# SYNTHESIS OF ALKYL QUATERNARY AMINO CELLULOSES AND AN INVESTIGATION OF THEIR POTENTIAL AS BILE ACID SEQUESTRANTS

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## ABSTRACT

One method of treatment for patients with elevated serum cholesterol levels (hypercholesterolemia) is the oral administration of an anion exchange resin to adsorb bile acids in the gastrointestinal tract. The resins used successfully to date have been mainly synthetic ones, whereas anion exchange derivatives of cellulose have not been shown to be effective.

Alkyl quaternary amino (QA) cellulose ion exchangers were prepared from hydroxypropylated regenerated cellulose (HP Indion$^{R}$). Their capacities for cholate anions were measured in the presence of a background of chloride ions at physiological concentration. It was shown that butyl QA cellulose was ineffective at binding cholate anions. However, alkyl QA celluloses with alkyl groups longer than butyl (octyl and dodecyl) bound cholate anions in preference to the chloride ions and greatly improved capacities were obtained. The capacities of the octyl and dodecyl QA celluloses were dependent on the cross-linking present in the HP Indion used. If the cross-linking was greater than the 15% used in HP5 Indion, then the capacity for binding cholate dropped off. A significant increase in substitution level and decrease in swollen volume were obtained by reprocessing the alkyl QA cellulose ion exchangers and this improved their cholate binding capacities.

The alkyl QA celluloses were all prepared by first attaching chemically reactive epoxide groups to HP Indion and then reacting those epoxide groups with a tertiary amine (N,N-dimethyl-N-alkylamine) to form the quaternary amino cellulose. These two steps were repeated for the reprocessed products. The optimum conditions for epoxidation of HP5 Indion were shown to be 48 hours at 4-6°C in 6% sodium hydroxide with excess epichlorohydrin. In the reprocessing step, where this reaction was used a second time, 24 hours was sufficient to reach maximum epoxidation levels. When coupling the N,N-

dimethyl-N-alkylamine to the epoxide cellulose it was necessary to use aqueous ethanol as a solvent. The ethanol concentration was dependent on the size of the alkyl group. For N,N-dimethyl-N-dodecylamine a concentration of greater than 60% ethanol was required. For larger amines considerable hydrolysis of the epoxide groups occured during coupling. This was avoided almost completely in the first coupling step by half-neutralising the amine with hydrochloric acid, to lower the pH and buffer the reaction. The optimum coupling time and temperature were shown to be 10 hours at 70°C.

As a result of optimising these processing steps the substitution level obtained for dodecyl QA cellulose was increased from 0.83 to 1.14 meq/g at the end of the first stage and from 1.18 to 1.50 meq/g with the reprocessed product.

A range of alkyl QA celluloses were prepared using the optimum conditions found for dodecyl QA cellulose. Octyl and dodecyl QA celluloses prepared on HP7 Indion were shown to have higher capacities for cholate than cholestyramine and colestipol (Colestid$^{R}$) at cholate concentrations less than 6 mmol/L. The dodecyl QA cellulose had superior capacities at all cholate concentrations (0-15 mmol/L) when measured on the basis of the volume of the ion exchanger used. In contrast, cholestyramine and colestipol had higher capacities for deoxycholate anions than either of the cellulose products, except when the residual concentration of deoxycholate was very low (less than 0.5 mmol/L). Cholestyramine and colestipol bound deoxycholate efficiently (97-99%) when the residual concentration was between 5-10 mmol/L, but not cholate (29-48%). However the alkyl QA celluloses bound deoxycholate and cholate with very high efficiency (82-138%).

To compare the efficiencies of the ion exchangers at low concentrations a more sensitive test was developed utilising a mixture of conjugated bile salts, similar in composition to that found in the human duodenum. This was equilibrated with

samples of each ion exchanger and then analysed by HPLC. Methyl and butyl QA celluloses were the least effective at reducing the concentrations of the bile salts, however they still performed as well as colestipol. The capacity of octyl QA cellulose was much better than the methyl or butyl QA celluloses, indicating the importance of the hydrophobic alkyl chain. Cholestyramine had a similar capacity to the octyl QA cellulose, except that it was not as effective at binding glycocholate. The most effective product was found to be dodecyl QA cellulose, which reduced the concentration of all of the bile salts to lower levels than any other ion exchanger.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

| | |
|---|---|
| BBS | Bicarbonate Buffer System |
| CHD | Coronary Heart Disease |
| CL | Cross-Linking |
| Conc. | Concentration |
| DEAE | 2-Diethylaminoethyl |
| DMB-Cellulose | Butyl Quaternary Amino Cellulose |
| DMDo-Amine | N,N-dimethyl-N-dodecylamine |
| DMDo-Cellulose | Dodecyl Quaternary Amino Cellulose |
| DMO-Cellulose | Octyl Quaternary Amino Cellulose |
| ECH | Epichlorohydrin |
| FH | Familial Hypercholesterolemia |
| GC | Glycocholate |
| GDC | Glycodeoxycholate |
| HDL | High Density Lipoprotein |
| HP Indion | Hydroxypropylated Regenerated Cellulose |
| HP3 Indion | 25% Cross-Linked HP Indion Cellulose |
| HP5 Indion | 15% Cross-Linked HP Indion Cellulose |
| HP7 Indion | 10% Cross-Linked HP Indion Cellulose |
| HPLC | High Performance Liquid Chromatography |
| LDL | Low Density Lipoproteins |
| meq | Milliequivalent/s |
| ODS | Octadecyl Silica |
| % aq | Percentage Aqueous (v/v) |
| QA | Quaternary Amino |
| RT | Room Temperature |
| SD | Standard Deviation |
| SP-Cellulose | Sulphopropyl Cellulose |
| TMA-Cellulose | Methyl Quaternary Amino Cellulose |
| TC | Taurocholate |
| TDC | Taurodeoxycholate |
| TPC | Total Plasma Cholesterol |
| UV | Ultraviolet |
| VLDL | Very Low Density Lipoproteins |

# CHAPTER 1. INTRODUCTION

## 1.1 OUTLINE

The object of this thesis was to produce a quaternary amino (QA) cellulose ion exchanger with potential for use as a bile acid sequestrant in the treatment of hypercholesterolemia.

This introduction backgrounds the debilitating effects of hypercholesterolemic disorders and gives a brief summary of the clinical methods which are used to treat them. Treatments involving bile acid sequestrants, their mode of action and chemistry are reviewed. The procedures previously developed at Massey University for the preparation of cellulose ion exchangers are summarised. A rationale for the type of exchanger to be synthesised is given and the modifications considered necessary for promoting the selective and efficient binding of bile acids are discussed.

## 1.2 EFFECTS OF ELEVATED PLASMA CHOLESTEROL

It is now widely accepted that elevated plasma cholesterol is associated with coronary heart disease (CHD)[1]. In normal caucasians the total plasma cholesterol (TPC) level is approximately 200 mg/dL[2], but only a small fraction of this circulates as free cholesterol in the blood. Between 50-70% of TPC is transported between tissues as a low density lipoprotein (LDL) fraction, 20-45% as a high density lipoprotein (HDL) fraction, 5-10% as a very low density lipoprotein (VLDL) fraction and an insignificant amount in the chylomicrons[3]. Abnormally high levels of LDL (β-lipoproteins), and therefore cholesterol, can be associated with a number of diseases. This condition is classified as hyper-β-lipoproteinemia and there are two recognised subtypes: type IIa and type IIb[4]. Both of these subtypes result in premature CHD[5].

Familial hypercholesterolemia (FH) is a relatively common inherited form of hyper-β-lipoproteinemia, which can be identified at birth. FH individuals have significantly elevated TPC and LDL levels (2-3 times greater than normal in heterozygotes and 4-6 times greater than normal in homozygotes). The clinical consequences of unmanaged FH are well documented and include arcus coronae, xanthomas, marked atherosclerosis and premature CHD[3].

## 1.3 GENERAL APPROACH TO CLINICAL MANAGEMENT OF HYPERCHOLESTEROLEMIA

Substantial reductions, sometimes resulting in normalisation of TPC and LDL concentrations, can now be made in all forms of hyper-β-lipoproteinemia except for homozygous FH[6]. Initial treatment involves adherence to a strictly controlled diet, followed by administration of hypolipidemic drugs where necessary (Table 1.1). Altered diets (which limit cholesterol intake to less than 300 mg per day and are low in saturated fat) can be expected to reduce TPC and LDL levels by up to 10%[7], but normalisation is still not achieved in some cases. Recent studies[6,8,9] indicate that the most promising treatment of severe hyper-β-lipoproteinemia probably lies with combined treatment regimens, incorporating a bile acid sequestrant with complimentary drugs.

TABLE 1.1 Hypolipidemic Drugs Used in the Management of Plasma Cholesterol[6]

| Drug | Mode of Action |
|---|---|
| Nicotinic acid | Inhibits secretion of VLDL (a precurser for LDL synthesis) |
| Compactin<br>Mevinolin | Cause competitive inhibition of the major rate limiting step of cholesterol biosynthesis |
| Clofibrate<br>Gemfibrozil | Cause hydrolysis of VLDL triglycerides by lipoprotein lipase |
| Neomycin | Inhibits reabsorption of bile acids (used where bile acid sequestrants are poorly tolerated) |

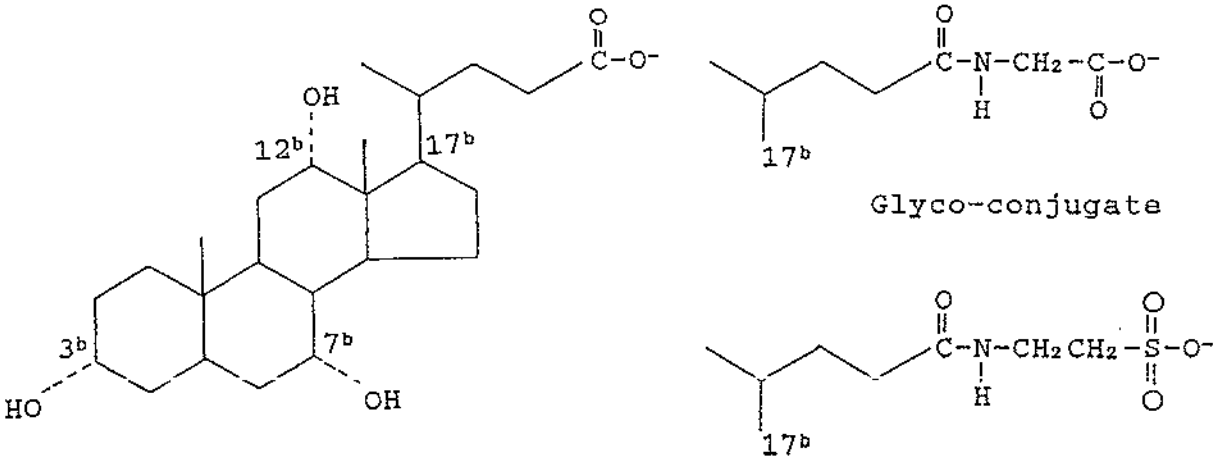
## 1.4 USE OF BILE ACID SEQUESTRANTS TO REDUCE PLASMA CHOLESTEROL LEVELS

One well established method of treating hypercholesterolemia involves oral administration of non-absorbable anion exchangers which function as bile acid sequestrants. These materials have basic functional groups attached to a semi-rigid polymer backbone and they operate by exchanging chloride ions for bile acid anions in the intestinal lumen[10,11].

Bile acids are secreted into the duodenum in response to food intake, where they promote the absorption of dietary fat. The structure of the major bile acids and their distribution in human bile is given in Figure 1.1[12]. Ingestion of bile acid sequestrants increases the faecal excretion of bile acids and lowers their concentration in the intestine. This results in decreased absorption of dietary cholesterol and other lipids[13]. It also causes an indirect decrease in plasma cholesterol, resulting from disruption of the enterohepatic circulation by which bile acids are reabsorbed. In healthy individuals approximately 95% of bile acids are actively reabsorbed from

the distal ileum and the remaining 5% are compensated for by new synthesis[12]. However, under the conditions of bile acid sequestrant therapy fewer bile acids are reabsorbed, so an increased rate of synthesis must occur. Plasma cholesterol (from LDL) is the precurser for all new liver synthesis of bile acids[14], therefore when bile acid sequestrants are used there is increased removal of plasma LDL and a corresponding decrease in TPC[15].

Figure 1.1 Major Bile Acids Present in Human Bile[a]




Cholate

| Bile Acid | 3[b] | 7[b] | 12[b] | Proportion (%) |
|---|---|---|---|---|
| Cholate | OH | OH | OH | 50 |
| Chenodeoxycholate | OH | OH | H | 30 |
| Deoxycholate | OH | H | OH | 15 |
| Lithocholate | OH | H | H | 5 |

[a] Bile acids are usually present in human bile as sodium or potassium salts, and they are conjugated with either glycine or taurine

[b] Numbers refer to positions in the sterol ring

## 1.5 BILE ACID SEQUESTRANTS CURRENTLY AVAILABLE

The ion exchangers which have been used for therapy are:

### A. Cholestyramine

Cholestyramine, the most commonly used bile acid sequestrant, is or has been available as Cuemid (83% cholestyramine[16]), Questran (45% cholestyramine[16]) and Quantalan[17]. It is a copolymer of styrene and 2% divinylbenzene, with quaternary amino groups attached using trimethylamine and balanced with chloride counterions (Figure 1.2A).

### B. Colestipol Hydrochloride

Colestipol hydrochloride is a copolymer of diethylenetriamine and epichlorohydrin with approximately 20% of the available nitrogens titrated with hydrochloric acid (Figure 1.2B). It is sold under the proprietary name Colestid[17].

### C. Diethylaminoethyl (DEAE) Sephadex

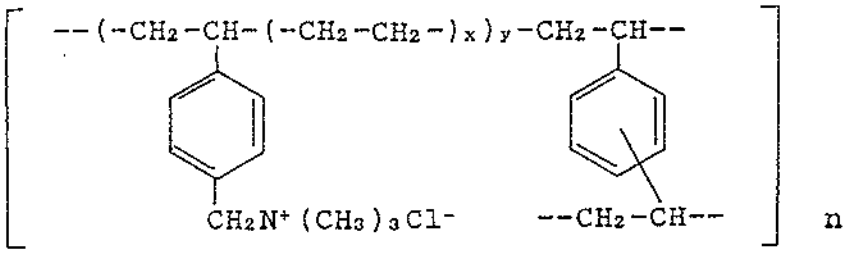
Polydexide or PDX chloride (formerly Secholex) is 79% DEAE-Sephadex[16]. It is a dextran cross-linked with epichlorohydrin and orthosubstituted with 2-diethylaminoethyl (DEAE) groups, some of which are quaternised with 2-diethylaminoethyl chloride[17] (Figure 1.2C).

Cholestyramine based bile acid sequestrants (Questran and Cuemid) are usually preferred over other products (Colestid and Secholex) because they have superior capacity and binding kinetics for bile acids in vitro[16,20]. Laboratory studies have demonstrated that Questran binds 40% more bile acids than Cuemid after mixing with bile for 2 hours at 37°C[16]. In contrast, clinical studies show that the different bile acid sequestrants produce similar hypolipidemic effects in vivo[21,-22,23]. Typically, the TPC in patients can be expected to drop by 20-25% more than that achieved with dietary control alone. This drop occurs in a very short time after administration of

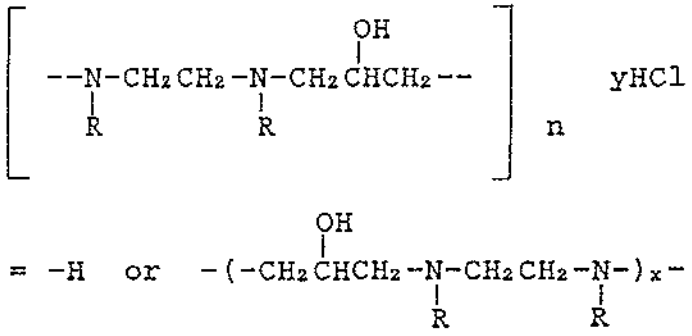
the sequestrant and can be sustained indefinitely with continued use.

Figure 1.2 Partial Structures of Bile Acid Sequestrants Used in Therapy

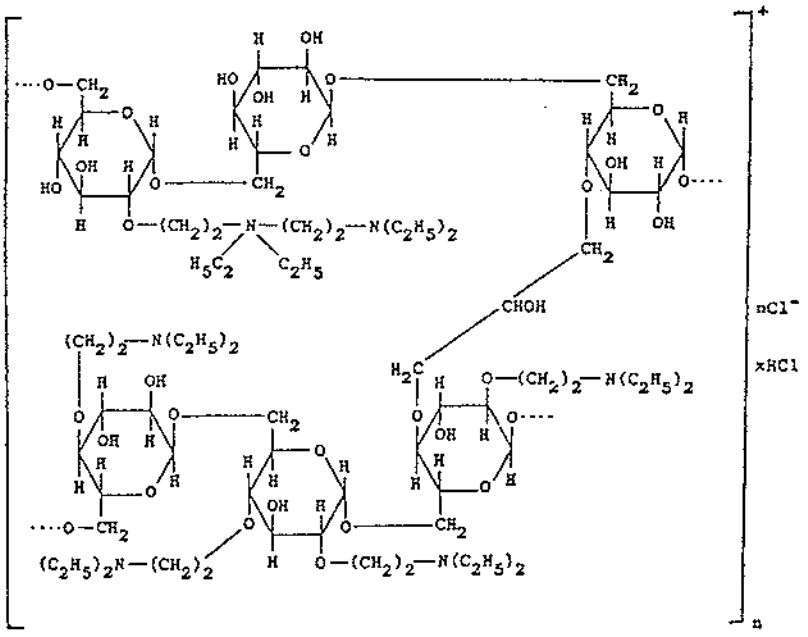
A. Cholestyramine[18]



B. Colestipol[18]



C. DEAE Sephadex[19]



## 1.6 SOME PROBLEMS ASSOCIATED WITH THE USE OF BILE ACID SEQUESTRANTS

Bile acid sequestrants are nonspecific and, as a result, a number of anions are able to compete with the bile acid anions for the available binding sites. The in vitro efficiency for binding bile acids in duodenal fluid is low for all of the sequestrants mentioned in Section 1.5, with only about 50% of the available ion exchange capacity being used under physiological conditions[16]. The efficiency may be even less in vivo. Also small reductions of plasma cholesterol are compensated for by an increase in cholesterol biosynthesis[15]. Thus for effective therapy prolonged and/or large dose rates are required[17].

Since the ion exchangers are not absorbed from the gastro-intestinal tract no systemic ill effects can be expected. Nevertheless, prolonged therapeutic treatment (e.g. 16-20 g cholestyramine per 24 hours) commonly results in constipation (10-50% incidence) and can sometimes lead to nausea, vomiting and other gastrointestinal discomfort. Because normal fat absorption is impaired, malabsorption of fat soluble vitamins may also occur, leading to side-effects including lypocalcemia, hypothrombonemia and/or folic acid deficiency[10,11]. Considerable work has therefore focused on making bile acid sequestrants more effective.

The bile acid binding capacity of cholestyramine is enhanced by the addition of taurine and its 'pharmaceutically accepted' salts[25], ascorbic acid[26] and some essential fatty acids[27]. Cholestyramine resins with different mesh sizes have been tested, however while finer particle sizes were less gritty and more palatable, their in vivo efficiency in binding bile acids was not improved over the standard grade[28]. More recent studies have attempted to make cholestyramine more palatable by the addition of CM-cellulose, Primogel, lactose and flavours[29]. Also, formulations incorporating dietary fiber and

citric acid have been shown to eliminate the mouthfeel and taste of ion exchange drugs[30]. These studies point to the need for the development of new sequestrants which have superior ion exchange capacity and higher selectivity for binding bile acids.

## 1.7 THE USE OF HP INDION CELLULOSE MATRIX FOR THE PREPARATION OF ION EXCHANGERS

Earlier work by Dr J.S. Ayers at Massey University has demonstrated that the chemical modification of cellulose can produce materials suitable for derivatisation and subsequent use as chromatography media. These studies demonstrated that hydroxypropylated regenerated cellulose (now available as HP Indion[a]) was an acceptable starting matrix for the preparation of ion exchangers[31]. Some of the HP Indion ion exchangers produced locally have proved to be ideal for large scale protein separation[32], because they are resiliant to both chemical and physical attrition, and have high capacities for binding macromolecules[31].

HP Indion is prepared from regenerated cellulose, which is first ground and then graded into different particle sizes. Next it is swollen with propylene oxide and cross-linked with epichlorohydrin in the presence of sodium hydroxide, according to the method first described by Sheerin[33]. Typical groups present in the HP Indion cellulose matrix are shown in Equation 1.1. HP Indion is available in a range of particle sizes and degrees of cross-linking[34].

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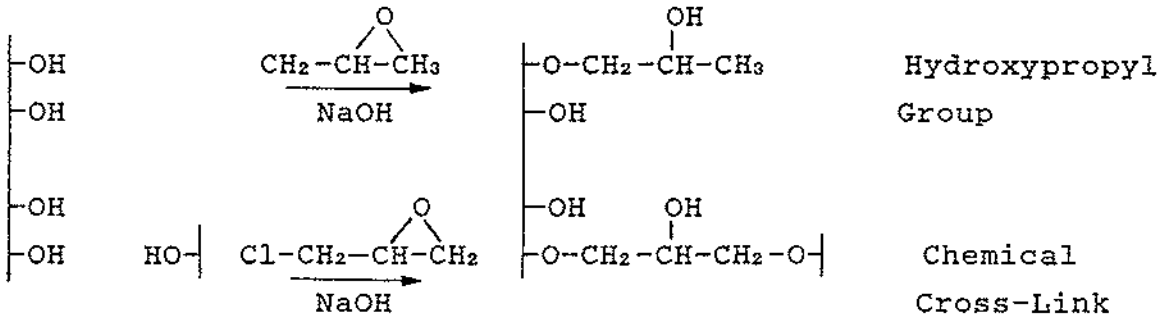
[a] Waitaki International Biosciences, Christchurch

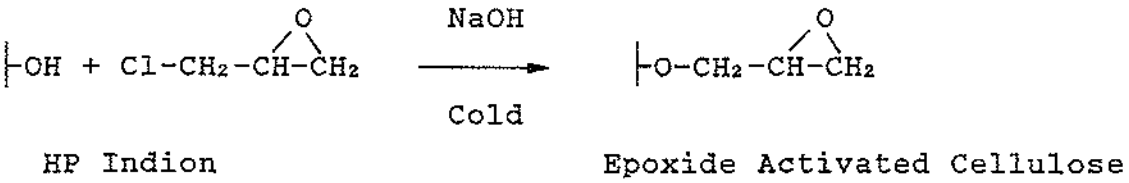
Equation 1.1 Preparation of HP Indion from Regenerated Cellulose




## 1.8 EPOXIDE ACTIVATION OF HP INDION CELLULOSE MATRIX

One very effective method of preparing ion exchangers from the cellulosic HP Indion starting matrix involves epoxide activation (Equation 1.2) and coupling (Equation 1.5).

Equation 1.2 Epoxide Activation

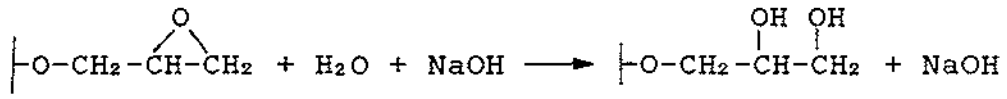



The conditions needed to substitute the maximum number of epoxide groups onto the cellulose matrix have been thoroughly investigated[35]. The critical factor affecting the level of epoxidation relates to the ratio of sodium hydroxide and epichlorohydrin used. Sodium hydroxide is consumed by epichlorohydrin in a 1:1 mole ratio in order to react with the cellulose matrix, and also through hydrolysis (Equation 1.3).

Equation 1.3 Hydrolysis of Epichlorohydrin

Once the sodium hyroxide has been consumed further activation is not possible no matter how much epichlorohydrin is used. However, if excess sodium hydroxide is used hydrolysis of the active epoxide groups occurs (Equation 1.4).

Equation 1.4 Hydrolysis of Activated Cellulose by Excess Sodium Hydroxide



Epoxide Activated Cellulose

Hydrolysis Product

In order to produce the maximum number of active epoxides a 25% molar excess of epichlorohydrin over sodium hydroxide must be used. A 6-8% w/v solution of sodium hydroxide is also essential: above this concentration extra weight is added to the cellulose without a significant increase in the number of epoxides produced (indicating that hydrolysis had occured); but when less than 6% soidum hydroxide is used limited epoxidation results. The actual volume of sodium hydroxide solution, and the number of active epoxides produced, ultimately depends on the physical properties of the HP Indion used. Other factors influencing the number of epoxides produced are the temperature and time of the reaction. Maximum epoxidation (up to 1.4 meq/g) is obtained by activating at low temperatures, however the time required for the reaction to reach completion at 4°C is in excess of 30 hours.

It is possible to couple a wide variety of functional groups to epoxide activated HP Indion (Table 1.2). Previous work demonstrated that amino cellulose ion exchangers were moderately effective at binding bile acid anions in vitro[36]. However, this work was limited and synthetic methods for optimising the ion exchange function were not investigated.

Table 1.2 Ion Exchangers Prepared from Epoxide Activated HP Indion

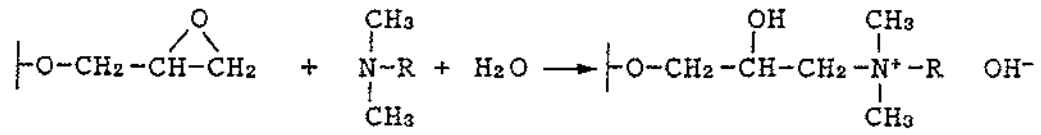
| Ion Exchanger | Structure | Function |
|---|---|---|
| Sulphopropyl (SP) cellulose | $-SO_3^-Na^+$ | Cation Exchange |
| Phosphate (PT) cellulose | $-PO_4^-Na^+$ | |
| Quaternary Amino (QA) cellulose | $-NR_3^+Cl^-$ | Anion Exchange |
| Tertiary Amino (DE) cellulose | $-NHR_2^+Cl^-$ | |
| Primary Amino (PA) cellulose | $-NH_3^+Cl^-$ | |

## 1.9 AIM OF THESIS

Bile acid sequestrants operate by exchanging negatively charged counterions (usually chloride ions) for bile acid anions, hence it was anticipated that quaternary amino (QA) cellulose ion exchangers would be effective because they remain charged at all pH levels. Therefore it was decided to prepare a range of dimethylalkyl QA celluloses by coupling N,N-dimethyl-N-alkylamines to epoxide activated HP Indion as shown in Equation 1.5.

The aim was to identify the most effective alkyl group, and then optimise the synthesis of the cellulose ion exchanger. It was hoped that the capacity of the resultant product to bind bile acids in vitro would match or exceed that of cholestyramine. Ultimately it was hoped that a cellulose based drug would alleviate some of the side-effects which occur with the synthetic polystyrene matrix of cholestyramine.

Equation 1.5 Preparation of Quaternary Amino (QA) Cellulose (Coupling)



Epoxide Activated Cellulose — Dimethylalkyl QA Cellulose