 10%) which accounts for the failure to detect unreacted ester groups in the IR spectra of the Mono-QDA6 resin. As described earlier, the ion-exchange capacity of the beads was determined by potentiometric titration, in which the Cl^- counter ions were replaced by nitrate anions, then titrated with silver nitrate. It is important to note that the small ions, such as Cl^- and nitrate, can travel easily through the areas of high degree of local cross-linking (regions I and II), which the amine functionality value reflects. However, bile salt anions cannot

travel easily into region I. As a result, the areas of the beads which are categorized as regions I and II do not make any contribution to the binding of bile salt anions.

2.3.2.2 Evaluation of Sorption Capacity for Mono-QDA6 And QDA6 Resins

The explanation for the second question is also related to the degree of local cross-linking within the PMA beads. As was established in the previous section, the polymeric ion-exchange beads consist of various areas with different degrees of local cross-linking (Figure 19). It was concluded that the most important areas within the beads are those of a low degree of local cross-linking, into which the bile salt anion can diffuse (region III).

The isotherms for the resins Mono-QDA6 and QDA6 have been re-plotted, in Figure 2.20, with the sorption capacities expressed on a mass basis to facilitate the evaluation of the sorption capacities of these resins. In spite of the lower density of functional sites, resin Mono-QDA6 has a higher capacity than resin QDA6 both at high and low Ceq. It should be recalled that the additional cross-linking created in resin QDA6 by the free diamine greatly suppresses its binding efficiency. It appears that the overall ion-exchange capacity of the resin is not as critical as the local ion-exchange capacity of specific areas such as region III. Using this kind of argument, it becomes apparent that the ion-exchange capacity within region III of resin Mono-QDA6 is greater than that of the resin QDA6.

These results suggest that the previous methods of functionalization actually create additional cross-linking within the beads, and the mono-protection method for functionalization greatly increases the binding efficiency of the resin.

2.3.3 Effect of Tris-HCl Buffer on Binding Efficiency

Many ionic species, such as Cl^-, Na^+, and HCO^-, exist in the gastrointestinal tract (1). The other anions in the gastrointestinal tract can compete with bile salt anions for the positively charged ammonium groups on the resin, and this causes a reduction of the

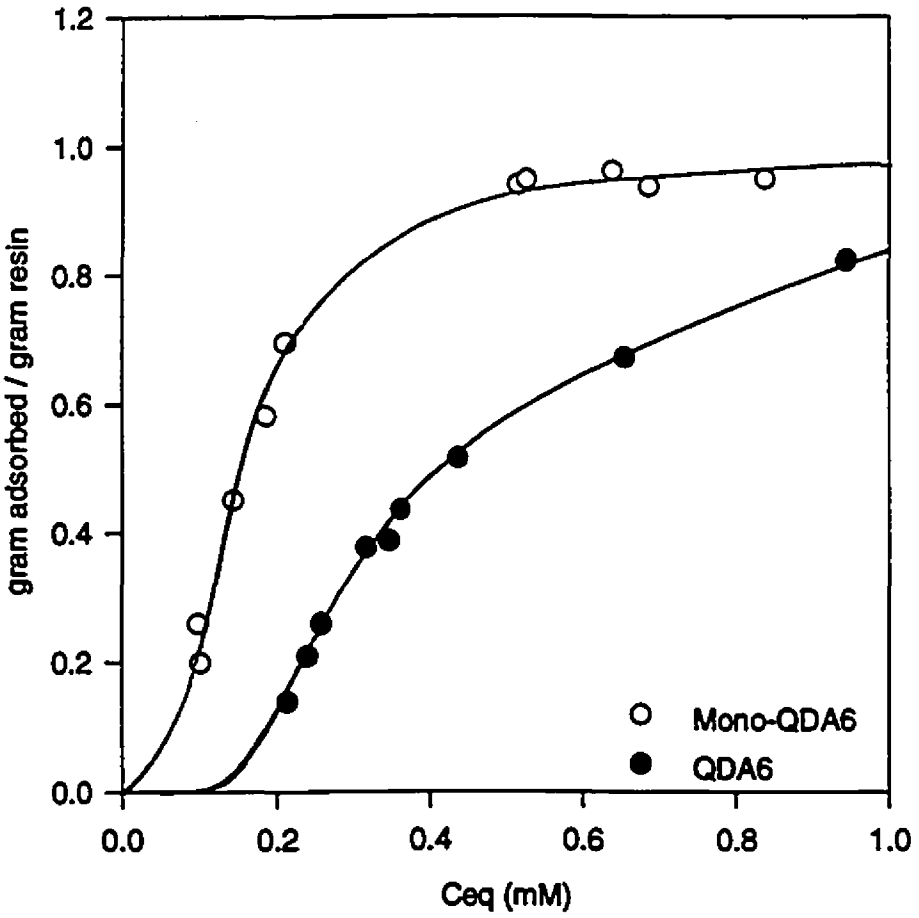


Figure 2.20 The isotherms for the sorption of cholate, on a mass basis, by the resins Mono-QDA6 and QDA6 in 2.5 mM Tris buffer (pH 7.2) at room temperature.

amount of bile acid anion bound by the resins (28,29). This is one of the reasons why cholestyramine is relatively inefficient (50-fold less potent *in vivo* than *in vitro*) (30). The buffer was used not only to perform the sorption experiment in the presence of other ionic species, but also to obtain solutions that corresponded to the pH range (7-8) of the gastrointestinal tract (1).

The presence of tris buffer introduces Cl^- anions and $(HOCH_2)_3CN^+H_3$ cations into the system. According to Wu (1), as the concentration of the buffer increases, the capacity of the resins to bind bile acid anions decreases. This decrease results from the non-specificity of electrostatic interactions of positively charged ammonium groups. To be more specific, the positively charged resin bind anions indiscriminately. The quaternary amine group competes with other positively charged species in the system for bile salts, and at the same time, bile salt anions compete with other anions in the system for positively charged groups in the resins. Figure 2.18 presents the isotherms for the sorption of cholate in 2.5 mM tris buffer solution by resins Mono-QDA6 and QDA6. For both resins the binding capacity is decreased by the presence of tris buffer solution.

2.3.4 Effect of Cross-Linking on Selectivity

The sorption experiment with cholate in 2.5 mM tris buffer solution shows somewhat of a decrease in the binding capacity of the resins. However, the relative degree by which binding is depressed is not the same for resins Mono-QDA6 and QDA6. In Figure 2.21A the isotherms for the sorption of cholate in 2.5 mM tris buffer and in water, at room temperature, by the resin Mono-QDA6 are re-plotted. Similarly Figure 2.21B shows those for resin QDA6, under the same conditions. Clearly, the decrease in sorption efficiency due to the presence of buffer is much greater for the more highly cross-linked resin (QDA6) than for the lower cross-linked resin (Mono-QDA6), particularly at low equilibrium concentration. For example, at an equilibrium concentration of 0.2 mM, the extent binding capacity of resin Mono-QDA6

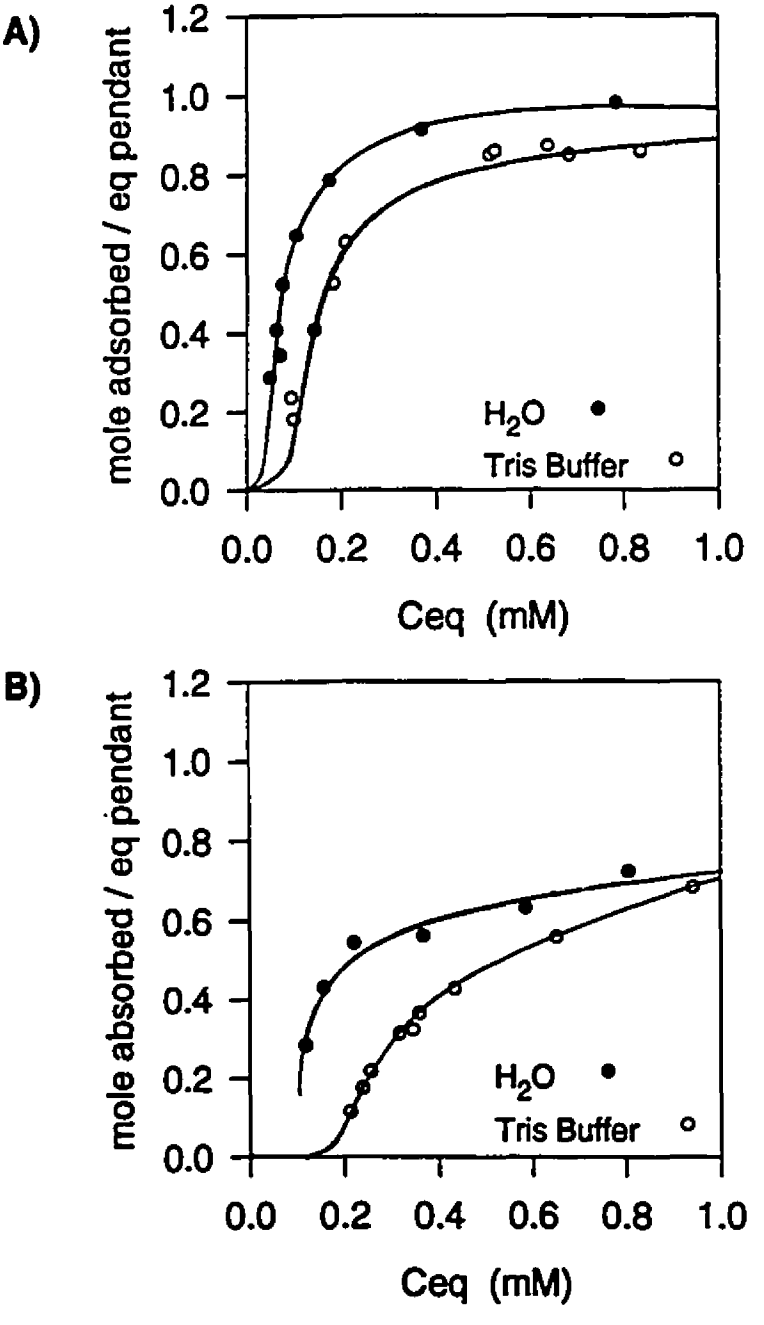


Figure 2.21 The isotherms for sorption of cholate in both water and 2.5 mM tris buffer (pH 7.2), at room temperature, by quaternized resins: A) Mono-QDA6 and B) QDA6.

is depressed by about 20% whereas that of resin QDA6 is depressed by 85%. Earlier in this chapter, it was demonstrated that the resin QDA6 has a higher degree of cross-linking due to additional cross-linking during functionalization. As indicated in (Table 2.3), the swellability of the resin Mono-QDA6 is 158% while that of the resin QDA6 is 117%.

In addition to the non-specificity of electrostatic interactions of positively charged groups on the resins, the size of competing anions is an important factor. If the anions are to interact with positive groups on the resins, they must diffuse inside the beads. For example, a resin with a relatively high degree of cross-linking exerts greater limitations on the movement of large anions, such as bile salt anions, than a resin with a lower degree of cross-linking. In other words, decreasing the limitation to the movement of cholate anions gives it a better opportunity to compete with other anions and this means that the selectivity of the resin for cholate is increased to some extent. Therefore, it can be concluded that the beads developed by new method of functionalization not only have a higher binding capacity for bile salt anions, but they also have higher selectivity for bile salt anions.

In addition to this comparison between resins Mono-QDA6 and QDA6, which emphasizes the importance of the degree of cross linking, further evidence is provided by the comparison of sorption behavior of these resins to that of the resins that were prepared by Wu (1). Figure 2.22 presents the isotherms for the sorption of sodium glycocholate in 2.5 mM tris buffer (pH 7.1), at 20.0 °C by quaternized resins Q-PDA12 and Q-PDA6 that were synthesized by Wu (1). For comparison, the isotherms for the sorption of cholate by resins QDA12, QDA6, and Mono-QDA6 are also re-plotted in (Figure 2.22). The binding capacity of the resins QDA6 and QDA12 is somewhat greater than that of the resins Q-PDA6 and Q-PDA12. This is due to differences in the degree of cross-linking of these resins. As indicated in (Table 2.3), the swellability of

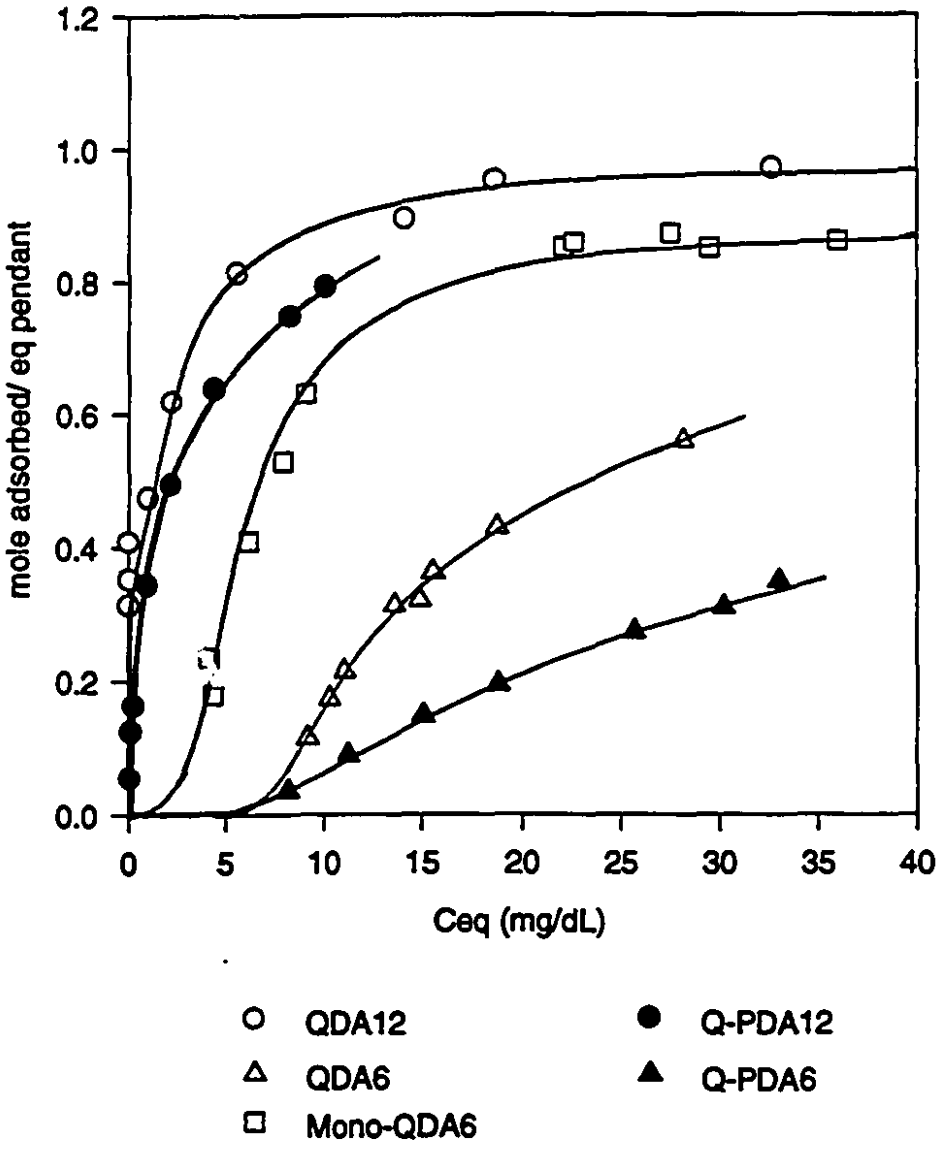


Figure 2.22 The isotherms for the sorption of NaGCA in 2.5 mM tris buffer (pH 7.1), at 20.0 °C, by quaternized resins Q-PDA12 and Q-PDA6 that were synthesized by Wu (Reference 1). For comparison, the isotherms for the sorption of cholate in 2.5 mM Tris buffer (pH 7.2) by quaternized resins QDA12, QDA6, and Mono-QDA6 are re-plotted.

Wu's PMA resin (the polymer support) was 16% while the swellability of PMA resin prepared in this study is 30%. Furthermore, the swellability of the resin QDA6 is 117% and that of the resin Q-PDA6 was 67%. These are indications of lower cross-linking density of the original PMA resin in this study.

The comparison between the sorption isotherms by resins Mono-QDA6, QDA6, and Q-PDA6 (which all have the same chemical structure but various degrees of cross-linking) indicates the importance of degree of cross-linking on the selectivity of the resins for bile acids. The rigidity of the polymeric matrix of lower cross-liked resin is less; therefore, it exerts less tension opposing the osmotic pressure in the interior of the beads, which results in a higher swellability. In any case, it can be concluded that the degree of cross-linking is an important factor in the binding behavior of polymeric resins for bile acids.

2.3.5 Effect of The Length of Hydrophobic Spacer

According to Wu (1), in addition to electrostatic interactions, hydrophobic interactions are of the importance in the binding of bile salt anions by ammonium-bearing resins. He demonstrated that the sorption capacity of poly(acrylamide) resins increases with an increase in the length of the hydrophobic spacer of the pendent groups (1,26). This is confirmed by the results of this study. Figure 2.23 presents the isotherms for the sorption of cholate in tris buffer, at room temperature, by the resins QDA12, QDA6, and Mono-QDA6. Not only does the resin QDA12 have the highest binding affinity, but it also shows the highest binding capacity for cholate anions. The shape of the isotherm of the resin QDA12 follows the sharp increase in binding capacity at low Ceq, whereas the isotherm of the resin QDA6 and Mono-QDA6 have a distinct S shape characteristic. The S shape indicates positive cooperativity. As shown in Figure 2.23, the degree of cooperativity in the resin Mono-QDA6 is greater than in the resin QDA6

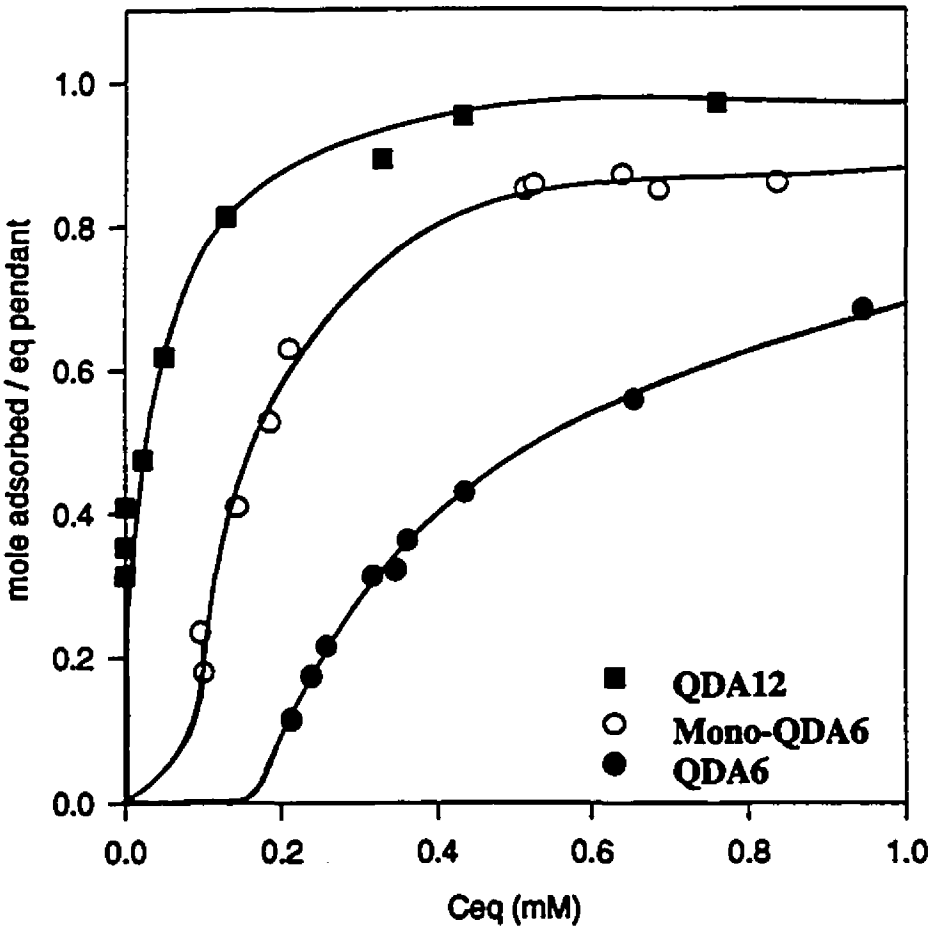


Figure 2.23 The isotherms for the sorption of cholate in 0.0025 M tris buffer (pH 7.2), room temperature, by quaternized resins, showing the effect of length of the hydrophobic spacer.

(the cooperativity parameters are presented in section 2.3.7). The isotherm of the resin Mono-QDA6 shows significant improvement as compared with the isotherm of the resin QDA6; however, it is not as effective as the resin QDA12. The isotherms of Mono-QDA6 and QDA12 resins reveal that both hydrophobic interactions and the degree of cross-linking make substantial contributions to the binding capacity of poly(acrylamide) resins. However, the hydrophobic effect seems to have a greater contribution than the degree of cross-linking.

2.3.6 Sorption Characteristics of QDA12 and QTA6 Resins

It was also established by Wu (1,26) that increasing the number of positively charged ammonium groups per pendent group increases the binding capacity of the resins. This finding shows the importance of electrostatic interaction for the sorption process. Figure 2.24 presents the isotherms for the sorption of cholate in water, at room temperature, by the resins QDA12 and QTA6, and Figure 2.25 shows the corresponding isotherms for cholate in 2.5 mM tris buffer solution.

As is shown in Figure 2.24 and 2.25, at low equilibrium concentrations resin QTA6 binds less cholate anions than resin QDA12; however, at higher equilibrium concentrations the binding of cholate by the resin QTA6 is higher than that by resin QDA12. Furthermore, the initial slope of sorption isotherms, at low Ceq, for the resin QTA6 is much smaller than the resin QDA12, indicating the hydrophobic interactions are less importance in the QTA6 resin. In the case of the resin QDA12, both electrostatic and hydrophobic interactions are important parameters; however, in the case of the resin QTA6, the hydrophobic interactions are of minimal importance. Interestingly, the total number of carbons in the hydrocarbon spacers of the pendent group of the QTA6 resin is the same as that for the QDA12 resin. However, the presence of the amine which divides the hydrocarbon spacer into two groups of 6 methylenes, dramatically decreases the hydrophobicity of pendent groups on the QTA6 resin. Therefore, for resin QTA6 the

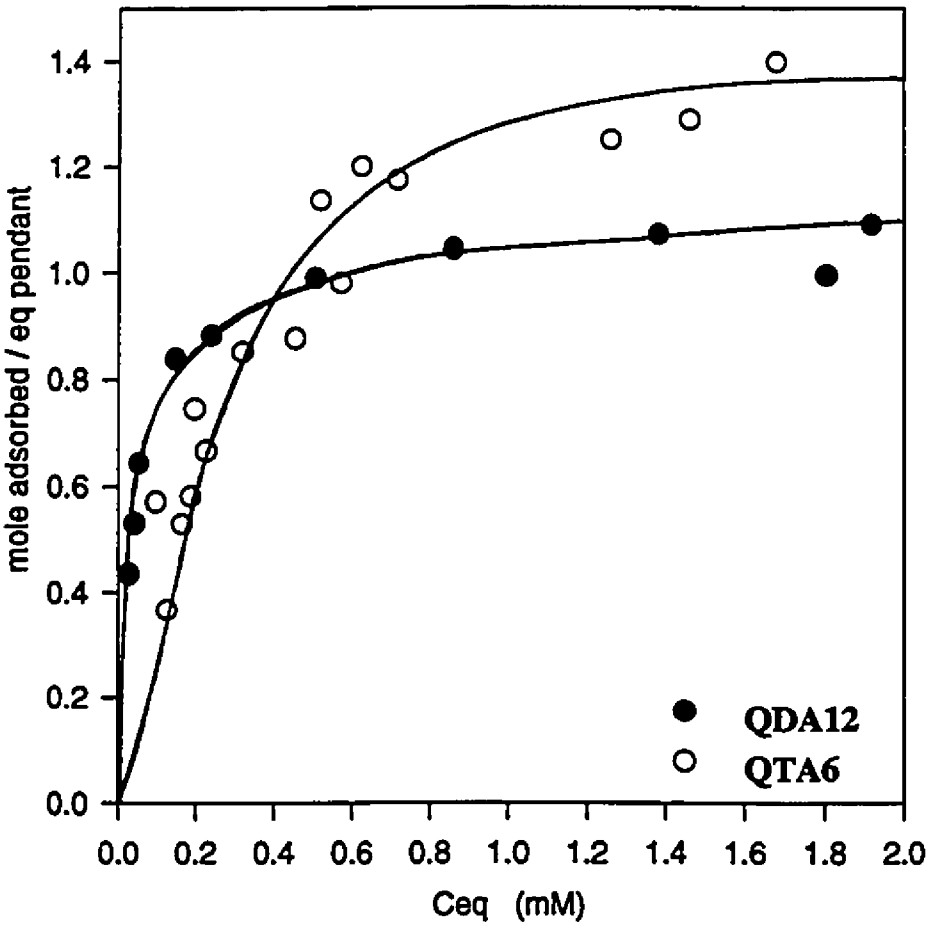


Figure 2.24 The isotherms for the sorption of cholate in water, at room temperature, by quaternized resins QDA12 and QTA6 showing the effect of the number of quaternized ammonium groups in each pendent group.

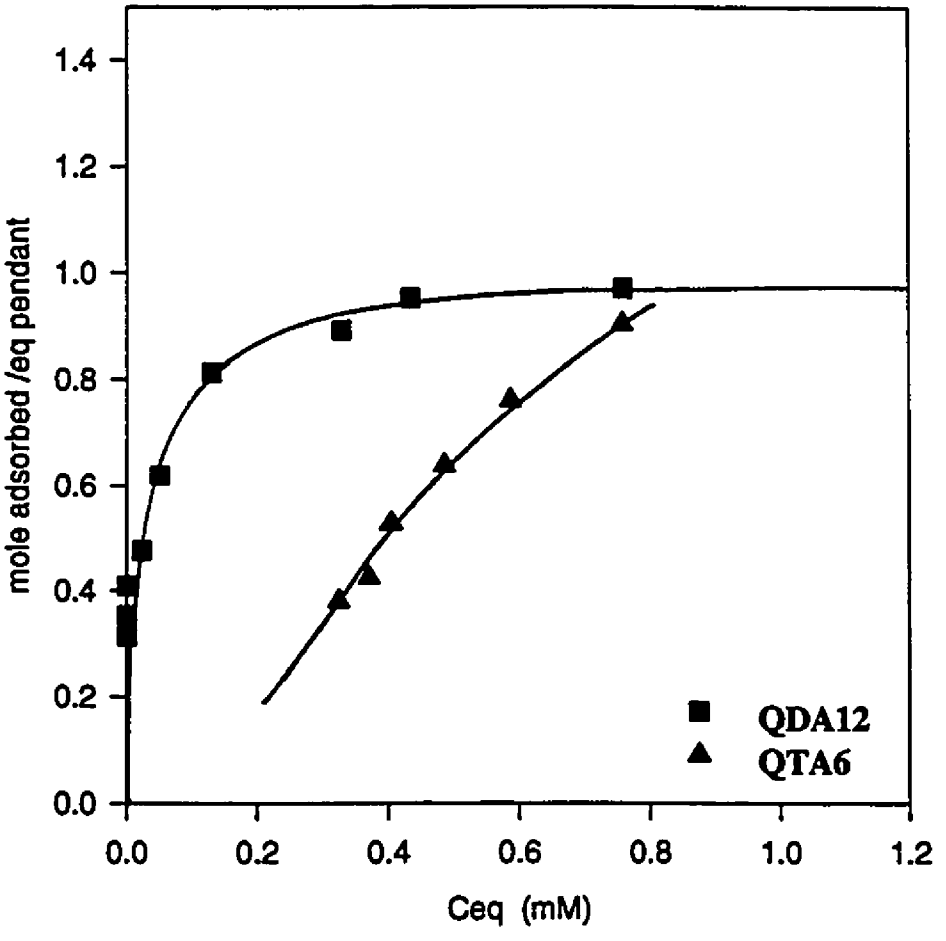


Figure 2.25 The isotherms for the sorption of cholate in 2.5 mM tris buffer (pH 7.2) at room temperature by resins QDA12 and QTA6.

hydrophobic effect is not as important as for the QDA12 resin. It is possible that in addition resin QTA6 may have a higher degree of cross-linking due to the larger number of amines per pendent group.

2.3.7 Cooperativity Parameter

The isotherms for the sorption of cholate in both water and tris buffer solution by the resins Mono-QDA6 and QDA6 show the sigmoidal characteristic (Figure 2.17 and 2.18). According to Wu, Brown, and St-Pierre (26), the S-shaped isotherms indicate positive cooperativity due to hydrophobic interactions and perhaps hydrogen bonding among bile salt anions bound at adjacent positions within the resin (1).

Furthermore, the cooperative binding process of surfactants on polymeric resins has been evaluated (31-33). According to Okazaki and Osada (31,32), when a surfactant undergoes a stoichiometric reaction with a polymer, the overall stability constant can be calculated as follows:

$$\text{Polymer} + \text{Surfactant} \overset{K}{\rightleftharpoons} \text{Complex}$$

$$K = (C_i - C_{eq}) / \{C_p - (C_i - C_{eq})\}C_{eq}$$

where C_p is the moles of functional group on the polymer in the total volume of the solution, C_i is the initial concentration of the surfactant, C_{eq} is the surfactant concentration surrounding the polymer at equilibrium, and K is the overall stability constant. On the basis of the Zimm-Bragg theory for helix-coil transition (34), Satake and Yang (35) derived the binding constant for cooperative binding. They obtained the following expression:

$$K = K_o u = 1/(C_s)_{0.5}$$

where K_o is the equilibrium constant for the binding of a surfactant molecule at an isolated binding site on a polymer network, "u" is a cooperativity parameter

characterizing the interaction between adjacent bound surfactants, K is the overall stability constant, and $(C_s)_{0.5}$ is the equilibrium free surfactant concentration at the half-bound point. In the Satake-Yang treatments the cooperativity parameter, u, expresses an equilibrium constant for the aggregation of bound surfactant molecules; for example, for a process:

$$2(OD) \rightleftharpoons OO + DD$$

$$u = (OO)(DD) / (OD)^2$$

where (OO) represents the two neighboring free sites and (D) an occupied site.
The cooperativity parameter can be determined from the slope of the binding isotherm at the half-bound point according to the following equation (35):

$$(d\beta/dC_s)_{0.5} = \sqrt{u}/4$$

where β is the degree of binding which is define as the molar ratio of the bound surfactant to the total binding sites on the polymer matrix.

Using the above equations, the equilibrium constants and the cooperativity parameters for the binding of cholate by the various poly(acrylamide) resins that were synthesized for this study were determined. Table 2.4 presents the resulting values for cooperativity parameters and binding constants for the sorption of cholate anions in both water and a tris buffer solution by resins Mono-QDA6, QDA6, and QTA6.

Since the pendent groups of resin Mono-QDA6 and QDA6 are small and the hydrophobic interaction between bile salt anions and pendent groups are of minimal important, the cholate anions are not able to align themselves with the pendent groups. Therefore, the binding of cholate at low equilibrium concentration is low. However,

when the concentration of cholate anions within the beads increase, they start to self-aggregate and create micelles.

Table 2.4 The binding constants and cooperativity parameters for the sorption of cholate by various poly(acrylamide) resins.

Resins	Solvents	K $L.mol^{-1}$ ($\times 10^{-3}$)	K_o $L.mol^{-1}$ ($\times 10^{-3}$)	Cooperativity parameter, u ($\times 10^{-2}$)
Mono-QDA6	H_2O	14	0.07	2.0
Mono-QDA6	Tris-buffer	6.7	0.06	1.1
QDA6	H_2O	7.3	0.06	1.3
QDA6	Tris-buffer	2.8	0.04	0.67
QTA6	H_2O	5	0.06	0.81
QTA6	Tris-buffer	2.6	0.04	0.67

The cross-linking density plays an important role in the degree of cooperativity for the binding of cholate by poly(acrylamide) resins. The cooperativity parameter of resin Mono-QDA6 is about 65% greater than that of resin QDA6. Furthermore, the values of K_o (the equilibrium constant for the binding of cholate at an isolated binding site) for both resins Mono-QDA6 and QDA6 are similar, as expected. However, the overall binding constant of resin Mono-QDA6 is almost twice that of resin QDA6. Clearly, this means that the binding of cholate at an isolated binding site of both resins Mono-QDA6 and QDA6 are almost identical. On the other hand, resin Mono-QDA6 with less cross-linking density allows a greater number of cholate within its bead cavities.

2.4 Conclusions

Ammonium-bearing sorbents for bile salt anions have been synthesized by two different methods of functionalization. The prime objective was to develop an alternative method for the functionalization of lightly cross-linked PMA resin that eliminates the additional cross-linking during the reaction. Poly(methyl acrylate) beads were functionalized by both the previous and the new method using 1,6-hexanediamine as the functional unit.

The strategy for the new method of functionalization was to attach a protecting group to one of the amines of functional units; the mono-protected diamine was then reacted with PMA. The mono-protected diamine (N-tert-butoxycarbonyl-1,6-hexanediamine) was successfully synthesized and the functionalization procedure was developed by setting up several reactions having various conditions. It was concluded that PMA can be functionalized successfully with mono-protected diamine, either in a solvent (such as DMF having a high concentration based on mono-protected amine) at 150-160 °C or without any solvent at 120-130 °C. The protecting Boc group was successfully removed by the reaction with hydrogen fluoride (HF).

The swelling characteristics of the synthesized poly(acrylamide) resins were studied, and the effect of temperature on the degree of swelling was evaluated. It was concluded that temperature does not have appreciable effect on the swellability of the resins QDA12 and QDA6. However, in the case of the resin Mono-QDA6, the swellability increases as the temperature increases.

Furthermore, the degree of swelling and shrinking of these resins that results from the sorption and desorption of cholate depends on the composition of the resin. For resin QDA6 the change in the volume due to sorption and desorption of cholate anions is insignificant. On the other hand, the change in the degree of swelling and shrinking of the resins QDA12 and Mono-QDA6 is substantial (about 10%). It should be noted that the binding capacities of resins QDA12 and Mono-QDA6 are relatively higher than that

of the resin QDA6. Therefore, it appears that there is a relationship between the binding capacity and the degree of swelling/shrinking of the resins.

The isotherms were determined for the sorption of cholate in tris buffer and water. These indicate the superiority of the Mono-QDA6 resin (which was developed by the new method) over QDA6 resin (which was prepared by the previous method). In spite of the higher sorption capacity of Mono-QDA6, its amine functionality value was observed to be lower than that of the QDA6 resin. This is thought to be a steric effect reflecting the degree of local cross-linking.

For further evaluation, a microstructure model of three-dimensionally cross-linked PMA beads was introduced, based on the idea of the degree of local cross-linking. The assumption was that the degree of cross-linking is not uniform throughout the polymeric beads; therefore, the beads possess a variety of degrees of local cross-linking throughout their matrix. Here, the polymeric beads were divided into 3 distinguishable regions according to the size of functional units and bile salt anions. Region I was assigned to the regions within the beads that permit the diffusion of the free diamine molecules, but which cannot be penetrated by either bile salt anion or mono-protected diamine. Region II was assumed to be the regions with a medium degree of cross-linking into which both free diamine and protected diamine can diffuse but bile salt anions cannot enter. Finally, region III was stated to be the areas of low cross-linking which are accessible to the large anions such as bile salt anions.

Additional cross-linking not only creates a higher overall degree of cross-linking but it also results in a lower density of positively charged ammonium groups within the region III of QDA6 resin. As was pointed out, the most important areas within the beads are the areas of a low degree of local cross-linking (region III). Basically, that is why the resin QDA6 has such a low binding capacity for bile salts.

One of the most important aspects of the sequestering of bile acid anions is the selectivity factor. Increasing the selectivity of resins for bile salt anions has been the

major area of research for many years. Here, it was determined that the degree of cross-linking might play an important role in the selectivity. The comparison between the depression in binding efficiency of resins Mono-QDA6 and QDA6 for cholate in a tris buffer indicates that the relative selectivity of the resin Mono-QDA6 for bile salt anions is higher than that of the resin QDA6. Thus, the higher degree of cross-linking decreases the selectivity of the resins. In other words, the selectivity of a resin for bile salt anions can be substantially improved by lowering the degree of cross-linking.

The effect of the number of ammonium groups per pendent group and the degree of hydrophobicity of the resins was also studied. The sorption characteristics of resins QTA6 and QDA12, which each have 12 hydrocarbon (CH_2) groups in their pendent group, were compared. Even though resin QTA6 has a higher number of ammonium groups per pendant, it possesses a lower binding capacity for sodium cholate than resin QDA12. The best explanation for this phenomenon is based on a consideration of the degree of hydrophobicity, in which the pendent group of QTA6 resin is less hydrophobic than that of QDA12 resin. Resin QDA12 not only has a higher binding capacity and efficiency, but it also has a higher selectivity for bile salt anions. These results indicate the superiority of hydrophobic effect over the number of ammonium groups per pendent group at low equilibrium concentrations.

The degree of cooperativity of the resins was calculated using the slope of the isotherms at half-bound point. The binding of cholate by resin Mono-QDA6 involves a greater degree of cooperativity than the resins QDA6 and QTA6. The results suggest that the resin with a lower degree of cross-linking has a greater cooperative binding with cholate.

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CHAPTER THREE

SORPTION OF BILE SALT ANIONS BY POLY(ACRYLAMIDE) RESINS HAVING VARIOUS ORGANIC COUNTER-IONS

3.1 Introduction

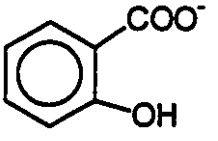
As described in Chapter One, the basic concept behind the sequestering of bile acid anions by the majority of insoluble organic sorbents is the ion-exchange process. Application of ion-exchangers as bile salt sequestrants cannot be fully exploited without an understanding of the physical and chemical principles that govern the exchange reactions. In order to develop an appropriate sequestrant which has a high selectivity for bile salt anions, the ionic properties of ion-exchanger must be understood, and their interactions with other anions should be investigated.

Until now, most of the experiments performed on the sorption of bile acid anions by insoluble organic sequestrants have used ion-exchange resins having chloride counter-ions (and in a few cases, iodide counter-ions). So far, the main strategy that scientists have been trying to follow is to increase the capacity and/or affinity of exchange resins for bile acid anions. This is a direct approach to improve the binding characteristics of the resins. However, another approach that might be considered is to load the exchanger with a counter-ion of low relative affinity. As a result, when a bile salt anion is introduced to the system, the counter-ion of low relative affinity would be displaced easily from the resin. In other words, loading the resin with an ion of lower selectivity than that of the bile salt anions would result in the preference of the exchanger for the bile salt anions. Therefore, an improvement in binding efficiency of the resin for bile acid anions might be expected.

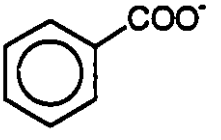
One of the important aspects which should be considered in the ion exchange process of the bile salt anions is that they are relatively large anions composed of hydrophobic and hydrophilic portions. Thus, the bile salt anion cannot be considered as a point charge, and the movement of ions in and out of the resin is not controlled solely by electrostatic interactions. Therefore, the exchange process of bile salt anions cannot be treated in the same way as that of small ions such as Cl^-. This chapter provides a simplified review of the selectivity of large organic ions, and the major factors that

promote the selectivity will be discussed. Furthermore, the important aspects of the ion-exchange process of large organic ions will be reviewed, and the significance of water structure and hydrophobic interactions will be discussed.

In this chapter, the selectivity of poly(acrylamide) resins having various kinds of counter-ions is presented. The sorption of bile salt anions by resins QDA12, QTA6, and Mono-QDA6 having organic counter-ions such as benzoate, salicylate, acetate, and propionate is evaluated. The selected ions are in the form of carboxylate anions, similar to the bile salt anions (Figure 3.1).



salicylate



benzoate

CH_3COO^-

acetate

$CH_3CH_2COO^-$

propionate

Figure 3.1. The organic anions used as the counter-ion in poly(acrylamide) resins.

Furthermore, a comparison will be made between the benzoate and salicylate counter-ions, and the effect of additional hydrogen bonding due to the hydroxyl group on the benzene ring will be discussed.

The interest of this work is to provide evidence that the organic counter-ions can affect the sorption of bile salts by poly(acrylamide) resins. This alteration is demonstrated to be such that the counter-ions of low affinity improve the sorption of bile salts, whereas the counter-ions of high affinity decrease the binding capacity of resins for bile salts.

3.2 Experimental

3.2.1 Replacement of Cl^- With Organic Counter-Ions

The counter-ions of poly(acrylamide) resins, prepared by the methods described in Chapter 3, were separately replaced by the organic counter-ions benzoate, salicylate, propionate, and acetate. The sodium salts of benzoic acid, salicylic acid, propionic acid, and acetic acid were obtained from Aldrich Chemicals, and they were used as received. To replace the Cl^- counter-ion on the resin, a few grams of cross-linked poly(acrylamide) beads were added to an aqueous salt solution (~0.5 M) and allowed to stir for 24 hours. The beads were then removed by filtration and washed with distilled water; they were placed in a fresh salt solution and allowed to stir for another 24 hours. This process was repeated 3 times. The beads were washed several times with warm, then cold, distilled water followed by 95% ethanol. At the end, the beads were dried in a vacuum oven (at 50 °C) for 2 days.

3.2.2 Sorption Procedure

The stock solutions of the sodium salt of cholic acid, NaCA, were prepared with a concentration of about 50 mg/dL in 2.5 mM aqueous tris (hydroxymethyl)-aminomethane-HCl buffer (pH 7.2) and in distilled water, separately. The resins were weighed (ranging from 5-15 mg) into a series of bottles of different sizes. The volume of the stock solution added to the bottles ranged from 8-32 mL. The samples were allowed to attain equilibrium under shaking with a mechanical shaker, at 120 oscillations per

minute for 12 hours. The concentrations of bile salt in the solutions were then analyzed by HPLC, following the procedures described in Chapter 2 (Section 2.3.1).

3.3 Theoretical Background

In this section, a simplified version of the theoretical aspects of the ion-exchange process of large organic ions is presented. To avoid complications, it is assumed that no other ionic species are present in the system.

3.3.1 Selectivity

In general, the simplest definition that can be extracted for selectivity is that *the resin exhibits some preference for one ion relative to other ions* (1). However, it should be remembered that the effect of the solution phase on the selectivity of the resin for an ion is one of the most important aspects. Therefore, the selectivity can be defined quantitatively as follow:

$$R\text{—}N^+C^- + CA^- \rightleftharpoons R\text{—}N^+CA^- + C^- \qquad \text{(eq. 3.1)}$$

where, R-N$^+$ is considered to be the anion exchange resin (the exchange site of PMA), CA$^-$ is the cholate anion, and C$^-$ is the counter-ion (exchangeable ions in the solution). The selectivity coefficient (or relative affinity coefficient), K, is defined by (2):

$$K_{CA^-/C^-} = (X^*_{CA^-} / X^*_{C^-})(X_{C^-} / X_{CA^-}) \qquad \text{(eq. 3.2)}$$

where X^* is the mole fraction of counter-ions in the ion-exchange phase, and X is the mole fraction of counter-ions in the solution phase; then,

$$X^*_{CA^-} + X^*_{C^-} = 1, \text{ and } \quad X_{CA^-} + X_{C^-} = 1$$

Since both anions are monovalent, either their molar or molal concentration can be used. Any value of K_{CA^-/C^-} that is different from unity indicates the relative difference in preference of the solution and ion-exchange phase for the two competing ions. For example: a K_{CA^-/C^-} value of less than 1 signifies that the resin binds C^- more strongly than CA^-, and a value of selectivity that is greater than one signifies that the resin prefers CA^- over C^-.

It should be emphasized that in an exchange process the relative affinity of both ions for both phases must be considered. In other words, strong interactions of an ion with a resin do not guarantee a high selectivity for that ion, unless there is not a similar strong interaction of that ion with the co-ion or solution in the external solution phase. Therefore, selectivity can be considered as the competition among the co-ions in the solution phase, the functional groups of the resin, and the solution phase.

The preference for one ion over another in a specific phase is strongly dependent on the effect of the thermodynamic properties of that phase. The thermodynamic effects can be summarized in the term *activity*, which actually is a measure of escaping tendency (2). Therefore, the higher the activity of an ion in solution, the greater its escaping tendency. For example, if the resin itself has no preference for CA^- or C^-, the interactions between the ions and solution phase determine the selectivity factor. Therefore, if α_{CA^-} is greater than α_{C^-}, then the ion-exchange resin will contain more CA^- ions than C^- ions because of the greater tendency of CA^- to escape from solution.

In addition to the activity, other factors that can affect the selectivity of a resins are the hydrated size of the ions and the physical properties of the resin, e.g., its mechanical (or elastic) properties (3,4). According to the elastic property of cross-linked resin, the resistance of the resin matrix to stretching causes the resin to prefer ions of smaller hydrated volume. Therefore, in the case of large ions and highly cross-linked resins, the elastic properties of the cross-linked resin and the hydrated size of the exchanging ion determine the course of the exchange process. These factors of cross-

linking and hydrated size can be expressed by the addition of an osmotic pressure-volume term to the free-energy change of exchange process (4):

$$RT \ln K'_{CA^-/C^-} = \Pi(V^*_{C^-} - V^*_{CA^-}) \qquad \text{(eq. 3.4)}$$

Where $V^*_{C^-}$ and $V^*_{CA^-}$ are the hydrated volumes of CA^- and C^- in the resin, respectively, and Π is the swelling pressure (which is called elastic counter-pressure) in the resin. As an example, if $V^*_{CA^-} > V^*_{C^-}$ then $K'_{CA^-/C^-} < 1$; in other words, of two exchangeable counter-ions, the one with the smaller hydrated ionic radius (the anion C^-) will be preferred by the exchanger. For highly swollen resin or small ions, this term is negligible (2).

3.3.2 Basic Thermodynamic Aspects of the Ion-Exchange Process of Large Organic Ions

The thermodynamic basis of ion-exchange phenomena has been well established by Donnan and Guggenheim. The detailed application of the Donnan theory in the ion-exchange process of bile acid anions has already been presented by Léonard (6). In the Donnan theory, the cross-linked polyelectrolyte network of an ion-exchange bead can be considered as a separate phase, and the forces in the network can be evaluated by the osmotic pressure difference between the outer aqueous and inner resin phase. The well-known Donnan equation for the exchange of a pair of monovalent ions, such as bile salt anion and C^- anion, has the form (5):

$$RT\ln K_{th} = RT\ln K_{CA^-/C^-} + RT\ln(\gamma''_{CA^-} / \gamma''_{C^-}) - RT\ln(\gamma'_{CA^-} / \gamma'_{C^-}) + P(V''_{CA^-} - V''_{C^-}) \qquad \text{(eq 3.5)}$$

where, K_{th} is the thermodynamic equilibrium constant, γ'' is the activity coefficient of ions in resin phase, γ' is the activity coefficient of ions in aqueous phase, P is the osmotic

pressure, and V" is the partial molar volume. The last term in this equation, containing the osmotic pressure and partial molar volume, does not contribute significantly to the exchange process of small ions; however, in the case of large organic ions, these parameters can become very important (7).

In the purely electrostatic models, the ion-ion interaction can be described by an activity coefficient; however, the non-ionic residue of an organic molecule introduces additional terms into equation 3.5.

$$RT\ln K_{th} = RT\ln K_{CA^-/C^-} + RT\ln(\gamma''_{CA^-} / \gamma''_{C^-}) - RT\ln(\gamma'_{CA^-} / \gamma'_{C^-}) + P(V''_{CA^-} - V''_{C^-}) + \Delta H_{ns} + \Delta H_{nn} - T\Delta S_{ns} - T\Delta S_{nn} \quad \text{(eq 3.6)}$$

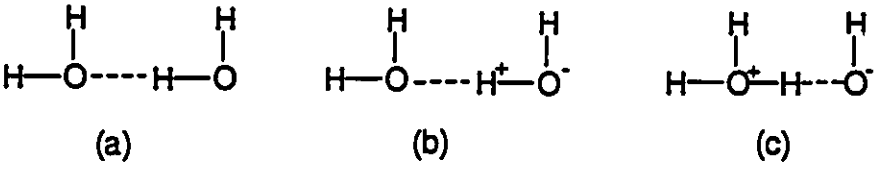
In the case of large organic counter-ions, the interactions in the two phases between the nonpolar part of the ion and the solvent are denoted by the subscript (ns), and the interactions between the nonpolar part of the ion and nonpolar part of the resin are denoted by the subscript (nn).

In the exchange process of large organic ions, the change in the structure of water, the hydrophobic interactions. and rotational hindrance play important roles. Basically, these phenomena arise as a result of the effect of the non-ionic residue of an organic ion. In addition to the ability of water to form clusters, the interactions of the nonpolar part of the organic ion with water molecules promote a change in water structure. Furthermore, there is a nonpolar-nonpolar interaction between the hydrophobic portion of the organic ion and the hydrophobic region of the resin matrix which creates the hydrophobic interactions. The interactions between the nonpolar part of the organic ions and resin matrix result in the loss of a degree of freedom of the organic molecule.

3.3.3 Factors Underlying the Ion-Exchange Process of Large Organic Ions

3.3.3.1 Change in Water Structure and Hydrophobic Bonding

Part of the complexity and diversity of the ion-exchange behavior in aqueous solutions is due to the unusual nature of liquid water. Compared to other common liquids, water molecules at room temperature form a highly hydrogen-bonded network with relatively strong intermolecular forces (7). In the water cluster, there are a maximum of four hydrogen bonds per water molecule, and it is assumed that the hydrogen bonded molecules are in equilibrium with free water molecules (8). Among the resonance structures of hydrogen bonded water molecules (a, b, and c), the contribution of structure c is the one which gives the covalent character of the water bonds (9).



(a) (b) (c)

The water network tends to reject other species unless these species have a hydrophilic group or a charged group. The water-water interactions have the tendency to force the large and poorly hydrated ions together to minimize their disturbance of water structure (10). In some respects, this tendency of water molecules creates ion-pairs between the ion and exchange sites on the exchanger matrix.

In hydrophobic bond formation, the main contribution to the free-energy change lies not in the various attractive energy changes but rather in an increase in entropy (9). The formation of hydrophobic bonds is attributed to the change of the water structure brought about by the organic molecules (8). According to Nemethy and Scheraga (8), the nonpolar organic molecules promote the structure of water and thereby bring about a

decrease in the entropy of the solution. However, when the nonpolar solute particles move into the resin phase, coalesce or adhere to other nonpolar parts of the system, there is an increase in entropy and therefore a decease in free energy of the system, thereby decreasing the probability of water structure formation. The removal of organic ions from the solution phase into the hydrocarbon (resin) phase then causes a decrease in water structure, which corresponds to an increase in entropy and as a result a decrease in the free energy of the system. Therefore, it can be concluded that a bond is hydrophobic when its formation is accompanied by an overall increase in entropy.

As was pointed out, besides the effect of water structure which promotes the removal of organic species from the aqueous phase, the transfer of organic ions from the aqueous phase to the resin matrix can result from the direct interaction between the hydrophobic portions of the organic ions and the resin matrix. The flexible structure of the hydrocarbon chain in the resin can promote the formation of enclosed pockets for organic species. Due to the loss of the translational and rotational degrees of freedom of the organic ion inside the matrix cavities, there is a strong decrease in entropy.

As a result of the special qualities of the ion-exchange process of organic ions, the transfer of an ion from an aqueous to a resin phase can be dominated by the change in the water structure as well as by direct interactions between the hydrocarbon parts of the organic ions and the resin matrix. The change in water structure is accompanied by an entropy increase, while the direct hydrophobic interaction involves a negative entropy change. It should be noted that the pure electrostatic interactions no doubt also play an important role in the exchange process of large organic ions.

3.3.3.2 Ionic Interactions

In the ion-exchange process of organic anions, electrostatic interactions are also important factors. In general, ions can lower their electrostatic free energy by attracting ions of opposite charge (10). In the case of smaller ions, each ion can be considered as a point charge on a spherical particle. However, since large organic ions consist of a

number of atoms, they cannot be considered as single point charges. This fact creates complexity in predicting the selectivity of the exchange process, and the full discussion of these interactions is beyond the scope of this chapter.

3.3.3.3 The Effect of Cross-Linking Non-Uniformity of Exchange Sites

It is known that cross-linking has a pronounced effect on the exchange process of a resin. In a simple manner, it is thought that the very large organic ions cannot physically diffuse into a highly cross-linked resin, and they are screened out (the *sieve effect*). However, in addition to the simple sieve effect, cross-linking affects the ion-exchange property of the resins by creating a non-uniform environment and by changing the swelling characteristics of the exchanger. It is well established that the magnitude of selectivity is a unique function of the average amount of water per exchange group (2). When the exchanger is highly swollen, the average number of water molecules per fixed grouping is large; for each exchange site, there will be a large configurational volume that counter-ions can attack.

The fixed groups within an exchange matrix do not all behave in an identical manner with respect to their exchange property (11). The variation in exchange property within a resin results from environmental differences at the exchange sites. One major factor which contributes to this environmental non-uniformity is the length of chain segments between neighboring cross-links (11). In other words, the degree of localized cross-linking around each group is not constant but varies to some extent. This is illustrated by the structural model presented in the previous chapter (Figure 2.19). It is obvious that the non-uniformity of exchange sites affects the "selectivity" property of different areas within the resin. In addition to the difference in the local degree of cross-linking, which creates non-uniformity within the resins, the conformational variations in and around the pendent group may also play an important role in the non-uniform property of the exchange sites.

3.4. Results and Discussion

3.4.1 Effect of Aliphatic Organic Counter-Ions

Figure 3.2 presents the isotherms for the sorption of cholate in distilled water, at room temperature, by reference resin QDA12 which shows the effect of replacing the Cl^- counter-ions with organic counter-ions (propionate and acetate) on the sorption efficiency. There is a dramatic increase in the binding capacity for cholate anions of the resin having organic propionate or acetate counter-ions. Compared to the resins with acetate, those with propionate counter-ions have a higher sorption capacity for bile salt anions, which represents a lower relative affinity of the resin for propionate anion. This result indicates that the propionate anions leave the resin phase more readily than acetate.

The ion-exchange sorption isotherms indicate that there is stronger affinity of the resin for the acetate anion than for the propionate. Since the organic part of the propionate is expected to form stronger hydrophobic interactions than that of the acetate anions, this is an unexpected result. In other words, it is expected that the acetate anions should leave the resin phase more easily than propionate anions. Even though the organic part of the acetate ion is smaller than that of propionate, the acetate ion seems to have a stronger interaction with resin matrix. It appears that although there may actually be somewhat stronger hydrophobic interactions between the resin matrix and propionate, the electrostatic interactions between the resin and propionate are weaker. The stronger intermolecular interactions between the acetate and exchanger are representative of a higher effective dielectric constant in the vicinity of carboxylic group of acetate ion (12).

As discussed previously, in water the organic ions promote structure formation. This is disrupted when the organic ions migrate into the resin phase and this results in an increase of entropy. Furthermore, it has been pointed out that there is a hydrophobic interaction between the resin matrix and organic ions. Keeping these facts in mind, it is expected that the organic ions would prefer the resin phase rather than aqueous phase.

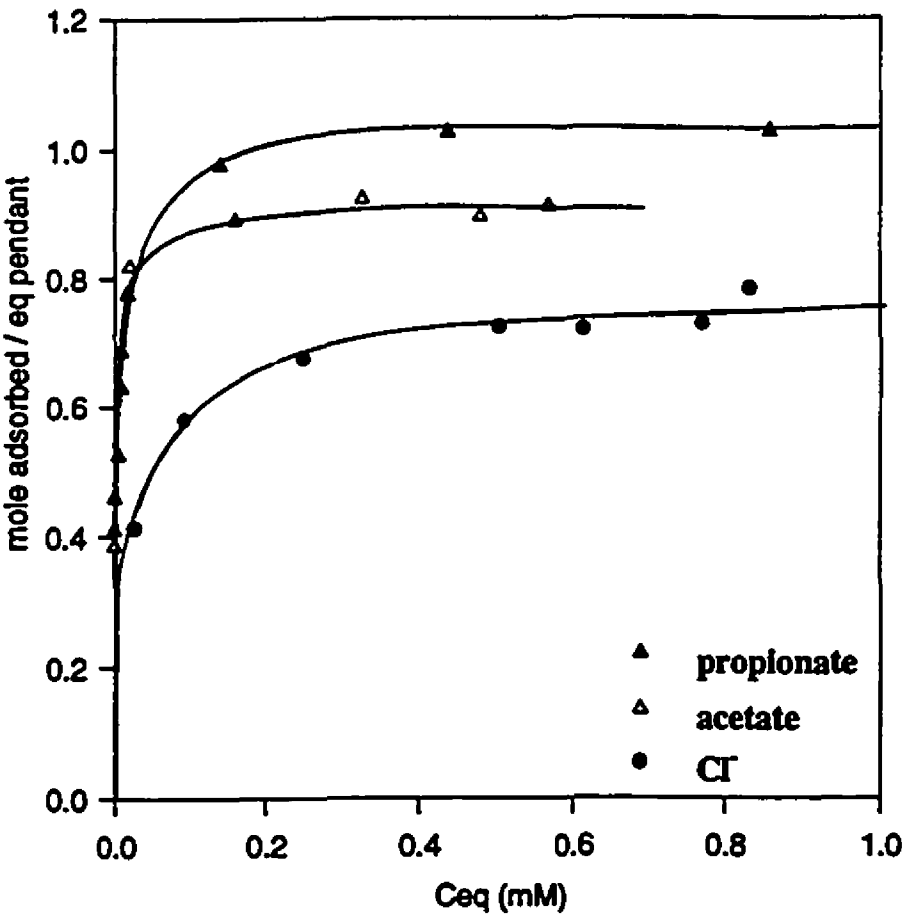


Figure 3.2 The isotherms for the sorption of cholate in distilled water at room temperature by the quaternized QDA12 reference resin, comparing the effect of aliphatic organic counter-ions with Cl^- counter-ion.

However, the sorption experiments suggest that propionate and acetate anions are more easily displaced form the resin phase to the aqueous phase than Cl^-. There has not been any particular explanation for this behavior in the literature; however, it seems likely that the reason for higher preference of the resin for Cl^- than propionate or acetate is related to the electrostatic characteristics of these ions. It appears that the electrostatic interaction between the resin and Cl^- is stronger than that with propionate or acetate. As a result it might be hypothesized that the hydrophobic and electrostatic interactions of the resin with propionate or acetate and the effect of entropy loss in the system due to the migration of propionate or acetate into the solution phase are all less important than the ionic interactions between the resin and Cl^-.

To pursue this matter further, it is helpful to look at literature results of the reverse process (sorption of aliphatic carboxylic acids by ion-exchange resins). Starobinets and Gleim (12) investigated the sorption of aliphatic carboxylic acids of various hydrocarbon chain lengths by Dowex-1 resin (which is also strong-base anion exchanger). Figure 3.3 shows the isotherms for the sorption of aliphatic carboxylic acids by Dowex-1 resin having Cl^- counter-ions. The results indicate a increase in sorption with hydrocarbon chain length, except for the formic, acetic, and propionic acids. The extent of ion-exchange was seen to decrease form formic to propionic acid and to increase again for the higher hydrocarbon chains. The increase in selectivity of the resin with hydrocarbon chain lengths may be due to the hydrophobic effect. On the other hand, the unexpected decrease in the sorption isotherms from formic to propionic is more difficult to interpret. The authors suggested that the reason for this decrease is the decrease in effective dielectric constant in the immediate vicinity of the carboxyl-group from formic to propionic acid. Perhaps the most important aspect of this results, as related to the present work, is that both acetate and propionate are only poorly bound to poly(acrylamide) resins.

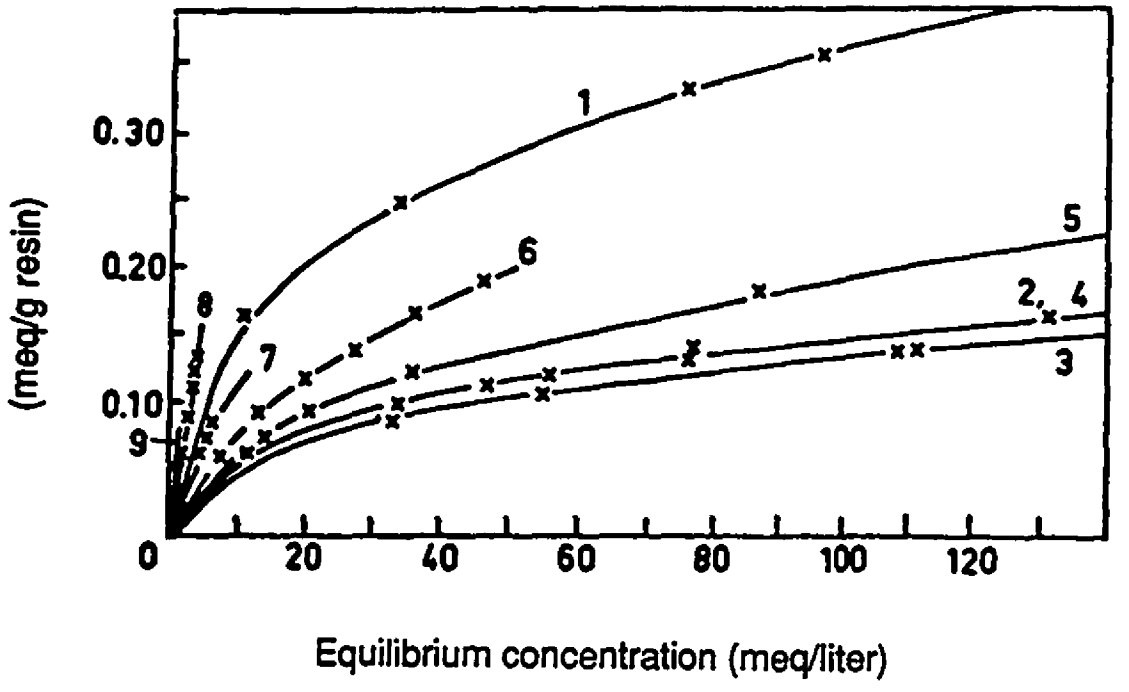


Figure 3.3 The isotherms for the sorption of 1) formic, 2) acetic, 3) propionic, 4) butyric, 5) valeric, 6) caproic, 7) heptanic, 8) capryric, 9) pellargonic acids by Dowex-1 resin having Cl^- counter-ion (Reference 12).

3.4.2 Effect of Aromatic Organic Counter-Ions

Benzoate and salicylate were used to study the effect of aromatic counter-ions on the sorption behavior of poly(acrylamide) resins. Figure 3.4 presents the isotherms for the sorption of cholate in distilled water, at room temperature, by reference resin QDA12 having benzoate and salicylate counter-ions. The reference resin QDA12 with benzoate or salicylate counter-ions has a lower binding capacity than the same resin with Cl^- counter-ions. This suggests stronger interactions between the resin and benzoate or salicylate than with the Cl^- anion. Furthermore, the sorption isotherms indicate that the resin with the salicylate counter-ion has the lowest binding capacity for cholate, indicative of a relatively strong affinity of the resin for salicylate anions. These anions have a similar structure (Figure 3.1) except that the salicylate has a hydroxyl group on the benzene ring. This hydroxyl group is available for hydrogen bonding involving salicylate anions bound at adjacent sites. As expected, the effect of hydrogen bonding greatly increases the preference of the resin for salicylate counter-ions.

Figure 3.5 presents the isotherms for the sorption of cholate in distilled water, at room temperature, by reference resin QDA12 having various kinds of organic counter-ions (both aliphatic and aromatic) in the same graph, and it compares their binding capacities with that of resins having a chloride counter-ion.

3.4.3 Effect of Organic Counter-ions and Low Degree of Cross-Linking

An attempt was made to evaluate and compare the isotherms for the sorption of cholate by Mono-QDA6, which has a low degree of cross-linking, having propionate and Cl^- counter-ions. Since reference resin QDA12 with propionate counter-ions showed the highest binding efficiency as compared to that with the other counter-ions, only the binding capacity of the resin Mono-QDA6 with propionate counter-ion was studied. Figure 3.6 presents the isotherms for the sorption of cholate in distilled water, at room

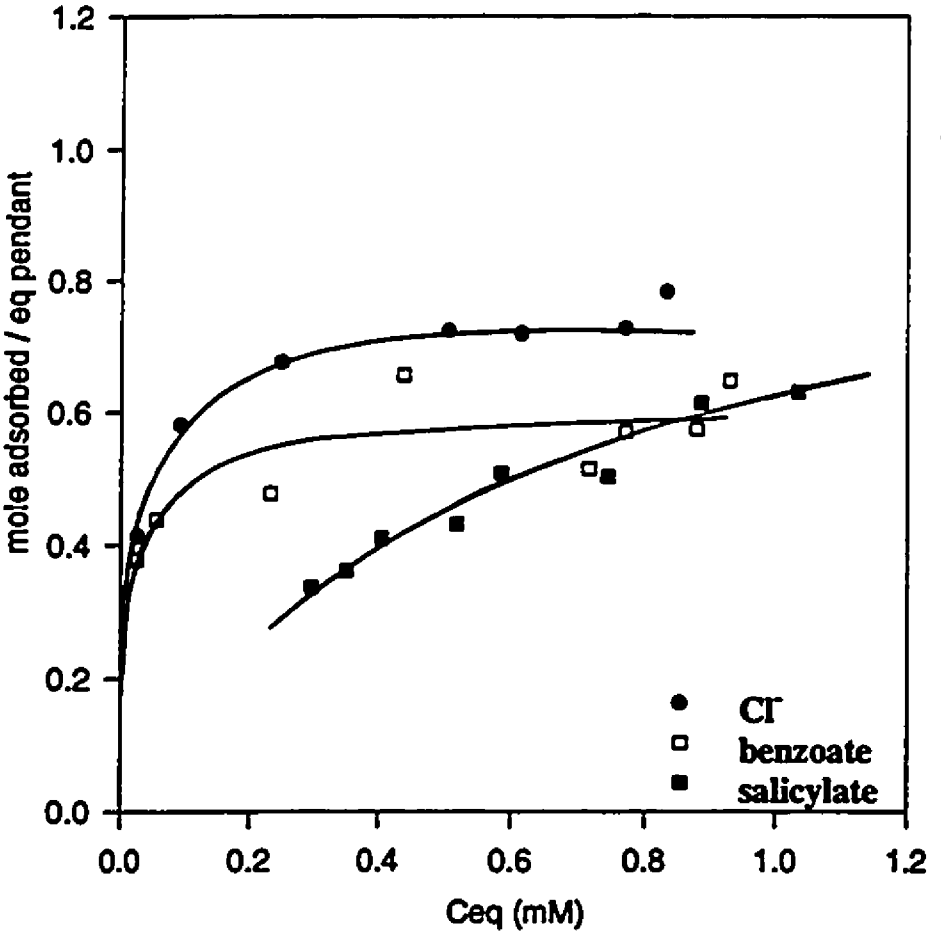


Figure 3.4 The isotherms for the sorption of cholate in distilled water at room temperature by quaternized QDA12 reference resin, comparing the effect of aromatic organic counter-ions with Cl^- counter-ion.

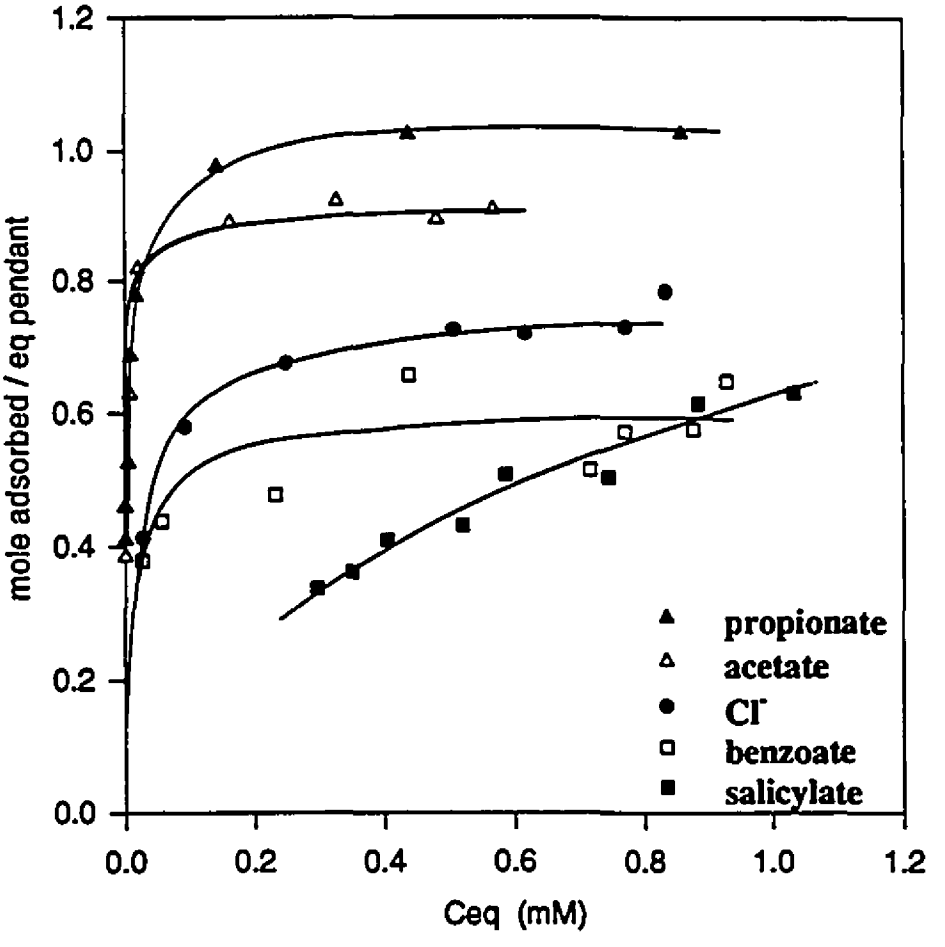


Figure 3.5 The isotherms for the sorption of cholate in distilled water at room temperature by reference resin QDA12, showing the effect of changing the counter-ions.

temperature, by resin Mono-QDA6 having propionate and Cl^- counter-ions. Although reference resin QDA12 with propionate shows a higher binding capacity than when it has chloride counter-ions, the isotherms for sorption of cholate by resin Mono-QDA6 with propionate or Cl^- counter-ions are very similar. It seems that the organic counter-ions do not have a significant effect on the sorption of cholate by low cross-linked resin. At this point, a conclusive explanation cannot be offered to account for this behavior. However, it is known that the less cross-linked beads have a higher degree of swelling; therefore, there is larger amount of water *inside* the resin matrix. It is questionable whether any water structure exists *inside* a cross-linked bead. However, if the water structure is assumed to form *inside* the highly swollen Mono-QDA6 resin, the interactions between the propionate anion and resin would be stronger. At the same time, the increase in entropy due to migration of bile salt anions from the aqueous phase to the resin phase would perhaps be very small.

3.4.4 Effect of Organic Counter-ion and the Number of Ammonium Groups per Pendent Group

To study the effect of organic counter-ions on poly(acrylamide) resins further, the sorption behavior of QTA6 resin having benzoate and acetate counter-ions for sodium cholate was evaluated. The isotherms for the sorption of cholate in distilled water, at room temperature, by resin QTA6 having acetate, Cl^-, and benzoate counter-ions are presented in (Figure 3.7). Even though the pendent groups of resin QTA6 are less hydrophobic than those of resin QDA12, there is a strong interaction between the QTA6 resin and benzoate anion. This fact is indicated by the relatively low uptake of cholate by resin QTA6 having benzoate counter-ion. On the other hand, QTA6 resin with acetate shows a dramatic improvement in sorption relative to that when Cl^- is the counter-ion. Thus, using acetate as a counter-ion improves the binding efficiency of

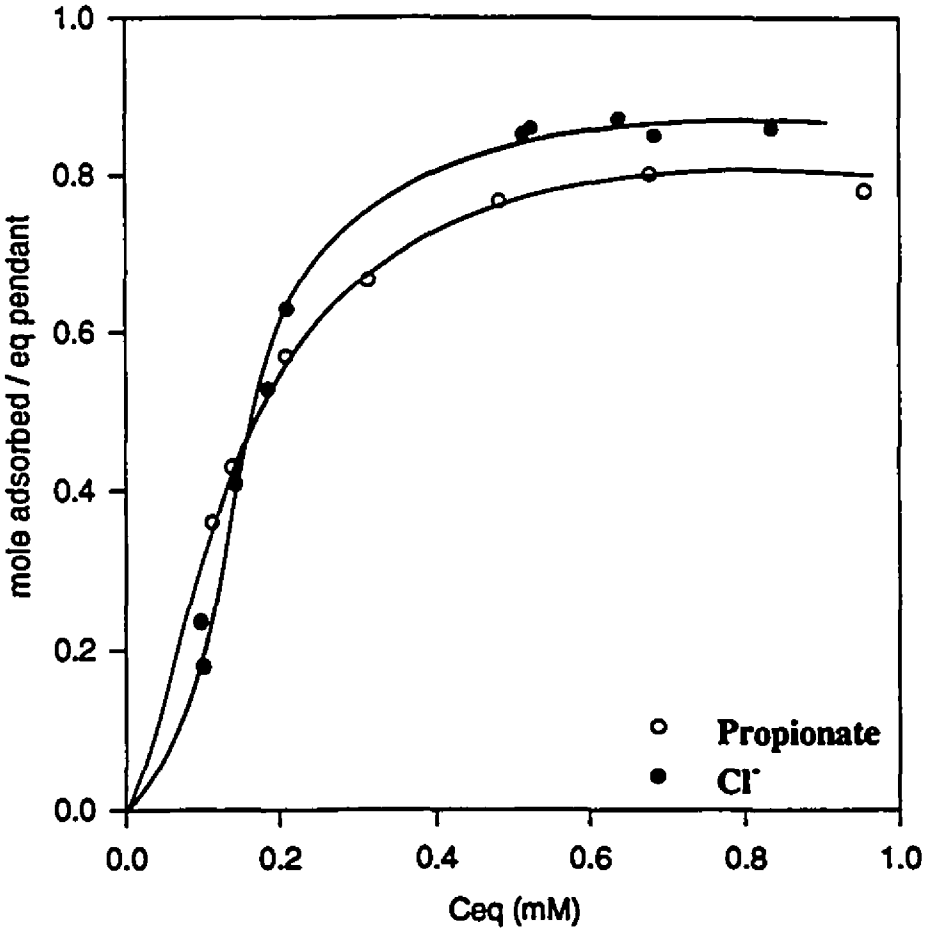


Figure 3.6 The isotherms for the sorption of cholate in distilled water, at room temperature, by Mono-QDA6 resin with a low degree of cross-linking, comparing the effect of organic counter-ions with Cl^-.

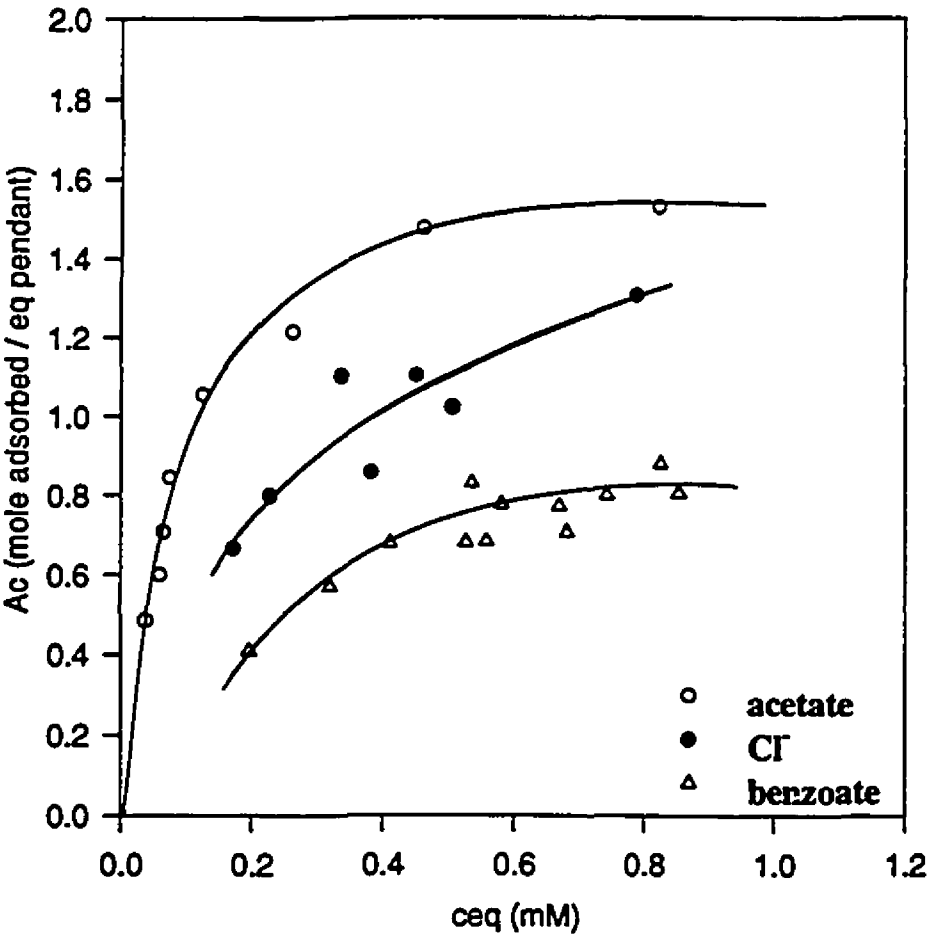


Figure 3.7 The isotherms for the sorption of cholate in distilled water, at room temperature, by resin QTA6, showing the effect of organic counter-ions.

QTA6 resin for bile salt anions. The improved binding efficiency is indicated by the increase in the slope of sorption isotherms at low equilibrium concentrations.

Figure 3.8 presents the isotherms for the sorption of cholate in 2.5 mM tris buffer, at room temperature, by QTA6 resin having acetate and propionate counter-ions. In tris buffer, the binding characteristic of the resin with either acetate or propionate is improved compared to that with chloride counter-ions; however, the sorption capacities of this resin with propionate and acetate counter-ions are identical. The reason for identical sorption behavior of QTA6 with acetate and propionate is not clear. Because of the higher number of positively charged ammonium groups per pendent group, hydrophobic interactions certainly play a smaller role in sorption process.

One of the main disadvantages of a dominant electrostatic interaction is the low selectivity of the resin for bile salt anions. This aspect can be evaluated by comparing the sorption isotherms for cholate in distilled water and in tris buffer by the resins QDA12 and QTA6. The isotherms for the sorption of cholate in distilled water and in 2.5 mM tris buffer (pH 7.2), at room temperature, by the reference resin QDA12 having acetate counter-ions are presented in (Figure 3.9A); in the same manner, the isotherms for the sorption of cholate in water and in 2.5 mM tris buffer (pH 7.2), at room temperature, by the resin QTA6 having acetate counter-ions are presented in (Figure 3.9B). In the case of resin QTA6, the depression of binding capacity in tris buffer represents a lack of selectivity of the resin for bile salt anion. In the case of the reference resin QDA12, a small decrease in binding capacity is seen at very low equilibrium concentration, but for equilibrium concentrations of greater than 1.5 mM the binding capacities for cholate in distilled water and tris buffer are almost identical. Since electrostatic interactions are predominant for resin QTA6, it binds anions indiscriminately. On the other hand, because of strong hydrophobic interactions in the case of resin QDA12, it shows a preference for cholate anions.

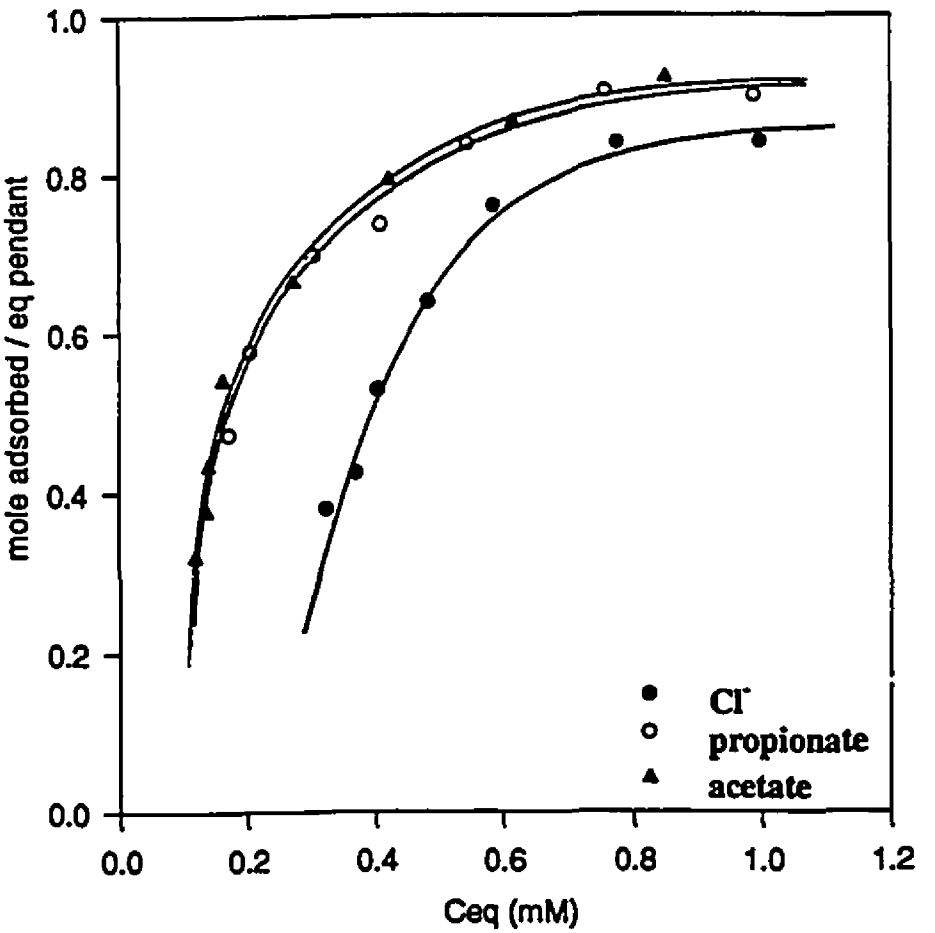


Figure 3.8 The isotherms for the sorption of cholate in 2.5 mM Tris buffer (pH 7.2), at room temperature, by the resin QTA6, showing the effect of aliphatic organic counter-ions.

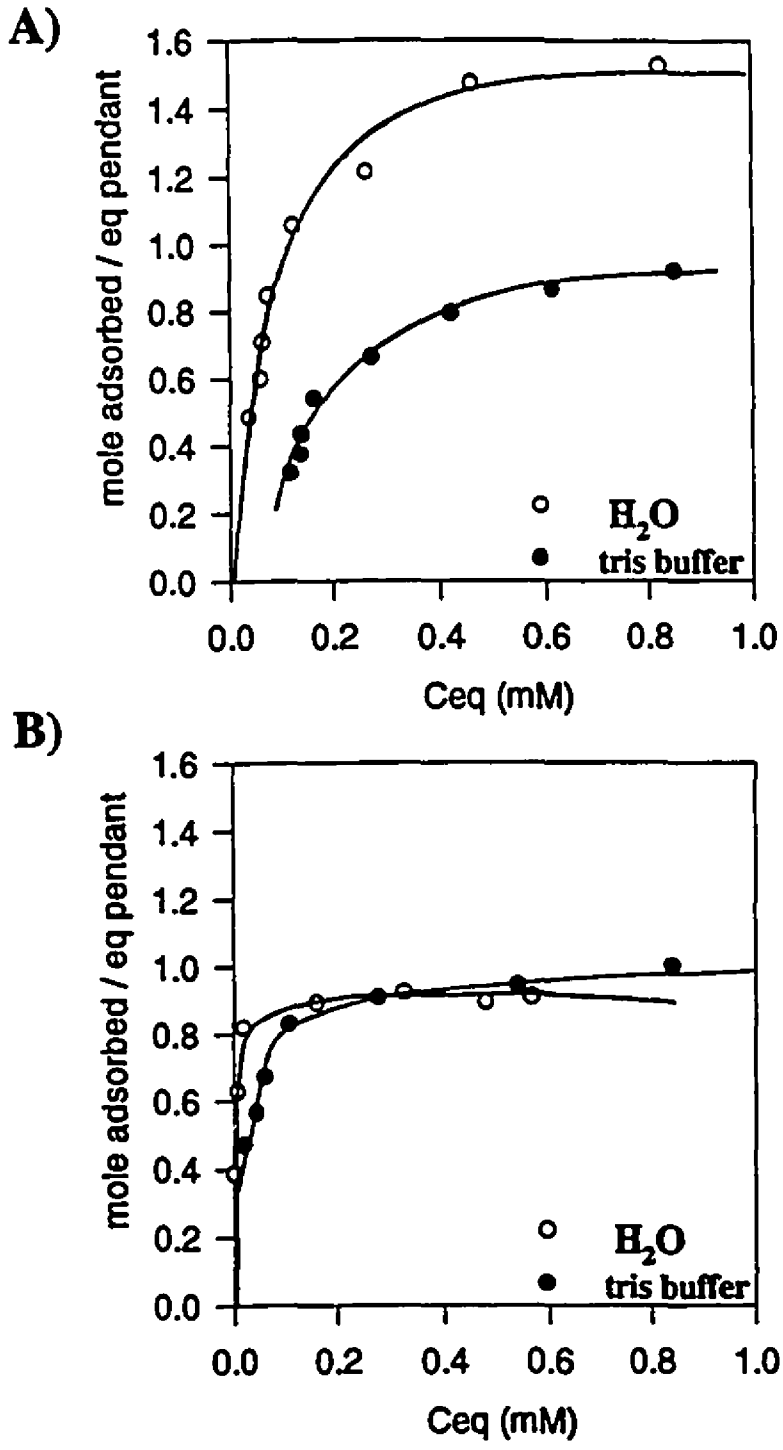


Figure 3.9 The isotherms for the sorption of cholate in both distilled water and 2.5 mM Tris buffer (pH 7.2) by A) QTA6 resin and B) QDA12 resin, carrying acetate as the counter-ion, comparing the sorption depression of these two resins as a result of tris buffer.

3.4 Conclusions

In this chapter, the factors that are operative in the ion-exchange process of large organic ions such as bile salt anions have been considered. In addition to their charge groups, organic ions contain the organic residue which appears to be of importance to the exchange process. The key interactions involved in the ion-exchange process of organic ions are: (1) the electrostatic interactions between the positively charged groups on the resin and the organic carboxyl groups, (2) the interactions between the non-polar part of the ion and the solvent, and (3) the interactions between the non-polar part of the ion and non-polar part of the resin. The interactions between the nonpolar part of the ion and the aqueous solvent bring about the change in the structure of water, and the interactions involving the nonpolar-nonpolar parts of the ion and resin give rise to hydrophobic interactions, which results in the loss in a degree of freedom of the organic ions.

The change in water structure is an important aspect in exchange process of large organic ions. The non-polar part of the organic ion promotes the formations of water clusters; thus resulting in a decrease in entropy. However, the migration of organic ions from aqueous phase to resin phase has the reverse effect on formation of water cluster, which results in an overall entropy increase.

A study was made of the effect of a few organic counter-ions on the sorption of cholate by poly(acrylamide) resins. The counter-ions which were used include salicylate, benzoate, acetate, and propionate. The overall results indicate that the highly cross-linked poly(acrylamide) resins (having hydrophobic pendent groups) have the highest relative affinity for salicylate ion and lowest relative affinity for propionate.

To evaluate the effect of aromatic organic counter-ions, the Cl^- counter-ions are replaced by salicylate and benzoate. As expected, reference resin QDA12 showed a high relative affinity for aromatic counter-ions in keeping with the strong hydrophobic interaction between the benzene ring of counter-ions and resin matrix. Also, a somewhat higher relative affinity of the resin for salicylate than benzoate anions indicates the

importance of hydrogen bonding in the ion-exchange process. These results were extracted from the isotherms for the sorption of cholate by reference resin QDA12. The isotherm for the sorption of cholate by reference resin QDA12 having benzoate counter-ion showed a greater binding capacity than that of the same resin with salicylate counter-ions, particularly at low equilibrium concentration. The lower selectivity of the resin for benzoate anion than salicylate is attributed to the lack of hydrogen bonding among the benzoate anions within the resin.

Furthermore, to evaluate the effect of aliphatic organic counter-ions, propionate and acetate were used as the counter-ions. In spite of the lower binding capacity of reference resin QDA12 with salicylate and benzoate than chloride counter-ions, with propionate and acetate counter-ions binding capacity improved dramatically, especially at low equilibrium concentrations. The relative affinity order of the reference resin QDA12 for propionate and acetate exhibits an unexpected feature. The reference resin QDA12 has a higher affinity for acetate than propionate. This was attributed to be strong electrostatic interaction between the acetate and the resin resulting from the higher dielectric constant.

Among the organic anions applied as counter-ions, reference resin QDA12 having propionate showed the best isotherms for the sorption of cholate. In summary, the binding capacity of resin QDA12 with various counter-ions has the following order:

propionate > acetate > Cl^- > benzoate > salicylate

In order for a resin to bind bile salt anions, it must release the counter-ions to the solution phase. The counter-ion which has the weakest interaction with resin leaves the resin phase most easily; thus the binding capacity for bile salt would be highest. Therefore, it can be concluded that the resin which has a low selectivity for its own counter-ion would result in the highest binding capacity for bile salt anions. Therefore, the relative affinity order of the resin QDA12 for these counter-ions is as follow:

salicylate > benzoate > Cl^- > acetate > propionate

This simply means that the resin has strongest interaction with salicylate and weakest interaction with propionate. This relative affinity order obtained is identical to data reported in literature (13). Dorfner collected a set of data from various studies which had been carried out on ion-exchange process, and he introduced a replacement series for some ions on strong-base anion exchanger (which is **Polymer-$N^+(CH_3)_3$**) as follow(13):

Benzene sulfonate > **Salicylate** > citrate > I^- > Phenate > HSO_4^- > ClO_3 > NO_3^- > Br^- > CN^- > HSO_3^- > BrO_3^- > NO_2^- > **Cl^-** > HCO_3^- > IO_3^- > Formate > **Acetate** > **Propionate** > F^- > OH^-.

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CHAPTER FOUR

CONCLUSION

4.1 Contributions to Original Knowledge

The research described in this thesis involves the following major aspects: 1) The introduction of a new method of functionalization of poly(methyl acrylate) resin for the synthesis of sorbents for bile acids. 2) A comparison of isotherms for the binding of cholate by these new resins with those for resins functionalized by the previous method in a study of the effects of cross-linking on binding capacity, swelling characteristics, and degree of cooperativity. 3) A study of the effects of organic counter-ions on the binding behavior of poly(acrylamide) resins with bile salt anions.

This research was based on the hypothesis that eliminating additional cross-linking during the functionalization procedure will increase the binding capacity of the resins by: (a) increasing the number of available functional groups and (b) favoring the degree of swelling and shrinking.

1) Synthesis

The objective was to eliminate or minimize the additional cross-linking during the functionalization procedure. The strategy used in the new method was to attach a protecting group to one of the amines of the diamine reactant. This mono-protected diamine was then reacted with PMA, and the Boc protecting group was removed by the reaction with hydrogen fluoride. The elimination or minimization of cross-linking resulted in a higher density of available functional groups on the resin; thus, it increased the ionic interactions.

2) Effect of Cross-linking

The main driving force for the sequestering of bile acids by anion-exchange resins is electrostatic interactions. It was demonstrated that lowering the overall cross-linking of a poly(acrylamide) resins increases the density of functional units available for interaction with bile acid anions.

(a) Binding capacity

The evaluation of sorption isotherms revealed that a lower degree of cross-linking not only results in a higher binding capacity, but it also improves the selectivity of the resins for bile salt anions. It was demonstrated that the binding capacities of resins synthesized according to the new method of functionalization are superior to those of resins synthesized via the previous method. Furthermore, the lower degree of cross-linking accounted for improved diffusion of bile salt anions inside the resins. Thus, the selectivity of the resins for bile salt anions can be substantially improved by lowering the degree of cross-linking.

(b) Swelling Characteristics

There is a direct relationship between the binding efficiency and the degree of swelling/shrinking of the polymeric resin. The resins with a lower degree of cross-linking have higher degree of swelling and shrinking as well as higher binding capacity. Resins with a lower degree of cross-linking exert less limitation on the diffusion of bile salt anions into the polymeric beads.

(c) Cooperative binding

The S-shaped isotherms for the sorption of bile salt anions by poly(acrylamide) resins imply a positive cooperativity in the binding. This suggests the positive effect of hydrophobic interactions among bile salt anions bound at adjacent positions within the resin beads. The degree of cooperativity of a resin having a lower degree of cross-linking is higher than a resin with a higher degree of cross-linking. This implies that there is a possibility that bile salt aggregates or mixed-micelles exist within the beads.

3) The Effects of Organic Counter-ions

The counter-ions play an important role in the selectivity of the resins for bile salt anions. The poly(acrylamide) resins with counter-ions of low affinity exhibit higher binding capacities than the same resin having the counter-ions of higher affinity. Counter-ions which have weak interactions with the resin leave the resin phase more

easily. Therefore, the binding capacity and selectivity of the resin for bile salt anions can be improved by loading the resin with low affinity, such as acetate and propionate anions. The relative affinity order of the resin QDA12 for few organic counter-ions is as follows:

salicylate > benzoate > Cl^- > acetate > propionate

4) Potential for application

The need to replace cholestyramine and cholestipol with a more biocompatible sorbent possessing greater selectivity and binding capacity for bile salt anions is an important aspect. The *in vitro* studies described in this thesis demonstrate that the resins with a lower degree of cross-linking exhibit a higher binding capacities as well as better selectivity for bile salt anions. These advantages make these resins promising drug candidates for the treatment of hypercholesterolemia. Furthermore, an *in vivo* study of minimally cross-linked poly(acrylamide) resins having counter-ions of acetate and propionate could provide valuable results for future development of lipid lowering agents.

6.2 Suggestions for Future Work

1. Development of a new class of bile acid sequestrants using self-assembling systems such as polymeric micelles. These polymeric micelles can be achieved with di- or tri-block copolymers having hydrophobic and hydrophilic blocks. The use of polymeric micelles as a bile acid sequestrants could overcome some of the physical and chemical inconveniences of current sorbents.

Such sorbents could be prepared from diblock copolymers consisting of a a block of styrene and another composed of an acrylate. The acrylate could be functionalized by reaction with diamines according to the methods developed in this study. This would

result in polymer micelles with a styrene core and hydrophilic coronas having quaternary amine groups. Since the polymeric micelles are much smaller size than polymeric beads, the surface area per unit weight would be much higher. Furthermore, the movement of the corona chains would provides excellent cavities for bile acids and even bile acid micelles. The potential of mixed micelles of di-block polymeric chains and bile acids is the most attractive aspect of this idea.

2. Combination of non-systemic agents can be another area of research for developing appropriate lipid lowering agents. For example, the polymeric resins or polymeric micelles could be synthesized to have the pendent groups of: (a) bile acid dimers, (b) various kinds of N-(cycloalkyl) alkyl amines, or (c) saponins such as soya saponins.

3 The functionalization of PMA with 1,12-dodecanediamine using the new method of functionalization. Since the poly(acrylamide) resin having pendent groups synthesized from 1,12-dodecanediamine is already one of the most potent resins developed in this laboratory, it would be valuable to see how the selectivity and biocompatibility of this resin can be improved by eliminating the additional cross-linking during the functionalization process.

4. The effect of counter-ions on the selectivity of the resins for bile salt anions should be investigated further, and more experiments are required to see the effect of various counter-ions. Animal tests are recommended to evaluate the poly(acrylamide) resins having acetate and propionate counter-ions.

5. The active transport mechanism in the ileum is so efficient that it tends to strip bile acids from the resin. An important point, which is often forgotten, is that loading

and unloading of bile acids with is not symmetrical. Loading happens mainly in the duodenum, and unloading happens in the ileum under very different conditions and pH. Therefore, it is very important to conduct further studies on the desorption of bile acids from the resins as well.

6.3 References

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Appendix

Experimental Data

Chapter 2

Figure 2.9

volume(mL)	signal(mV)	volume(mL)	signal(mV)
0.000	-102.400	4.460	-143.700
5.000e-3	-104.800	4.560	-144.900
0.010	-106.500	4.660	-146.100
0.015	-107.200	4.860	-148.900
0.024	-109.900	4.960	-150.600
0.075	-111.900	5.060	-152.200
0.140	-113.800	5.160	-153.900
0.180	-114.500	5.260	-155.700
0.260	-115.400	5.360	-157.600
0.360	-116.200	5.460	-159.700
0.460	-116.800	5.660	-164.500
0.560	-117.500	5.760	-167.500
0.660	-118.000	5.860	-170.400
0.860	-119.000	5.960	-173.900
1.160	-120.500	6.060	-177.700
1.260	-121.000	6.150	-182.300
1.460	-122.100	6.210	-185.400
1.560	-122.600	6.285	-190.000
1.660	-123.100	6.350	-194.600
1.760	-123.600	6.415	-201.600
1.960	-124.700	6.480	-210.300
2.060	-125.300	6.545	-222.600
2.260	-126.400	6.610	-247.600
2.460	-127.500	6.740	-326.800
2.660	-128.700	6.805	-340.800
2.760	-129.300	6.880	-349.900
2.960	-130.600	6.950	-356.500
3.060	-131.300	7.020	-361.600
3.160	-132.000	7.090	-365.700
3.260	-132.700	7.185	-370.200
3.360	-133.500	7.280	-374.000
3.460	-134.300	7.380	-376.800
3.560	-135.100	7.480	-380.200
3.660	-135.800	7.580	-382.800
3.760	-136.600	7.680	-385.300
3.860	-137.600	7.780	-387.200
3.960	-138.500	7.880	-389.200
4.160	-140.400	7.980	-390.900
4.360	-142.600	8.080	-392.400
		8.180	-394.200

Figure 2.10

volume(mL)	signal(mV)	volume(mL)	signal(mV)
0.000	151.500	1.375	-155.600
5.000e-3	151.700	1.475	-157.800
0.010	151.400	1.575	-160.000
0.015	151.100	1.675	-162.500
0.025	150.400	1.775	-165.400
0.045	148.500	1.875	-168.300
0.075	145.200	1.975	-172.800
0.110	141.000	2.035	-175.500
0.140	137.000	2.120	-180.200
0.165	133.200	2.170	-182.900
0.190	128.900	2.245	-188.000
0.210	124.900	2.285	-191.600
0.230	120.000	2.320	-194.800
0.240	117.700	2.365	-199.700
0.260	110.200	2.390	-203.100
0.270	106.800	2.415	-207.100
0.285	97.600	2.440	-211.500
0.295	91.500	2.465	-216.400
0.305	80.800	2.490	-223.100
0.315	60.600	2.515	-239.300
0.325	-8.800	2.540	-259.400
0.335	-50.600	2.565	-284.900
0.345	-85.700	2.590	-306.900
0.355	-108.800	2.615	-319.000
0.365	-123.400	2.640	-326.900
0.375	-132.000	2.665	-332.800
0.385	-136.300	2.690	-337.400
0.395	-138.100	2.720	-341.900
0.405	-139.000	2.750	-345.400
0.420	-139.800	2.795	-349.600
0.450	-140.900	2.855	-354.900
0.505	-141.900	2.890	-357.600
0.575	-143.100	2.955	-360.800
0.675	-144.500	3.055	-365.800
0.775	-145.800	3.115	-368.200
0.875	-147.300	3.215	-371.400
0.975	-148.800	3.315	-373.900
1.075	-150.200	3.415	-376.100
1.175	-152.000	3.515	-378.200
1.275	-153.800	3.615	-379.700

Figure 2.13

(a) Mono-QDA6 (in H_2O)

Temp. (°C)	Swellability (%)
12.160	168.627
16.300	169.753
20.500	173.315
25.000	173.505
30.000	178.108
35.000	182.220
41.500	184.233
45.000	190.871
52.000	194.008
55.000	197.537
60.000	208.636
75.000	236.411

(b) QDA12 (in H2O)

Temp. (°C)	Swellability (%)
0.600	83.057
5.500	83.356
10.700	83.049
15.600	83.040
21.600	83.530
25.600	83.655
30.300	83.042
35.800	80.625
41.800	81.823
45.400	83.318
54.000	84.238
49.000	85.204
63.700	86.060
60.000	87.887
65.000	86.658
70.000	89.999
75.000	91.902

(c) QDA6 (in H2O)

Temp. (°C)	Swelability (%)
12.160	107.043
16.300	107.861
20.500	108.352
25.000	105.23
30.000	109.941
35.000	111.535
41.500	109.198
45.000	109.991
52.000	110.796
55.000	111.850
60.000	117.012
75.000	127.944

(d) Mono-QDA6 (in NaCA solution)

Temp. (°C)	Swellability (%)
12.160	157.717
16.300	161.532
20.500	164.926
25.000	164.184
30.000	168.330
35.000	171.618
41.500	172.077
45.000	174.764
52.000	177.567
55.000	180.659
60.000	185.238
75.000	204.833

(b) QDA12 (in NaCA solution)

Temp. (°C)	Swellability (%)
0.600	66.338
5.500	71.328
10.700	70.347
15.600	70.395
21.600	70.802
25.600	68.855
30.300	70.432
35.800	70.072
41.800	69.402
45.400	70.239
54.000	71.229
49.000	69.690
63.700	70.781
60.000	70.232

	65.000	68.870	20.500	96.534
	70.000	70.783	25.000	92.215
	75.000	70.336	30.000	96.266
			35.000	97.601
(c)	QDA6 (in NaCA solution)		41.500	97.598
	Temp.	Swellability	45.000	97.445
	(°C)	(%)	52.000	97.877
	12.160	92.624	55.000	98.133
	16.300	94.363	60.000	98.693
	20.500	96.534	75.000	98.994

Figure 2.14

(A) QDA12

(a) H_2O (temp. increase)

Temp. (°C)	Swellability (%)
10.0	81.262
20.0	81.252
30.0	82.914
40.0	81.799
50.0	83.448
60.0	83.469

(b) Change to NaCA solution (temp. increase)

Temp. (°C)	Swellability (%)
10.0	75.843
20.0	76.042
30.0	78.375
40.0	78.377
50.0	78.903
59.0	78.427

(c) NaCA (temp. decrease)

Temp (°C)	Swellability (%)
50.0	79.897
40.0	80.362
30.0	80.311
20.0	80.325
10.0	80.075

(B) QDA6

(a) H_2O (temp. increase)

Temp. (°C)	Swellability (%)
2.0	105.212
11.5	109.283
20.0	107.109
30.0	107.782
40.0	109.784
48.0	108.489
60.0	111.718

(b) Change to NaCA solution (temp. increase)

Temp. (°C)	Swellability (%)
20.0	108.694
30.0	108.788
40.0	109.601
50.0	110.206
58.0	108.544

(c) NaCA (temp. decrease)

Temp (°C)	Swellability (%)
50.0	109.777
40.0	113.047
30.0	113.660
20.0	112.743
10.0	110.721

(C) Mono-QDA6

(a) H_2O (temp. increase)

Temp. (°C)	Swellability (%)
10.0	180.009
20.0	179.261
30.0	181.733
40.0	182.948
50.0	183.196
60.0	183.709

(b) Change to NaCA solution (temp. increase)

Temp. (°C)	Swellability (%)
10.0	168.413
20.0	166.637
30.0	170.891
40.0	171.754
50.0	170.963
59.0	173.144

(c) NaCA (temp. decrease)

Temp (°C)	Swellability (%)
50.0	173.475
40.0	173.215
30.0	172.258
20.0	171.817
10.0	171.763

Figure 2.15

(A) QDA12

(a) NaCA solution (temp. increase)

Temp. (°C)	Swellability (%)
10.0	77.983
20.0	77.977
30.0	77.983
40.0	79.213
50.0	79.215
60.0	82.281

(b) Change to H_2O (temp. increase)

Temp. (°C)	Swellability (%)
10.0	85.204
20.0	85.263
30.0	85.652
40.0	86.235
50.0	85.631
59.0	86.848

(c) H_2O (temp. decrease)

Temp (°C)	Swellability (%)
50.000	88.522
40.000	87.434
30.000	86.852
20.000	86.852
10.000	87.594

(B) QDA6

(a) NaCA solution (temp. increase)

Temp. (°C)	Swellability (%)
1.5	95.901
2.0	95.556
11.5	97.136
20.0	93.020
30.0	94.410
40.0	97.188
48.0	98.702
60.0	98.766

(b) Change to H_2O (temp. increase)

Temp. (°C)	Swellability (%)
10.0	90.021
20.0	90.123
30.0	89.428
40.0	91.677
50.0	92.452
58.0	90.120

(c) H_2O (temp. decrease)

Temp (°C)	Swellability (%)
50.0	96.584
40.0	97.188
30.0	96.110
20.0	96.614
10.0	95.519

(C) Mono-QDA6

(a) NaCA solution (temp. increase)		(b) Change to H_2O (temp. increase)		(c) H_2O (temp. decrease)	
Temp. (°C)	Swellability (%)	Temp. (°C)	Swellability (%)	Temp (°C)	Swellability (%)
10.0	171.426	10.0	184.383	50.0	202.600
20.0	172.789	20.0	187.186	40.0	203.204
30.0	176.689	30.0	191.716	30.0	199.686
40.0	179.303	40.0	194.655	20.0	197.331
50.0	181.260	50.0	198.883	10.0	196.034
60.0	186.577	59.0	201.887		

Figure 2.16

(a) H_2O

Concentration ($M \times 10^{-5}$)	Peak area
0.000	0.000
5.825	155.611
11.650	349.047
23.300	639.841
34.950	1001.217
46.600	1295.647
58.300	1627.031
69.900	1980.424
81.550	2308.450
93.200	2653.959
104.900	2997.146
116.500	3308.622

(b) Tris-buffer solution

Concentration ($M \times 10^{-5}$)	Peak area
0.000	0.000
5.792	159.031
11.584	318.063
22.168	636.125
34.752	954.183
45.336	1272.250
57.920	1590.313
69.504	1908.376
80.088	2226.438
92.622	2543.128
104.256	2872.564
113.640	3180.742

Figure 2.17

(a) QDA6

Ceq (mM)	Sorption (mol/eq)
0.119	0.283
0.157	0.432
0.222	0.542
0.369	0.560
0.587	0.632
0.809	0.721

(b) Mono-QDA6

Ceq (mM)	Sorption (mol/eq)
0.050	0.287
0.072	0.344
0.064	0.408
0.079	0.524
0.108	0.646
0.177	0.786
0.371	0.914
0.786	0.979

Figure 2.18

(a) Mono-QDA6

Ceq (mM)	Sorption (mol/eq)
0.102	0.179
0.097	0.235
0.144	0.408
0.185	0.527
0.211	0.629
0.514	0.850
0.639	0.871
0.526	0.857
0.686	0.850
0.837	0.860

(b) QDA6

Ceq (mM)	Sorption (mol/eq)
0.346	0.321
0.362	0.361
0.436	0.428
0.655	0.558
0.945	0.683
0.214	0.114
0.239	0.173
0.258	0.214
0.317	0.312

Figure 2.20

(a) MONO

Ceq (mM)	Sorption (g/g resin)
0.102	0.198
0.097	0.259
0.144	0.451
0.185	0.582
0.211	0.695
0.514	0.939
0.639	0.961
0.526	0.946
0.686	0.938
0.837	0.949

(b) QDA6

Ceq (mM)	Sorption (mol/eq)
0.346	0.321
0.362	0.361
0.436	0.428
0.655	0.558
0.945	0.683
0.214	0.114
0.239	0.173
0.258	0.214
0.317	0.312

Figure 2.21 A)

(a) Mono-QDA6 (in Tris buffer)

Ceq (mM)	Sorption (mol/eq)
0.102	0.179
0.097	0.235
0.144	0.408
0.185	0.527
0.211	0.629
0.514	0.850
0.639	0.871
0.526	0.857
0.686	0.850
0.837	0.860

(b) Mono-QDA6 (in H_2O)

Ceq (mM)	Sorption (mol/eq)
0.050	0.287
0.072	0.344
0.064	0.408
0.079	0.524
0.108	0.646
0.177	0.786
0.371	0.914
0.786	0.979

Figure 21 B)

(a) QDA6 (In H_2O)

Ceq (mM)	Sorption (mol/eq)
0.119	0.283
0.157	0.432
0.222	0.542
0.369	0.560
0.587	0.632
0.809	0.721

(b) QDA6

Ceq (mM)	Sorption (mol/eq)
0.346	0.321
0.362	0.361
0.436	0.428
0.655	0.558
0.945	0.683
0.214	0.114
0.239	0.173
0.258	0.214
0.317	0.312

Figure 2.22

(a) Q-PDA12

Ceq (mg/dL)	Sorption (mol/eq)
0.040	0.055
0.150	0.125
0.260	0.164
0.920	0.345
2.150	0.495
4.390	0.637
8.300	0.747
10.060	0.792

(b) Q-PDA6

Ceq (mg/dL)	Sorption (mol/eq)
8.190	0.036
11.290	0.089
15.090	0.147
18.820	0.194
25.710	0.272
30.210	0.309
33.030	0.348

(c) QDA6

Ceq (mg/dL)	Sorption (mol/eq)
14.915	0.321
15.570	0.361
18.790	0.428
28.184	0.558
40.695	0.683
9.197	0.114
10.300	0.173
11.092	0.214
13.627	0.312

(d) Mono-QDA6

Ceq (mg/dL)	Sorption (mol/eq)
4.375	0.179
4.188	0.235
6.194	0.408
7.977	0.527
9.090	0.629
22.142	0.850
27.516	0.871
22.656	0.857
29.528	0.850
36.023	0.860

(e) QDA12

Ceq (mg/dL)	Sorption (mol/eq)
0.000	0.314
0.000	0.353
0.000	0.408
0.992	0.475
2.193	0.619
5.578	0.813
18.672	0.952
32.706	0.970
14.151	0.893

Figure 2.23

(a) Mono-QDA6

Ceq (mM)	Sorption (mol/eq)
0.102	0.179
0.097	0.235
0.144	0.408
0.185	0.527
0.211	0.629
0.514	0.850
0.639	0.871
0.526	0.857
0.686	0.850
0.837	0.860

(b) QDA12

Ceq (mM)	Sorption (mol/eq)
0.000	0.314
0.000	0.353
0.000	0.408
0.023	0.475
0.051	0.619
0.130	0.813
0.434	0.952
0.760	0.970
0.329	0.893

(c) QDA6

Ceq (mM)	Sorption (mol/eq)
0.256	0.025
0.325	0.274
0.346	0.321
0.362	0.361
0.436	0.428
0.655	0.558
0.945	0.683
0.214	0.114
0.239	0.173
0.258	0.214

Figure 2.24

(a) QDA12

Ceq (mM)	Sorption (mol/eq)
0.028	0.435
0.054	0.643
0.148	0.838
0.241	0.882
0.504	0.990
0.859	1.043
0.043	0.530
1.381	1.071
1.806	0.994
1.918	1.089

(b) QTA6

Ceq (mM)	Sorption (mol/eq)
0.127	0.367
0.165	0.529
0.187	0.581
0.227	0.666
0.455	0.876
0.519	1.134
0.318	0.851
0.570	0.980
0.623	1.200
0.715	1.174
0.099	0.570
0.198	0.744
1.259	1.250
1.461	1.287
1.679	1.398

Figure 2.25

(a) QDA12

Ceq (mM)	Sorption (mol/eq)
0.000	0.314
0.000	0.353
0.000	0.408
0.023	0.475
0.051	0.619
0.130	0.813
0.434	0.952
0.760	0.970
0.329	0.893

(b) QTA6

Ceq (mM)	Sorption (mol/eq)
0.325	0.380
0.371	0.425
0.405	0.528
0.485	0.638
0.587	0.759
0.758	0.904

Chapter 3

Figure 3.2

(a) (Cl^-)

Ceq (mM)	Sorption (mol/eq)
0.093	0.579
0.616	0.718
0.771	0.727
0.834	0.782
0.027	0.413
0.250	0.675
0.506	0.723

(b) (Propionate)

Ceq (mM)	Sorption (mol/eq)
0.000	0.410
0.000	0.460
0.005	0.525
0.008	0.686
0.017	0.776
0.141	0.976
0.437	1.024
0.858	1.026

(c) (Acetate)

Ceq (mM)	Sorption (mol/eq)
0.000	0.385
0.0083	0.629
0.020	0.819
0.162	0.890
0.327	0.922
0.480	0.894
0.568	0.910

Figure 3.3

(a) (Salicylate)

Ceq (mM)	Sorption (mol/eq)
0.295	0.336
0.350	0.362
0.404	0.409
0.519	0.431
0.586	0.507
0.744	0.503
0.884	0.614
1.033	0.630

(b) (Cl^-)

Ceq (mM)	Sorption (mol/eq)
0.093	0.579
0.616	0.718
0.771	0.727
0.834	0.782
0.027	0.413
0.250	0.675
0.506	0.723

(c) (Benzoate)

Ceq (mM)	Sorption (mol/eq)
0.056	0.438
0.026	0.379
0.232	0.477
0.770	0.570
0.716	0.514
0.929	0.647
0.877	0.575
0.438	0.656

Figure 3.4

(a) (Salicylate)

Ceq (mM)	Sorption (mol/eq)
0.295	0.336
0.350	0.362
0.404	0.409
0.519	0.431
0.586	0.507
0.744	0.503
0.884	0.614
1.033	0.630

(b) (Propionate)

Ceq (mM)	Sorption (mol/eq)
0.000	0.410
0.000	0.460
0.005	0.525
0.008	0.686
0.017	0.776
0.141	0.976
0.437	1.024
0.858	1.026

(c) (Benzoate)

Ceq (mM)	Sorption (mol/eq)
0.056	0.438
0.026	0.379
0.232	0.477
0.438	0.656
0.770	0.570
0.716	0.514
0.929	0.647
0.877	0.575

(d) Acetate

Ceq (mM)	Sorption (mol/eq)
0.000	0.385
0.008	0.629
0.020	0.819
0.162	0.890
0.327	0.922
0.480	0.894
0.568	0.910

(e) (Cl^-)

Ceq (mM)	Sorption (mol/eq)
0.093	0.579
0.616	0.718
0.771	0.727
0.834	0.782
0.027	0.413
0.250	0.675
0.506	0.723

Figure 4.5

(a) Mono-QDA6 (Cl^-)

Ceq (mM)	Sorption (mol/eq)
0.102	0.179
0.097	0.235
0.144	0.408
0.185	0.527
0.211	0.629
0.514	0.850
0.639	0.871
0.526	0.857
0.686	0.850
0.837	0.860

(b) Mono-QDA6 (propionate)

Ceq (mM)	Sorption (mol/eq)
0.113	0.360
0.139	0.429
0.210	0.569
0.315	0.666
0.483	0.765
0.679	0.801
0.955	0.779

Figure 3.6

(a) QTA6 (Benzoate)

Ceq (mM)	Sorption (mol/eq)
0.196	0.409
0.319	0.569
0.411	0.679
0.558	0.683
0.580	0.776
0.668	0.771
0.680	0.706
0.743	0.800
0.854	0.805
0.526	0.680
0.535	0.829
0.826	0.879
0.320	0.569

(b) QTA6 (Cl^-)

Ceq (mM)	Sorption mol/eq
0.173	0.666
0.228	0.798
0.384	0.857
0.337	1.100
0.452	1.101
0.506	1.020
0.790	1.305

(c) QTA6 (Acetate)

Ceq (mM)	Sorption (mol/eq)
0.038	0.484
0.061	0.599
0.066	0.708
0.076	0.845
0.125	1.055
0.265	1.214
0.464	1.474
0.824	1.527

Figure 3.7

(a) QTA6(Cl^-)

Ceq (mM)	Sorption (mol/eq)
0.325	0.380
0.371	0.425
0.405	0.528
0.485	0.638
0.587	0.759
0.777	0.839
0.998	0.840

(b) QTA6(Propionate)

Ceq (mM)	Sorption (mol/eq)
0.173	0.473
0.205	0.577
0.304	0.697
0.410	0.736
0.546	0.838
0.758	0.904
0.988	0.898

(c) QTA6 (Acetate)

Ceq (mM)	mol/eq
0.119	0.320
0.137	0.377
0.140	0.433
0.162	0.540
0.274	0.663
0.422	0.793
0.616	0.866
0.850	0.921

Figure 3.8 A)

(a) QTA6 (in Tris-buffer)

Ceq (mM)	Sorption (mol/eq)
0.119	0.320
0.137	0.377
0.140	0.433
0.162	0.5
0.274	0.663
0.422	0.793
0.616	0.866
0.850	0.921

(b) QTA6 (in H_2O)

Ceq (mM)	Sorption (mol/eq)
0.038	0.484
0.061	0.599
0.066	0.708
0.076	0.845
0.125	1.055
0.265	1.214
0.464	1.474
0.824	1.527

Figure 3.8 B)

(a) QDA12 (In Tris-buffer)

Ceq (mM)	Sorption (mol/eq)
0.020	0.470
0.045	0.565
0.063	0.672
0.110	0.829
0.279	0.908
0.543	0.944
0.841	1.001

(b) QDA12 (In H_2O)

Ceq (mM)	Sorption (mol/eq)
0.000	0.385
0.008	0.629
0.020	0.819
0.162	0.890
0.327	0.922
0.480	0.894
0.568	0.910