Evaluation of colominic acid as a steric stabilisation agent for liposomes

Ioannis Papaioannou

School of pharmacy
University of London

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This thesis describes research conducted in the School of Pharmacy, University of London between the 15th of October 2001 and the 30th of September 2004, under the supervision of Professor Gregory Gregoriadis. I certify that the research described in original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature

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Abstract

Conjugation of PEG to liposomes has been shown to prolong their retention in the blood (steric stabilisation). Colominic acid (α-2/8 polysialic acid), a capsular polysaccharide from K1 antigen positive *Escherichia coli* strains has been proposed as an alternative to PEG. A previous study concluded that colominic acid was able to prolong the circulation time of egg-PC:Cholesterol liposomes, to a small (but significant) extent. In this study, the potential of colominic acid as a steric stabilisation agent was more thoroughly evaluated. Colominic acid of narrowly defined molecular weight was obtained by fractionating the polydisperse commercially available preparations using anion exchange chromatography. Liposomes with saturated lipids were used and the conjugation chemistry was changed to Maleimide chemistry, to obtain a higher degree of grafting. The steric stabilisation capabilities of small (6.2kDa) and large (22.7kDa) colominic acid, conjugated to liposomes in this manner, were investigated by looking at partitioning in PEG-dextran two phase systems, the binding of plasma proteins in vitro and by measuring the half-life of the polysialylated liposomes in vitro. It was found that polysialylated liposomes partitioned in the dextran layer more than control liposomes and their partitioning was not altered by the presence of plasma proteins. Polysialylated liposomes were very effective at blocking the deposition of proteins to the liposome including mouse immunoglobulins and complement C3. Small colominic acid was much more effective than large colominic acid in this respect. In vivo the polysialylated liposomes were cleared faster than control liposomes, but when the mice were pre-injected with free colominic acid prior to dosing them with the liposome formulation, the picture was reversed and CA-liposomes circulated longer than control liposomes.
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Abbreviations

AcOH  Acetic acid
aco-HAS  Polyaconylated human serum albumin
AEC  Anion exchange chromatography
asGM₁  Asialoganglioside M₁
BMPS  N-(β-maleimido-propionic acid)-hydroxysuccinimide ester
BS³  Bis(sulfosuccinimidyl)suberate
BSA  Bovine serum albumin
C₁q  Complement 1q
C₃  Complement 3
C₃b  Complement 3b
CA  Colominic acid
CASH  Thiol-colominic acid
CA-NHS  N-hydroxysuccinimidyl colominic acid
CHO  Chinese Hamster Overy cells
CL  Cardiolipin
CF  5(6)-carboxyfluorescein
Chol  Cholesterol
DCP  Dicetylphosphate
DMSO  Dimethylsulphoxide
DMPG  Dimyristoyl-phosphatidylglycerol
1,4-DNPH  1,4-Dinitrophenylhydrazine
DODAC  Dioleoyldimethylammonium chloride
DPPC  Dipalmitoyl-phosphatidyl ethanolamine
DSG  Disuccinimidyl glutarate
DSPC  Distearoyl-phosphatidyl choline
DSPE  Distearoyl-phosphatidyl ethanolamine
DSPM  N-(β-maleimidopropionyl)-distearoyl-phosphatidyl ethanolamine
DSPS  Distearoyl-phosphatidyl serine
DTNB  5,5'-dithio-bis(2-nitrobenzoic acid)
DTT  Dithiothreitol
EDC  1-ethyl-3(3-dimethyl-aminopropyl carbodiimide
EDTA  Ethylenediaminetetraacetic acid
EGTA  Ethyleneglycoltetraacetic
FPLC  Fast protein liquid chromatography
FPLC-AEC  Fast protein liquid chromatography – Anion exchange chromatography
GD1  Ganglioside D1
GH  Growth Hormone
GM1  Ganglioside M1
GPC  Gel permeation chromatography
GT1  Ganglioside T1
HDL  High density lipoprotein
HRP  Horseradish peroxidase
HPLC  High pressure liquid chromatography
HPTS  8-hydroxyppyrene-1,3,6-trisulphonic acid
HSA  Human serum albumin
IgG  Immunoglobulin G
2-IT 2-iminothiolane (Traut’s reagent)
LDL Low density lipoprotein
LUV Large unilamellar vesicles
MeOH Methanol
MLV Multilamellar vesicles
MW Molecular weight
NCAM Neuronal cell adhesion molecule
NS-DPPE N-succinyl-dipalmitoyl-phosphatidyl ethanolamine
PA Phosphatidic acid
PAA Polyacrylic acid
PAGE Polyacrylamide gel electrophoresis
PBS Phosphate buffered saline
PC Phosphatidyl choline
PCS Photoc correlation spectroscopy
PE Phosphatidyl ethanolamine
PEG Polyethylene glycol
PEG-PE Polyethylene glycol-phosphatidyl ethanolamine
PEG-DSPE Polyethylene glycol-distearyl-phosphatidyl ethanolamine
PG Phosphatidyl glycerol
PI Phosphatidyl Inositol
PIP Phosphatidyl inositol-phosphate
PS  Phosphatidyl serine
PSA  Polysialic acid
PSA-NCAM  polysialated neuronal cell adhesion molecule
PVDF  polyvinylidene fluoride
RBC(s)  Red blood cell(s)
RES  Reticuloendothelial system
SA  Stearylamine
ScR(s)  Scavenger Receptor(s)
SDS  Sodium dodecyl sulphate
SDS-PAGE  Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC  Size exclusion chromatography
SLPC  1-stearoyl-2-linoleyl-phosphatidyl-choline
SM  Sphingomyelin
SUV  Small unilamellar vesicles
Tc  Crystalline Transition Temperature
tdGPC  Tripple detection gel permeation chromatagraphy
TEA  Triethylamine
TLC  Thin layer chromatography
TNBS  4, 6 trinitro-benzene sulfonic acid
TNB  5-thio-2-nitrobenzoc acid
UV  Ultraviolet
VLDL  Very low density lipoprotein
Chapter 1

Introduction
1.1 Liposomes and their components

A liposome (figure 1.1b) can be defined as a closed, membranous, self-sealing, water-filled vesicle, composed of one or more lipid bilayer membranes, similar in structure to biological membranes. Lipids are a diverse class of molecules of biological origin (i.e. encountered in living beings) that are sparingly soluble in water, but soluble in organic solvents, which are hence used to extract lipids from biological matter (Voet and Voet 1997).

Many lipids have an amphiphilic nature and a tendency to form macromolecular aggregates in water. Some lipids prefer to form micelles (e.g. taurocholate), while other lipids (most notably the glycerophospholipids) have a tendency to form bilayer membranes. Glycerophospholipids are the most important type of bilayer forming lipids and the principal component of all biological membranes. They are usually the most important constituents of liposomal membranes as well. Glycerophospholipids are phosphoester derivatives of phosphatidic acid (sn-glycerol-3-phosphate esterified at C1 and C2 with fatty acids), with an alcohol of a polar organic molecule known as the “head group”. For example phosphatidyl choline (figure 1.1a) is a phosphoester of phosphatidic acid with the alcohol group of choline. Glycerophospholipids, due to the presence of the two fatty acid chains (known as the “tails”), which sterically hinder packing into spherical micellar structures, prefer to form bilayer-membrane structures instead.
Figure 1.1 a) Chemical structure of phosphatidylcholine. b) Conceptualised diagram of a liposome, shown in cross-section c) Space filling model of a typical phosphatidylcholine bilayer structure. The polar heads are exposed to the aqueous environment while the lipid chains are sequestered internally. (Figure adapted from Lodish et al 1999)
A phospholipid bilayer membrane (figure 1c) is an extended bimolecular leaflet, consisting of two layers of phospholipids arranged so that the fatty acid tails are in contact with each other and the polar head groups are exposed to the solvent on either side of the leaflet (Voet and Voet 1997).

This arrangement with the lipids roughly in parallel is more favourable than a micelle because there is no steric hindrance and packing of the lipid tails is maximised. The bilayer is actually a two-dimensional fluid, as the lipids are free to move laterally (Voet and Voet 1997). Transverse motion is limited to slow exchange (flip-flop) between the inner and outer leaflets (Voet and Voet 1997). Below a certain temperature, the hydrophobic packing forces become powerful enough to hold the lipids in place, causing the bilayer to become semi-solid. This is called the crystalline transition temperature or Tc (figure 1.2).

The fluidity of the bilayer is determined by the ability of the fatty acid tails to maximise packing. Saturated long fatty acid tails are straight and maximise packing, so phospholipids with long saturated fatty acid tails make bilayers that are more solid and rigid with a high Tc. Unsaturated fatty acids are not straight, because the double bonds introduce bends along the
hydrocarbon chain. The bends prevent maximal packing so that the bilayers formed by phospholipids with shorter unsaturated fatty acid chains, tend to be more fluid and deformable with a low Tc. Other components aside from phospholipids can also be included in the bilayer (and hence in liposomal membranes) such as cholesterol or stearyl amine. Amphiphilic molecules can become part of the bilayer depending on their particular properties (figure 1.3). These other components can also influence the packing forces and hence affect the Tc and other physical properties of the liposome. For example inclusion of cholesterol increases the packing forces between the phospholipids making the liposomes more rigid (Kirby et al 1979). Some typical glycerophospholipid components of liposomal membranes are phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl glycerol and phosphatidyl inositol. When the phospholipid comes from a natural source (e.g. egg yolk) the name of the fatty acid chains is omitted, because the fatty acid chains are a mixture of different fatty acids including a number of unsaturated ones. When the phospholipid is synthesised, so both the fatty chains on the lipid molecule are the same, the composition of the fatty acid chains is also included. For example 1,2 distearoyl phosphatidyl choline, is esterified with two stearic (C18, no double bonds) acid chains. Other components of liposomal membranes are cholesterol, stearylamine, gangliosides and sphingomyelin. In particular certain gangliosides, have been shown to have an important effect when incorporated into liposomes (see below). The structure of some common phospholipids is shown in figure 1.4. The structure of gangliosides GM₁ and GT₁ is shown in figure 1.5
Figure 1.3 Schematic drawing of a bilayer that includes non phospholipid components, such as sphingolipids and cholesterol. These components are also amphiphilic and pack into the bilayer in a similar way to the phospholipids (Figure adapted from Voet and Voet 1997)
Figure 1.4 Common phospholipids components of biological membranes.
Figure 1.5 (a) The structure of gangliosides GM$_1$ and GT$_1$, two typical sphingolipid components of mammalian membranes.

Figure 1.5 (b) The glycosyl moiety of GM$_1$, shown in greater detail (from http://www.lipidlibrary.co.uk/Lipids/gang/)
1.2 Liposomes as drug delivery vehicles.

Over the past 30 years, ever since the idea of using liposomes as drug delivery vehicles was conceived (Gregoriadis et al 1971), a great deal of research has gone into liposomal formulations of various therapeutic agents, including among others chemotherapy agents (e.g. doxorubicin) (Gabizon 1992a), antifungal agents such as amphotericinB (Perez et al) 1993 and vaccines (Gregoriadis et al 1996, 1999, 2002). The aim of a drug delivery system is to increase the efficacy and decrease the toxicity of the delivered substance (Allen et al 1995). The main reasons for using liposomes as a drug delivery vehicle are stability, improved pharmacokinetic and improved biodistribution profile over the free molecule (Woodle 1995). Substances entrapped in a liposome are protected from the outside environment as the membrane excludes a large number of solutes from the liposome interior. More importantly, for as long as a compound remains within the liposome, it assumes the pharmacokinetics and biodistribution of the liposomal carrier (Allen et al 1995). For example by entrapping doxorubicin in a liposome, not only is excretion of the drug slowed down dramatically (Gabizon 1992a), but in addition the therapeutic index increases and its biodistribution shifts away from the cardiac muscle, greatly reducing cardiac toxicity since the heart has a greatly reduced uptake of liposomal doxorubicin compared to free doxorubicin (Papahadjopoulos et al 1991, Abraham et al 2005) It is obvious that by entrapping a substance in a liposome, its pharmacokinetics and biodistribution can be altered without physically modifying the substance itself. The behaviour of the liposome can further be improved by altering its composition (Gregoriadis and Senior 1980, Senior and Gregoriadis 1982a, 1982b, Gabizon and Papahadjopoulos 1992b, Ghosh and Bachhawat 1992) or directly modifying its surface (Klibanov et al 1990), which again does not require
any modification of the encapsulated drug. For this reason, the clearance of liposomes from the circulation has been studied extensively in an attempt to understand the underlying clearance mechanisms and consequently how to improve the in vivo behaviour of liposomes.

1.3 Clearance of liposomes from the circulation

The clearance of liposomes from the circulation is not yet fully understood, though great progress has been made towards this end. A number of processes have been implicated, but clearance is also dependent to a large extent upon the particular characteristics of the liposomes, including size, fluidity, surface charge, the presence or absence of particular lipids and the grafting of polymers onto the liposomal surface. There is a large body of evidence suggesting that the cells of the reticuloendothelial system (RES) are the major route of liposome elimination (Allen et al 1995), particularly in the liver (Kupffer cells) and the spleen (Segal et al 1975). Other processes that have been implicated include extravasation into tissues and uptake by non-RES cells (e.g. liver parenchymal cells) (Scherphof and Kamps 2001), the action of plasma lipoproteins (e.g. HDL) (Kirby et al 1980), the complement system (Devine and Bradley 1998) and receptor interactions (e.g. the scavenger receptor) (Scherphof and Kamps 1998). Some of the liposome clearance mechanisms are depicted in figure 1.6.
Figure 1.6 The effect of liposome physicochemical properties on liposome clearance from the circulation. (See text for references and more detail).

In general, most liposome formulations exhibit dose dependent, saturation-type (Michaelis-Menten-like) clearance kinetics (Allen et al. 1995). At low doses a fast $\alpha$-half-life and a slower $\beta$-half-life, can be observed (Allen et al. 1995). The $\alpha$-half-life is believed to represent uptake into the RES, which is fast but has a limited capacity (Allen et al. 1995, Woodle and Lasic 1992, Woodle 1995). The $\beta$-half-life on the other hand is thought to represent elimination by the remaining mechanisms, which are slower but have a much higher capacity (Allen et al. 1995, Woodle et al. 1995). This model however is a simplification as the clearance of liposomes is a more complex process than that of small molecules. An alternative hypothesis suggests that the typical bimodal clearance profiles of liposomes are
also caused by the size heterogeneity of liposome preparations as the larger liposomes are cleared faster, while smaller liposomes are cleared more slowly.

At high dose a few liposome formulations, exhibit log-linear clearance (Senior and Gregoriadis 1982b), but their clearance is still dose dependent and it is not log-linear at lower doses. On the other hand liposomes which have been especially formulated to avoid the RES more effectively (stealth liposomes), appear to have a log-linear, elimination profile even at low doses (non dose-dependent clearance) (Allen et al 1995, Woodle et al 1995). It should be noted that the clearance of PEG-liposomes is not always log-linear.

1.3.1 The effect of size.

The half-life and biodistribution of liposomal formulations is intimately linked to their size. Juliano and Stamp (1975) showed that smaller liposomes have a significantly improved half-life, a result which was also confirmed by other studies (Senior and Gregoriadis 1982a, 1982b). It is thought that larger vesicles (300nm or more in diameter) are effectively retained by the spleen through a filtration mechanism and are more susceptible to phagocytosis by the liver and spleen macrophages (Huang 1987), while smaller liposomes, are more adept at avoiding the spleen. It was shown by Harashima and co-workers (1994) that complement mediated uptake of liposomes in a perfused liver system, is enhanced with increasing size in the range of 200 to 800 nm. Liposomes whose diameter is 100 nm or less, are in addition able to extravasate in tissues with fenestrated endothelium and particularly the liver, high pressure sites such as the extremities (Charois and Allen 2003) and more importantly at sites of injury/inflammation or tumour sites (Gregoriadis et al 1977, Gabizon 1992a, Huang et al 1991), where the vasculature is newly formed and consequently more “leaky” (Dvorak et al
1988). This difference in behaviour has important consequences. Uptake by the RES, is rapid, but easily saturated (low capacity). Hence larger liposomes which are cleared effectively by the RES, are removed from the circulation quickly. Smaller liposomes, which are more adept at evading uptake, exhibit longer circulation times. Despite their more rapid clearance large liposomes are still useful for the delivery of therapeutic agents such as vaccines which need to be targeted to the RES. For the delivery of encapsulated drugs to locations other than the tissues of the RES however it is clear that small liposomes must be used. Although this has not been studied extensively, it is likely that extravasation into tissues and subsequent uptake by non-RES cells is an important component of the secondary clearance mechanisms responsible for the β-half-life. For example it was shown that stealth liposomes incorporating PEGylated lipids, have a much increased tendency to accumulate in the extremities, the skin and sites of injury or inflammation (Charrois and Allen 2003, Lasic 1995). Due to their ability to extravasate into tissues with “leaky” vasculature, small liposomes, with a diameter of 100 nm or less, are passively targeted to tumours and sites of inflammation (Gabizon 1992a, Ishida et al 1999). The minimal uptake of such small liposomes by many other tissues that do not have leaky vasculature and the increased plasma circulation times, all serve to increase the tumour targeting effect, making small liposomes attractive candidates for the delivery of chemotherapy agents. Although it is generally true that large liposomes cannot extravasate, this is not always the case. It appears that large size (200-400 nm), low cholesterol, phosphatidylyl-serine (PS) containing liposomes have access to the liver parenchymal cells as well, but if the PS in the formulation is replaced by phosphatidylyl-glycerol (PG), there is no substantial uptake by the liver parenchymal cells (Scherphof and Kamps 2001). In addition substitution of the PS and
PC in the preparation with the fully saturated distearoyl versions of the same lipids (DSPC and DSPS), also reduces the uptake by the liver parenchymal cells. It was suggested (Scherphof and Kamps 2001) that liposomes can also be forced through the liver endothelium fenestrations in the narrow capillaries of the hepatic sinusoids by the passing red blood cells, provided that the liposomes are able to transiently interact with the endothelium. PS containing liposomes can bind the liver endothelium, while PG liposomes cannot.

1.3.2. The effect of surface charge.

The effect of surface charge on the clearance of liposomes from the circulation is not straightforward. Studies by Gregoriadis and Neerunjun (1974), and by Juliano and Stamp (1975), showed that inclusion of negatively charged lipids such as PS resulted in accelerated clearance. It turns out however that the exact nature of the negatively charged lipid is just as important as the charge. Thus although incorporation of PS, dicetylphosphate (DCP), cardiolipin and PA, in the liposomes resulted in enhanced clearance compared to neutral liposomes (Gregoriadis and Neerunjun 1974, Juliano and Stamp 1975, Gabizon and Papahadjopoulos 1988), it was shown that the inclusion of PI or ganglioside GM₁ can have the opposite effect (Allen and Chon 1987, Gabizon and Papahadjopoulos 1992b). Furthermore, the less negatively charged asialoganglioside GM₁ (asGM₁), which lacks a galactose linked sialic acid group is not effective at prolonging the half-life of liposomes in the blood (Allen and Chon 1987, Gabizon and Papahadjopoulos 1992b). It was further shown that in contrast to PI and GM₁, the very similar lipids phosphatidyl-inositol-phosphate (PIP) and ganglioside GT₁ are less effective in prolonging liposome circulation times (Gabizon and Papahadjopoulos 1992b). The difference between PIP and PI is that PIP has a
phosphate group on the inositol moiety of the head group. The difference between GM\textsubscript{1} and GT\textsubscript{1} is that GT\textsubscript{1} has 3 terminal negatively charged sialic acid groups compared to 1 sialic acid group on the side chain for GM\textsubscript{1}. Thus the common features between PIP and GT\textsubscript{1} compared to PI and GM\textsubscript{1} is greater and more accessible negative charge. The difference between PI and GM\textsubscript{1} compared to PS, PA, DCP and other negatively charged lipids that increase liposome clearance is the presence of a more bulky, hydrophilic head group, that can sterically hinder access of large molecules to the surface. The actual amount of negatively charged lipid included is also critical. Increasing for example the amount of PI to 41mol\% from 23mol\% resulted in a dramatic increase in liposome clearance rates and a shift of liposome accumulation to the liver but away from the spleen (Gabizon and Papahadjopoulos 1992b). It appears that bulky hydrophilic groups can counteract the negative impact of a negatively charged group as long as the charged group is buried under the bulky group.

The results of various ex-vivo and in vitro experiments studying the effects of negatively charged lipids on the uptake of liposomes by cell lines, phagocytes and the perfused liver, are complicated and in a few cases contradictory. This is likely the result of the presence of multiple mechanisms for the removal of such liposomes, which can become dominant or non-functional depending on the conditions. For example in was shown by Hu and Liu (1996) that serum-dependent and non-serum-dependent processes both have a role in the uptake of negatively charged liposomes by the perfused liver. Also Lee and co-workers (1992a, 1992b, 1993) have shown that monkey kidney CV-1 cells take up negatively charged liposomes a great deal faster than the murine macrophage-like J774 cells and that while uptake of negatively charged liposomes by CV-1 cells, was relatively insensitive to serum and polyanions, the presence of serum or polyinosinic acid had a strong inhibitory effect on
J774 cells. Additional lines of evidence suggested that the two cell lines take up PS-containing liposomes, through different routes (Lee 1992a, 1993). It is difficult to extrapolate the results of such experiments in vivo as there may be co-operation or even competition between the different mechanisms resulting in behaviour that is quite unpredictable. It is however well documented now that one of the major reasons for the different behaviour of liposomes incorporating negatively charged lipids is their interaction with plasma proteins and in particular opsonins. For example apolipoprotein H (β2-glycoprotein) can interact strongly with phosphatidic acid (PA), PS or cardiolipin (CL) containing liposomes (Chonn et al 1995). Treating the serum with EDTA, EGTA/Mg\(^{2+}\) or heating at 56°C, conditions which inactivate complement, also abolished the enhancing effect of serum on the uptake of liposomes in a perfused liver system (Hu and Liu 1996). Incubation of serum with an excess PA, dicetylphosphate (DCP) or PG containing liposomes, and then removing the liposomes reduced the ability of the treated serum to promote uptake of PS-containing liposomes by the perfused liver to different degrees (Hu and Liu 1996). The presence of such competitive effects suggests that there are serum components that mediate liver uptake of negatively charged liposomes that are common for more than one type of lipid. Bradley et al (1999) have obtained evidence that this may be due to direct activation of the classical complement pathway, by accessible negatively charged residues but this will be discussed later on.

An intriguing possibility, which has not been so far well investigated, is that liposomes with a high negative surface charge density are also cleared by additional and different mechanisms from those mediating the clearance of liposomes with a low negative surface charge density.
1.3.3 The effect of lipid composition.

In addition to size and charge, the exact nature of the liposome components is crucial in determining their behaviour in vivo. As discussed in sections 1.3.1 and 1.3.2 for example PIP is a great deal less effective at prolonging liposome circulation, compared to the closely related lipid PI. Also, while GT, does not have a significant impact, on the plasma half-life of liposomes, GM, has proven to be quite effective in this respect. There are other physical properties of the liposome aside from size and charge that have proven to be important in determining in vivo behaviour. For example a large body of evidence has now been accumulated suggesting that liposomes with more rigid gel-type bilayers circulate for longer than liposomes with deformable, fluid bilayers do (Gregoriadis and Davis 1979, Gregoriadis and Senior 1980, Senior and Gregoriadis 1982a, 1982b, Kirby et al 1979, 1980, Allen 1981, Allen and Everest 1983). One of the most important lipids determining the in vivo behaviour of liposomes is cholesterol. Incorporation of cholesterol in the bilayer, increases its rigidity resulting in improved half-life and in vivo stability (Kirby et al 1979) It has also been shown that cholesterol makes the liposomes resistant to the action of plasma lipoproteins (HDL), especially hindering lipid exchange and/or removal (Kirby et al 1980, Allen 1981).

Studies of the interaction of serum proteins with negatively charged multilamellar liposomes containing varying amounts of cholesterol (Moghimi and Patel 1988), revealed significant differences in protein binding and subsequent interaction with splenic and hepatic cells: Rat hepatic and splenic cells both showed a preference in the absence of serum for taking up cholesterol-free and cholesterol-poor (20 mol%) liposomes compared to cholesterol-rich (46.6 mol%) liposomes. The uptake decreased with increasing cholesterol. However upon addition of serum there was a dramatic shift in the uptake values. The uptake of cholesterol
free liposomes was strongly suppressed for both cell types, while the uptake of cholesterol-poor liposomes was mildly enhanced. On the other hand the uptake of cholesterol rich liposomes by splenic cells was strongly enhanced by serum, but reduced albeit to a small extent for hepatic cells, which was surprising. Other experiments have also shown that serum components, which are able to promote uptake of a liposomal formulation by some cell types can suppress uptake by other cells (reviewed in Moghimi and Patel 1998). This was demonstrated by treating (see below) or fractionating serum with ammonium sulfate and investigating the effect of the treated serum or the ammonium sulfate fractions on the uptake of liposomes by different cell types. Fresh serum increased the uptake of cholesterol poor liposomes by liver phagocytes and of cholesterol rich liposomes by spleen and peritoneal phagocytes. Serum treatment involved heating it to 56°C, dialysis or addition of EGTA. Such treatment abolished or reduced the enhancing effect of serum on the uptake of cholesterol rich liposomes by spleen and peritoneal macrophages. In contrast the same treatment further increased the ability of serum to enhance the uptake of liposomes by liver phagocytes. It is intriguing that adding calcium chloride to EGTA treated serum or adding the dialysate to the dialysed serum reversed the effect of such treatments on the ability of serum to influence the uptake of liposomes by liver and peritoneal macrophages, whereas it did not reverse it for spleen macrophages. It appears therefore that the clearance of the negative liposomes used in these studies, is mediated by competing mechanisms for the different groups of phagocytic cells. It is likely that the same specific lipid components of these liposomes are recognized both by the proteins that enhance uptake by the liver phagocytes and those that enhance uptake by the spleen phagocytes, or that the components of the liposome that are recognised by the liver cells are masked by the binding of proteins that target to the spleen, so that there
is competition between the two systems. The spleen specific factors are able to bind more strongly and hence prevent the binding of the liver specific factors or mask the components that are recognised by the liver phagocytes. A possible mechanism for this competition, based on differential interactions with complement and the scavenger receptors, will be discussed later in this chapter. As discussed in section 1.3.2, it is possible that a crucial factor, which was not investigated by these researchers, is the actual density of the negative charge on the liposome surface. It must be noted that the liposomes used in these studies are negatively charged and multilamellar and hence these results cannot necessarily be extrapolated to the behaviour of many other liposome formulations. The real value of these results is the demonstration that the recognition of liposomes by phagocytes does not necessarily use a common process and in fact competition between the different processes may exist, as well as that cholesterol can have a profound impact on the interaction of liposomes with plasma proteins and phagocytic cells.

The role of using saturated high Tc phospholipids has also been the subject of investigation. It is now well documented that using saturated phospholipids (for example using hydrogenated PC or DSPC) improves both the stability and the circulation times of liposomes (Gregoriadis and Senior 1980, Senior and Gregoriadis 1982a, 1982b). As discussed in section 1.3.1 it was shown in the perfused liver system that while 200-400nm liposomes composed of egg-PC were able to access the liver parenchymal cells, liposomes of similar size composed of DSPC were not. Senior and Gregoriadis (1982b) have identified a formulation with an exceptionally long half-life. It appears that a liposome formulation composed of equimolar DSPC and cholesterol, at a dose of 1.8-2.4 μmol of phospholipid per 20-25 g mouse has log-linear clearance and a half-life approaching 20 hours. However even
when it comes to liposomes composed of fully saturated phospholipids and cholesterol, clearance is still dose dependent (Senior et al 1991). The same authors found that liposomes composed of DSPE, DSPC and cholesterol at a 1:1 ratio between phospholipid and cholesterol did not have log-linear clearance at doses of 0.4 or 0.8 µmol phospholipid. The β-half-life of these liposomes approached 12h and the fast initial clearance phase was strongly reduced by increasing the phospholipid dose from 0.4 to 0.8 µmol. The same liposomes conjugated with 5 kDa PEG, showed the same clearance profile at both doses (Senior et al 1991). It is thus clear that both the identity and the physical properties of the lipids used in liposome formulations can influence the in vivo fate of the liposomes, as a result of both specific interactions and non specific interactions brought about by the effect of the lipids on the physical properties of the liposome.

In conclusion the lipid composition has been shown to be critical in determining the half-life of liposomes in the circulation. The presence of cholesterol, the use of high Tc lipids, the presence of bulky hydrophilic head groups and the presence of accessible charged groups can greatly affect the interaction of liposomes with components of the in vivo environment and their subsequent clearance.

1.3.4 Receptor and non-receptor mediated liposome clearance.

It is clear that a number of receptors are involved in some of the liposome clearance pathways. Specific ligands such as mannose (Espuelas et al 2003), lectins (Vodovozova et al 2000), galactose (Gregoriadis and Senior 1984) or antibodies (Gregoriadis and Neerunjun 1975, Gregoriadis et al 1977, Huwyler et al 1996) can be used to target liposomes to specific receptors, but in addition to that, there seem to be receptors involved in the clearance of
liposomes that do not have specific targeting groups on their surface, either due to the interaction of liposomal components with plasma components, which can then interact with a receptor or via direct receptor binding. The evidence for the presence of these reactions has been reviewed by Scherpof and Kamps (1998). Three different groups of receptors have been implicated in liposome clearance: the scavenger receptors (Fukasawa et al 1996, Fujiwara et al 1996, Rigotti et al 1995, Nishikawa et al 1990, Murphy et al 2005, Shirai et al 1999, Terpstra et al 1998) the complement receptors (Devine and Bradley 1998, Bradley et al 1999, Moghimi and Patel 2002, Harashima et al 1994, Liu et al 1995, Roerdink et al 1983) and the plasma lipoprotein receptors (Scherpof and Kamps 1998, Guo et al 1980).

1.3.4.1 The scavenger receptors

Scavenger receptors (ScRs) were discovered through studying the uptake of cholesterol from low density lipoprotein (LDL) in atherosclerotic plaques. ScRs are a diverse class of transmembrane receptors which are responsible for the uptake of modified LDL such as oxidised or acetyl LDL, though they are also able to bind a wide range of lipid or lipoprotein-based ligands (Shirai et al 1999). The scavenger receptor family is divided into 5 classes from A to H (Murphy et al 2005), but only classes A (type I and II), B and C have so far been implicated in liposome uptake (Scherpof and Kamps 1998). Class A type I and II ScRs can bind, in addition to modified LDL, polynucleic acids, bacterial components and some carbohydrate based ligands (Murphy et al 2005). Class B receptors recognise native and modified LDL, HDL and VLDL, collagen, fatty acids, anionic phospholipids, thrombospondin and apoptotic cells (Murphy et al 2005, Terpstra et al 1998, Fukasawa et al 1996). Class C receptors, have two complement control protein domains and have been
shown to bind to acetyl-LDL as well as whole bacterial cells and a wide range of polyanionic ligands (Murphy et al 2005). The recognition of polyanionic ligands appears to be a common feature among the ScR family. ScRs are found in abundance only in a small subset of tissues that include macrophages and liver sinusoid endothelial cells (Murphy et al 2005). In particular, the type B scavenger receptor SRB1 was shown to recognise negatively charged liposomes and apoptotic cells (Fukasawa et al 1996). It has been demonstrated that PS liposomes can inhibit binding of red blood cells (RBCs) that have suffered oxidation damage to mouse macrophages (Terpstra et al 1998). Apoptosis and oxidation damage on RBCs have been shown to result in an increase in the amount of PS displayed in the outer lipid leaflet of the plasma membrane (Sambrano and Steinberg 1995, Fadok et al 1992). Sambrano and Steinberg (1995) found that the binding of oxidatively damaged RBCs to mouse macrophages was inhibited by oxidised LDL, implicating a member of the scavenger receptor family and showed that the appearance of PS on the RBC surface is important. This finding was confirmed by the same group who also showed that the responsible receptor strongly binds PS containing liposomes (Ramprasad et al 1995), adding weight to the evidence pointing out that the scavenger receptor recognises exposed PS. The importance of the lipid moiety in the binding to the scavenger receptor was further proven by Terpstra et al (1998) who demonstrated that oxidised 1-stearoyl-2-linoleyl-phosphatidyl-choline (SLPC) liposomes (SLPC:cholesterol 2:1 molar ratio, 80-120 nm in diameter) and a lipid micro-emulsion prepared from oxidised LDL were able to inhibit the binding of oxidatively damaged RBCs to mouse macrophages, while non-oxidised SLPC liposomes and a lipid micro-emulsion from native LDL caused no inhibition. This suggested that the scavenger receptors can also recognise the oxidised unsaturated phospholipids. It is possible that there
may exist an additional clearance mechanism for liposomes with unsaturated lipids, involving oxidation of the fatty acid tail double bonds and uptake by the scavenger receptors. However oxidation is a slow process and hence this mechanism is unlikely to be particularly significant.

The scavenger receptors have been shown to have an important role in the clearance of liposomes whose surface was modified with polyanionic macromolecules. Fujiwara et al (1996) investigated the interaction between cultured RAW macrophages (Murine leukaemic monocyte macrophage cell line) and liposomes whose surface was modified with polyacrylic acid (PAA), a polymer that contains multiple carboxyl groups (one in every repeat). After 18 hours of incubation with either egg-PC:Cholesterol SUV liposomes conjugated with PAA or control egg-PC:cholesterol SUV, the macrophages took up about 5 times more of the PAA-liposomes than the control liposomes. The uptake of liposomes showed saturation behaviour in accordance with a Michaelis–Menten like model, suggesting that a specific receptor is involved. The liposomes were almost exclusively found in the acidic compartment of the cells after about 1 hour of incubation, estimated by including HPTS in the liposomes and looking at the HPTS spectra and fluorescence of the cells after uptake of the HPTS liposomes. The uptake of PAA-liposomes by RAW macrophages could be inhibited by dextran sulfate, BSA derivatised with maleic acid groups (maleyl-BSA), LDL (to a small extent) and acetyl-LDL. It could not be inhibited with poly-cytidine, which is a polyanion but is not a ligand for the scavenger receptor or heparin. Kamps et al (1997), showed that liposomes modified with polyacetylated HSA (aco-HSA) were found almost exclusively in the liver (80% of the injected dose), with less than 5% remaining in the serum just 30 minutes after injection in rats. In contrast, 80% of the control liposomes remained in the
serum at the same time point. When the animals were injected, prior to the injection of the liposomes, with 5 mg of polyinosinic acid, a known inhibitor of the scavenger receptors, the serum levels of aco-HSA liposomes after 30 minutes increased to 60% of the injected dose and the liver retention decreased 4-fold. Kupfer and liver sinusoid endothelial cells accounted for 90% of the hepatic uptake of aco-HSA, with the endothelial cells taking up about 2.5 times more label than the Kupfer cells. Pre-injection with polyinosinic acid resulted in a strong decrease in the uptake by the Kupfer and endothelial cells, but it also decreased the label uptake by the liver parenchymal cells. In contrast control liposomes are taken up predominately by the Kupfer cells with little or no contribution from the endothelial cells. Both groups showed clearly that macromolecules with a large number of carboxyl groups (polyacrylic acid, aco-HSA and maleyl-BSA) can be recognised by the scavenger receptors resulting in receptor-mediated endocytosis.

The importance of the scavenger receptors in liposome clearance has been clearly demonstrated both in vivo and in vitro. The work carried out so far suggests that the scavenger receptors are important in the clearance of negatively charged liposomes (especially liposomes containing PS) and can result in rapid clearance of liposomes modified with certain polyanionic ligands.

1.3.4.2 Complement and complement receptors

Complement is well known to be important in liposome clearance (reviewed in Devine and Bradley 1998). Complement activation results in deposition of complement C3b, which is an efficient opsonin, as it is recognised by the macrophage complement receptor and promotes phagocytosis (Devine and Bradley 1998, Alving and Wassef 1992). Liposomes can activate
complement either via the classical pathway, or via the alternative pathway (Cunningham et al 1979). The classical pathway involves binding of naturally occurring antibodies to liposomal components (Alving and Wassep 1992) or the interaction of C-reactive protein (Pentraxin pathway) with phosphatidylcholine and sphingomyelin (Volonakis and Narkates 1981, Richards et al 1977). Additional evidence for the activation of the Pentraxin pathway by liposomes has been presented by Schwalbe et al (1992) through specific interaction of the Pentraxin family proteins (C-reactive protein and serum amyloid P component) with phosphatidylcholine and phosphatidyl ethanolamine. Some liposomal components (such as cardiolipin) can also specifically activate the classical pathway in an antibody independent fashion (Kovacsovics et al 1985). It was proven by Bradley et al (1999) that C1q can directly bind cardiolipin (CL) containing liposomes and that the peptide consisting of residues 14-26 from the C1qA chains can inhibit this binding. The inhibition was shown to be sensitive to the amount of positive charge present on the peptide rather than its specific sequence, suggesting that charge is important for the inhibition while structure is not. No C1q binding to neutral liposomes was observed. Coupled with the observation that PS molecules are taken up by the scavenger receptor this can be used to explain the results of Moghimi and Patel discussed in section 1.3.3. Moghimi and Patel (1998) concluded that there was a dialyzable, heat-labile, Ca\(^{2+}\) dependent serum component that could target the liposomes to the spleen and whose removal resulted in increased liver uptake. Since liposomes with accessible negative charges can directly bind C1q and in addition are good targets for the scavenger receptors, a clear competition mechanism can be established: binding of C1q to the accessible negative charges results in deposition of complement and opsonisation, but at the same time it masks the negatively charged residues and could be effectively blocking the
interaction between the negatively charged components and the scavenger receptors. Hence, binding of C1q and the rest of the complement components may be enhancing the uptake of liposomes by the splenic macrophages, but reducing the rapid scavenger receptor mediated uptake by the liver sinusoidal endothelial and Kupfer cells.

1.3.4.3 Lipoprotein receptors
The lipoprotein receptors have an important role in the transport and redistribution of lipids such as cholesterol and triglycerides in the body. They are known to recognise lipoprotein particles (such as LDL), which generally consist of a layer of phospholipids and proteins (known as apolipoproteins) surrounding a core that is essentially an agglomerate of non-polar compounds such as triglycerides and cholesteryl esters (Brown and Goldstein 1986). Typically the apolipoproteins are the components that are recognised by the lipoprotein receptors (Salter and Brindley 1988) and these proteins have been shown to have an affinity for liposomes (Guo et al 1980). Evidence has been presented that the apolipoprotein apoE is important in the elimination of neutral PC:cholesterol liposomes by the liver, by using apoE deficient mice (Scherpof and Kamps 1998). The uptake of PC:cholesterol liposomes by the spleen was identical in the control and apoE deficient mice, but the uptake by the liver was reduced 6-fold, with a concomitant 6-fold increase in the blood retention in the apoE deficient mice compared to the control mice. In contrast, the liver uptake of 10%PS liposomes was only slightly different and in fact slightly increased for the apoE deficient mice, despite the fact that PS liposomes adsorb significant amounts of apoE (Scherpof and Kamps 1998). The authors suggested that, possibly the uptake of PS liposomes by the
apolipoprotein receptors is too slow compared to uptake via complement-mediated or scavenger receptor-mediated phagocytosis to have any significant impact.

1.4 Stealth liposomes

Given the importance of the RES in the clearance of liposomes from the circulation, methods to reduce or prevent liposome uptake by the RES have been the subject of intense investigation. Liposomes which are able to avoid the RES and have prolonged circulation half-lives are termed stealth liposomes (Allen 1992). Stealth liposomes by evading the RES can be targeted for delivery to other tissues either passively to tissues with leaky vasculature (e.g. tumours) or actively by exploiting specific interactions, such as that between the OX26 mAb and the transferrin receptor (Huwyler et al 1996) as well as between folate and the folate receptor (Ni et al 2002). Many attempts have been made to shield the liposomal surface by conjugating hydrophilic polymers particularly carbohydrates, spurred by the apparent ability of PI and GM₁ to prolong liposome circulation times (Gabizon and Papahadjopoulos 1992b, Allen and Chonn, 1987). However most of these attempts have been met with at best limited success, with a single exception. Grafting polyethylene glycol to the liposome surface has proven exceptionally effective at prolonging the plasma half-life of many liposome formulations (Klibanov et al 1990, Senior et al 1991, Allen et al 1992). The mechanism through which this is accomplished is still under investigation. The experimental evidence obtained by studying GM₁ and PEGylated liposomes will be discussed in this section.
1.4.1 Ganglioside GM\textsubscript{1} incorporating liposomes.

Glycolipids and the sialogangliosides in particular are important components of biological membranes, serving a wide variety of functions ranging from signal transduction to cell-cell recognition and adhesion (Barrier 2003, Tettamanti and Riboni 1994, Cheresh et al 1986). The term sialoganglioside was first used by Ernst Klenk in 1942 to describe a family of newly isolated lipids from ganglion cells that contained sialic acid (Klenk 1970). Gangliosides are typically composed of ceramide attached to a core chain consisting of galactose, glucose, and for most gangliosides N-acetylglucosamine. One or more galactose residues are linked at their 3-hydroxyl group with sialic acid (N-acetylneuraminic acid). A role for sialic acid in the membranes of circulating blood cells has been established by showing that removal of sialic acid results in rapid uptake of circulating cells by the Kupfer cells in the liver (Woodruff and Gesner 1969). It is now known that this interaction is mediated by the galactose receptors, as removal of sialic acid exposes galactose residues (Schauer 1990, Gregoriadis and Senior 1984). The potential of gangliosides in improving the circulation times of liposomes was first demonstrated by Allen and Chonn (1987). These researchers found that inclusion of ganglioside GM\textsubscript{1} in eggPC:cholesterol (2:1) liposomes (160-170nm in diameter – large unilamellar vesicles or LUVs) drastically reduced the uptake of liposomes by the liver at 2h post injection, in a fashion dependent on the GM\textsubscript{1} content. The reduction in liver uptake and the liposome blood levels 2 h post-injection, reached a plateau at 5 mol% GM\textsubscript{1}, as greater GM\textsubscript{1} incorporation did not result in significant improvement. Spleen uptake showed a slight increase as the amount of GM\textsubscript{1} is increased. A more pronounced effect was observed when sphingomyelin (SM) was included in the bilayer. A dramatic decrease in liver uptake and a modest decrease in spleen uptake with a
concomitant large increase in blood levels was seen in eggPC:SM liposomes incorporating GM₁, when the amount of GM₁ was increased above 7mol%. Some very interesting results were also obtained with asialoganglioside asGM₁. It was found that PC:cholesterol LUVs with 7mol% asGM₁ had a higher blood/RES ratio, 2 h post-injection, than PC:cholesterol LUVs. Compared to the liposomes with 7 mol% GM₁, the asGM₁ liposomes had a more than 2-fold decreased blood/RES ratio 2 h post injection. Even more interesting is the finding that asGM₁ failed to improve the blood/RES ratio of SM:PC liposomes, while GM₁ was very effective. This result once again demonstrates the importance of the interplay between different clearance mechanisms. While it is clear that both asGM₁ and GM₁ can block some of the liposome elimination mechanisms (discussed below), asGM₁ also introduces a new clearance mechanism (uptake via the galactose receptors). For PC:cholesterol liposomes, which are cleared rapidly, inclusion of asGM₁ results in prolonged half-life, as clearance via the galactose receptor is slower than the clearance of PC:cholesterol liposomes without asGM₁, so that overall there is a net improvement. For SM:PC liposomes on the other hand, which are cleared more slowly, inclusion of asGM₁ does not result in prolonged half-life because clearance via the galactose receptor is not much faster than the clearance of SM:PC liposomes without asGM₁, so that overall there is no improvement. This complex interplay needs to always be taken into account when studying liposome stabilisation methods.

Further work with gangliosides clearly showed that many other gangliosides, like the disialoganglioside GD₁₈ and trisialoganglioside GT₁₈ failed to produce results similar to GM₁ (Gabizon and Papahadjopoulos 1992b, Allen and Chonn, 1987, Chonn and Cullis 1992). This may reflect a trade-off, between the stabilising effect of the carbohydrate on the liposome surface and the surface-accessible negative charge introduced by the gangliosides.
In addition it was shown that GM$_1$ liposomes could slow down but not prevent agglutination of biotinylated liposomes by streptavidin and it did not inhibit the binding of 34A monoclonal antibody-bearing immunoliposomes to lung endothelial cells (Mori et al 1991). Chonn and Cullis (1992), also demonstrated that while PC:cholesterol:PG liposomes can deplete complement, the same formulation with 10 mol% GM$_1$ could not. The same researchers studied the binding of complement C3 and IgG onto liposomes with and without GM$_1$. They found that 2 minutes after intravenous injection, no C3/C3b could be detected bound on SM:PC or SM:PG liposomes with 10 mol% GM$_1$, in contrast to various formulations without GM$_1$ which quickly associate with C3/C3b and IgG to a greater or lesser extent. Incidentally PS and cardiolipin liposomes showed the greatest attachment of IgG and C3.

1.4.2 Polyethylene glycol grafted liposomes.

To date the most successful method for creating stealth liposomes is the incorporation of PEGylated lipids. Typically the lipid used is PE, whose amine group is covalently linked to a PEG chain ranging in molecular weight from 2000 to 5000 Da. Drawing on results showing that PEG was highly effective in prolonging the blood circulation of proteins, a number of groups investigated the ability of PEG to do the same for liposomes. (Woodle et al 1990, Klibanov et al 1990, Senior et al 1991, Allen et al 1991, Papahadjopoulos et al 1991).

1.4.2.1 Polyethylene glycol prolongs the circulation time of liposomes

Klibanov and co-workers (1990) showed that incorporation of 7.4 mol% of PEG-PE in PC:cholesterol liposomes resulted in a plasma half-life of 5 h compared to 1.5 h for a similar
preparation containing GM₁ and less than 30min for the control PC:cholesterol liposomes. The molecular weight of PEG was found to be very important. PEG of less than 1900 Da molecular weight was not very effective at increasing the plasma retention time of liposomes, while PEG1900 produced the same effects as PEG5000 (Allen 1992). Inclusion of the PEG lipid into liposomes showed saturation behaviour and the limit was dependent on the PEG molecular weight (Allen 1992). The optimum molecular weight and optimum incorporation level of PEG-lipid for liposome stabilisation was determined to be 2000 Da and 5 mol% respectively (Woodle and Lasic 1992).

1.4.2.2 Interactions of proteins, cells and other surfaces with PEG-liposomes

Experiments characterizing the effect of PEG on liposomes showed that PEG can prevent the streptavidin induced aggregation of liposomes containing biotinylated lipid in contrast to GM₁, which could only slow the process down, but not stop it (Mori et al 1991). PEGylated immunoliposomes (PEG5000), derivatised with the 34A monoclonal antibody (highly specific to pulmonary endothelial cells), were found to be unable to bind to the lung endothelial cells, when both the antibody and the PEG were grafted to the liposome surface, once again in contrast to results obtained with GM₁ (Mori et al 1991). PEG750 and PEG2000 did not reduce antibody binding (Mori et al 1991). PEGylated immunoliposomes, where the antibody is attached to the end of the liposome grafted PEG chain instead of the being attached directly to the liposome surface are in contrast able to bind their target. Such liposomes bearing the OX26 activating monoclonal antibody to the rat transferrin receptor, were able to bind their target and activate it since the immunoliposomes could cross the blood brain barrier, a fact that can best be explained by transferrin receptor mediated
transcytosis (Huwyler et al 1996). These results strongly suggest that PEG is able to hinder surface to surface contact as well as contact between molecules displayed on the liposome surface with molecules displayed on other surfaces, in a manner that is dependent on PEG chain length. GM₁ in contrast does not have this ability. A number of studies have been published suggesting that grafting of PEG to the liposome surface can reduce protein binding (Du et al 1997, Chiu et al 2001). Theoretical calculations on the ability of PEG-grafted on the liposome surface to repel both proteins and other surfaces (e.g. other liposomes, or cell membranes) have been also been published (Lasic et al 1992, Needham et al 1992). It has been proposed that the presence of PEG can sterically hinder access to the liposome surface (Lasic et al 1992) an effect which has been termed steric stabilisation (Papahadjopoulos et al 1991, Lasic et al 1991). The stability of liposomes in suspension has been described theoretically (LeNeveu et al 1976, 1977, Sato 1980) by modifying the DVLO theory (Verwey et al 1948) for colloidal suspensions. Based on this model, liposomes remain in solution rather than aggregate and precipitate, due to a balance between a mixture of attractive and repulsive forces. The attractive forces are van der Waals forces and hydrophobic interactions. The repulsive forces are electrostatic repulsions, a repulsive hydration force and steric repulsion. The hydration force arises from the presence of water of solvation that is structured and tightly held in place around the colloid particle or liposome by hydrogen bonds. This water needs to be “squeezed” out of the way before another molecule can approach, a process that requires energy and hence raises the energy barrier that must be overcome before getting through to the surface past the hydration shell. The presence of both attractive and repulsive forces results in there being an energy minimum when the colloid (or liposome) surfaces are at a certain non-zero distance (i.e. the particles
staying apart is more energetically favourable than aggregation) as well as an energy barrier for another surface or large molecule in approaching the colloid (or liposome) surface. The presence of PEG, raises this energy barrier by contributing to both the hydration force and the steric repulsion forces. The PEG chain in solution acquires strongly bound water of solvation, which results in a much more ordered and tightly bound hydration shell around the liposome. Furthermore the physical presence of PEG sterically hinders other molecules or surfaces from approaching. This effect of PEG has been shown to depend upon the conformation that PEG adopts on the liposome surface, which in turn depends on the length of the PEG chain and the density of the grafted PEG chains on the particle surface (Needham et al 1992, Lasic et al 1992). Larger PEG chains cannot achieve high density on the liposome surface and hence the chains adopt a mushroom like conformation (Borisov et al 1995), with most of each individual PEG chain collapsed into a roughly spherical structure that is connected to the liposome by an extended segment of the chain. Smaller PEG chains, have a much reduced tendency to collapse into more compact structures and in addition can achieve high densities on the liposome surface. This results in the chains adopting an extended conformation and forming a brush-like arrangement that surrounds the surface (Borisov et al 1995). The brush seems to be a more effective arrangement in sterically hindering access to the liposome surface. It is likely that PEG, in addition to slowing down protein adsorption (Senior et al 1991), can directly prevent interactions between cells and liposomes (Du et al 1997).
1.4.2.3. The mechanism of steric stabilisation by PEG.

It was initially suggested that PEGylation acts mostly by blocking interactions with plasma proteins, and consequently serum opsonins, thus rendering the liposomes "invisible" to the immune system (Chonn 1992, Allen 1992). However the actual mechanism, by which PEG prolongs liposome circulation in the blood turned out to be more complex.

It was found that PEG-liposomes can actually activate complement (Szebeni et al 2002, Laverman 2001) and can elicit an immune response that is apparently PEG specific (Shroda et al 2005, Ishida et al 2003), showing that PEG is not completely non immunogenic as will be discussed later in this section.

Senior and co-workers (1991) studied the affinity of PEG-liposomes for plasma proteins using a two-phase system and showed that incubation with plasma shifted the partitioning of PEG-liposomes to nearly match the partitioning of control liposomes incubated with plasma. It took a longer time for the partitioning to be completed, compared to the control liposomes. A different study indicated that to obtain high inhibition (>50%) of prothrombin binding to PS liposomes, a PEG-lipid incorporation that exceeds 10 mol% is required and that 5 mol% PEG-lipid did not result in good inhibition (Chiu et al 2001). In contrast 5 mol% PEG-lipid is sufficient and in fact optimum for prolonging liposome half-life in vivo (Woodle and Lasic 1992). In contrast to proteins cell-liposome interactions can be inhibited by only 0.5 mol% PEG lipid (Chiu et al 2001, Du et al 1997).

Johnston et al (2001) studied the association of mouse serum proteins with DSPC:cholesterol liposomes containing negatively and positively charged lipids (with and without PEG) and their ex-vivo uptake by bone marrow macrophages in the presence and absence of serum. They found by total protein measurements and SDS-PAGE analysis on liposomes purified by
gel permeation chromatography after incubation with serum, that PEG decreased the binding of plasma proteins to DSPC:cholesterol liposomes only to a small extent, in good agreement with the observations of Senior et al (1991). Surprisingly PEG actually increased the binding of plasma proteins for DSPC:cholesterol:PS liposomes and for the positively charged DSPC:cholesterol:DODAC liposomes. With the cell-line experiments it was found that without PEG or serum pretreatment, DSPC:cholesterol had the lowest uptake by bone marrow macrophages, followed by DSPC:cholesterol:PS liposomes, while DSPC:cholesterol:DODAC liposomes had the highest uptake. When plasma was added, the uptake of DSPC:cholesterol and DSPC:cholesterol:DODAC remained unchanged, while the phagocytosis of DSPC:cholesterol:PS liposomes, was decreased by approximately 20-fold. Without serum pretreatment, addition of 5 mol% DSPE-PEG2000 to the liposomes, resulted in all 3 formulations having the same uptake. Surprisingly the uptake level was actually increased for DSPC:cholesterol, while for the other two formulations it was drastically decreased. The results after serum pretreatment of PEG liposomes were even more surprising. The uptake of DSPC:Cholesterol:PEG liposomes was drastically reduced to a level lower than that seen without PEG. The uptake of the other two PEG formulations also decreased drastically, but to a lesser extent than DSPC:cholesterol:PEG liposomes especially for the DODAC formulation. The authors concluded that PEG does not efficiently block the binding of plasma proteins in good agreement with the results by Chiu et al (see above). More importantly they found that addition of plasma reduces the uptake of PEG liposomes by bone marrow macrophages.

Levchenko et al (2002) studied the in vivo clearance of PC:cholesterol liposomes incorporating 6 mol% SA, PA and PS with and without 6 mol% PEG750 or PEG5000. They
found that addition of PEG5000 to PC:cholesterol liposomes with 6mol% SA resulted in a blood clearance and a liver elimination profile that was identical to that of PC:cholesterol liposomes with 6 mol% of PEG5000 or PEG750, i.e. the negative impact of SA on the liposome clearance was completely reversed. Addition of PEG750 to SA containing liposomes on the other hand greatly reduced the negative impact of SA as well, but did not eliminate it. PEG2000 at 6 mol% was able to reverse to a large extent though not completely the negative impact of incorporating PA in the liposomes, while PEG750 was not effective. Neither PEG2000 nor PEG750 at 6 mol% was effective at reducing the negative impact of PS. These results clearly support the results obtained by Chiu et al (see above) who concluded based on the inhibition of PS-mediated prothrombin activation that 5 mol% PEG is not sufficient to prevent interaction of proteins with surface PS lipids.

It appears that PEG, at least at the optimum incorporation level for prolonging circulation, is not very effective at reducing the binding of proteins to the liposome or at blocking the access of proteins (including complement) to the liposome surface. More importantly the results reported by Johnstone and co-workers (2001) seem to suggest that the bound proteins are important in preventing the phagocytosis of PEGylated liposomes, as plasma seems to inhibit bone marrow macrophage uptake of PEGylated liposomes instead of enhancing it. On the other hand PEG could counteract the negative impact of including PS in the liposome formulation, in the ex-vivo phagocytosis experiments. Given that PEG even at very low incorporation level is effective at hindering surface to surface contacts, the data described here suggest that PEG may be inhibiting phagocytosis, more by preventing binding of liposomes to phagocytes, than by preventing attachments of proteins.
1.4.2.4 The biodistribution of PEGylated liposomes

The effect of PEG on liposome biodistribution has also been investigated (Mori et al 1991, Ishida et al 1999, Awasthi et al 2003). Mori and co-workers (1991) found that although 6mol% of PEG750, PEG 2000 and PEG5000 was effective at prolonging the circulation time of PC:cholesterol liposomes, the liver and spleen were still the major clearance route for the liposomes. Ishida et al (1999) reported that the biodistribution of DSPC:cholesterol liposomes with 4.8 mol% PEG2000, in solid tumour-bearing mice was strongly size dependent. For liposomes with an average diameter of 120nm addition of 4.8 mol% DSPE-PEG2000 resulted in longer a 4-fold increase of liposome blood level, a 4-fold increase in tumour localization and a drastic reduction in the uptake of liposomes by the liver and spleen, at 6 hours post injection. In contrast, for DSPC:cholesterol liposomes with 4.8 mol% PEG and an average diameter of 400nm blood levels at 6 hours post-injection, were much lower than both the PEGylated and the non-PEGylated 120 nm liposomes. Tumour levels were similar to those for non-PEGylated DSPC:cholesterol liposomes. Even though liver retention was decreased significantly compared to the 120 nm liposomes, retention by the spleen was strongly increased. The biodistribution of 400 nm and 120 nm liposomes with PEG2000 was also studied after splenectomising the mice. For the 120 nm liposomes, blood circulation did not change significantly and neither did tumour localization, while liver retention was increased to a small extent. For the 400 nm on the other hand, splenectomy increased the blood levels sharply to a level comparable with the smaller liposomes, while tumour and liver localization remained unchanged. Even more interesting is the observation that the small liposomes extravasating in the tumour were exclusively localized in the tumour infiltrating macrophages and not in the cancer cells themselves. The circulation half-life seen
by Ishida and co-workers was quite low compared to what was reported by Chiu et al 2001 and Senior et al 1991, who saw half-lives in excess of 18 hours. This is probably due to differences in dosing. Chiu et al used a dose of 2 μmol phospholipid per mouse and Senior et al used a dose of 2.5 μmol phospholipid per mouse, while Ishida et al used a dose of only 0.3 μmol phospholipid per mouse. The results described above, indicate that the spleen is the major route of liposome clearance for large size PEGylated liposomes, that even small PEGylated liposomes are still cleared predominately by the RES (albeit more slowly), even though they are quite effective at avoiding the spleen. Interestingly, large PEG liposomes had a similar blood clearance with the small PEG liposomes after removal of the spleen, however no increase in tumour targeting was observed, showing the important of small size for passive targeting to tumours.

Awasthi et al (2003) also studied the effect of size in the biodistribution of DSPC:cholesterol:α-tocopherol liposomes with 5 mol% DSPE-PEG in rabbits, using large doses (>10 mg of lipid per animal). They found that as liposome size increases from 130 to 320 nm, a gradual increase in spleen uptake and a reduction in circulation times is seen when the liposome size is increased past 165 nm in diameter. Other organs with significant uptake were the skin, muscle, bone marrow and the liver. Increasing the size of the liposomes generally resulted in decreased uptake by the skin and the liver, but no clear cut trend could be seen.

More evidence of the importance of the RES in PEG-liposome uptake, comes from the observation that PEGylated liposomal doxorubicin formulations, can still induce an RES blockade (Lim et al 2000).
The evidence from the biodistribution studies, confirms that PEG-liposomes are not as invisible to the immune system as was previously suggested. Although clearance via the spleen and liver is slowed down, the spleen remains the major route of elimination especially for larger liposomes.

1.4.2.5 Interactions between PEGylated liposomes and the immune system

A very interesting result was obtained by Dams et al (2003), who reported that intravenous injection of PEGylated liposomes alters the clearance profile of subsequent injections of PEGylated liposomes, in both rats and rhesus monkeys. They also showed that the frequency of dosing is critical and obtained evidence of the involvement of a heat labile transfusable serum component. Ishida et al (2003) showed that the reduction in circulation time was maximal when the second injection was administrated 5 days after the first one and that liver uptake quadrupled for the second dose, while spleen uptake did not change significantly. No effect was seen when the time between the injections was 14 days or more. They confirmed that the factor responsible for this effect was transfusable, and heat labile, by transfusing heated and non-heated serum from a rat dosed with PEG-liposomes, 5 days prior to the transfusion experiment. They also showed that the second dose does not increase complement consumption.

Philips and Dahman (1995) investigated the immunogenicity of IgG2a immunoliposomes. Rat IgG2a was coupled with avidin and non-covalently linked to liposomes made from DPPC, DMPG and DPPE-biotin with or without 5 mol% PEG-distearate (Mr = 400) through the biotin-avidin linkage. Free rat IgG2a was not immunogenic in mice, while Alum-adsorbed Ig2a was only weakly immunogenic. Liposome associated Ig2a on the other hand
produced an anti-rat IgG2a antibody titer that was about 5-fold higher than that obtained with
the alum formulation. Surprisingly the PEG containing immunoliposomes were about 2-fold
more immunogenic than the liposomes without PEG. The antibody response was specific to
IgG2a with little cross-reaction with IgG2b. Minor antibody responses to the biotin-
phospholipid linkage were also seen. Tardi et al. (1997) found that Ovalbumin covalently
linked to DSPC:cholesterol liposomes with 5 mol% PEG produced a more than 4-fold
increased anti-ovalbumin titer compared to free ovalbumin. In addition they demonstrated
that encapsulating doxorubicin in the liposomes prevents an immune response to this protein.
A potential mechanism of action is that PEG slows down uptake of the liposomes by
macrophages in the spleen, giving the liposomes more time to present the antigens to the
spleen B-cells directly, in addition to being taken up for antigen processing and presentation
by phagocytes. It is quite relevant that the size of the liposomes used by Phillips and
Dahman, had an average diameter of 560 nm, which would result in high spleenic retention
as demonstrated by Ishida et al. (1999) and Awasthi et al. (2003) This would explain why
doxorubicin included in the liposome blocks the immune response. The liposomes increase
the antigenicity of ovalbumin, by carrying the protein to the spleen and remaining intact
longer (due to slower phagocytosis) displaying it to the antigen producing cells. In the same
way if doxorubicin is included in the liposomes, it can kill the cells in the spleen being
activated by the ovalbumin liposomes and hence block the immune response. An interesting
possibility is that the membrane bound PEG due to its amphiphilic properties can interact
with the protein, altering the tertiary protein structure and exposing buried epitopes. The two
possibilities suggested are not mutually exclusive and in fact it is more likely that they would
be synergistic. It is therefore evident that PEGylated liposomes, are still perfectly able to
interact with the immune system and in fact they can actually enhance the antigenicity of proteins present in the liposome surface.

1.4.2.6 PEGylated liposomes: conclusions.

It would appear that the mechanism of action by which PEG prolongs the retention of liposomes in the blood, is not as straightforward as it was originally thought. The in vitro data suggest that the optimal incorporation levels of PEG for improving the liposomal plasma half-life, are sufficient to strongly inhibit cell-liposome interactions but insufficient to strongly inhibit protein association with the liposomes. In fact as discussed above it was shown that binding of plasma proteins to PEG-liposomes can strongly inhibit uptake by bone marrow macrophages. The in vitro data are supported by the observations from the in vivo work. PEG-liposomes are taken up more slowly by the RES, and distribution into sites of inflammation and injury or in tissues with “leaky” vasculature (such as tumours) is increased. Nevertheless they still accumulate mostly in the RES, which explains why doxorubicin PEG-liposomes induce an RES blockade and why an RES blockade can prolong the circulation times of PEG-liposomes. The apparent immunogenicity of liposome-grafted PEG and proteins attached to PEG-liposomes suggests a possible and obvious mechanism for PEG localization in the RES. It is important to note that PEG-liposomes extravasating into tumours seem to be taken up mostly by tumour infiltrating macrophages. Finally proteins bound to PEG liposomes become more immunogenic and inclusion of doxorubicin into these liposomes can abolish that response and PEG could not prevent complement activation (complement is an important opsonin), but it could inhibit the binding of immunoliposomes to their target.
These results strongly suggest that the inhibition of the cell to liposome interactions is the most important component of the improved in vivo behaviour of PEG-liposomes. Such cell-liposome interactions (i.e. strong binding of liposomes to phagocytic cells) are necessary before cells of the MPS can take up liposomes (Johnstone et al 2001), hence it is likely that PEG can inhibit uptake by macrophages regardless of whether proteins can reach and bind the liposome surface. Even if proteins manage to reach the liposome surface, their interaction with receptors on RES cells, which is necessary for uptake, will be hindered by the presence of PEG.

Johnstone et al 2001 proposed that PEG and plasma proteins act together to inhibit liposome-macrophage uptake. They suggested that even though PEG at 5 mol% is insufficient to block protein access to the liposome surface, protein binding to the liposome surface may result in PEG undergoing a structural change, adopting an arrangement that is optimal for inhibiting cell to cell interactions, they also suggested that the PEG itself might actually provide a scaffold for the binding of proteins. The model of the protein-induced conformation change in PEG, is not in good agreement with the observed immunogenicity of proteins linked to PEG-liposomes, as the extended configuration should have blocked the interaction of the linked antigen, although this can also be explained as the result of the PEGs used (400 and 2000) not being long enough. An intriguing hypothesis is that PEG due to its amphiphilic nature, may actually (as suggested by Johnstone et al) be able to bind proteins directly in a more or less non-specific manner, thus coating the liposome with plasma proteins that have no function in clearing foreign substances from the circulation. These proteins, may compete with and prevent the binding of proteins that can accelerate liposome clearance, effectively rendering the liposome invisible to the immune system using a coat of “self”-proteins. Such a
mechanism is sometimes utilized by bacteria to evade the immune system. For example the 
*S. aureus* protein A that is displayed on the bacterial surface results in massive deposition of 
IgG on the bacterium, bound through its Fc portion (Mims et al 1998). In this way the 
bacterium acquires a coating of non-functional IgG (the Fc portion is blocked), which 
actually shields the bacterium from antibodies against other components of its protein coat 
(Mims et al 1998).

1.4.3 Colominic acid.

Another polymer that has shown great potential in prolonging the half-life of proteins in 
plasma is colominic acid (Jain et al 2003, Gregoriadis et al 2005, Fernandes and Gregoriadis 
1996, 1997, 2001). Colominic acid is a naturally occurring, linear, α2-8 linked homopolymer 
of sialic acid (see figure 1.4) and is hence one of the family of polymers known as polysialic 
acids (Roth et al 1993). It is a major polysaccharide capsule constituent of the *E.coli* K1 
strain and the group-B *meningococci*. 

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The presence of colominic acid in the capsules of these bacteria is a strong determinant of pathogenicity (Mushtaq et al 2005). It has been shown that bacteria with colominic acid are resistant to complement fixation and phagocytosis, while bacteria without colominic acid are quite vulnerable to complement fixation and complement mediated opsonisation (Mushtaq et al 2005). The reason behind this property of colominic acid is that it occurs naturally in most mammals, including humans (Roth et al 1993). For example short colominic acid chains are found in the neuronal cell adhesion molecule (NCAM) (Cremer et al 2000, Kudo et al 1996). The polysialic acid chains on NCAM have been shown to have a vital role in neuronal development, axon growth and fasciculation as well as various cell migration processes (Cremer et al 1997, 1998, Yoshida et al 1999). It is also an important regulator of cell interactions that involve membrane contact (Rutishauser et al 1988). In addition polysialylated NCAM (PSA-NCAM) has been implicated in the regulation of long-term
potentiation at the hippocampal mossy fiber synapses (Cremer et al 2000). Hence, for most mammalian species polysialic acid is a self antigen, which explains the extreme difficulty of raising antibodies specific for the *N.meningitis* group B and *E.coli* K1 capsules (Berry et al 2005, Muhlenhoff et al 1998, Nato et al 1991). This property of colominic acid makes it a potentially very useful molecule for making "stealth" liposomes.

1.4.4 Polysialylated liposomes

The polysialylation of liposomes and the properties of the conjugate have been investigated previously by Zhang (Zhang 1999). Colominic acid was covalently linked to PC:cholesterol:PE containing liposomes by reductive amination. The CA was shown to be stable on the liposome surface after incubation for 2 hours in plasma. The polysialylated liposomes did not change in size even after 4 weeks storage at 4°C, while the control liposomes increased in size from less than 200 nm to more than 4 μm. Incubation of the liposomes in 0.5% albumin did not affect the polysialylated liposomes, but liposomes with 10 mol% PE increased in size by 7-8fold and the 20 mol% PE liposomes increased in size by about 2-fold. Similar results showing a size increase for non-polysialylated liposomes and no change for polysialylated liposomes were also obtained upon incubation with plasma. Grafting of colominic acid was found to reduce protein binding by 2-3fold. On the other hand it was shown that liposomes grafted with colominic acid were more "leaky", especially upon incubation with plasma. The polysialylated liposomes released about 8 to 12% of encapsulated label depending on the amount of PE in the liposomes compared with 2-3% for the control liposomes, when incubated at 37°C in PBS for 24 hours. Upon incubation with plasma this increased to 25-35% for the polysialylated liposomes compared with about 5%
for the control liposomes. It is very interesting to note here that there appears to be an effect dependent on the PE incorporation level, on the stability of liposomes in plasma even though it was clearly shown that the degree of polysialylation was identical for all preparations containing more than 10 mol% PE. Interesting results were also obtained from the in vivo studies in mice. Using a dose of 3.2 μmol phospholipid per 20-25 g mouse, injected in the tail-vein, at 6 hours post-injection, 35.1% of the polysialylated liposomes remained in circulation compared with 10% of the control liposomes. At 3 hours post injection about 42% of the polysialylated liposomes remained, compared with 12% for the control. Using a dose of 1.6 μmol per mouse and at 3 hours post injection 22.8% remained for the polysialylated liposomes, compared with 9.2% for the control liposomes. Upon investigation of the biodistribution of the polysialylated liposomes it was found that after 3 hours, the main route of elimination for all formulations was the liver, with the kidney being the second organ with the greatest uptake (the marker was 3H-PC). A decrease in the amount present in the liver was seen for polysialylated liposomes with 10% PE compared to 10 mol% PE liposomes without colominic acid. A decrease was also seen in the kidney and lung uptake. There was no reduction in the splenic accumulation, but there was a 2-fold increase in the blood retention for the polysialylated liposomes compared to the control. It is interesting that polysialylated liposomes with 20 mol% PE had a number of small differences in their biodistribution profile, despite having the exact same degree of grafting. For the 20% PE CA-liposomes, splenic uptake was less than that of both the other preparations. Kidney and lung uptake was more than that of 10 mol% PE liposomes, but less than that of the control. Some differences were seen in the blood and liver uptake. The differences are large in magnitude,
but the standard error is also quite large, so the differences may not be statistically
significant.

A number of questions were left unanswered. The extent of conjugation was still very low
compared to the incorporation levels found optimal for PEG. Approximately 0.1 mol% of
polysialylated lipid was formed, compared to 5 mol% for PEG lipids. It was hence not
determined, whether increasing the extent of colominic acid conjugation to levels comparable
with the amount of PEGylated lipid required for optimal performance, can improve the
performance of the liposomes. The use of egg PC:cholesterol based formulations, means that
results cannot be extrapolated to more stable formulations, which are more likely to be useful
and it complicates the interpretation of the data. For example as explained in section 1.4.1
Allen et al (1987) observed a significant stabilisation effect with asialoGM₁ with
eggPC:cholesterol based formulations, even though it is well known that asialoGM₁ is taken
up by the galactose receptor (see section 1.4.1). On the other hand asialoGM₁ failed to
prolong the circulation of SM:PC liposomes, which are more stable. Thus if colominic acid
activates a new clearance mechanism, its effect may be missed if a formulation that is very
rapidly cleared is used. The size of the polysialylated liposomes was well over 120 nm (140-
150 nm), which would have a significant impact on the results. Protein binding to
polysialylated liposomes was decreased, but only 2-3 fold. The pattern of protein binding to
the liposomes was not investigated. Conjugation of colominic acid on the liposomes resulted
in greater carboxyfluorescein (CF) leakage compared to the control liposomes, both in the
presence and in the absence of plasma. No explanation was offered for the increased leakage
in the absence of plasma. For the increase in the presence of plasma it was proposed the
hydrolysis and removal of colominic acid might allow proteins to increase the leakage, but
that is inconsistent with the results, as this cannot account for an increase in leakage compared to control liposomes. It has been calculated theoretically (Hristova and Needham 1995) and verified by physical measurements (Garbuzenko et al 2005) that addition of large polymers to bilayers results in a decrease in the bilayer internal pressure. Hence conjugation of large colominic acid chains to liposomes, may have decreased the bilayer internal pressure, making the liposomes inherently more "leaky", but also more susceptible to infiltration by proteins that can insert into bilayers, further promoting leakage of the liposome contents. Liver uptake was reduced but not dramatically, as is the case with GM₁ and PEG liposomes. The spleen uptake was reduced, yet an even more pronounced decrease was observed in the kidney. Another important issue is that the molecular weight of the colominic acid supplied by Sigma at the time was underestimated by about 5 kDa.

Although the circulation times of PC:cholesterol liposomes was increased, the response was strongly dose dependent, as the percentage of the injected dose remaining at 3 hours post injecting decreased by nearly 50% when the dose was decreased to 1.6 μmol phospholipid from 3.2 μmol phospholipid. Greatly decreased dose dependency is on the contrary observed with GM₁ and PEG (Allen 1992). The half-life of the polysialylated liposomes is also rather low, due to the use of egg-PC in the preparation. It took less than 3 hours to remove 50% of the liposomes from the circulation, which is quite low when compared to the 18-20 hours it takes to remove 50% plain DSPC:cholesterol:DSPE formulation at the same dose or 4-6 hours at 1/8th the dose.
1.5 List of objectives

In order to more thoroughly evaluate the steric stabilisation capabilities of colominic acid. It was decided to polysialylate DSPC:Cholesterol formulations, which already have a very good in vivo behaviour (Senior and Gregoriadis 1982b). First of all colominic acid of better defined molecular weight needed to be prepared, to ensure that the effect of the colominic molecular weight can be investigated given the crucial effect that PEG molecular weight has for PEGylated liposomes (Maruyama 1991). Other conjugation chemistries aside from reductive amination had to be explored to increase the extent of grafting and decrease the size of the final product. The effect of colominic acid on the surface also required further investigation and the interactions of polysialylated liposomes with plasma proteins had to be characterised in more detail. It was hoped that the results would shed some more light into the mechanism by which colominic acid can prolong the half-life of liposomes in the circulation.

A parameter which needs to be considered carefully is the charge of the colominic acid. Accessible, non-sterically hindered negative charges are believed to have a detrimental effect on the circulation times of liposomes. The scavenger receptor, which has been implicated in the clearance of liposomes from the circulation, is well known to recognize negatively charged polymers with a broad specificity, and it may also recognise liposomes modified with colominic acid (see section 1.3.4.1). For example it was demonstrated that the scavenger receptor is responsible for the rapid removal from the circulation by liver endothelial cells of liposomes coupled to polyacryltylated human serum albumin (Kamps 1996, 1997) and liposomes modified with polyacrylic acid (Fujiwara 1996). Both these compounds contain multiple negatively charged carboxylic acid groups, like colominic acid does, so it is possible
that colominic acid may also be recognized by the scavenger receptor. It is not possible to
determine if any such interaction occurs from the result of the study by Zhang (Zhang 1999),
due to the use of PC:cholesterol formulations and the low conjugation ratio. It was thought
that the use of DSPC:cholesterol based formulation, would make the presence of such an
interaction easier to observe.
Chapter 2

*Materials and methods*
2.1 Assays: Materials
Colominic acid (linear α-(2→8)-linked *E. coli* K1 polysialic acid) 22.7kDa average molecular weight (1.34 polydispersity) was purchased from Camida, Ireland. Resorcinol (1,3 dihydroxy-benzene), copper sulphate, 2, 4, 6 trinitro-benzene sulfonic acid (TNBS), borax, glycine 5,5'-dithio-bis(2-nitrobenzoic acid) referred to as DTNB or Ellman's reagent, cysteine, ethylene-diamine-tetra-acetic acid (EDTA), sodium hydrogen phosphate, ammonium isothiocyanate, iron(III) chloride hexahydrate, potassium ferricyanide, Ferric ammonium sulphate, sodium carbonate, sodium dodecyl sulphate (SDS), glucose, 2,4 dinitrophenylhydrazine (2,4-DNPH) solution (1mM in 1N HCl) and other chemicals were purchased from Sigma-Aldritch, UK. Hydrochloric acid, chloroform, ethanol, acetic acid and other solvents were purchased from VWR international, UK. Phospholipids were obtained from Lipoid, UK. Deionised water was obtained from an Elgastat Option 4 water purification unit (Elga Limited, UK). Non-sterile microtitre 96 well plates (sterilin) were obtained from VWR, international. A plate reader (Dynex Technologies, UK) was used for spectrophotometric determinations in aqueous solutions. A Beckman DU650 spectrophotometer was used for spectrophotometric determinations in organic solvents.

2.1.1 Determination of colominic acid with the resorcinol assay
The mechanism of colour formation in the Svennerholm resorcinol assay (1957) has not been completely elucidated. It is believed that the basis of the resorcinol assay is the reaction between the acid degradation products of the saccharides with resorcinol (Ferrier and Collins 1972). When heated in mineral acids free sugars may degrade through dehydration to form furan derivatives, followed by break down of these furan derivates, resulting in the formation of various carbonyl-group containing molecules (Ferrier and Collins 1972). Resorcinol is
well known to form polyaromatic structures known as resorcinarenes following acid catalysed reactions with aldehydes (Schneider and Schneider 1994, Timmerman et al 1996).

Scheme 2.1: The formation of resorcinarenes from the reaction between resorcinol and an aldehyde (scheme from St.Luce 2004)
The resorcinarenes themselves are coloured and may be the chromophore, but an additional possibility is that the actual chromophore is a xanthene dye. It has been claimed that xanthene dyes can form when a resorcinarene is heated in DMSO formed by ring opening followed by dehydration and oxidation of non-cyclic resorcinarene (St. Luce 2004).

![Chemical structure](attachment:image.png)

Scheme 2.2: Formation of Xanthenes from resorcinarenes

The resorcinol assay was carried out according to the method of Svennerholm (1956), with modifications. In more detail the resorcinol reagent was prepared by mixing 20 ml of 1% (w/v) resorcinol in water with 80 ml of concentrated hydrochloric acid. The sample to be determined was diluted if necessary to a colominic acid concentration ranging from 40 to 200 µg/ml to remain within the detection limits of the assay. 100 µl of sample were mixed with 100 µl of resorcinol reagent and heated at 95°C for 15 minutes. After cooling down 400 µl of ethanol were added, 200 µl were transferred to a 96 well microtitre plate and the absorbance
at 560 nm determined with a plate reader. The assay was calibrated with colominic acid 22.7 kDa (1.34 polydispersity) standards, of 40, 80, 100, 120, 160 and 200 µg/ml.

2.1.2 Determination of amine groups (TNBS assay)

![Scheme 2.3](image)

The TNBS assay is based upon the substitution reaction of 2,5,7-trinitrobenzene sulfonic acid (TNBS) with primary amines, where the amine replaces the sulfonic acid group creating an orange coloured product.

The TNBS assay was carried out according to the non-quenching method of Snyder and Sobocinski (1975), with modifications. The detection limits for this assay are 0.1 to 1 mM amine groups. In more detail, a working solution was prepared by diluting the 15 mM TNBS stock 1 in 180 in 0.1 M borax pH 9.25 (natural unadjusted pH of 0.1M borax). 180 µl of the working solution were added to 20 µl of unknown sample in the well of a 96well microtitre plate and after mixing the mixture was allowed to stand for 50 minutes, prior to reading absorbance at 405 nm. If necessary, prior to assaying the sample was diluted to a colominic acid concentration ranging from 0.1 to 1 mM prior to reading. The assay was calibrated with glycine standards at 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 and 1 mM concentration.
2.1.3 Determination of thiol groups (The Ellman’s assay)

Scheme 2.4: Thiols react with DTNB by splitting the dithiol and releasing the TNB ion, which is the chromophore.

The Ellman’s assay (Ellman, 1959) was carried out according to Hermanson (1996), with modifications. DTNB was dissolved in 0.1 M phosphate with 1 mM EDTA at a concentration of 4 mg/ml. A working solution was prepared by diluting the stock DTNB solution 1 in 50 in 0.1 M phosphate pH 8.0 (pH adjusted with conc. HCl) containing with 1 mM EDTA. 150 μl of the stock were added to 15 μl of sample in a well of a microtitre 96well plate. The assay mixture was allowed to stand for 30 minutes before reading the absorbance at 405 nm. If necessary, prior to assaying, the sample was diluted to adjust the thiol concentration so that it lies within the range of 0.1 to 1mM, which is the detection range for this assay. The assay was calibrated with cysteine standards at 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 1 mM concentration.

2.1.4 Determination of phospholipids (The Stewart assay)

The Stewart assay was carried out as described by Stewart (Stewart, 1980), with modifications. The assay is based on the complexation of phospholipids with iron. An aqueous iron solution is added to a volume of CH3Cl. Lipids can either be already dissolved
in the chloroform or a small volume of liposomes in aqueous buffer can be added to the iron solution. Upon mixing, iron forms complexes with phospholipids that are coloured and partition almost exclusively in the chloroform layer. This results in the organic layer becoming coloured. In more detail, a stock solution of 0.1 M iron(III) thiocyanate (ferrithiocyanate) solution was made by mixing 20 ml of 0.2 M iron(III) chloride hexahydrate with 20 ml of 0.8 M ammonium isothiocyanate (27.03 g/l FeCl₃·6H₂O and 30.4 g/l NH₄SCN final concentration). 300 μl of lipid in chloroform was mixed with 150 μl of 0.1 M ferrithiocyanate solution and vortexed for 1 minute. The two phases were separated by centrifugation at 10,000 rcf for 1 minute. 200 μl were withdrawn from the lower organic phase, diluted to 400 μl and measured for absorbance at 488 nm. The lipid sample was diluted to a concentration ranging from 0.03 to 0.2 mM phospholipids, if necessary. It is necessary to use the same phospholipid mixture to calibrate the assay as each phospholipid has a different propensity to form complexes with the ferrithiocyanate ion. Typically solutions of 0.03, 0.06, 0.09, 0.12 and 0.16 and 0.2 mM solutions of phospholipid are used.

For the determination of lipid concentration in liposome solutions two methods were used. The first more accurate method consisted of freeze-drying 50 to 200 μl of liposome solution, dissolving the powder in 0.5 ml chloroform, centrifuging at 10,000 rcf for 5 minutes to spin down buffer salts and determining the lipid concentration in the supernatant. The second method, which is faster but less accurate, consisted of adding 10-30 μl of aqueous liposome suspension to 300 μl of chloroform, vortexing for 1 minute, adding 150 μl of 0.1 M iron (III) thiocyanate and processing as above.
2.1.5 Determination of maleimide groups

A chemical method for determining maleimide concentration was developed, based on a modification of the Ellman’s assay. The maleimide group is detected through its reaction with the 5-thio-2-nitrobenzoate (TNB) ion, resulting in the disappearance of the TNB yellow colour, i.e. a decrease in the absorbance at 405nm of the solution (see Scheme 2.5).

\[
\text{COOH} \quad \text{COOH} \\
\text{R} \quad \text{N} \\
n- \quad \text{NO;} \\
\text{R} \quad 1
\]

Scheme 2.5: Maleimide can react with the thiol of the TNB ion, resulting in the disappearance of the yellow colour of the TNB ion.

The assay was carried as follows: A stock of reduced Ellman’s reagent was prepared by dissolving DTNB in 0.1 M phosphate pH 8.0, with 1 mM EDTA at 4 g/l and reducing with 2.5 mM DTT for 1 hour at 37°C. The reduced DTNB stock was then diluted 1 in 50 in phosphate pH 8.0, with 1 mM EDTA and added to 15 μl of sample in the well of a microtitre 96 well plate. After 1 hour at room temperature, the absorbance at 405nm was measured. The sample was diluted to a maleimide concentration if necessary ranging from 0.1 mM to 0.6 mM. The assay was calibrated with maleimido-PEG 5000 standards of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM concentration.
2.1.6 Determination of aldehyde groups on colominic acid (ferricyanide assay and 2,4 DNPH assay)

The aldehyde groups created by periodate oxidation on colominic acid were determined by using a method for the determination of aldoses developed by Park and Johnson (1949). The Park and Johnson assay is based on the reduction of hexacyanoferrate(III) (ferricyanide) \([\text{Fe(CN)}_6]^{3-}\), to hexacyanoferrate(II) (ferrocyanide) \([\text{Fe(CN)}_6]^{4-}\), by aldehyde groups. Ferrocyanide is then converted to prussian blue (ferric ferrocyanide or iron(III) hexacyanoferrate(II)), by addition of iron(III) ions (Southgate 1976). Prussian blue is not soluble, but a colloidal suspension is formed by using SDS (Park and Johnson 1949). The amount of aldehyde present can then be determined by measuring the Prussian blue spectrophotometrically. The mechanism is illustrated in scheme 2.6.

\[
\begin{align*}
[\text{Fe(CN)}_6]^{3-} & \xrightarrow{\text{Na}_2\text{CO}_3, 95^\circ \text{C}} \ [\text{Fe(CN)}_6]^{4-} & \xrightarrow{\text{Fe}^{3+}, \text{H}_2\text{SO}_4, \text{SDS}} & \text{Fe}_4[\text{Fe(CN)}_6]_3 \\
\text{ferricyanide} & \xrightarrow{\text{RCHO}} \text{ferrocyanide} & \xrightarrow{\text{Fe}^{3+}, \text{H}_2\text{SO}_4, \text{SDS}} & \text{prussian blue}
\end{align*}
\]

Scheme 2.6: The chemical mechanism of the Park and Johnson assay.

In more detail stock solutions of potassium ferricyanide (0.5 g/l in water), sodium carbonate (5.3 g/l in water) and ferric ammonium sulphate (1.5 g/l in 0.05N H₂SO₄ with 1 g/l SDS) were prepared. Then 100 μl of sample were mixed with 100 μl of ferricyanide and 100 μl of sodium carbonate solution. The mixture was heated to 95°C for 15 minute and 0.5ml of ferric ammonium sulphate with SDS was added. Then after 15 minutes 250 μl from each sample were transferred to a well on a 96-well plate and the absorbance at 630nm was determined using a plate reader. As the hemi-ketal at the reducing end of colominic acid also reacts to a
small extent, it is necessary to have a negative control of non-oxidised colominic acid so that the colour generated due to the reducing end hemi-ketal can be subtracted.

In addition, 2,4 dinitrophenyl hydrazine (2,4 DNPH) was used to detect the presence of aldehyde groups by reacting aldehyde colominic acid with a solution of 1 mM in 1 N HCl for 30 minutes at 37°C and observing the formation of the hydrazone which forms a yellow crystalline precipitate (Normal and Waddington 1983 – scheme 2.7). 5 mg of aldehyde-colominic acid are typically dissolved in 200 µl of 2,4DNPH solution. The reducing end also reacts with 2,4 DNPH to a small extent, but the colour is different and less of the precipitate is formed. It is thus necessary to have a negative control with non-oxidised colominic acid for comparison. The amount of hydrazone formed can be determined quantitatively by adding two volumes (400 µl) of 1 M NaOH and reading the absorbance at 450 or 490 nm. The reaction is shown in scheme 2.7.

\[
\text{2,4 dinitrophenylhydrazine} + \text{aldehyde group} \xrightarrow{1 \text{N HCl, } 37°C} \text{hydrazone chromophore}
\]

Scheme 2.7: The chemical mechanism of the 2,4 DNPH assay.
2.2.1 Fractionation of colominic acid: Materials

Colominic acid (linear α-(2→8)-linked *E. coli* K1 polysialic acid) 22.7 kDa average molecular weight (1.34 polydispersity) was purchased from Camida, Ireland. Tris-Glycine or Tris-Borate-EDTA 4-20% minigels, gel tanks, and electrophoresis buffers were obtained from Invitrogen, UK. Sepharose Q FF anion exchange matrix, HiTrap Q FF 1 ml and 5 ml pre-packed columns (sepharose Q and MonoQ 5/50GL), XK16 and XK50 columns for chromatography and pump tubing from Amersham Pharmacia, UK. Dialysis tubing 3 kDa molecular weight cutoff from Pierce, UK. Hydrochloric acid, ethanol, isopropanol, acetonitrile and other organic solvents were purchased from VWR international, UK. Ammonium bicarbonate, sodium chloride, triethanolamine, alcian blue, acetic acid, glycine, sodium nitrate and other chemicals from Sigma-Aldrich, UK. Deionised water was obtained from an Elgastat Option 4 water purification unit (Elga Limited, UK). Non-sterile microtitre 96 well plates (sterilin) were obtained from VWR, international. A plate reader (Dynex Technologies, UK) was used for spectrophotometric determinations in aqueous solutions.

2.2.2 Fractionation of colominic acid: Anion exchange chromatography

A series of buffers containing 20 mM triethanolamine pH 7.4 and NaCl at a concentration ranging from 25 to 500 mM with 25 mM steps were prepared. Anion exchange columns were prepared by packing an XK50 column with 900 ml of Sepharose Q FF, or an XK16 column with 75 ml of Sepharose Q FF. The flow rate for the XK16 and XK 50 columns was 0.056 ml/ml for each 1 ml of matrix, i.e. 50 ml/min for the XK50 and 4.2 ml/min for the XK16. For the pre-packed HiTrap columns a flow rate of 1ml/ml was used for the 1 ml column and 2 ml/min for the 5 ml column. The pump was placed after the column (pulling) and not before
the column (pushing). Columns were equilibrated with 5 column volumes of 20 mM triethanolamine pH 7.4 at the appropriate flow rate. The sample was applied to the column via a syringe port and then the column was washed with 5 column volumes of 20 mM triethanolamine pH 7.4. Following the wash a step gradient was used to elute the bound colominic acid. The column was washed with 1.5 column volumes of each of the NaCl 25-500 mM buffers in a sequence from the least to the highest concentration of NaCl. After the step gradient any remaining bound species were removed by washing with 5 column volumes of 20 mM triethanolamine pH 7.4 with 1 M NaCl. During the step gradient, 1.5 column volume fractions were collected all the washes were also collected.

2.2.3 Fractionation of colominic acid: Polyacrylamide gel electrophoresis (PAGE) of colominic acid

For the tris-glycine gels the running buffer was prepared by dissolving 14.4 g of glycine and 2.4 grams of Tris in 1 L of deionized water and verifying that the pH is 8.7. For the tris-borate-EDTA gels, the running buffer was purchased as a 20X stock and an appropriate volume was diluted with deionized water prior to use. Samples were prepared by mixing 20 μl of 10-40 mg/ml colominic acid dissolved in water with 2X native PAGE sample buffer (purchased). 20 μl of each sample were loaded on the gel. After loading a current of 50 volt was applied to the gel for 5 hours or until the dye in the sample buffer reached two thirds of the way down the gel. Following electrophoresis the gel was removed from its casing and stained for 30 minutes with a solution of 5% alcian blue in 2% acetic acid. The gel was then de-stained for 15 minutes with 2% acetic acid.
2.2.4 Fractionation of colominic acid: Recovery of colominic acid from the anion exchange fractions

For medium or low scale fractionation, each fraction was concentrated by ultrafiltration or diafiltration to a volume of 10 ml or less and then dialysed against three 2 L changes (at least 7 hours for each change) of 50 mg/L ammonium bicarbonate. After dialysis the colominic acid was recovered by freeze drying.

For large scale fractionation where there each fraction is more than 1 L in volume, it is necessary to concentrate to around 200 ml with ultrafiltration and diafiltration and then re-dissolve the powder in the minimum volume of water and dialysing it in 50 ml portions.

2.2.5 Triple detection linked gel permeation chromatography

Colominic acid samples were dissolved in 0.2 M sodium nitrate, and 10% acetonitrile and were chromatographed on over 2x GMPWXL columns with detection by refractive index and light scattering for molecular weight determination (GPC system: VE1121 GPC solvent pump, VE3580 RI detector and collation with Trisec 3 software (Viscotek Europe Ltd). Samples (5 mg/ml) were filtered over 0.45 μm nylon membrane and run at 0.7 cm/min with 0.2 M NaNO₃ and 10% CH₃CN as the mobile phase.

2.2.6 FPLC-AEC fractionation of colominic acid

A 1 ml MonoQ column was equilibrated at a flow of 1 ml/minute in the HPLC system with 20 mM triethanolamine pH 7.4 (buffer A). 100 μl of 100 mg/ml colominic acid was then injected and the column washed with 5ml of buffer A. Then the bound colominic acid was eluted with a NaCl linear concentration gradient using a gradient mixer and two buffers:
buffer A and buffer B. Buffer B consisted of 20 mM triethanolamine pH 7.4 with 1 M NaCl. Buffer B was gradually increased from 0 to 50% over 60 minutes. The column was then washed for 5 minutes with 100% buffer B to remove everything that remained bound to the column.

2.3.1 Modifications of colominic acid: Materials

Colominic acid (linear α-(2→8)-linked *E. coli* K1 polysialic acid) 22.7 kDa average molecular weight (1.34 polydispersity) was purchased from Camida, Ireland. PD-10 columns pre-packed with Sephadex G25, Sephadex 25 gel permeation chromatography matrix, XK16 columns for chromatography and pump tubing from Amersham Pharmacia, UK. Dialysis tubing 3 kDa molecular weight cutoff, disuccinimidyl-glutarate (DSG) from Pierce, UK. Ethanol, DMSO, dioxan and other organic solvents were purchased from VWR international, UK. Ammonium bicarbonate, ammonium chloride sodium chloride, sodium phosphate, phosphate buffered saline (PBS – 20 mM phosphate) tablets, sodium hydroxide pellets, sodium cyanoborohydride, cystamine, sodium periodate, EDTA, 2-iminothiolane, 3-maleimidopropionic acid N-hydroxysuccinimide ester (BMPS), disulfosuccinimidyl-subberate (BS³) and other chemicals from Sigma-Aldrich, UK. Deionised water was obtained from an Elgastat Option 4 water purification unit (Elga Limited, UK). Non-sterile microtitre 96 well plates (sterilin) were obtained from VWR, international. Spin column concentrators 0.5, 6 and 20 ml were obtained from Vivaspin, Germany.
The structure of colominic acid is shown in scheme 2.8

Scheme 2.8: The structure of colominic acid.

Throughout this chapter this structure will be illustrated schematically as shown below

The entire reaction series presented in this section is summarised in scheme 2.9
Scheme 2.9: Modification of colominic acid with functional groups.
2.3.2 Periodate oxidation of colominic acid

Colominic acid was dissolved in deionised water at 10-50 mg/ml depending on the molecular weight, to ensure a maximum concentration of 3.3 mM colominic acid and mixed with 0.5 volumes of freshly prepared 60 mM sodium meta-periodate (final concentration 20 mM). For efficient oxidation it is necessary to have at least a 6 fold molar excess of sodium periodate over the colominic acid. The reaction was carried out in the dark at 25°C for exactly 15 minutes. At the end of the 15 minutes one of two possible purification methods was used.

In the first purification method the reaction was stopped by the addition of a 100-fold molar excess of ethylene glycol. The colominic acid was then dialysed against two 2 L changes of 50 mg/L ammonium bicarbonate (each for at least 8 hours) and then against two 2 L changes of deionised water set to pH 7 with sodium hydroxide.

In the second purification method the colominic acid was precipitated by the addition of ice-cold ethanol to 75%. The precipitate was recovered by centrifugation at 3000 rcf in a desktop centrifuge and re-dissolved at an approximate concentration of 200 mg/ml colominic acid. The new solution was precipitated again by adding ice-cold ethanol to 75% and cooling at minus 20°C for 5 minutes. The precipitate was recovered by centrifugation at 15000 rcf in a beckman ultracentrifuge, redissolved at an approximate concentration of 25 mg/ml and freeze-dried.

2.3.3 Reductive amination of oxidised colominic acid with ammonium chloride

A stock sodium cyanoborohydride solution was made by dissolving sodium cyanoborohydride at 5 M concentration in 1 M NaOH. This stock solution was stored at -20°C for a period of up to 2 months. Colominic acid was dissolved at 20 mg/ml in water and
a 300-fold molar excess of ammonium chloride was added along with one 15 μl of NaCNBH₃ stock per ml of reaction mixture. The pH was set to 8 with NaOH if necessary and the reaction was allowed to proceed for 60 hours at 37°C. The reaction was purified either by ethanol precipitation as described in section 2.3.2, or by dialysis against two 2 L changes of 50 mg/L ammonium bicarbonate, then against two 2 L changes of water set to pH 7 with NaOH followed by freeze drying.

2.3.4 Gel permeation chromatography on Sephadex G25

Sephadex G25 column (either pre-packed PD10s or XK16 columns packed with 75 ml of matrix) was equilibrated with 5 volumes of mobile phase. For analytical purposes the mobile phase was PBS, pH 7.4. For preparative purposes the mobile phase was 0.15 M ammonium bicarbonate pH 7.8. For PD10 columns the sample was applied in a volume of 0.25 to 1 ml and 0.5ml fractions were collected. For XK16 columns the sample was applied in a volume of 1 to 2 ml and 2 ml fractions were collected. For preparative purposes the fractions containing the product were freeze-dried, as ammonium bicarbonate is volatile and is for the most part removed during freeze drying. Alternatively for preparative purposes the product containing fractions were concentrated by ultrafiltration with the vivaspin 500 μl concentrators. This was accomplished by adding 0.5 ml to the concentrator centrifuging at 13000 rcf for 10 minutes in a benchtop microfuge, then repeating the process with more 0.5 ml portions of the pooled fractions until the entire sample has been concentrated to about 100 μl.
2.3.5 Thiolation of amine-CA with 2-iminothiolane

15 mg of amine-CA were dissolved in 300 µl of PBS pH 8 with 1 mM EDTA. To this 100 µl of PHB-EDTA containing 7 mg of 2-iminothiolane was added and the reaction was allowed to proceed for 90 minutes. The product was purified by gel permeation chromatography on Sephadex G25 followed by concentration with ultrafiltration as described in section 2.3.4. Prior to use 0.5 µl of sample were diluted 100 times and 2 µl were assayed with the resorcinol assay (section 2.1.1) to determine the concentration of colominic acid.

2.3.6 Thiolation of aldehyde CA using cystamine

Aldehyde-CA was converted to cystamine-CA by reductive amination of aldehyde-CA with cystamine. The reductive amination reaction was set up as described in section 2.3.3, using a 100-fold molar excess of cystamine instead of ammonium chloride. At the end of the reaction and before purification the disulfide bond in cystamine was reduced with 100 mM DTT for 1 h at 37°C to unmask the aldehyde. Purification was accomplished either by dialysis as described in section 2.3.3 for samples larger than 100 mg or for samples less than 100 mg by gel permeation chromatography on Sephadex G25 eluting with ammonium bicarbonate and freeze-drying the CA fractions as described in section 2.3.4.

2.3.7 Synthesis of maleimide-colominic acid from amine colominic acid with BMPS

For small scale production of maleimide CA, 25 mg of amine CA in 0.2 ml of PBS pH 7.4 was mixed with a 15-fold molar excess of BMPS in 0.3 ml DMSO. After 1 hour at 25°C, CA maleimide was purified with a PD10 column using PBS as the mobile phase and concentrated, ultracentrifugation as detailed in section 2.3.4. For production at the 100 mg
scale, 100 mg of colominic acid dissolved in 0.8 ml of PBS was mixed with a 15 fold molar excess of BMPS dissolved in 1.2 ml of DMSO. After 1 hour at 25°C, CA maleimide was purified by gel permeation chromatography on an XK16 column packed with Sephadex G25 with ammonium bicarbonate as the mobile phase as described in section 2.3.4. The colominic acid containing fractions were pooled and freeze dried.

2.3.8 Synthesis of NHS-colominic acid from amine colominic acid with BS³

Colominic acid (25 mg) was dissolved in 300 μl of PBS pH 7.4 and mixed with a 50-fold molar excess of BS³ in 100 μl of PBS pH 7.4. After 30 minutes at 25°C the NHS-colominic acid was purified by gel permeation chromatography using a PD-10 column with PBS as the mobile phase as described in section 2.3.4. The CA fractions were immediately used without freeze-drying or concentrating by ultrafiltration to prevent hydrolysis of the NHS ester.

2.3.9 Synthesis of NHS-colominic acid from amine colominic acid with DSG

Colominic acid (25 mg) was dissolved in 30 μl of PBS pH 7.4 (50 μl final volume) in a 2 ml eppendorf centrifuge tube and mixed with a 50-fold excess of DSG, in 600 μl of DMSO. After 30 minutes at 25°C the colominic acid maleimide was precipitated by the addition of 1.4 ml of dioxan. The precipitate was recovered by centrifugation in a benchtop microfuge, at 13000 rfc for 5 minutes. The pellet was redissolved in 600 μl of DMSO and precipitated again with 1.4 ml of dioxan. After 3 precipitations, the final CA pellet was dissolved in the conjugation buffer prior to use.
2.3.10 Reaction of NHS colominic acid with human growth hormone

NHS colominic acid was prepared as described in sections 2.3.8 and 2.3.9. Purification was accomplished by GPC. To each void column fraction immediately after purification 0.5mg of protein was added. Prior to addition of the protein a small sample of each fraction was tested with the resorcinol assay to determine the amount of colominic acid present in each fraction. The reaction was carried out at room temperature overnight (~16 h), before analysis by SDS-PAGE (see section 2.6.5). To account for the effect of the NHS crosslinker alone on the protection controls were set up by adding the appropriate amount of NHS crosslinker to 0.5 mg of protein in 0.5 ml of PBS to get the same cross-linker to protein ratio as the colominic acid to protein ratio in the reaction mixture.

2.4.1 Liposome activation: Materials

1,2-distearoyl-sn-glycerol-phosphatidyl-choline (DSPC) and 1,2-distearoyl-sn-glycerol-phosphatidyl-ethanolamine (DSPE) were purchased from lipoid, UK. Cholesterol, N-succininy1-1,2-dipalmitoyl-sn-glycerol-phosphatidyl-ethanolamine (NS-DPPE), 3-maleimidopropionic acid N-hydroxysuccinimide ester (BMPS), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-iminothiolane (2IT), phosphate buffered saline (PBS – 20mM phosphate) tablets, ethylene-diamine-tetra-acetic acid (EDTA), acetic acid, molybdenum blue 5% solution in sulphuric acid and other chemicals from Sigma-Aldrich, UK. Glass beads, thin layer chromatography plates (silica matrix derivatised with UV fluorescent groups) Ethanol, Methanol, DMSO, triethanolamine, chloroform and other organic solvents were purchased from VWR international, UK. Deionised water was obtained from an Elgastat Option 4 water purification unit (Elga
Limited, UK). Non-sterile microtitre 96 well plates (sterilin) were obtained from VWR, international. Spin column concentrators 0.5, 6 and 20ml were obtained from Vivaspin, Germany.

2.4.2 Modification of DSPE with a maleimide group using BMPS

40 µmol of DSPE, were dissolved in 1.84 ml of choloform: methanol (MeOH): triethylamine (TEA) 40:5:1 (1.6ml chloroform, 200 µl MeOH, 40 µl TEA), by heating the mixture at 35°C and 25 mg (94 µmol) of solid BMPS were added. The reaction mixture was vortexed until all the BMPS dissolves and gently agitated for 2hours. The excess BMPS and the reaction by products were extracted once with 1 ml of PBS and twice with 1 ml of 1:1 PBS:MeOH. The remaining solvent was removed by rotary evaporation and the lipid was redisolved in 1 ml of chloroform.

2.4.3 Thin layer chromatography

0.5 to 5 µl of sample were spotted on a thin layer chromatography (TLC) silica plate and air dried. The bottom portion of the plate was immersed in the mobile phase, making sure that the mobile phase does not reach the spotted sample and the mobile phase was allowed to move towards the top until it reached 1cm from the top end of the plate. The plate was then air dried and illuminated under short wavelength UV light. The areas that did not fluoresce (UV shadow) were marked with pencil. Following UV illumination the plate was immersed briefly in 5% molybdenum blue (molybdenum oxide) in concentrated sulphuric acid. As soon as the plate was developed it was scanned to record the result.
2.4.4 Liposome preparation

An appropriate lipid mixture e.g. 20 μmol of DSPC and 20 μmol of cholesterol or 20 μmol of DSPC and 20 μmol of cholesterol and 2 μmol of DSPE, was prepared in a round bottom rotary evaporator flask. The appropriate combinations of the following stock solutions were used: 100 mM cholesterol, 100 mM DSPC, 5 mM DSPE, 5 mM NS-DPPE and 30-38 mM DSPM all in CHCl3 and additional CHCl3 was added to a final volume of 2 ml. The solvent was evaporated in a 50ml or 100ml rotary evaporator flask, under vacuum (creating using a running water) with a rotary evaporator to form a thin lipid film on the sides of the flask. The film was hydrated slowly at 60°C with 2 ml of the appropriate buffer (e.g. PBS pH 7.4 or PBS with 0.2 M carboxyfluorescein) in 0.2 ml aliquots, with the help of glass beads, forming a suspension of multilamellar vesicles (MLVs). The MLVs are converted to small unilamellar vesicles (SUVs) by sonicating 4 times for 1 minute with 1 minute intervals in between. The sonication needs to be carried at 60°C or more, so water and not ice was used as the temperature sink. The sonication was carried out in a 10 ml beaker, with a 2 cm thick titanium probe, at 5 microns amplitude in a Sony Soniprep 150 sonicator. Following sonication the SUV suspension was centrifuged at 13000 rcf for 10 minutes to spin down titanium particles from the probe. The physical characteristics of the liposomes were then measured by photon correlation spectroscopy.

2.4.5 Activation of DSPE liposomes with BMPS

Liposomes consisting of DSPC:cholesterol:DSPE 10:10:1 (20 mM DSPC, 2 mM DSPE) were prepared as described in section 2.4.4, hydrating them with PBS pH 7.4. To 0.5 ml of liposome suspension, 50 μl of 200 mM BMPS in DMSO were added (20 mM final
concentration – 10-fold molar excess). The BMPS precipitates partially, so the mixture was agitated by doing the reaction on a rotating wheel. After 3 hours at 25°C, the unreacted BMPS and DMSO were removed by gel permeation chromatography using column (Sephadex G25) with PBS pH 7.4, using PBS pH 7.4 as the mobile phase according to the method described in section 2.3.4. The most concentrated liposome fraction (3-3.5 ml elution volume) was used in further steps and the other fractions were discarded.

2.4.6 Activation of NS-DPPE liposomes with EDC/NHS

Liposomes consisting of DSPC:cholesterol:NS-DPPE 10:10:1 (20 mM DSPC, 2 mM NS-DPPE) were prepared as described in section 2.4.4, hydrating them with PBS pH 7.4. To 0.5 ml of liposome suspension solid EDC and NHS were added to a final concentration of 25 mM EDC and 5 mM NHS. After 30 minutes at 25°C, the reaction by-products and unreacted EDC/NHS were removed by gel permeation chromatography using a column (Sephadex G25) with PBS pH 7.4 as the mobile phase, according to the method described in section 2.3.4. The most concentrated liposome fraction (3-3.5 ml elution volume) was used immediately to avoid hydrolysis of the NHS group. The remaining fractions were discarded.

2.4.7 Activation of DSPE liposomes with 2IT

Liposomes consisting of DSPC:cholesterol:DSPE 10:10:1 (20 mM DSPC, 2 mM DSPE) were prepared as described as described in section 2.4.4, hydrating them with PBS pH 7.4. To 0.5 ml of liposome suspension, solid 2-IT was added to a final concentration of 50 mM. After 1 h at 25°C, the reaction by-products and unreacted 2-IT were removed by gel permeation chromatography using a PD10 column (Sephadex G25) with PBS pH 7.4 as the
mobile phase, according to the method described in section 2.3.4. The most concentrated liposome fraction (3-3.5 ml elution volume) was used in further steps and the other fractions were discarded.

**2.4.8 Extraction of lipids from liposomes**

Lipids were extracted from a liposome solution in an organic phase by adding 1ml of chloroform followed by 0.5 ml of methanol to 0.5 ml of liposomes. The mixture was vortexed for 1 minute, subjected to centrifugation at 13000 rpm for 5 minutes, the aqueous (top) phase was removed and extracted in the same way with 2 more mls of chloroform, to recover the remaining lipid.

**2.5.1 Conjugation of the colominic acid derivatives to liposomes: Materials**

1,2-distearoyl-sn-glycerol-phosphatidyl-choline (DSPC) and 1,2-distearoyl-sn-glycerol-phosphatidyl-ethanolamine (DSPE) were purchased from lipoid, UK. Cholesterol, N-succininy1-1,2-dipalmitoyl-sn-glycerol-phosphatidyl-ethanolamine (NS-DPPE), 3-maleimidopropionic acid N-hydroxysuccinimide ester (BMPS), 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC), N-hydroxy succinimide (NHS), 2-iminothiolane (2IT), phosphate buffered saline (PBS – 20 mM phosphate) tablets, ethylene-diamine-tetra-acetic acid (EDTA), and other chemicals from Sigma-Aldrich, UK. Glass beads, Ethanol, Methanol, DMSO, triethanolamine, chloroform and other organic solvents were purchased from VWR international, UK. Deionised water was obtained from an Elgastat Option 4 water purification unit (Elga Limited, UK). Non-sterile microtitre 96 well plates (sterilin) were
obtained from VWR, international. Spin column concentrators 0.5, 6 and 20 ml were obtained from Vivaspin, Germany.

2.5.2 Conjugation of amine colominic acid to NHS/EDC activated liposomes

Amine-CA was prepared as described in section 2.3.3. DSPE containing liposomes were activated as described in section 2.4.6. Any residual ammonium ions were removed from amine-CA by purification with a PD10 column (Sephadex G25), using PBS as the mobile phase and concentrating the void volume fractions as described in section 2.3.4. 100 μl of the concentrate containing the colominic acid were added to 0.5 ml of activated liposomes. If necessary the pH was set to 7.4 and the reaction was allowed to proceed for 16 hours at 25°C. The conjugated liposomes were purified by gel filtration in a PD-10 column, packed with 7 ml of Sepharose CL-4B, using PBS as the mobile phase. 0.5 ml fractions were collected and the first 1.5 ml were discarded. All fractions were analysed with the resorcinol assay for colominic acid and the Stewart assay for lipid. The two most concentrated liposome fractions were pooled and used for further work.

2.5.3 Conjugation of NHS-colominic acid to DSPE liposomes

NHS-CA was prepared as described in section 2.3.7 (DSG) and 2.3.8 (BS³). For the NHS-CA made by the DSG approach the CA-NHS pellet was quickly dissolved in 0.5 ml of DSPE liposomes consisting of DSPC:cholesterol:DSPE 10:10:1 (10 mM DSPC). After reaction for 16 hours at 25°C, the liposomes were purified as described in section 2.5.2. For the NHS-CA made by the BS³ approach, 0.5 ml of liposomes were concentrated to 100 μl with a 0.5 ml concentrator and added directly to the purified NHS-CA as soon as it is eluted from the
2.6.1 In vitro and in vivo characterisation: Materials

Colominic acid (linear α-(2→8)-linked *E. coli* K1 polysialic acid) 22.7 kDa average molecular weight (1.34 polydispersity) was purchased from Camida, Ireland. PD-10 columns and sepharose CL-4B gel permeation chromatography matrix from Amersham Pharmacia, UK. Dialysis tubing 3 kDa molecular weight cutoff, Pierce, UK. Dextran T500 (technical dextran 500kDa molecular weight), PEG5000, PEG8000, sodium chloride, sodium phosphate, heparin, phosphate buffered saline (PBS − 20 mM phosphate) tablets, sodium hydroxide pellets, bovine serum albumin, tween 20, 3-3’-diaminobenzidine enhanced liquid substrate system kit and other chemicals from Sigma-Aldrich, UK. Blotting paper and PVDF membranes from Sigma-Aldrich, UK. HRP-conjugated Rabbit anti-Mouse immunoglobulin antibody was purchased from DAKO, UK, HRP-conjugated rabbit anti-goat IgG antibody was purchased from Sigma-Aldrich, UK, Goat anti-complement C3 (human) antibody was obtained from Calbiochem, UK. Tris-glycine 4-20% polyacrylamide minigels, gel tanks, electrophoresis buffers and colloidal Coomassie blue gel staining kit were obtained from Invitrogen, UK. The blotting apparatus was purchased from BDH. Deionised water was obtained from an Elgastat Option 4 water purification unit (Elga Limited, UK). Non-sterile microtitre 96 well plates (sterilin) were obtained from VWR, international. Spin column concentrators 0.5, 6 and 20 ml were obtained from Vivaspin, Germany. A plate reader (Dynex Technologies, UK) was used for spectrophotometric determinations in aqueous solutions. A Perkin-Elmer, LS 50B fluorescence spectrometer, was used for fluorescence measurements.
column. Following reaction for 16 hours at 25°C, the liposomes were purified as described in section 2.5.2.

2.5.4 Conjugation of thiol-colominic acid to BMPS activated DSPE liposomes
DSPE liposomes were activated with BMPS as described in section 2.4.5. Thiol-CA was made from amine-CA with 2IT as described in section 2.3.5 or from cystamine-CA as described in section 2.3.6. 0.1 ml of thiol-CA concentrate was added to 0.5 ml of activated liposomes in PBS pH7.4. The pH was set to 7.4 if necessary. After 16 hours of reaction at 25°C, the liposomes were purified as described in section 2.5.2.

2.5.5 Conjugation of thiol colominic acid DSPM liposomes
Thiol-CA was made from amine-CA with 2IT as described in section 2.3.5, or from aldehyde-CA with cystamine as described in section 2.3.6. 0.1 ml of concentrated solution of 2IT thiolated CA or reduced cystamine-CA was added to 0.5 ml of 1:1 DSPC:cholesterol liposomes containing DSPM at a ratio over the DSPC of 1 in 20, 1 in 10 or 1 in 5 in PBS pH 7.4. After 8 or 16 hours of reaction the liposomes were purified as described in section 2.5.2.

2.5.6 Conjugation of maleimide CA to DSPE liposomes activated with 2IT
DSPE liposomes were thiolated with 2-iminothiolane (2IT) as described in section 2.4.7. Maleimide-CA was made from amine-CA with BMPS as described in section 2.3.7. 15 mg of solid maleimide CA was dissolved in 0.5 ml of thiolated liposomes. The pH was set to 7.4 if necessary. After 16 hours of reaction at 25°C, the liposomes were purified as described in section 2.5.2.
2.6.2 Mouse plasma

Blood was obtained from female balb/c mice, 6-10 weeks old by cardiac puncture, using heparin (100 units/ml) as an anticoagulant. The blood cells were separated by centrifugation at 1500 g for 5 minutes at room temperature. The plasma was removed and centrifuged again at 1500 g for 5 minutes to ensure complete removal of blood cells. The plasma was separated into 100 µl aliquots and stored at -80°C prior to use.

2.6.3 Dextran-PEG two-phase systems

Two systems were used dextran T500-PEG8000 in 20mM phosphate buffered saline (PBS) pH 7.4 or dextran T500-PEG5000 in 20 mM sodium phosphate pH 7.6. The PEG8000 system was made by mixing equal volumes of 10% (w/w) Dextran T500 and 10% (w/w) PEG8000 in PBS pH 7.4. The solutions were mixed with a vortex mixer for 2 minutes and allowed to separate at 25°C for about 2 h. To ensure complete separation the tube was centrifuged at 1000 g for 2 minutes and then allowed to stand for 30 minutes at 25°C. The layers were then separated and stored at 4°C. The PEG 5000 system was made by mixing the appropriate volumes of 20% (w/w) solutions of Dextran T500 and PEG5000 in deionised water, 0.6 M sodium phosphate, 0.64 M sodium chloride and deionised water to give a final concentration of 5% (w/w) Dextran, 5% (w/w) PEG5000, 10 mM sodium phosphate and 160mM sodium chloride. The layers were separated as before. For both systems, prior to use 250 µl of each layer was added to a 1.5 ml eppendorf tube and vortexed for 2 minutes, then allowed to separate at 25°C for 30 minutes. 50 µl of liposomes entrapping quenched CF (see section 2.4.4) were added to the system, diluted as appropriate to have a fluorescence signal of about 300 fluorescence units per µl after addition of TritonX-100 to 1% and PBS to a total
volume of 2 ml. After vortexing for 2 minutes the sample was allowed to stand at 25°C for 30 minutes, then centrifuged for 1 minute at 1000 g and allowed to stand for another 15 minutes. After separation 200 µl of each layer were transferred to a separate test tube and the fluorescence quantified after addition of TritonX100 to 1% and PBS to total volume of 2 ml. For plasma affinity experiments the liposomes were diluted to 600 fluorescence units per µl, then mixed 1:1 with mouse plasma (section 2.6.2) and incubated at 37°C for the appropriate time period before adding 50µl to the two phase system.

2.6.4 Isolation of plasma proteins that bind to liposomes

Plasma proteins with an affinity for liposomes were separated by incubating the liposomes with plasma and then removing unbound proteins by gel permeation chromatography. In detail, 100 µl of liposome solution containing 2.5 mM DSPC was mixed with 100 µl mouse plasma and incubated for 3h at room temperature. Mouse plasma was obtained as described in section 2.6.2. Following the incubation, the liposomes and their associated proteins were separated from the bulk of plasma proteins, by passing through a PD-10 column packed with sepharose CL-4B, using PBS, pH 7.4 as the mobile phase. The void volume fractions were concentrated by ultrafiltration to a final volume of 50 µl, using a vivaspin 500 spin column. If necessary the volume was adjusted to 50 µl with PBS, pH 7.4.

2.6.5 Analysis of liposome associated plasma proteins by SDS-PAGE

20 µl of liposome and associated proteins isolated after incubation with plasma as described in section 2.6.5, were mixed with 20 µl of 2x SDS, tris-glycine loading buffer and heated at 85°C for 1 minute, then 28 µl were loaded onto the well of a tris-glycine 4-20%
polyacrylamide minigel. After electrophoresis at 150 volts for 1 hour the gels were stained with the colloidal Coomassie blue staining kit according to the manufacturer's instructions.

2.6.6 Analysis of liposome associated plasma proteins by Western blotting

An unstained SDS-PAGE gel of the liposome associated proteins prepared as described in section 2.6.5 was blotted onto a PVDF membrane as follows: The gel was placed onto a piece of blotting paper pre-wetted with transfer buffer (0.192 M glycine, 25 mM tris base, 20% methanol) and a PVDF membrane pre-wetted with methanol and then soaked in transfer buffer, was carefully placed onto of the gel ensuring there are no air bubbles. Another wetted piece of filter paper was placed on top of the PVDF membrane to complete the filter-membrane “sandwich”. The filter-membrane sandwich was placed into the blotting apparatus, which was filled with transfer buffer and the proteins were transferred to the membrane by applying a current of 400 mA for 4 hours. After the transfer the PVDF membrane was removed and incubated for 2 hours with blocking buffer: 5% (w/v) bovine serum albumin in phosphate buffered saline (20 mM phosphate) pH 7.4 containing 0.1% Tween 20 (PBS-Tween). The blocking buffer was decanted and the membrane was incubated at 25°C for 1 hour with either anti-complement C3 antibody diluted 1:500 in blocking buffer or with horseradish peroxidase (HRP) conjugated anti-mouse immunoglobulin antibodies diluted 1:1000 in blocking buffer. The non-specifically bound antibody was washed off with three 10 minute washes of PBS-Tween. For the C3 antibody the membrane was then incubated with HRP conjugated anti-goat antibody dilute 1:1000 in blocking buffer and washed again with 3 changes of PBS-Tween. The presence of HRP was detected using the 3,3’-diaminobenzidine enhanced liquid substrate system according to the manufacturer’s
instructions. Briefly 5 ml of the substrate solution were added directly onto the membrane and allowed to stand with gentle rocking until satisfactory band intensity was obtained.

2.6.7 In vivo pharmacokinetics of liposomes injected intravenously

The pharmacokinetic studies were carried out on female 6-8 weeks old balb/c mice (20-25 g). The dose of lipid used in each experiment is stated in the appropriate figures in the text. Polysialylated liposomes were prepared as described in section 2.5. 100 µl of liposome solution entrapping quenched CF were injected via the tail vein. At the appropriate time points, 40µl of blood were collected from the mouse tail and added to 460 µl of phosphate buffered saline (PBS) containing 100 units per ml Heparin as an anticoagulant. The blood cells removed by centrifuging at 1500 g for 5 minutes and the plasma transferred to a clean centrifuge tube. 100 µl of plasma was diluted to 2 ml with PBS 20% (v/v) TritonX-100 was added to a final concentration of 1%. The samples were mixed and allowed to stand for 10 minutes and then the fluorescence was determined, using an excitation wavelength of 495nm and an emission wavelength of 520nm. A sample of mouse plasma isolated from untreated mice and diluted appropriately with and without triton was used as the blank. The fluorescence of undiluted plasma was calculated by multiplying by 20, since the sample was diluted 1 in 20 prior to reading. The total volume of blood in each mouse was calculated by assuming a blood volume (µl) to weight (g) ratio of 55 hence the total blood volume was 55*(mouse weight). The input (fluorescence immediately after the injection) was calculated by determining the fluorescence of the injected material multiplying by the volume of liposomes injected and dividing by the total blood volume. The fluorescence remaining was calculated by dividing the fluorescence of undiluted plasma with the input. For experiments
with CA pre-injection, the mice were injected with 5 mg of 22.7kDa colominic acid via the tail vein, 5 minutes prior to injection of the liposome sample.
Chapter 3

Anion exchange fractionation of colominic acid
3.1 Introduction

The standard method for producing colominic acid is extraction from a culture of a suitable \textit{E.coli} strain (Roth et al 1993). Colominic acid produced in this fashion is highly variable in size and in addition the higher molecular weight species are often attached to lipid molecules (Gregoriadis et al 1993). The average size of the preparation is controlled by altering the incubation period and the strain used, but the variability is always high, with a wide mixture of molecular weights being present. Very small colominic acids (<10kDa) are quite difficult to prepare with this approach. High polydispersity and lack of control over the size are undesirable properties in any product intended for use in pharmaceutical preparations. Also the efficacy of polyethylene glycol (PEG) in sterically stabilizing liposomes is dependent on the polymer’s size (Woodle and Lasic 1992). The best results are obtained when PEG between 2 and 5kDa is used. The results obtained with PEG suggest that when studying the capability of a polymer to sterically stabilise liposomes, size is a very important parameter. Hence it is necessary to produce colominic acid of more defined molecular weight for the purposes of this study. A relatively simple and straightforward method for accomplishing this is to fractionate the polydisperse material. Various techniques have been used for the fractionation of polydisperse polymers, including gel permeation chromatography (GPC), especially the GPC variant known as high osmotic pressure chromatography (Luo and Teraoka 1998, Xu et al 1999 and ion exchange chromatography (for charged polymers) (Hallenbeck et al 1987, Zhang et al 1997, Ravenscroft et al 1999), fractionation by precipitation (Lis and Schleif 2004) and fractionation by diafiltration (Sudareva et al 1989). Colominic is a highly charged polymer, with a negatively charged carboxyl group in every repeating unit, hence anion exchange chromatography is well suited for fractionating this
polymer. In this chapter the use of anion exchange chromatography in producing narrow polydispersity fractions from the commercially available polydisperse preparations of colominic acid will be described.

3.1.1 Anion exchange chromatography

In anion exchange chromatography the negatively charged sample is bound onto a positively charged matrix in a low ionic strength buffer and then eluted from the column either using high ionic strength (which weakens ionic interactions) or by altering the pH, which affects the ionization state of the sample and/or the matrix. Anion exchange chromatography (AEC) has been used in the past to partially fractionate negatively charged bacterial polysaccharides for vaccine purposes (Costantino et al 1999) and has been shown to be an effective approach for the analytical separation of sialic acid homopolymers (Zhang et al 1997).

For the separation of different species with AEC, a difference in the binding constants of the species to be separated is necessary. The higher the binding constant the slower the sample migrates through the column at any given ionic strength. A theoretical study of the interaction of polyacrylic acid with charged particles under different pH and ionic strength conditions has been carried out by Laguecir and Stoll (2005). In addition the adsorption of weak polyelectrolytes on curved charged surfaces has been modelled theoretically by Muthukumar, von Goeler and Kong (von Goeler and Muthukumar 1994, Kong and Muthukumar, 1998). This model is clearly valid for the binding of macromolecules to ion exchange matrices. According to this model the binding constant is dependent on the polymer's charge, the chain length, molecular weight, conformation, temperature and the ionic strength of the solution. The strength of electrostatic interactions decreases
exponentially with the distance and this effect is even more pronounced in an aqueous environment. Hence, the strength of binding is greatest when the polymer can adopt an extended conformation so that all available charges can be in close proximity to the charged surface. Larger chain length and less extended conformations prevent all the charged atoms on the polymer from being in close contact with the matrix and hence the contribution of these atoms to the binding strength is decreased (increased distance). Increased molecular weight also decreases the binding as it increases the average distance of the polymer from the surface. Increased ionic strength results in increased shielding of the charge on the polymer and matrix, which decreases the binding constant. Increasing the polymer’s charge on the other hand increases the strength of the binding. Colominic acid contains a charged carboxylic acid atom in every repeating unit, therefore its charge and consequently the binding constant increases in proportion to the chain length.

At low chain length colominic acid is able to adopt an extended conformation allowing all the charges to come into close contact with the surface. At larger chain length, the polymer is less able to adopt an extended conformation so that not all the charges are able to come into close contact with the surface. In addition, as the chain length increases the contribution of each additional subunit in proportion to the total charge becomes progressively smaller. The result is that past a certain point the binding constant does not increase in a linear fashion with chain length. Instead the increase in the binding constant afforded by each additional subunit becomes progressively smaller, so that the resolution between species that differ by one subunit decreases constantly with increased polymerization number. While the chain length is low, the binding constant increases rapidly with each subunit allowing good resolution between species with a different degree of polymerization. As the chain length
increases, the resolution between species that differ by one subunit becomes smaller and smaller, until at very high chain length the difference in the binding constants becomes negligible so that the species can no longer be resolved.

As charged molecules enter the ion exchange column they displace ions on the matrix and adsorb onto it. While the charged molecules are bound to the matrix they cannot move with the mobile phase. When the top of the column becomes saturated, the charged molecules can no longer bind and they move further down the column binding onto the as yet non-occupied areas of the matrix. If the entire column is saturated, the charged molecules come straight through the column (breakthrough). As a result, when a charged molecule (in this case colominic acid) is loaded onto the column, it forms a band at the top whose width increases in proportion to the amount loaded. Since colominic acid is in equilibrium between being bound on matrix and being dissolved in the mobile phase (adsorption is reversible), some of the unbound colominic acid is carried down the column by the buffer flow. This colominic acid cannot bind the saturated matrix in the middle part of the band so it moves past and binds the matrix again when it reaches the bottom portion of the band, where there available matrix. As a result the buffer flow causes a constant migration of molecules from the top of the band to its bottom, effectively causing the band to move downwards. The speed of this migration depends on the binding constant. The greater the binding constant the more the equilibrium shifts towards adsorption and the fewer the molecules that are available for the flow to carry down the column. If the constant is very high, there is effectively no migration. Increasing the ionic strength decreases the binding constant making the molecules partition more in the mobile phase and migrate faster. If the ionic strength is high enough the equilibrium shifts completely towards desorption and the molecule no longer binds the
matrix. A difference in binding constants allows separation of molecules by setting the ionic strength of the mobile phase to a value that decreases the binding constants enough for the species with the low binding constant to desorb leaving the species with the high binding constant adsorbed. Even if the species do not have such a large difference in the binding constants, the one with the low binding constant will desorb more and hence migrate faster down the column. For separations of complex mixtures an ionic strength gradient is used, i.e. the ionic strength of the mobile phase increases over time. This allows all species in the preparation to be eluted. However each species will only begin to migrate when the ionic strength reaches a certain value which depends on the binding constant. Therefore species with different binding constants will come out of the column at different times, allowing them to be separated.

A step gradient approach has been used to fractionate colominic acid. A step gradient involves washing the column with a fixed volume of a series of buffers with progressively increasing ionic strength. A step gradient works in a similar way to a linear gradient, only because the ionic strength remains fixed for short periods of time at a progressively higher and higher value, each step elutes a mixture of species that are closely related in size even more so than what is allowed by a linear gradient. The actual size and polydispersity obtained can be controlled by varying the number of steps, the volume of each step and the maximum and minimum ionic strength. The amount of colominic acid loaded is also very important.

3.2 Small scale fraction of 22.7 kDa colominic acid using a linear gradient elution

So far in the literature, high resolution separation of charged polymers has been achieved using high pressure conditions (HPLC). To determine if anion exchange fractionation of
colominic acid can be carried out under medium pressure conditions (FPLC) it was attempted to fractionate 10 mg of colominic acid using a linear NaCl gradient elution from 0 to 500 mM (see section 2.2.6.) on a 1ml high resolution Mono-Q sepharose column. Figure 3.1, shows the chromatogram obtained. Detection of the polymer was accomplished by monitoring absorbance at 214 nm.

**FPLC-AEC fractionation of Colominic acid**

![Chromatogram](image)

**Figure 3.1.** Fractionation of Colominic acid by Anion exchange using a linear gradient from 0 to 500 mM. 10 mg of colominic were injected in the column in 100 µl of 20 mM triethanolamine pH 7.4 and then the column was washed for 5 min, prior to applying the gradient. Fractions were collected every minute starting at 5.5 minutes.

To determine whether fractionation had occurred, selected fractions were concentrated by ultrafiltration and analysed by polyacrylamide gel electrophoresis (PAGE). The results are presented in figure 3.2. It is clear from figure 3.2 that anion exchange is very effective at fractionating colominic acid, as distinct bands are obtained, instead of a smear like the one observed with the stock preparations. Measurement of the recovery using the resorcinol showed that each fraction contains a very small amount (1-5%) of the colominic acid loaded.
PAGE analysis of the FPLC-AEC fractionated CA

Lanes:
1) 35.5-36.5 minutes
2) 40.5-41.5 minutes
3) 42.5-43.5 minutes
4) 45.5-46.5 minutes
5) 48.5-49.5 minutes
6) 55.5-56.5 minutes
7) 22.7 kDa stock
8) 39 kDa stock
9) 11 kDa stock

Figure 3.2. PAGE analysis of the 10 mg CA fractionated by FPLC-AEC on a 1 ml MonoQ column. Selected 1 min fractions were concentrated to less than 50 µl by ultrafiltration, and 13 µl were loaded onto the gel (see section 2.2.3). The gel is a 4-20% tris-borate-EDTA polyacrylamide gel and was stained for colominic acid with alcian blue. The standards used are the commercially available 11 kDa, 22 kDa and 39 kDa, preparations.

3.3 Small scale fractionation of 22.7 kDa colominic acid using step gradient elution

The resolution of the linear gradient was greater than what is necessary for the purposes of this study, which results in low recovery for each fraction. In addition scaling up to large amounts of colominic acid (>100 mg) is difficult and requires expensive equipment. Instead of a linear gradient a step gradient elution procedure, was used with the lower resolution but cheaper sepharose Q matrix, to separate the polymer into a smaller number of broader fractions with greater colominic acid content. To determine the effectiveness of this method in fractionating colominic acid, initially small (50-200 mg) amounts of colominic acid were
applied to 5 ml of ion exchange matrix and fractionated as described in section 2.2.2. It was found that breakthrough (see above) occurs when 200 mg of colominic acid are loaded on the column but not when 150 mg are loaded. Apparently 200 mg is sufficient to fully saturate the column. Figure 3.3 shows the results of the fractionation for 200 mg of colominic acid with the 5ml ion exchange column.

![Fractionation of colominic acid (22.7KDa)](image)

**Figure 3.3.** Fractionation of 200 mg of 22.7 kDa colominic acid using a 5 ml sepharose Q anion exchange column. After binding, the column was washed with 5 ml of 20 mM triethanolamine pH 7.4. A step gradient was then applied ranging from 0 to 500 mM NaCl. At each step the concentration was increased by 25 mM (20 steps in total). The volume of each step was one column volume (5 ml). After the end of the gradient, the column was washed with 5 ml of 1 M NaCl to remove everything still bound to the

<table>
<thead>
<tr>
<th>Buffer</th>
<th>20mM Triethanolamine pH 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step gradient range</td>
<td>0 to 500 mM NaCl</td>
</tr>
<tr>
<td>Increment per step</td>
<td>25mM</td>
</tr>
<tr>
<td>Volume of each step</td>
<td>5 ml</td>
</tr>
<tr>
<td>Post gradient wash</td>
<td>1 M NaCl</td>
</tr>
<tr>
<td>Post gradient wash volume</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

110
column (fraction 21). All fractions were analysed by the resorcinol assay for the presence of colominic acid.

As can be seen in figure 3.3, the step gradient approach results in the colominic acid eluting from the column in many fractions. To determine whether fractionation had occurred, each fraction was concentrated by ultrafiltration and analysed by polyacrylamide gel electrophoresis (PAGE). After concentration it was found that nearly all fractions contained some colominic acid even though for some of them the amount was rather low. The results are shown in figure 3.4

**Figure 3.4.** PAGE analysis of the 200 mg CA fractionated with a 5 ml column. All samples were concentrated by ultrafiltration, dialysed to remove salt and freeze-dried. ~30 μg of colominic acid were loaded onto the gel (see section 2.2.3). The gel is a 4-20% tris-glycine polyacrylamide gel and was stained for colominic acid with alcian blue. The standards used are the commercially available 11 kDa, 22 kDa and 39 kDa, preparations. Numbers in parenthesis for the standards denote the polydispersity index. Note that the colominic acid in the 50 mM and 75 mM fractions is small (<7 kDa) and it runs off the gel. The 25 mM fraction also contains some large species.
Selected fractions were analysed by triple detection gel permeation chromatography (tdGPC) to determine their molecular weight and polydispersity. These results are shown in table 3.1.

<table>
<thead>
<tr>
<th>Fraction (NaCl concentration)</th>
<th>Molecular weight (kDa)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM</td>
<td>8.06</td>
<td>1.104</td>
</tr>
<tr>
<td>100 mM</td>
<td>12.49</td>
<td>1.067</td>
</tr>
<tr>
<td>125 mM</td>
<td>14.47</td>
<td>1.298</td>
</tr>
<tr>
<td>175 mM</td>
<td>16.02</td>
<td>1.558</td>
</tr>
<tr>
<td>225 mM</td>
<td>20.457</td>
<td>1.280</td>
</tr>
<tr>
<td>275 mM</td>
<td>23.457</td>
<td>1.470</td>
</tr>
<tr>
<td>325 mM</td>
<td>31.200</td>
<td>1.206</td>
</tr>
<tr>
<td>375 mM</td>
<td>34.49</td>
<td>1.411</td>
</tr>
<tr>
<td>425 mM</td>
<td>39.08</td>
<td>1.346</td>
</tr>
</tbody>
</table>

**Table 3.1**: Triple detection GPC analysis of selected colominic acid fractions from those shown in figure 3.4

The tdGPC analysis clearly shows that fractionation has occurred, but the polydispersity still seems quite high. It is clear that overloading the column and using one column volume for each step, results in insufficient removal of the low molecular weight species. This causes a “tailing” effect, in the higher fractions, i.e. the fraction contains mostly larger species but also a “tail” of smaller molecular weight species. This is what increases the polydispersity of the fractions as the large species keep the average molecular weight (Mw) high, while the small species drag the number average molecular weight (Mn) down, which greatly increases the polydispersity index (Mw/Mn). Not surprisingly the greatest polydispersity is seen in the
middle fractions. This is because at the highest fractions none of the very small species remain in the column.

To address this issue the experiment was repeated using 150 mg of colominic acid and 1.5 column volumes for each step of the gradient (i.e. 7.5 ml instead of 5 ml). The results are shown in figure 3.5

Figure 3.5: PAGE analysis of the preparations obtained from the fractionation of 150 mg CA with a 5 ml column using 1.5 column volumes for each step of the gradient. Comparing this result with the one shown in figure 3.4, it is immediately apparent that the fractions obtained are a great deal narrower in terms of molecular weight distribution (smaller polydispersity). Again the 75 mM fraction runs off the gel.
Again selected fractions were analysed by tdGPC to verify that the polydispersity has indeed decreased as can be seen in Table 3.2.

<table>
<thead>
<tr>
<th>Fraction (NaCl concentration)</th>
<th>Molecular weight (kDa)</th>
<th>Polydispersity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 mM</td>
<td>7.412</td>
<td>1.065</td>
</tr>
<tr>
<td>125 mM</td>
<td>12.310</td>
<td>1.076</td>
</tr>
<tr>
<td>175 mM</td>
<td>14.120</td>
<td>1.013</td>
</tr>
</tbody>
</table>

**Table 3.2:** Triple detection GPC analysis of selected colominic acid fractions from those shown in figure 3.5

As predicted, the polydispersity decreases greatly when the volume of each step in the gradient is increased to 1.5 column volumes. It is immediately apparent from comparing the gel in figure 3.5 with that in figure 3.4 that this because the “tailing” effect due to incomplete elution at each step is no longer an issue. The fractions also seem to be slightly smaller in terms of average molecular weight. This is attributed to the decreased amount of sample loaded. At 200 mg the column becomes saturated and larger species are eluted more easily.

To determine whether the volume of each step in the gradient is more important than the amount of sample loaded, the experiment was repeated with 200 mg of colominic acid and 1.5 column volume steps. The results obtained (not shown) are very similar, with most of the fractions having a polydispersity of less than 1.1. It can therefore be concluded that 1.5 column volume steps are necessary for obtaining narrow molecular weight fractions.

### 3.4 Large scale fractionation of 22.7kDa colominic acid

To produce usable quantities of fractionated colominic acid the process was scaled up to 3 g of material using a 75 ml column. Again the volume of each step was set to 1.5 column
volumes, to ensure the polydispersity is low. Since the smaller species are less accurately measured by tdGPC, more difficult to process and harder to visualize by PAGE, the focus was shifted to the larger molecular weight species for optimisation purposes, so as to minimize the number of samples processed. This was accomplished by replacing all the steps using elution buffers with a salt concentration below 325 mM, with a single 3 L wash with a 325 mM salt buffer to elute all the low molecular weight species off the column. The results are shown in figure 3.6

![Fractionation of high molecular weight species from 3g of CA with a 75ml column](image)

**Figure 3.6:** Analysis of the fractions from the separation of high molecular weight species from 3 g of 22.7 kDa, PD1.34 colominic acid. The 3 L of the 325 mM wash contain 70% of the population and is not shown on this graph. Each of the remaining fractions is 112.5 ml in volume.

The fractions were also analysed by PAGE, to verify that fractionation has occurred and to compare the fractions with the samples from the small scale fractionations (see figure 3.7) and by tdGPC (see table 3.3) to determine the average molecular weight and polydispersity.
Figure 3.7: PAGE analysis of the fractions shown in figure 3.6. The gel is a tris-glycine 4-20% polyacrylamide gradient gel. The colominic acid is visualised by staining the gel with alcian blue for colominic acid. Two samples from the small scale purification (purified by concentration dialysis and freeze dried) are also included for comparison.

Figure 3.7 shows that the fractionation process works well when the production is scaled up as long as the ratio of colominic acid to the matrix is the same and the 1.5 column volume per step ratio is maintained. The fractions seem to be slightly smaller in size compared to the small scale fractionation. This is because the extensive wash at 325 mM removes all the small molecules and some of the intermediate molecules as well. As explained in the Introduction, even low salt concentration washes can cause high molecular weight bands to migrate albeit slowly. The size of the samples was measured by tdGPC. The results are shown in table 3.3, the GPC traces for the 400 and 450 mM samples are shown in figure 3.8.
### Table 3.3: Tripple detection GPC analysis of the fractions obtained from the fractionation of 3 g of colominic acid (22.7 kDa, 1.34 pd). The fractions are the same as those shown in figure 3.7.

<table>
<thead>
<tr>
<th>Elution step (NaCl concentration)</th>
<th>Average molecular weight (kDa)</th>
<th>Polydispersity index</th>
<th>% Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>325 mM</td>
<td>12.586</td>
<td>1.091</td>
<td>77.4%</td>
</tr>
<tr>
<td>350 mM</td>
<td>20.884</td>
<td>1.037</td>
<td>3.2%</td>
</tr>
<tr>
<td>375 mM</td>
<td>25.542</td>
<td>1.014</td>
<td>5.0%</td>
</tr>
<tr>
<td>400 mM</td>
<td>28.408</td>
<td>1.024</td>
<td>4.4%</td>
</tr>
<tr>
<td>425 mM</td>
<td>Not measured</td>
<td>Not measured</td>
<td>7.4%</td>
</tr>
<tr>
<td>450 mM</td>
<td>43.760</td>
<td>1.032</td>
<td>2.3%</td>
</tr>
<tr>
<td>475 mM</td>
<td>42.921</td>
<td>1.096</td>
<td>0.2%</td>
</tr>
</tbody>
</table>

**Figure 3.8 (a):** Tripple detection GPC analysis of the 400 mM fraction. The peak obtained is narrow and symmetrical and the refractive index peak coincides with the light scattering peak, clearly showing that the sample has very low polydispersity.
Figure 3.8 (b): Tripple detection GPC analysis of the 450 mM fraction. As before the peak is narrow and symmetrical and the refractive index peak coincides with the light scattering peak, showing that the polydispersity is low. The peak is slightly shifted (about 0.4mL) to the left, verifying that the 450 mM fraction is larger than the 400 mM fraction.

Figure 3.8 (c): Tripple detection GPC analysis of the polydisperse starting material (22.7 kDa). In this case the peak is much broader and the refractive index peak does not coincide with the light scattering peak, due to the high polydispersity of the starting material.
Once again the polydispersity of the fractions is very low, even in the first fraction, but most of all in the intermediate molecular weight fractions. This result lends more support to the argument that complete removal of as many of the low molecular weight species as possible is crucial in obtaining the narrowest possible polydispersity. It is also clear that two relationships affect the fractionation. One is the relationship between the amount of colominic acid that can be fractionated and the column volume. The other is the relationship between the volume of matrix used and the volume of each elution step. The results suggest that a value of 40 mg colominic acid (or less) per ml of matrix for the former and 1.5 ml of each elution buffer per ml of matrix for the latter, work very well for the purposes of fractionation. One interesting note is that the 475 mM wash is slightly smaller than the previous one (450 mM) and more polydisperse. It is also clear from the gel that it contains some intermediate molecular weight species. This is not unexpected since the reversibility of the binding means that some colominic acid will always remain bound on the matrix unless the ionic strength is raised so high that the binding constants fall to very low values.

To produce material for the conjugation studies 3 g of colominic acid are fractionated with a 75 ml column or 25 g of colominic acid are fractionated with a 900 ml column using the full elution step range from 50 to 500 mM NaCl with 25 mM steps. A typical chromatogram obtained is shown in figure 3.9.
Fractionation of 3g of CA with a 75ml column

Figure 3.9: Chromatogram obtained from the complete fractionation of 3 g of CA (22.7 kDa 1.34 pd) with a 75 ml column and the full step gradient range of 25 to 500 mM with 25 mM elution steps.

The chromatogram is similar to the one shown in figure 3.6 only the peak at 375 mM is greatly increased, while the peak at 400 mM is slightly higher. This yet again shows that the 3 L wash with 325 mM elutes some of the intermediate species and even a small amount of the higher species, as explained in the Introduction.

Results obtained with PEG have shown that it is the small molecular weight PEGs (less than 5 kDa) that perform best in sterically stabilising liposomes. Hence for this purpose the 50m M, 75 mM and 100 mM fractions are the most promising ones for sterically stabilising liposomes. The 75 mM fraction that was used in further experiments had a molecular weight of 6.2 kDa and a polydispersity index of 1.15.
Chapter 4

Production of colominic acid modified with reactive groups
4.1 Introduction

The reactivity of native colominic acid is limited to the hemi-acetal group at its reducing end. This group can be modified with hydrazines (Lipoxen unpublished observations), but generally it is neither reactive enough nor versatile enough to be used as is for efficient conjugation of colominic acid to macromolecules or other structures. Hence, there is a need to derivatise colominic acid, in order to introduce reactive groups that can be used for efficient conjugation to the desired target. The introduction of reactive groups in polysaccharide molecules has been extensively studied and many methods are described in the literature (Svenson and Lindberg 1979, Seppälä and Mäkelä 1989, Fattom et al 1992, de Velasko et al 1993, Gupta et al 1995, Pawlowski et al 1999). There are a number of groups in colominic acid that present themselves as suitable targets, such as the carboxyl groups for condensation reactions with alcohols and amines (using carbodiimide catalysts) and the N-Acetyl groups, which can yield amine groups upon deacetylation (Jennings et al 1985), but the most attractive target is the extracyclic vicinal diol at the non-reducing end. Colominic acid contains no other vicinal diol groups. Unlike the carboxyl and N-Acetyl groups, which are present in every repeat, the vicinal diol is unique and can be used to introduce a single reactive group onto the colominic acid molecule. This vicinal diol can be specifically oxidised by NaIO₄ without modifying other groups (Jennings and Lugowski 1981, Zhang 1999).

Several modifications of colominic acid were explored in this study. Following oxidation by periodate to create the aldehyde, a method described by Pawlowski et al (1999) was adapted to introduce amine and thiol groups to colominic acid. Briefly the aldehyde was converted to an amine via reductive amination with ammonium chloride forming amino-colominic acid (amino-CA) or with cystamine forming cystamine-colominic acid (cystamine-CA). Amino-CA was reacted with 2-iminothiolane (2IT – Trout’s reagent) to
convert the amine to a thiol, or cystamine-CA was reduced with DTT to unmask the
disulphide. In addition methods were devised for introducing a maleimide group and an
N-hydroxysuccinimide (NHS) ester onto amino-CA, using small molecule crosslinkers. A
reaction tree detailing all the colominic acid modifications is presented in figure 4.1.

**Figure 4.1:** The modifications of colominic acid (scheme 2.9 from methods section 2.3.1)
4.2 Periodate oxidation

The scheme below presents a detailed mechanism for the oxidation of the vicinal diol groups at the non-reducing end of colominic acid by the periodate ion.

\[
\begin{align*}
\text{H} & \text{H} \quad \text{OH} \\
\text{H} & \text{H} \quad \text{OH} \\
\text{R} & \text{R}
\end{align*}
\]

\[
\text{HO} + \text{O}_3\text{O}^- \rightarrow \text{HO} + \text{O}_3\text{O}^- + \text{H}_2\text{O}
\]

The periodate ion attacks one of the diol carbon atoms and the oxygen of the second diol group attacks the periodate ion leading to the formation of a cyclic intermediate with the release of a water molecule. The cyclic intermediate decomposes to two aldehyde molecules and an iodate ion (Guthrie and Honeyman 1964).

In addition to vicinal diols periodate can oxidise other groups that contain oxygen or nitrogen atoms on adjacent carbons, but the oxidation rates for different groups vary quite significantly. Hence periodate concentration, temperature and reaction time can be used to limit oxidation to the more susceptible groups (vicinal diols). Colominic acid contains a single vicinal diol at its reducing end and thus sodium periodate can be used to selectively oxidise it and thus introduce a single aldehyde group on colominic acid.
It has been reported that 1 mM periodate at 4°C for 1 hour specifically oxidises the diols on the exocyclic arm of sialic acid residues of various proteoglycans (Hermanson 1996). The reason why the exocyclic arm is more susceptible to oxidation, is that the carbon bonds in the exocyclic arm have a greater degree of rotational freedom. The greater rotational freedom allows the oxygen atoms of the diol group to adopt with greater ease the optimal conformation for a productive collision with the periodate ion. In the vicinal diols of the ring structures, optimal positioning of the oxygen atoms is hindered by the decreased rotational freedom of the ring carbon-carbon bonds. Colominic acid having no other vicinal diol groups except the one on the exocyclic arm of the non-reducing end residue, can withstand much higher concentrations of periodate at higher temperature, than many others carbohydrate polymers without breakage of the glycosidic bonds occurring. In previous work with colominic acid (Zhang 1999 and Lipoxen technologies unpublished observations) 100mM periodate for 15 minutes at 25°C was used and proven to be effective. Triple detection gel permeation chromatography (tdGPC – described in chapter 3) analysis has shown that for large polymers, these conditions can result in a small but significant reduction in molecular weight (Lipoxen technologies unpublished data), coupled by an increase in polydispersity.

At higher temperature or for reaction times greater than 15 minutes the glycosidic bonds begin to break as well, as observed by a small decrease in size and increase in polydispersity during triple detection GPC analysis (Lipoxen, unpublished observations). However it was found in this study that complete oxidation can be obtained using only 10-20mM periodate for 15 minutes at room temperature as long as the periodate to colominic acid molar ratio is 8 or greater. A decreased concentration of periodate facilitates purification and practically eliminates the chance that breakage of the glycosidic bonds may occur in the process. Periodate reactions can be stopped by the
addition of a large excess of ethylene glycol to the reaction. Ethylene glycol reacts with the remaining periodate and eliminates it quickly, producing formaldehyde as a side product. Removal of the formaldehyde and the remaining ethylene glycol requires extensive dialysis (as described in chapter 2). Where possible this step has been circumvented by using 70% ethanol to selectively precipitate colominic acid (as described in section 2), leaving the remaining periodate in solution. Two precipitation steps result in almost complete removal of periodate from the final product. Periodate is light sensitive so it needs to be protected from light at all times when in solution. Exposure to light is undesirable as the photochemical reactions of periodate may produce other reactive species that can react with colominic acid directly.

The recovery with the ethanol precipitation technique is dependent on the concentration of colominic acid in the original solution, the salt concentration and polymer molecular weight.

Low molecular weight colominic acid or colominic acid that has been ethanol precipitated several times is harder to precipitate and forms a much thinner precipitate that requires greater centrifugal force to sediment. In such cases precipitation may require cooling or increasing the ethanol concentration, to avoid loss of the low molecular weight species. The same issue exists with colominic acid that has been dialysed extensively. Repeated precipitations or extensive dialysis remove too much of the salt in the solution making precipitation difficult. The increased solubility of lower molecular weight colominic acid in 70% ethanol is expected since it is a function of the molecule’s overall charge.

The yield of the double precipitation method is comparable to the quenching and dialysis method (see below) but only for the larger molecular weight polymers (13 kDa). Colominic acid of molecular weight less than 13 kDa is difficult to precipitate effectively.
The aldehyde group was detected through its reaction with 2,4 dinitro-phenylhydrazine (2,4-DNPH) and determined quantitatively using the reduction of the ferricyanide ions by reducing sugars, as described by Park and Johnson (1949) (see section 2.1.6). 2,4-DNPH reacts with carbonyl groups in acidic conditions to form an insoluble brightly coloured hydrazone. The colour, solubility and other physical properties of the hydrazone are dependent upon the chemical entity which contained the carbonyl group. In the case of oxidised colominic acid, the hydrazone is bright yellow. Non-oxidised colominic acid can also react with 2,4 DNPH, via the reducing end, because in a small proportion of molecules the residue at the reducing end is open exposing a ketone. The extent of reaction is very small however. The hydrazone can be solubilised by adding 1 M sodium hydroxide, which allows its concentration to be determined by monitoring absorbance at 450 nm. The ketone at the reducing end gives a weak signal, so a non-oxidised control is necessary to account for it.

The ferricyanide assay (ferricyanide is a trivial name for hexacyanoferrate(III)), is based on the reduction of ferricyanide to ferrocyanide (hexacyanoferrate(II)). Aldehydes (or aldoses) are reacted with ferricyanide by heating in an alkaline solution. This results in the aldehyde reducing ferricyanide to ferrocyanide. The ferrocyanide ion is then detected by acidifying the solution and adding ferric ammonium sulphate in the presence of SDS. Addition of ferric (trivial name for iron(III)) ions to ferrocyanide results in the formation of prussian blue(iron(III) hexacyanoferrate(II) or ferric ferrocyanide) (Southgate 1976). The presence of SDS slows down the formation (and precipitation) of large Prussian Blue crystals allowing the absorbance of the solution to be recorded (Park and Johnson 1949). The ketone at the reducing end gives a weak signal, so a non-oxidised control is necessary to account for it.
Table 4.1 shows the results of oxidizing 22.7 kDa polydisperse and various types of monodisperse colominic acid. All polymers were oxidised with 20 mM periodate. It is clear that 20 mM periodate is quite sufficient for complete oxidation of the non-reducing end vicinal diol. Ethanol precipitation produces equivalent results to quenching and dialysis (the traditional approach) for the large molecular weight polymers. With 6.2 or 7.5 kDa polymer however, the recovery is very poor for ethanol precipitation.

<table>
<thead>
<tr>
<th>Molecular weight (kDa)</th>
<th>No of aldehyde groups per molecule (± Std error)</th>
<th>Purification method</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>1.196±0.042</td>
<td>Ethanol precipitation</td>
<td>88±6</td>
</tr>
<tr>
<td>22</td>
<td>1.146±0.012</td>
<td>Ethanol precipitation</td>
<td>81±11</td>
</tr>
<tr>
<td>19</td>
<td>1.0167±0.008</td>
<td>Ethanol precipitation</td>
<td>92±5</td>
</tr>
<tr>
<td>6.2</td>
<td>0.9934±0.051</td>
<td>Quenching and dialysis</td>
<td>96±3</td>
</tr>
<tr>
<td>7.5</td>
<td>1.057947*</td>
<td>Ethanol precipitation</td>
<td>57*</td>
</tr>
</tbody>
</table>

**Table 4.1**: Oxidation and recovery of size fractionated colominic acid. The number of aldehyde groups was estimated using the Park and Johnson ferricyanide assay. The values are averages from the analysis of different oxidation batches of the same polymer, except for the values marked with *, which are from a single experiment. Typically 300-500 mg of colominic acid are oxidised in each batch.

### 4.3 Reductive amination of oxidised colominic acid

Reductive amination is the conversion of an aldehyde to an amino group via the formation and reduction of the Schiff base, as outlined in the scheme below. Formation of the Schiff base occurs via nucleophilic attack of the amine nitrogen on the carbonyl carbon, forming a tetrahedral intermediate. The intermediate is unstable and decomposes
to the imine (Schiff base), with elimination of a water molecule. The imine is reduced to the amine by using sodium cyanoborohydride (NaCNBH₃).

Sodium cyanoborohydride is a substituted metal hydride reducing agent with a significantly reduced reactivity compared to the non-substituted version - sodium tetrahydroborate. While sodium tetrahydroborate (sodium borohydride) readily reduces carbonyl groups, sodium cyanoborohydride exhibits a preference for the imine (Schiff base). Metal hydrides react with aldehydes by donating a hydride ion to the carbon atom, while the oxygen atom of the aldehyde forms a bond with the metal. The complex can react with more aldehydes, until all its hydrides have been donated. Hydrolysis of the metal oxygen bonds then releases the alcohol molecules. Metal hydrides substituted with
electron sequestering groups are less reactive because they are less nucleophilic. This is why the cyanoborohydride ion is much less reactive than the borohydride ion. Sodium cyanoborohydride still reduces carbonyl groups albeit very slowly. This reduction can compete with the formation of the Schiff base at lower pH. Hence maximising reaction yield depends upon minimising the reduction of the aldehyde by cyanoborohydride. This is achieved by carrying out the reaction at a slightly basic pH and driving the reaction forward with a very large excess of amine or ammonium.

The pH of the reaction is important, as the reducing power of Sodium Cyanoborohydride in reactions with C=X bonds (where X=O, N) increases with decreasing pH, because high concentration of H⁺ promotes protonation of the non-carbon atom, thus making the carbon atom more susceptible to nucleophilic attack. At pH 3-5 cyanoborohydride readily reduces aldehydes and ketones, while at pH above 6 it is more selective for the iminium ion and not carbonyl groups. Formation of the Schiff base on the other hand increases with increasing pH. Nucleophilic attack on the carbonyl carbon by the amino group requires a free electron pair and thus the charged ammonium group cannot react with the carbonyl. At higher pH the equilibrium shifts towards the unprotonated form and hence the rate of the Schiff base formation increases. There is therefore yet another trade-off. Higher pH increases the rate of Schiff base formation, but decreases the reactivity of the cyanoborohydride.

The reaction was carried out using either ammonium chloride (NH₄Cl) or cystamine (NH₂(CH₂)₂S-S(CH₂)₂NH₂) as described in sections 2.3.3 and 2.3.6 respectively.

4.3.1 Reaction with ammonium chloride

Using ammonia/ammonium chloride in an aqueous environment is inconvenient because of the high pKa of ammonia compared to organic amines, necessitating a higher pH, but
was chosen because it does not add any more carbon atoms. The product was analysed by gel permeation chromatography (GPC) (figure 4.2). Amine groups were detected quantitatively with the trinitrobenzenesulfonic acid (TNBS) assay. The GPC results clearly show co-elution of the amine groups with colominic acid. This would not be the case if the amine signal was simply due to the presence of free ammonium ions in the purified product, as the ammonium ions are retained longer in the column and do not elute in the void volume like colominic acid. This result suggests that an amine group has indeed been created on the oxidised colominic acid molecule.

![Reductive amination with ammonium chloride: Sephadex G25 elution profile of the product.](image)

**Figure 4.2:** The purified product of the reductive amination reaction with ammonium chloride was analysed by gel filtration on Sephadex G25 matrix. The fractions were monitored with the resorcinol assay for colominic acid and with the TNBS assay for the amine groups.

The yield of this reaction is 85%. Ammonium ions are indeed present and begin to elute at an elution volume of 5.5 ml. About 40% of the amine signal in the freeze-dried
powder after purification with the double ethanol precipitation method is due to ammonium ions. Given that ammonium ions react less with the TNBS reagent, it is likely that a significant amount of ammonium is still present. When the presence of ammonium ions is undesirable the purification protocol is changed to a single ethanol precipitation, in order to concentrate the sample, followed by dialysis against ammonium carbonate. The ammonium carbonate is necessary to buffer the pH as colominic acid degrades quickly at acidic pH. It is volatile and sublimes during freeze-drying. If it is necessary to remove ammonium ions as completely as possible, the sample is dialysed against deionised water set to pH 7 with NaOH. Colominic acid of less than 13 kDa was purified by dialysis against two 2 L changes of changes of 50 mg/ml ammonium carbonate and one 2 L change of deionised water at pH7.0.

4.3.2 Reaction with cystamine

Reaction with cystamine is easier since it is an organic amine, so a smaller excess is used. Since cystamine has two amine groups per molecule there exists the possibility that two colominic acid molecules may react with one molecule of cystamine and become linked. This is not detrimental as cystamine is subsequently reduced to unmask the sulfhydryl group, so linked colominic acid molecules will be broken apart. Dimers which are not broken will be unable to participate in any further reactions. Figure 4.3, shows the analysis of the reductive amination reaction mixture by GPC. The fractions were analysed for thiol groups as well as amines and colominic acid to see if NaCNBH₃ is sufficient to reduce the disulphide bond of cystamine.
Reducive amination with cystamine: Sephadex G25 elution profile of the reaction mixture.

![Elution profile graph](image)

**Figure 4.3:** The reaction mixture from the reductive amination reaction with cystamine was analysed by gel filtration chromatography. The fractions were monitored with the TNBS assay for amine groups, with resorcinol for colominic acid and with the Ellman’s assay for thiol groups (see section 2.1 for all assays).

Once again the amine signal co-localises with the colominic acid in the void volume fractions, while the remaining cystamine elutes at a higher elution volume, suggesting that colominic acid has been successfully modified. The amine signal past fraction 4 comes from the free cystamine which is small enough to be in the fractionation range of the column. The reaction yield is 95%. NaCNBH₃ does not appear to reduce the disulphide bond of cystamine effectively. Only a very small amount of thiols can be detected. A more effective disulphide reducing reagent is necessary to unmask the thiol groups.

In order to observe the kinetics of the reaction the loss of aldehyde over the course of the reaction was monitored with the 2,4 DNPH assay. The results are shown in figure 4.4.

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Loss of aldehyde groups over time, during reductive amination of oxidised CA with cystamine

Figure 4.4: Loss of aldehyde groups during reductive amination with cystamine. At each time point a 300 µl sample is withdrawn and colominic acid is recovered by gel permeation chromatography and concentrated by ultrafiltration. The samples are tested for aldehyde with the 2,4 DNPH assay and for CA with the resorcinol assay. A colominic acid control with 30 mg/ml non-oxidised colominic acid was also tested with the 2,4 DNPH assay. The 2,4DNPH signal was normalised for the colominic content for all samples and the control after subtracting the blank. Then the control signal was subtracted from all the samples and % aldehyde remaining was calculated by taking the signal at time 0 as 100%.

It is clear that after 24 hours the aldehyde signal drops to a negligible level. The decrease in aldehyde cannot be used to track the degree of product formation, as cyanoborohydride even at pH8.0 also reduces the aldehyde to a small extent. It was found that after 30 hours, 30% of the aldehyde is lost under these reaction conditions even if no amine is present in the reaction mixture. Reductive amination competes with aldehyde reduction, so it is unlikely that much of the aldehyde is reduced to the alcohol through this side reaction.
4.3.3. The effect of temperature on the reductive amination reaction

The yield of the reaction is significantly affected by temperature. At 25°C the yield with ammonium chloride is between 60 and 70% (these values represent the minimum and maximum that has been obtained over a large number of reactions under these conditions). At 37°C the yield of the reaction is 80-90%. A possible explanation is that the formation of the Schiff base is an endothermic reaction, while the reduction of the aldehyde is exothermic, so that product formation is increased while the rate of the competing reaction is reduced.

4.4 Thiolation of colominic acid

In the following sections the two approaches were used to form a thiol group on colominic acid will be described. This was accomplished either using Traut’s reagent or cystamine.

4.4.1 Thiolation with 2-iminothiolane (2-IT or Traut’s reagent)

Traut’s reagent (Hermanson 1996) is an amine specific thiolation reagent. It was used to introduce a thiol group on amino-colominic acid. The reaction between amino-colominic acid and 2-IT is shown in the scheme below.
The thiolation of amino-CA with Traut's reagent (2-IT) proceeds via nucleophilic attack of the amine on the carbon of the thio-imidoester group of 2-IT. This results in breakage of the bond between the imine carbon and the sulphur atom and opens the ring producing a thiol linked to colominic acid via an amidine group.

2IT is specific for amines at pH8 and quite efficient. A yield of more than 85% is routinely reached. Use of 2IT also creates a 5-atom spacer (4 carbons and 1 nitrogen) between the thiol group and the carbon 7 of the non reducing end sialic acid residue of colominic acid. The spacer is connected to colominic acid via an amidine linkage. The amidine linkage is stable at pH8 or below but hydrolyses rapidly at high pH. 2-IT is very slowly hydrolysed at pH8 producing a small molecular weight thiol-containing molecule that can form a disulphide bond with the thiol-colominic acid. The other possible by-product is disulphide linked thiolated colominic acid dimers, or the disulphide formed between thiolated colominic acid and the hydrolysed 2-IT. This is why the actual yield decreases if the 2IT molar excess is increased past 50-fold or if the reaction time is increased past 2 hours. A 50-fold molar excess of 2IT is sufficient for the reaction to go to completion within 1 hour at room temperature. The results of the analysis of the reaction mixture by GPC are shown in figure 4.5.
Figure 4.5: Thiolation of amino-CA with 2-IT. The thiolation reaction was loaded onto a PD-10 packed with Sephadex G25 and eluted with PBS-EDTA. The fractions were monitored with the Ellman’s assay for thiol groups and the resorcinol assay for colominic acid. The TNBS can’t be used to determine amine in this case. A negative control reaction was also run using oxidised colominic acid instead of amino colominic acid.
A thiol signal can be seen in the void volume co-localising with the colominic acid, while the remaining thiols elute at a later elution volume (after 5 mL). No thiol signal can be seen in the void volume in the negative control. The extent of thiolation is ~60% (~75% of the amines modified).

4.4.2 Reduction of cystamine-colominic acid

Thiolation of colominic acid was also accomplished by reducing the disulphide added to colominic acid after reductive amination with cystamine, as detailed in section 2.3.6. Yields of 90-95% are routinely obtained with this method, which also has the added advantage of only requiring one purification step as the reduction of the disulphide can be accomplished by the addition of DTT straight into the reductive amination reaction mixture. This produces a 2 atom non-cleavable spacer between the thiol and colominic acid. A disadvantage of this approach is the increased probability of reducing end modification.

As can be seen in figure 4.6, the reduced colominic acid elutes in the void volume, while DTT and the small molecule by-products elute after 5 mL as can be seen by the strong thiol signal in the 6mL fraction. As can be seen in figure 4.3, prior to DTT reduction cystamine-CA has negligible thiol activity. Following DTT reduction ~85% of the colominic acid molecules contain a thiol group.

Contrary to the results published by Pawlowski et al (1999) for dextrans, it appears that for colominic acid, the most effective thiolation method is the cystamine approach, with a final yield of 86% compared to 60% for the 2-IT approach. Dextrans are neutral polysaccharides and contain more than one aldehyde group per molecule. Hence when these aldehydes are converted to thiols, the resultant thiolated dextrans have a significant propensity towards crosslinking. Thiols formed with 2-IT contain the changed amidine
group and hence have a decreased tendency to crosslink. Loss of thiol groups upon freeze
drying and storage is greater for cystamine thiolated dextran compared to 2-IT thiolated
dextran. Colominic acid on the other hand is already highly charged and does not have a
strong tendency to crosslink, so the cystamine approach proved advantageous, with little
loss of thiol upon freeze-drying and storage.

Unmasking of thiol groups in cystamine-CA by reduction with β-
mercaptoethanol

Figure 4.6: Purification of thiol-colominic acid after reduction of cystamine-CA by
β-mercaptoethanol. Excess β-mercaptoethanol and reaction by-products were
removed by gel permeation chromatography on a PD10 column packed with
Sephadex G25. The fractions were tested with the resorcinol assay for colominic
acid and the Ellman’s assay for thiol.

4.5 Modification of amino-colominic acid with maleimide

The scheme below details the condensation reaction between the heterobifunctional cross-
linker BMPS (3-maleimidopropionic acid N-hydroxysuccinimide ester) and amino
colominic acid. The amine nucleophilically attacks the activated ester carbon atom,
forming an amide group and releasing N-hydroxysuccinimide (NHS) in the process as shown in the scheme below.

\[
\text{CA} + \text{BMPS} \rightarrow \text{CA-maleimide} + \text{NHS}
\]

The N-hydroxy-succinimidyl (NHS) ester group is strongly reactive towards amines at pH 7.4, so the reaction is straightforward and effective. A 10-fold excess of BMPS over amino-CA was found to be sufficient. Increasing the excess of BMPS past 10-fold did not improve the final yield. The maleimide group is highly reactive with and specific for thiol groups, at pH 6.5 to 7.5 as depicted in the scheme below:

\[
\text{R}_2\text{SH} + \text{maleimide} \rightarrow \text{thioether bond} \quad \text{pH 6.5-7.5}
\]

The reaction proceeds via an addition reaction of the thiol onto the maleimide ring double bond, producing a stable thioether linkage. The maleimide group is stable in solution over short periods of time, but it can still hydrolyse, especially at pH above 8 (Hermanson 1996). Overnight incubation at 4°C resulted in loss of more than 70% of the maleimide activity. The reactivity of the NHS ester is improved at higher pH on the other hand, so the reaction time can be decreased. Hence the pH was set at 7.4 and the reaction time to 1h to obtain maximum reactivity for the NHS ester, while minimising the hydrolysis of the maleimide group.
Chapter 5

Formation of liposomes bearing reactive groups
5.1 Introduction

The plasma circulation times of liposome formulations vary greatly depending on the lipid composition (e.g. degree of phospholipids saturation, inclusion of cholesterol, lipid charge), and the size of the liposomes. Half-lives in mice, range from a few minutes to over 20 hours (Senior and Gregoriadis 1992b). Small unilamellar vesicle (SUV) liposomes consisting of equimolar DSPC and cholesterol, whose diameter is around 100nm or less, have an exceptional pharmacokinetic profile, exhibiting very long circulation times. For this reason it was decided to polysialylate DSPC: cholesterol based liposome formulations, in order to compare the polysialylated liposomes to the current “gold standard” and to obtain the maximum possible half-life. In the previous chapter the synthesis of more reactive colominic acid derivatives was described, yet even these more reactive variants are unable to modify DSPC: cholesterol liposomes, which are relatively inert chemically. Before conjugation can occur the liposomes must also be chemically activated, yet neither DSPC nor cholesterol offer themselves as attractive targets for this purpose. Hence, other lipids must be included in the formulation, which can either be modified in situ with groups able to react with the activated colominic acid, or bear such groups already. There are many examples of liposome activation in the literature (Hansen et al 1995, Skalko et al 1998, Bendas et al 1998, 1999). Typical methods involve the use of heterobifunctional crosslinkers to modify the amine groups of liposomes containing phosphatidyl-ethanolamine (PE) lipids. Additionally a PE lipid can be reacted directly with a crosslinker and then included in the liposomes. PE lipids themselves can also be targeted directly by using amine reactive groups, such as esters of carboxylic acids with N-hydroxysuccinimide, on the molecule that is to be conjugated to the liposomes. In this chapter, the four approaches that have been used to create activated liposomes will be described. They are: 1) Direct modification of 1,2-distearoyl-sn-glycerol-3-
phosphatidylethanolamine (DSPE) with maleimide to form a maleimide lipid (DSPM), which can then be directly incorporated into liposomes, 2) in situ modification of liposomes with maleimide, 3) in situ modification of liposomes with thiol groups and 4) in situ activation of liposomes with N-hydroxysuccinimide ester groups.

5.2 Modification of DSPE with maleimide

DSPE was reacted with the heterobifunctional crosslinker BMPS as described in section 2.4.2. The reaction is shown in the scheme below.

The free amino group of DSPE attacks the carbonyl carbon of the N-hydroxysuccinimide ester forming an amide bond with it. This creates N-([β-maleimidopropionyl]-1,2-distearoyl-sn-glycerol-3-phosphatidyl-ethanolamide (DSPM) and releases N-hydroxysuccinimide in the process. The solvent system was selected because of its ability to dissolve DSPE at a manageable temperature. DSPE is only partially soluble in
chloroform below 60°C and does not dissolve at temperatures below 60°C in DMF, DMSO and THF. DSPE is more soluble in chloroform-methanol mixtures than in pure chloroform. It is likely that the large hydrophobic tail and small zwitterionic head allow DSPE to form very stable layered crystals, which are more soluble in a mixture of polar and apolar solvents, than apolar solvents alone. Adding triethylamine, further increases the solubility of DSPE in the mixture. DSPC in contrast, which contains a more bulky head group, is a lot more soluble in chloroform. The solvent system used, chloroform: methanol: triethylamine (TEA) 8:1:0.2, dissolves DSPE at 30°C. Triethylamine is a good proton acceptor and its function is to deprotonate DSPE. This is necessary not only for the dissolution of DSPE at a lower temperature but also for the reaction with the NHS-ester, which can only react with the unprotonated form. The reaction was monitored with thin layer chromatography (TLC) in figure 5.1.

As can be seen in figure 5.1, all the highly mobile bands that stain with molybdenum blue (i.e. they contain phospholipids) cast a UV shadow. The presence of both lipid and UV absorbance suggests that this band is the DSPM lipid. BMPS migrates just below the DSPM lipid. It has a UV shadow but does not stain with molybdenum blue. DSPE does not cast a UV shadow, but it stains with molybdenum blue as expected. It is evident that the reaction of DSPE with BMPS is very fast and practically quantitative. With a 2.5 fold excess of BMPS, in just 15 minutes, there is no more DSPE, detectable by TLC. It is clearly not necessary to use a higher excess of BMPS as a 2.5 fold excess is able to drive the reaction to completion. To ensure that there is as little free DSPE as possible, the reaction was allowed to carry on for 2 hours, before purification by phosphate buffered saline (PBS)-methanol extraction and rotary evaporation of the remaining organic solvent as described in section 2.4.2. Three extractions are sufficient to remove any unreacted BMPS, as well as the reaction by-products (free NHS). The washings are monitored by TLC and UV spectroscopy. Both BMPS and the released NHS, have a strong UV
absorbance. The 3rd washing has no remaining UV absorbance and produces no UV absorbing band when analysed by TLC.

Figure 5.1: TLC analysis of the reaction of DSPE with BMPS. The lipids are stained with molybdenum blue. UV absorbing species are detected by illuminating with UV light. The areas of UV-shadow are marked with pencil on the plate prior to molybdenum staining. Three reactions were set up with 2.5, 5 and 10 fold excess of BMPS. The reaction with a 2.5 fold molar BMPS, was sampled at 5, 15, 30 and 60 minutes, to determine how the reaction progresses over time. The DSPE negative control has no BMPS. It was heated to 40°C before spotting onto the TLC plate to avoid precipitation, as the mixture cools down rapidly while pipetting. The BMPS reactions were not heated prior to sampling to avoid possible breakdown of the maleimide group and because it would be difficult to determine the effect of heating on the reaction kinetics. The 5 minute sample was sampled immediately after complete dissolution of the BMPS in the reaction mixture. Unavoidably the remaining DSPE precipitates, producing a streak. The TLC plate was developed with CHCl₃:MeOH:AcOH (15:4:1).
Figure 5.2, shows a TLC plate with the final purified product after extraction with PBS-methanol, removal of the reaction mixture solvent and dissolution in CHCl$_3$.

![TLC plate](image)

<table>
<thead>
<tr>
<th></th>
<th>DSPM purified (9.85mM)</th>
<th>DSPE (10.2mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 5.2:** Analysis of the purified DSPM lipid by TLC. The concentration of the lipid is calculated by TLC. The lipids are stained with molybdenum blue. Prior to staining the UV shadow of any UV absorbing bands are marked with pencil on the plate. The DSPM lipid band is the only spot on the plate that has a UV shadow. The DSPE negative control has no BMPS. The TLC plate was developed with CHCl$_3$:MeOH:AcOH (15:4:1). DSPM moves with the solvent front, but no other compound in the reaction mixture or any reaction by product can react with the molybdenum blue stain. Disappearance of the starting material shows that all the DSPE has reacted.

The highly mobile lipid band in lane 1 is the only area of UV shadow. No BMPS band is seen under UV illumination. No DSPE band is visible in lane 1 upon molybdenum blue staining. It can be concluded that a 2.5-fold ratio of BMPS is enough to drive the reaction to completion within 1 hour and 3 extractions with PBS-MeOH are sufficient to remove the unreacted BMPS from the reaction mixture. To quantify DSPM, the Stewart assay was used. However since the colour is highly dependent on the physical characteristics of
the lipid, DSPE and DSPC standards cannot be used for this purpose. Taking advantage of the fact that the reaction goes to completion the reaction mixture was assayed in triplicate after 2 hours, as all the lipid has been converted to DSPM and its concentration is known. It was also assayed in triplicate after the extractions and after the lipid was re-dissolved in CHCl₃ following removal of the reaction mixture solvent by rotary evaporation (see section 2). The recovery was calculated by dividing the value obtained after each purification step by the value obtained just before purification after the reaction was completed. The effect of methanol and TEA in the sample was accounted for, by comparing the result obtained with DSPE dissolved in CHCl₃ or in CHCl₃: MeOH: TEA 40:5:1. It was found to be negligible as very small amounts are assayed. BMPS alone gives no signal with the Stewart assay. Comparing the values at each point, showed little loss of lipid during the purification, with a yield of over 95%.

The removal of free BMPS was also monitored via TLC and measuring the UV absorbance of the washes. Wash 3 gives a very low UV absorbance reading and no shadow can be seen when a sample is run on a TLC plate. This is in contrast to washes 1 and 2, which do have a significant UV-absorbance and produce a UV absorbing band when spotted onto a TLC plate.

In the control reaction, without BMPS, as the mixture rapidly cools to room temperature, DPSE precipitates out. So the control reaction becomes turbid within 30 minutes. In the reaction mixture with BMPS on the other hand the reaction is rapid enough to modify DSPE before it comes out of solution, so no turbidity is seen even after 2 h. The lipid product is quite soluble in CHCl₃ at any temperature and also in DMF and DMSO at room temperature, suggesting that the addition of the N-(β-malemido)-propionyl group to DSPE greatly improves its solubility. The modified lipid was introduced into the liposomes simply by including it in the lipid mixture prior to formation of the lipid film, by rotary evaporation. The incorporation of the maleimide lipid was traced by TLC
analysis and by measuring UV absorbance at 280 nm after extraction of the lipids with PBS-methanol.

5.3 Activation of preformed liposomes

In addition to direct lipid activation as described in the previous section, three more approaches were explored, involving formation of active groups directly, by modifying the liposomal surface. All strategies used in activating the liposomes are shown in scheme 5.1.

![Scheme 5.1](image)

The lipids utilised in this study as targets for activation are DSPE and N-succinyl-DPPE (NS-DPPE). Direct activation of the surface is not always needed. DSPM and DSPE
containing liposomes already display maleimide and amino groups on their surface, which can react with the thiol- and NHS- derivatives of colominic acid. The bifunctional crosslinker N-(β-maleimidopropionyl)-succinimide ester, can attach a β-maleimidopropionyl group onto the amine groups of DSPE, via a condensation reaction between the N-succinimide ester (NHS group) of BMPS and the amine of DSPE forming DSPM (see section 5.2). BMPS modified liposomes therefore bear maleimide groups on their surface. Maleimide groups are reactive with thiolated colominic acid. The carbodiimide EDC (1-ethyl-3(3-dimethyl-aminopropyl carbodiimide) and N-hydroxysuccinimide (NHS) can be used to form an active N-succinimide ester (NHS ester) with the carboxyl group of N-succinyl-DPPE (NS-DPPE) lipids. The NHS ester is then reactive with amines and can hence react with amino-colominic acid. The final approach was to use Traut's reagent (2-iminothiolane or 2-IT) to convert the amine groups of DSPE liposomes to thiol groups (The reaction has been described in section 4.4). Thiol groups are reactive with maleimide and can react with maleimido-colominic acid.

5.3.1 Activation of DSPE incorporating liposomes with BMPS

The heterobifunctional crosslinker, BMPS can be reacted with DSPE containing liposomes to create maleimide-DSPE, *in situ* on the liposome surface as described in section 2.4.5. The details of the reaction between DSPE and BMPS were described in section 5.2. The maleimide liposomes are reactive with thiol-colominic acid. The maleimide lipid has a single negative charge due to loss of the positive charge on the ethanolamine group of DSPE through the reaction with BMPS. The negative charge of the maleimide lipid is the same as that for phosphatidylinositol. Reaction with BMPS thus imparts a negative charge on the liposomes as well (see table 5.1 later in this chapter for the exact value). The charge depends on the extent of BMPS derivatisation. Each
molecule of DSPE derivatised imparts to the liposome as much charge as a molecule of phosphatidyl glycerol. The activation was monitored by TLC.

Figure 5.3: TLC analysis of the reaction between BMPS and DSPE liposomes. DSPE liposomes reacted with BMPS were analysed by TLC to detect the modified lipid. The lipids were stained with molybdenum blue. The TLC plate was developed with CHCl₃: MeOH: AcOH (15:4:1). The controls used were a negative control with DSPE liposomes and no BMPS, DSPE lipid reacted with 0.6 equivalents of BMPS in CHCl₃: MeOH: TEA (see section 5.2) and DSPE in CHCl₃: MeOH: TEA. BMPS does not stain with molybdenum blue. It can be visualised as an area of shadow in a fluorescent background, by shining UV light on the plate, (see section 5.2). It migrates just below the maleimide lipid (DSPM).

It is not clear whether BMPS is able to cross the membrane and modify the amino groups of DSPE lipids in the inner lipid leaflet of the bilayer. This can be easily prevented by using a sulfonated derivative of BMPS, which is more water soluble requiring no DMSO for it to go into solution and it is unable to cross the bilayer because of its high negative charge.
The reaction mixture was purified by gel permeation chromatography as described in section 2.3.4. The liposome containing fractions were also analysed by TLC to ensure that the maleimide lipid can still be detected after removal of BMPS.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-3.5mL fraction</td>
</tr>
<tr>
<td>2</td>
<td>3.5-4mL fraction</td>
</tr>
<tr>
<td>3</td>
<td>4-4.5mL fraction</td>
</tr>
<tr>
<td>4</td>
<td>4.5-5mL fraction</td>
</tr>
<tr>
<td>5</td>
<td>Negative control (DSPC:chol:DSPE liposomes)</td>
</tr>
</tbody>
</table>

**Figure 5.4**: TLC analysis of the purified BMPS activated liposomes. The liposomes were purified by gel filtration chromatography on a PD-10 column packed with Sephadex G25. 5 μl of each fraction was spotted on the TLC plate, which was then developed with CHCl₃: MeOH: AcOH (15:4:1). Illumination of the plate with UV light showed no area of shadow, except two very faint spots near the solvent front, in lanes 2 and 3, co-localising with the DSPM bands.

To get some indication as to the extent of derivatisation of the surface the liposomes were extracted with 2:1 chloroform: methanol and the UV 280 absorbance was measured. The reading was compared with that of control liposomes and with maleimide lipid standards. To determine the efficiency of the extraction two concentrations of liposomes with preformed maleimide lipid were also extracted. The extraction is very efficient: 92% of the lipid is found in the chloroform layer. The lipid was estimated with the Stewart assay. The percentage of lipid estimated to be maleimide lipid was 3.7%. The starting liposomes
consisted of 1:1:0.2 DSPC: cholesterol: DSPE, hence in the starting preparation 9.1% of the total lipid was DSPE. Thus it can be concluded that ~41% of the DSPE lipid reacted with the BMPS reagent.

5.3.2 Activation of NS-DPPE incorporating liposomes with EDC/NHS

The carbodiimide EDC and NHS facilitate the reaction between a carboxylic acid and an amine, which results in the formation of an amide bond joining the two groups. EDC is effective on its own, but its efficacy can be further enhanced by using it in conjunction with N-hydroxysuccinimide. The reaction mechanism is outlined in Scheme 5.2.

One of the carboxyl oxygens attacks the EDC diimide carbon forming the O-acylisourea intermediate (active EDC ester), NHS then attacks the carboxylic acid carbon, releasing the isourea group as a byproduct and forming the active N-acyl-succinimide intermediate (active NHS ester). The active NHS ester can react rapidly with amines via nucleophilic attack of the amine on the carboxyl carbon, forming the final amide product and releasing the N-succinimide group, which then picks up a proton to reform NHS. The active EDC

---

**Scheme 5.2**

One of the carboxyl oxygens attacks the EDC diimide carbon forming the O-acylisourea intermediate (active EDC ester), NHS then attacks the carboxylic acid carbon, releasing the isourea group as a byproduct and forming the active N-acyl-succinimide intermediate (active NHS ester). The active NHS ester can react rapidly with amines via nucleophilic attack of the amine on the carboxyl carbon, forming the final amide product and releasing the N-succinimide group, which then picks up a proton to reform NHS. The active EDC
ester is also strongly reactive with amines, however it is much less stable than the active NHS ester with respect to hydrolysis. In addition it can self inactivate as one of the isourea group nitrogens can attack the carboxyl carbon, which will leave the isourea product permanently attached to the carboxyl carbon.

Formation of the amide will remove the charge from the carboxyl group making the liposomes less negatively charged. This is important as surface exposed negative charges might have a negative impact on the plasma half-life (Gregoriadis and Neerunjun 1974, Juliano and Stamp 1975, Gabizon and Papahadjopoulos 1988). After conjugation however, the charge will be buried under the colominic acid chain, in a similar manner to phosphatidyl inositol, which does not have a negative impact on the liposomal plasma retention times (Gabizon and Papahadjopoulos 1992b). It is difficult to quantitatively determine the extent of the reaction, given the inherent instability of the NHS ester and for the same reason it was not possible to accurately determine the surface charge of the NHS liposomes since hydrolysis of the ester will regenerate the starting lipid while the measurement is in progress. The size before the activation reaction and after purification of the activated liposomes was however measured. All the data of the physical measurements are presented in table 5.1 later in this chapter. The progress of the reaction was monitored by TLC. Figure 5.5 shows an analysis of the reaction mixture compared to a control without EDC/NHS.
Figure 5.5: TLC analysis of the EDC/NHS activation reaction mixture. The TLC plate was developed with CHCl$_3$: MeOH: AcOH 30: 9: 1. Lipids were visualised with molybdenum blue. The NHS lipid band casts a UV shadow upon UV light illumination.

A new species, with UV absorbance, that migrates faster than NSPPE can be seen. In addition, the activated liposomes were extracted once with 2:1 chloroform:methanol (500 µl sample, 500 µl methanol and 1 ml chloroform) and the optical density at 250 nm of the organic layer was measured. The absorbance was 0.48 for the NHS liposomes and 0.08 for the control. Since it is difficult to observe the NHS group on the liposomes directly and given the inherent stability of the NHS ester, the functionality was tested by conjugating to IgG, which has been successfully coupled to liposomes using EDC chemistry (Bendas et al 1998). The results are shown in figure 5.6.
Conjugation of IgG to EDC/NHS activated liposomes

Figure 5.6: Analysis of the conjugation reaction mixture (IgG with NHS liposomes) by gel permeation chromatography. The matrix used was Sepharose CL-6B. 1 mg of IgG was mixed with 500 μl of NHS liposomes (4mg total lipid per ml), in phosphate buffered saline pH 7.4 and allowed to react for 2 hours. The entire reaction mixture was then applied to a PD-10 column packed with Sepharose CL-6B, collecting 0.5 ml fractions. The fractions were analysed with the BCA assay for protein and the Stewart assay for phospholipids. A significant amount of protein (about 10%) was found to co-elute with the liposomes in the column void volume. A control reaction was also prepared with non activated DSPC: cholesterol: DSPE liposomes 1:1:0.2, to account for non-specific binding. Approximately 5% of the protein was found to co-localise with the liposomes in the column void volume. The ratio of protein to lipid in the void volume fractions for the control reaction was half as much as that for the actual conjugation reaction mixture.

5.3.3 Activation of DSPE liposomes with 2-iminothiolane

The surface amino groups of DSPE-containing liposomes can be converted directly into thiol groups by reacting them with 2-IT as described in section 2. The thiol groups are then reactive with maleimide colominic acid (section 4.4). Reaction of 2-IT with the lipid does not alter the charge of DSPE, because the amidine linkage also has a positive charge and hence preserves the positive charge of the amino group.

Figure 5.7, shows the detection by TLC of the modified lipid in the liposomes after their purification from free 2-IT.
Figure 5.7: TLC analysis of the purified DSPE liposomes activated with Traut’s reagent. The lipids were stained with molybdenum blue and the plate was developed with CHCl₃: MeOH: AcOH 15:3.5:1.5. The reaction mixture was applied to a Sephadex G25 packed PD10 as described in section 2. The most concentrated lipid fractions were pooled. 5 µl of the pooled fractions and 5 µl from both the remaining two lipid fractions were spotted on the TLC plate. 2 µl of the original DSPC: cholesterol: DSPE 1:1:0.1 liposomes were also loaded as a negative control. A third band in addition to the DSPE and DSPC bands can be seen in the most concentrated lipid fractions, suggesting the formation of a new lipid entity.

The yield does not seem to be particularly high, but only a small degree of derivatisation is needed. Measuring the exact number of thiol groups present is challenging since the Ellman’s assay does not work well in the presence of liposomes. Presumably the aromatic rings of DTNB get incorporated in the bilayer. However the reaction between 2IT and amines, is sufficiently reliable and well characterised to allow the assumption, with a certain degree confidence, that the new lipid is indeed the thiolated lipid, as no other side reactions can occur to give a different lipid product. The amidine linkage itself is very stable at pH 7.4, so no significant breakdown is expected.

The physical characteristics of the liposomes before and after activation are described in table 5.1 in the next section.
5.4 Physical characteristics of the activated liposomes

The physical characteristics (size and zeta-potential) of the liposomes incorporating activated lipid and the liposomes whose surface was activated chemically *in situ* are presented in table 5.1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Formulation (molar ratio)</th>
<th>Zeta size (nm ± std error)</th>
<th>Zeta potential (mV ± std error)</th>
<th>Polydispersity index (± std error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSPC liposomes</td>
<td>DSPC:cholesterol 1:1</td>
<td>85.8 ± 0.8</td>
<td>2.2 ± 0.1</td>
<td>0.17 ± 0.008</td>
</tr>
<tr>
<td>DSPE liposomes</td>
<td>DSPC:cholesterol:DSPE 1:1:0.1</td>
<td>89.9 ± 0.9</td>
<td>2.5 ± 0.1</td>
<td>0.102 ± 0.026</td>
</tr>
<tr>
<td>NS-DPPE liposomes</td>
<td>DSPC:cholesterol:NS-DPPE 1:1:0.1</td>
<td>58.8 ± 0.3</td>
<td>-7.2 ± 1.7</td>
<td>0.143 ± 0.028</td>
</tr>
<tr>
<td>BMPS liposomes</td>
<td>DSPC:cholesterol:DSPE 1:1:0.1 reacted with BMPS</td>
<td>88.7 ± 2.8</td>
<td>-3.3 ± 0.2</td>
<td>0.158 ± 0.022</td>
</tr>
<tr>
<td>NHS liposomes</td>
<td>DSPC:cholesterol:NS-DPPE 1:1:0.1 reacted with EDC/NHS</td>
<td>62.5 ± 1.0</td>
<td>-5.9 ± 3.8</td>
<td>0.222 ± 0.025</td>
</tr>
<tr>
<td>Thiolated liposomes</td>
<td>DSPC:cholesterol:DSPE 1:1:0.2 reacted with 2-IT</td>
<td>93.6 ± 0.8</td>
<td>1.7 ± 0.08</td>
<td>0.213 ±0.03</td>
</tr>
<tr>
<td>DSPM liposomes</td>
<td>DSPC:cholesterol:DSPE 1:1:0.1</td>
<td>76.7 ± 0.6</td>
<td>-4.3 ± 0.1</td>
<td>0.175 ± 0.025</td>
</tr>
</tbody>
</table>

Table 5.1: Physical characteristics of the activated liposomes

The values given in the table are the actual values from the PCS analysis (average of 3 measurements) of the formulations used for polysialylation reactions. For the BMPS, EDC/NHS and 2IT activated liposomes the starting material was the DSPE and NS-DPPE batches also described in this table, so that direct comparisons can be made.

The data presented in table 5.1 suggest that the activation has a very small effect on the size of the preparations. None of the three *in situ* activation methods changes the liposome size to any significant extent. However there seems to be an effect on the
polydispersity index. The BMPS activated liposomes do not change significantly compared to the starting material (DSPE liposomes) as the differences are within the standard error, however both NHS and 2IT activated liposomes seem to have a significantly higher polydispersity than the starting material. Nevertheless the polydispersity is still quite low.

Zeta potential changes are as predicted. DSPC and DSPE liposomes are effectively uncharged, NS-DPPE liposomes have a significant negative charge and DSPM liposomes have a small negative charge, which is expected since NS-DPPE and DSPM are negatively charged lipids. Activation with 2IT, does not affect the zeta-potential. This is expected since 2IT introduces a charged amidine linker, preserving the positive charge of amino group of DSPE. The zeta potential measurements on the EDC/NHS activated liposomes are not reliable, since the active group hydrolyses rapidly. Hence the value cannot be compared to that of the starting material and has no real significance. Activation with BMPS imparts a negative charge on the liposomes, since it converts the neutral DSPE to the negatively charged DSPM.
Chapter 6

Conjugation of the modified colominic acids to liposomes
6.1 Introduction

In the previous chapters colominic acid (CA) was fractionated into narrow polydispersity fractions and chemically modified with active groups. In addition DSPC based liposome formulations were created that were able to react with the activated colominic acid. In this chapter the results of the actual polysialylation reaction will be described. Two distinct chemistries have been used to this effect: Maleimide – thiol chemistry and N-hydroxysuccinimide ester (NHS ester) – amine chemistry. The reaction of NHS esters with amines has been described in section 4.6. Briefly amines nucleophilically attack the carboxyl carbon of NHS esters to form amide adducts. There are two possible combinations, react NHS-CA with liposomes incorporating a lipid with an amine group or react amino-CA with liposomes incorporating a lipid with an NHS ester group. The reaction of maleimide with thiols has been described in section 4.5. Briefly thiols give an addition reaction with the double bond between carbons 2 and 3 of the maleimide group forming a stable thioether adduct. There are two possible combinations: React thiol-CA with maleimide liposomes or react maleimide-CA with thiol-liposomes. Both approaches with both chemistries were explored. Unless otherwise indicated the colominic acid used is the 22.7 kDa stock (1.34 polydispersity).

6.2 Incubation of CA with liposomes

In order to determine whether incubation with colominic acid has any effect on the liposomes themselves, 500 µl of DSPC: cholesterol 1:1 liposomes (20 µmol total lipid per ml) were incubated for 16 hours at room temperature with 15, 35 and 100 mg of colominic acid. The starting liposomes were also allowed to stand at room temperature for 16 h, to control for the
effect of the incubation. Following incubation with 35 or 100 mg of colominic acid the liposomes were visibly more turbid and a pellet was seen after centrifugation at 13000 ref. No increase in turbidity and no pellet was seen after centrifugation of the control or in the liposomes incubated with 15 mg of colominic acid. The pellet and the supernatants were analysed with the Stewart assay to determine the liposome content. The results are shown in table 6.1.

<table>
<thead>
<tr>
<th>Colominic acid incubated with the liposomes (mg)</th>
<th>% of starting lipid in pellet</th>
<th>% of starting lipid in supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.07</td>
<td>99.93</td>
</tr>
<tr>
<td>15</td>
<td>0.95</td>
<td>99.05</td>
</tr>
<tr>
<td>35</td>
<td>17.4</td>
<td>82.6</td>
</tr>
<tr>
<td>100</td>
<td>48.8</td>
<td>51.2</td>
</tr>
</tbody>
</table>

*Table 6.1: Sedimentation of liposomes incubated with CA after centrifugation at 13000g. All tubes were centrifuged for 5 minutes, then the supernatant was removed and 0.5ml of PBS was added to the tube and vortexed to resuspend the pellets. The resuspended pellet and the supernatant were assayed for phospholipids with the Stewart assay. Total lipid was calculated as the sum of the reading in the pellet and the supernatant and the percentage lipid was calculated by dividing by total lipid and multiplying by 100.*

Table 6.1 confirms the visual observations. There is no sedimentation of liposomes for the control and the 15 mg samples. However there is a significant amount of sedimentation with the 35 and the 100 mg sample. To determine if there is any difference in size and polydispersity between the 15 mg sample and the control, the size and polydispersity of both preparations was measured with PCS. The results are shown in figure 6.1.
Figure 6.1: PCS analysis of DSPC liposomes incubated for 16h in PBS or in PBS with 15 mg of 22.7 kDa polydisperse colominic acid. Three independent measurements were taken for each preparation and the average is reported. Each measurement consists of the average of 10 acquisitions of the distribution. The graph presented is that for the 3rd measurement.

The PCS results show only a slight change in average size, but the polydispersity index nearly doubles. The distribution graphs suggest that very large species, well over 600 nm in diameter appear, while the largest species seen in the control do not exceed 300 nm. It can be concluded that incubation with just colominic acid at a concentration of 30 mg/ml or greater for 16 hours, causes a certain degree of aggregation. It appears that non-covalently attached colominic acid past a certain concentration has a destabilizing effect on liposomes. A similar effect has been observed for PEG (Hui et al 1999), who showed experimentally that PEG of 8-10 kDa drives liposome aggregation at low concentration by osmotic forces due to depletion of PEG from the water-liposome interface. At high concentrations it has been...
demonstrated that PEG drives aggregation and fusion by sequestering the free water, which removes part of the liposome hydration shell and decreases the repulsive hydration force (Hui et al 1999). It has also been shown that the inner liposome surface competes for the available water with entrapped sucrose, demonstrating that competition between the liposome surface and solutes for free water can exist. Therefore a possible explanation for the results observed here, is that large concentrations of colominic acid cause a change in the structure of water because the polymer sequesters a lot of the free water when it becomes hydrated. The decrease in the available free water could then reduce the repulsive hydration shell around the liposomes consequently promoting liposome aggregation. Conjugation of colominic acid to the liposome surface is expected to protect liposomes against such an occurrence, by acting as a steric barrier, but also due to its high charge, which should create an electrostatic repulsion between the liposomes and compete more effective with free colominic acid for the available water.

6.3. Separation of 22.7 kDa CA from liposomes by gel permeation chromatography

Gel permeation chromatography (GPC) has been used to separate macromolecules from liposomes after conjugation reactions with PEG or from serum proteins (Senior et al 1991, Chonn and Cullis 1992, Johnstone et al 2001). However in order for complete separation to be achieved, it is necessary to determine if there is an interaction between the two species to be separated that can cause them to co-elute during GPC. Examples of such interactions are passive adsorption or internalization into the liposome in the case of colominic acid since it can cause liposome aggregation. Hence an experiment was set up to determine how effectively colominic acid can be separated from liposomes using GPC on a sepharose CL-
4B matrix. The experiment consisted of incubating liposomes with colominic acid and then applying the mixture to a sepharose CL-4B column. Liposomes are very large and elute in the void volume fractions. Colominic acid on the other hand is within the fractionation range of sepharose CL-4B and elutes at a higher elution volume. It is only through association with the liposomes such as passive adsorption or internalization that colominic acid can appear in the void volume fractions. The results are shown in figure 6.2.

![Separation of liposomes from CA by gel permeation chromatography](image)

**Figure 6.2**: Separation of liposomes from colominic acid by gel permeation chromatography on sepharose CL-4B. CL-4B was packed into a PD10 column under gravity. To this column, 0.5 ml of liposomes (DSPC:cholesterol 1:1; 10 μmol/ml DSPC) mixed with 15 mg colominic acid and incubated for 4 hours, were applied and eluted with PBS collecting 0.5 ml fractions. Some of the fractions were pooled (such as the dead space volume fractions), since they were unlikely to contain essential information. The void volume fractions were all assayed individually. The fractions were analysed with the resorcinol assay for colominic acid and the Stewart assay for phospholipids.

It is apparent from the chromatogram of figure 6.2 that colominic acid does not adsorb onto the liposomes or becomes internalised when the liposomes are exposed to a concentration of 30 mg/ml (15 mg in 0.5 ml) colominic acid 22.7 kDa (1.34 polydispersity) for 4 hours at
room temperature. With 70 or 200 mg/ml colominic acid around 2-7% of the colominic acid appears in the column void volume. Almost all of this colominic acid can be recovered in the pellet after centrifugation of the void volume fractions at 13000 rcf, clearly showing that a small amount of CA is retained by aggregated liposomes because it’s only aggregated liposomes that are pelleted by centrifugation.

Thus it can be concluded that GPC can be used for determining the results of a conjugation reaction between liposomes and colominic acid, by applying the reaction mixture to a sepharose CL-4B column and monitoring the void volume fractions for the appearance of colominic acid, then comparing with a control mixture containing non reactive colominic acid or non reactive liposomes.

6.4 NHS chemistry.

Two approaches were attempted. Conjugation of NHS-colominic acid to DSPE liposomes or conjugation of amine-colominic acid and NHS liposomes.

6.4.1 NHS chemistry. Reaction between amine-CA and NHS liposomes

Amine-CA was synthesized by reductive amination of aldehyde-CA, as described in sections 4.3 and 2.3.3. Liposomes incorporating NS-DPPE were activated with NHS and EDC as described in sections 5.3.2 and 2.4.6. The reaction between the activated liposomes and amine-CA was set up as described in section 2.5.2. Briefly 15 mg of amine-CA was passed through a PD-10 column packed with Sephadex G25 to remove any remaining ammonium ions and the CA fractions were concentrated by ultrafiltration. The concentrate was used without freeze-drying. About 12mg of colominic acid were recovered after purification and
concentration of CA-amine, as estimated using the resorcinol assay. Immediately after purification of the activated liposomes by gel permeation chromatography, the concentrated CA-amine solution was added to the most concentrated liposome fraction, between 3 and 3.5 elution volume (0.5 ml). The reaction was carried out at room temperature for 16 hours. The liposomes were then purified from any unreacted colominic acid by gel permeation chromatography on sepharose CL-4B as described in section 6.2. All fractions were assayed with the resorcinol assay. All liposome fractions were measured for size and zeta potential by PCS. The chromatogram was very similar to that of figure 6.2. No colominic acid could be seen to co-elute with the liposomes in the void volume. All the colominic acid eluted between 6 and 12 ml elution volume, while all the liposomes eluted at 3 to 5 ml elution volume (void volume). The PCS data showed a small Zeta-size increase of about 15 nm and the polydispersity increased from ~0.220 to 0.383. Similar results were obtained for the control reaction using unmodified colominic acid instead of CA-amine. The evidence suggests that conjugation did not occur. The polydispersity did increase as observed before due to the presence of colominic acid.

A likely explanation is that the kinetics of the reaction are too slow, due to the large size of the liposomes and colominic acid, so that the ester hydrolyses before reaction can occur. Another possibility is that the linker between the liposome surface and the colominic acid is not long enough, resulting in steric hindrance, which discourages the reaction between amine colominic acid and the NHS esters on the liposome surface. The latter explanation does not very well accommodate the finding that IgG (see section 5.3.2) can be conjugated to liposomes with the same chemistry. The difference however is that the epsilon amino groups have a 5 atom spacer from the protein backbone and that IgG can adsorb onto the liposome.
surface (see section 5.3.2), while colominic acid cannot. The reactivity of the NHS form of
colominic acid was clearly proven by its reaction with growth hormone (see section 4.6). So
the reaction between NHS-colominic acid and DSPE liposomes was also investigated to
determine if the presence of the NHS on CA rather than on the liposome and the addition of a
longer spacer might promote the reaction.

6.4.2 NHS chemistry: Reaction between NHS colominic acid and DSPE liposomes

The extent of reaction between NHS liposomes and colominic acid is limited by the number
of available NHS groups on the liposome surface. When an NHS ester group hydrolyses, the
liposomes permanently lose a reactive group and the maximum possible extent of reaction is
instantly limited. If on the other hand NHS is placed on colominic acid and amine containing
lipids (DSPE) are included in the liposome formulation then even if many of the NHS groups
hydrolyse, the maximum possible extent of the reaction is not limited. NHS-colominic acid
will also have a 4 to 8 atom spacer (depending on the chemistry used – see chapter 4), which
will solve any steric hindrance problems. Hence to determine if this variation of the NHS
chemistry approach can be used for the conjugation of colominic acid to liposomes, NHS-CA
prepared as described in sections 4.6, 2.3.8 and 2.3.9 was reacted with liposomes
incorporating DSPE. Briefly 15 mg of amine CA was reacted with a homobifunctional NHS
crosslinker, either BS³ or DSG and then purified by gel permeation chromatography (BS³), or
triple dioxan precipitation from DMSO (DSG). For the NHS colominic acid prepared with
BS³, the fraction containing the greatest amount of CA (7 mg, 0.5 ml) was mixed with 0.5
ml of DSPC: cholesterol: DSPE liposomes 10:10:2 (10 μmol/ml DSPC) and allowed to react
for 16 hours. For the NHS-colominic acid prepared with the dioxan precipitation, the dioxan
pellet was redissolved in 0.5 ml liposomes consisting of DSPC: cholesterol: DSPE 10:10:2 (10 µmol/ml DSPC) and allowed to react for 16 hours. The reaction mixtures were then analysed by gel permeation chromatography on sepharose CL-4B as described in section 6.2. Once again, no evidence of conjugation was seen. This result lends support to the hypothesis that the hydrolysis of the NHS ester is too fast compared to the reaction between liposomes and colominic acid. It appears therefore that the NHS chemistry is not a very efficient method for the conjugation of CA to liposomes even though it is a generally effective and attractive chemistry to use due to its rapidity and simplicity.

6.5 Maleimide chemistry.

Two approaches were tested. Conjugating thiol-colominic acid to maleimide liposomes and conjugating maleimide-colominic acid to thiolated liposomes. The maleimide liposomes were formed either by activating DSPE liposomes in situ or by incorporating a maleimide lipid.

6.5.1 Maleimide chemistry: Thiol-Colominic acid and maleimide liposomes

Initially thiol colominic acid was conjugated to liposomes incorporating DSPE that had been derivatised in situ with maleimide using the bifunctional crosslinker BMPS (see section 5.3.1 and section 2.4.5). As described in chapter 4, thiol CA can be synthesised either by thiolation of amine CA with 2-iminothiolane (2-IT) or by reductive amination of CA-aldehyde with cystamine and reduction of the disulfide in cystamine (see sections 2.3.5 and 2.3.6). Briefly 8mg amine-colominic acid was reacted with 2IT in PBS-1 mM EDTA, pH8.0 and purified by gel permeation chromatography. The CA-containing fractions were then concentrated to
~100 µl by ultrafiltration. Alternatively, CA-aldehyde was reacted with cystamine for 50 hours at pH 8.0 with 2.5 g/l NaCNBH₃, and then reduced with 0.1 M DTT prior to purification by dialysis and freeze-drying. The reaction between BMPS-activated liposomes and thiol-CA was set up as detailed in section 2.5.4. Briefly, 0.5 ml of DSPC:cholesterol:DSPE liposomes (10:10:1 – 20 µmol/ml DSPC), were reacted with BMPS and purified by gel permeation chromatography. The fractions with the greatest concentration of lipid (as determined by the Stewart assay), were mixed either with the thiol-CA (22.7 kDa) concentrate (6.5 mg recovered) produced by reaction with 2-IT, or with 6 mg of the freeze-dried reduced CA-cystamine (22.7 kDa). The reaction was allowed to proceed for 20 hours and the liposomes separated from unreacted colominic acid by gel permeation chromatography on sepharose CL-4B. Figures 6.3 and 6.4 respectively show the chromatograms obtained.

Conjugation of CA-SH to maleimide Liposomes

![Graph showing the conjugation of CA-SH to maleimide liposomes](image)

**Figure 6.3:** The polysialylation reaction between maleimide liposomes and thiol-CA (22.7 kDa) formed with 2-IT was analysed by gel permeation chromatography. Phosphate buffered saline at pH 7.4, was used as the mobile phase. All fractions were analysed by the resorcinol assay. The presence of phospholipids was confirmed by the Stewart assay.
The void fractions in the chromatogram of figure 6.3 contain a significant amount of colominic acid, suggesting that colominic acid has indeed associated with the liposomes in this case. Colominic acid does not have a tendency to adsorb to liposomes as shown in section 6.2 and with the NHS chemistry experiments (see section 6.3). It is highly likely that this association is the formation of a thioether bond between the maleimide groups on the liposome and the thiol group on the colominic acid. The void volume fractions (elution volume 3 – 4.5 ml) contained 0.724 mg of colominic acid, which corresponds to 32 nmole of colominic acid. The most concentrated fraction (elution volume 3.5) had a concentration of about 0.034 mM colominic acid. The lipid in each fraction was compared to the starting preparation to determine the recovery of liposomes after the reaction and purification. The void volume fractions contained a total of 4.5 μmole of DSPC and 0.45 μmole of DSPE. Assuming that each molecule of colominic acid present in the void volume is conjugated to a single DSPE lipid that has been modified with BMPS then the number of moles of DSPE modified with CA should equal the number of moles of CA recovered in the void volume i.e. 32nmoles. The extent of derivatisation of DSPE with CA can be determined by dividing the number of moles of modified DSPE by the total number of DSPE moles in the void volume fractions and multiplying by 100. Therefore 7.1% of the DSPE was modified with colominic acid. Dividing the amount of modified DSPE lipid by the total amount of lipid and multiplying by 100 was used to calculate that the liposomes contained 0.33mol% polysialylated lipid.

To ensure that colominic acid cannot form a different sort of interaction with maleimide liposomes a negative control reaction was also set up in which stock colominic acid was reacted with 2IT and then mixed with BMPS activated liposomes. Analysis of this reaction
mixture with gel permeation chromatography showed that no CA appeared in the void volume fractions of the control reaction. It was hence concluded that in the polysialylation reaction mixture, the CA that eluted in the void volume was likely to be covalently bonded to the liposomes.

The results shown in figure 6.4 are essentially identical to the results in figure 6.3. The negative control in this case consisted of non-reduced CA-cystamine, in which case the amount of CA present in the void volume fractions was negligible. In this instance 7.4% of the DSPE lipid was derivatised with CA and the liposomes contained 0.35mol% polysialylated lipid. The calculations were done as described above, by assuming that each molecule of colominic acid found in the void volume fractions was conjugated to a single molecule of modified DSPE.

**Conjugation of reduced CA-cystamine to maleimide liposomes**

![Graph](image)

**Figure 6.4:** The polysialylation reaction between maleimide liposomes and reduced CA-cystamine (22.7 kDa) was analysed by gel permeation chromatography. Phosphate buffered saline at pH 7.4, was used as the mobile phase. All fractions were analysed by the resorcinol assay. The presence of phospholipids was confirmed by the Stewart assay. Once again the amount of CA in fractions 11 to 13 was beyond the limits of detection and was hence underestimated.
It can be concluded that both methods for producing thiol-CA are equivalent as far as the polysialylation reaction is concerned, meaning that the extra 2 carbons on the spacer produced by IT do not make any appreciable difference in the reaction yield. The cystamine approach however is advantageous in that it is simpler and more practical, it has a higher yield of thiol and the spacer group is not labile. Nevertheless, due to the availability of a stock of amine-CA, the 2IT approach was used to produce thiol-CA in all further work.

6.5.2 Maleimide chemistry: Use of maleimide lipid

Activation of liposomes with BMPS has the advantage of reduced modification of the inner lipid leaflet of the bilayer. This can even be completely avoided by using the sulfo-NHS variant of BMPS. However using a cross-linker to activate the liposome has a distinct disadvantage in that there is a requirement for purifying the activated liposome from the cross-linker, which complicates the process. It is also very difficult to control the amount of maleimide lipid present, when the crosslinker approach is used. To avoid this extra liposome purification step and to control the amount of maleimide lipid present more stringently, a maleimide lipid (DSPM) was created and incorporated into liposomes for conjugation with thiol-CA. Figure 6.5 shows the results of conjugating thiol-CA to DSPC:cholesterol liposomes incorporating 5mol% DSPM (the reaction conditions are given in the legend of figure 6.5). The amount of CA used was increased to 15 mg in an attempt to increase the grafting. The extent of grafting was calculated separately for the two most concentrated liposome fractions (3-3.5 ml and 3.5-4 ml). Each fraction was assayed in triplicate to determine the standard error. The average value is 12.2 ± 0.3% of DSPM modified with colominic acid, which corresponds to 0.58 ± 0.01mol% of polysialylated lipid (see section
6.5.1 page 167 for the calculation method). This figure is higher with that obtained with the BMPS activated DSPE liposomes, and the difference is beyond the limits of experimental error.

**Conjugation of thiol-CA to DSPM liposomes**

![Graph showing conjugation of thiol-CA to DSPM liposomes](image)

**Figure 6.5:** Conjugation of thiol-CA to DSPM liposomes. 6.5 mg of thiol-CA were reacted with 0.5 mL of DSPC: cholesterol: DSPM liposomes (10:10:1 – 10 μmol/ml DSPC) in phosphate buffered saline pH 7.4 for 16 hours. The liposomes were separated from unreacted CA by gel permeation chromatography on sepharose CL-4B. The void volume fractions were assayed with the resorcinol assay for colominic acid and with the Stewart assay for total phospholipid, from which the DSPM concentration can be calculated (one 11th of total phospholipids).

It is apparent that only a very small amount of lipid actually reacts with the activated colominic acid. It also seems that liposomes with 5mol% DSPM react more than liposomes
with 5mol% DSPE activated by BMPS. This is expected as its unlikely that all of the surface accessible DSPE will be modified by BMPS.

In order to determine the importance of the amount of maleimide lipid present in the liposomes, a conjugation with liposomes containing 2.5mol% DSPM (i.e. DSPC: cholesterol: DSPM 20:20:1) was attempted. In addition, to ensure that any differences in the reaction yield are not due to kinetic factors, the amount of colominic acid was increased to 15 mg and a second reaction was also set up that was analysed at 8 hours instead of 16 hours. The results are shown in table 6.2

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>% DSPM derivatised (± standard error)</th>
<th>mol% polysialylated lipid (± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>12.4 ± 0.8</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>16</td>
<td>12.4 ± 0.5</td>
<td>0.31 ± 0.01</td>
</tr>
</tbody>
</table>

Table 6.2: Conjugation to 2.5mol% DSPM liposomes. (See section 6.5.1 page 167 for the calculation method)

The percentage of DSPM derivatised with colominic acid was not decreased yet the polysialylated lipid content (mol%) of the final product had decreased. In addition, it seems that the reaction does not proceed much further after 8 hours. This result suggests that the limiting factor in this reaction is the maleimide group, as increasing the colominic acid, while decreasing the amount of maleimide lipid, results in a lesser extent of grafting. The most likely explanation is that the reaction slows down continuously as more and more colominic acid attaches to the surface, because the negative charge of the liposomes increases. It is expected that there is a limit to how much colominic acid can be incorporated to the liposome before the accumulation of negative charge and steric hindrance effects from the polymer.
block any further reaction. Since the maleimide also hydrolyses (see chapter 5), the chance of reaction decreases greatly as the reaction progresses and as the maleimide becomes more and more limiting. In order to reach the saturation point, there has to be enough initial maleimide, so that a higher chance of reaction is maintained long enough. In addition, the amount of colominic acid added to the liposomes cannot exceed a certain limit as it has been shown, in section 6.2 that the liposomes do not tolerate very high concentrations of colominic acid. This model was put to the test, by reacting 15 and 30 mg of colominic acid with liposomes containing 10% DSPM for 16 hours. The results are shown in table 6.3

<table>
<thead>
<tr>
<th>Colominic acid (mg)</th>
<th>% DSPM derivatised (± standard error)</th>
<th>mol% polysialylated lipid (± standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>5.79 ± 0.3</td>
<td>0.526 ± 0.03</td>
</tr>
<tr>
<td>30</td>
<td>5.73 ± 0.1</td>
<td>0.521 ± 0.01</td>
</tr>
</tbody>
</table>

**Table 6.3:** Conjugation of 15 or 30 mg CA to 10mol% DSPM liposomes. (See section 6.5.1 page 167 for the calculation method)

Comparing these results with those for 5mol% DSPM liposomes (see table 6.2) shows that the reaction does not progress much further even though there is twice as much available DSPM and 2 to 4 times more thiol-CA. As predicted, the reaction reaches a point where no more colominic acid can be added onto the liposomes, presumably due to charge repulsion and steric repulsion effects. It was concluded that 5mol% DSPM and 15 mg of colominic acid are sufficient to maximise the grafting of colominic acid to the liposome surface.

To verify this conclusion another reaction was set up with 15 mg of thiol-CA and 5mol% DSPM liposomes. The results are shown in figure 6.6. The results are very similar to those obtained from the reaction of figure 6.5. The proportion of DSPM reacted is 11.8% ± 0.04
and hence the liposomes contain $56.1 \pm 0.02$ mol% polysialylated lipid (see section 6.5.1 page 167 for the calculation method).

![Gel permeation chromatography on CL4B: Polysialylated liposomes.](image)

**Figure 6.6:** Polysialylation of 5mol% DSPM liposomes with 15 mg thiol CA (22.7 kDa 1.34 polydispersity). The liposomes consisted of DSPC: cholesterol: DSPM (10:10:1). 0.5 ml of liposomes (10 μmol DSPC per ml) were reacted with 15 mg of thiol CA concentrated to 50μl by ultrafiltration. The reaction was allowed to proceed for 14 hours.

It was shown earlier (section 6.2) that incubation of colominic acid with liposomes can lead to liposome aggregation. It was expected that grafting colominic acid would protect against such aggregation. Therefore to determine if the presence of colominic acid in the reaction mixture has an adverse effect on liposome size and polydispersity of the polysialylated liposomes, in the most concentrated fraction after purification was analysed by PCS. The value was then compared to that obtained for the starting material just before the reaction was set up. The results are shown in figure 6.7
Figure 6.7: PCS analysis of liposomes before and after polysialylation. DSPC:cholesterol:DSPM 10:10:1 liposomes (10 μmol DSPC per ml) just before the reaction are shown on the left and the same liposomes reacted with 15 mg thiol-CA for 14 hours and purified by gel permeation chromatography on sepharose CL-4B are shown on the right.

PCS analysis shows that the 14 hour incubation with colominic acid had no adverse effect on the polydispersity of the liposomes. In fact the polydispersity seems to have decreased as the smaller species (less than 30 nm) do not appear to be present in the purified polysialylated material. The size increases from 77 nm to 93 nm. It is possible that the small species are lost during the chromatographic separation of the liposomes from the reaction mixture. The chromatogram in figure 6.6 clearly shows that a small proportion of the liposomes elutes after the low volume and liposomes smaller than 30 nm should be within the fractionation range of sepharose CL-4B. Nevertheless it can be concluded that polysialylation protects the
liposomes from the aggregation induced by high concentrations of colominic acid and that the polysialylated product still has a satisfactory size and polydispersity.

In addition to size, PCS was used to measure the zeta potential of the liposomes. The starting material has a zeta potential of $-4.3 \pm 0.1$ mV, while the polysialylated material has a zeta potential of $-40.5 \pm 11.0$ mV. The large decrease in zeta potential to a high negative value was expected as polysialic acid is a highly negatively charged polymer.

**6.5.3. Maleimide chemistry: Conjugation of maleimide-colominic acid to thiol-lipid containing liposomes**

To fully investigate the maleimide chemistry, the effect of inverting the reactive groups used i.e. maleimide on colominic acid and thiol on the liposomes, was investigated. The reaction was set up as described in section 2.5.6. Briefly, liposomes containing DSPE at 5mol% were reacted with 2IT to convert the surface amino groups to thiols as described in section 5.3.3. After thiolation and purification by gel permeation chromatography, the most concentrated liposome fraction was reacted with 15 mg of maleimide colominic acid synthesised as described in section 4.5. The results are shown in figure 6.8. The percentage of DSPE derivatised was 10.1%, which equates to 0.48mol% of polysialylated lipid in the liposomes. The results are better than those obtained with BMPS activation of DSPE liposomes and thiol-CA (see section 6.4.1), but not as good as what is obtained by using DSPM liposomes (see section 6.4.2).
Conjugation of maleimide CA to thiol-liposomes

Figure 6.8: Polysialylation of thiol-liposomes with maleimide CA (22.7 kDa 1.34 polydispersity). 0.5 ml of liposomes (DSPC:chol:DSPE 10:10:1 – 10 μmol DSPC per ml), were reacted with 2IT, purified with gel permeation chromatography and reacted with 15 mg of maleimide CA. For the negative control amine-CA was used.

6.6 Conjugation with low molecular weight colominic acid

Steric stabilisation with polyethylene glycol (PEG) is most effective within a narrow range of molecular weights and extent of incorporation (Woodle and Lasic 1992). It was found, that incorporation of PEGylated lipid into liposomes exhibits saturation behaviour, i.e. that the incorporation cannot exceed a certain limit. A similar observation was made with colominic acid. Work with PEG showed in addition that large molecular weight PEG (e.g. 20kDa) does not stabilise liposomes as well as smaller PEGs do (Woodle and Lasic 1992). This is thought to occur because the smaller number of large chains results in a steric cloud that extends further but is not as dense as that created by a larger number of smaller chains. This results in decreased shielding of the liposome from the binding of plasma proteins. The optimum steric
stabilisation effect with PEG is seen with 5-10mol% of PEG with MW ranging from 2kDa to 5kDa (Allen 1992, Woodle and Lasic 1992).

Because PEG is incorporated as a lipid conjugate during bilayer formation, PEG exists in both sides of the bilayer. With colominic acid conjugated to preformed liposomes all the colominic acid is grafted on the solvent exposed side of the bilayer. Therefore when making comparisons with PEG, it must be kept in mind that for the extent of surface grafting to be equivalent, the amount of polysialylated lipid (in mol%) should be slightly higher than half the amount of PEG-lipid included in the formulations (The distribution of lipids between the inner and outer bilayer is asymmetrical). Thus liposomes with 0.6mol% of lipids derivatised with 22kDa colominic acid should have an equivalent degree of polymer grafting to their surface as liposomes incorporating 1mol% of 20kDa PEGylated lipid.

It is not straightforward to predict the behaviour of colominic acid in terms of steric stabilisation. Large polysialic acids (>20kDa) in the E.coli capsule perform very well at reducing the attachment of complement and inhibiting phagocytosis (Mushtaq et al 2005). On the other hand the extent of grafting with large polysialic acids seems to reach saturation at a low value, which may be insufficient to effectively cover the liposome surface. With small polysialic acids, the extent of grafting may reach a much higher level, which could result in more effective shielding of the liposome surface. To test this hypothesis, liposomes incorporating 5mol% of DSPM were reacted with 6.2 kDa (1.15 polydispersity) colominic acid and with 22.7kDa (1.34 polydispersity) colominic acid. The 6.2kDa colominic acid was the 75mM salt fraction from the anion exchange fractionation of the 22.7kDa (1.34) stock material – see chapter 3. The reactions were carried out as before using 15mg of colominic acid in both cases. It should be noted that this corresponds to ~3.5 times more colominic acid
chains for the 6.2 kDa preparation compared to the 22.7kDa preparation. Since the conditions were chosen in order to obtain the maximum possible degree of grafting, this was thought to be of no consequence to the final results. The results are shown in table 6.4.

<table>
<thead>
<tr>
<th>Molecular Weight (kDa)</th>
<th>% DSPM derivatised</th>
<th>mol% polysialylated lipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.2</td>
<td>54.4</td>
<td>2.59</td>
</tr>
<tr>
<td>22.7</td>
<td>10.9</td>
<td>0.52</td>
</tr>
</tbody>
</table>

**Table 6.4:** Conjugation of 6.2kDa (1.15 polydispersity) and 22.7kDa (1.34 polydispersity) to 5mol% DSPM liposomes. (See section 6.5.1 page 167 for the calculation method)

It can be seen in table 6.4, that conjugation with 6.2kDa CA resulted in a dramatic improvement of the grafting efficiency, with almost complete derivatisation of the outer bilayer leaflet DSPM with colominic acid. It should be noted, that the outer bilayer leaflet contains more lipid than the inner leaflet and the difference increases as the liposome size gets smaller. Hence more than 50% of each lipid is displayed on the liposome surface, which is why the proportion of DSPM reacted can be greater than 50%. It may be possible to increase the grafting further with the small polymer by adding more DSPM. The size and zeta potential of the 6.2kDa CA-liposomes were in the range of -35 to -40mV and 100 to 130nm respectively which is not appreciably different from that of the 22.7kDa CA-liposomes (see section 6.4.2 page 164).
Chapter 7

In vitro and vivo in characterisation of the polysialylated liposomes
7.1. Introduction

In chapter 6 the liposome polysialylation reaction and the purification of the colominic acid-liposome conjugates (CA-liposomes) were described, as well as certain of their physical properties such as a size, zeta-potential and polydispersity. In this chapter the experiments conducted to further characterise the product and determine the steric stabilisation capability of colominic acid will be described. As discussed in section 1.4, steric stabilisation by surface attached polymers is thought to be achieved through an increase in the repulsive hydration force and through direct steric inhibition, by the presence of the polymer, resulting in inhibition of protein-liposome interaction as well as the contact between the liposome surface and other surfaces. In order to determine how effective colominic acid is in this respect, a two-phase system was used to observe the effect of polysialylation on the surface properties of the liposomes. In addition the protein binding profiles of the polysialylated and the control liposomes were investigated.

7.2 Two phase-system experiments

A two phase system is formed by dissolving in the same solution two compounds that are incompatible with each other (Persson et al 1999, Li et al 2002), meaning that at a certain concentration upon standing they form separate phases, with each compound partitioning almost exclusively in one of the two phases. The two phase system used in these experiments was an aqueous dextran - polyethylene glycol (PEG) system (Walter et al 1990). High molecular weight dextrans and PEG are incompatible due to the differences in their solvation properties. Dextran structure water around them in a way that is not compatible with the structure water forms around PEGs. Hence the water that is structured around PEG cannot
form the maximum possible number of hydrogen bonds with the water that is structured around dextran. Past a certain concentration, when the available water becomes limiting, the presence of both PEG and dextran in a solution results in a reduced hydrogen bond formation, i.e. in partial disruption of the hydrogen bonding network. This is energetically unfavourable compared to individual solutions of PEG or dextran, where the hydrogen bonding network is not disrupted. Thus solutions containing high concentrations of PEG and dextran tend to separate into an upper PEG phase and a lower dextran phase. This is more energetically favourable than the mixed solution despite the large decrease in entropy, because of an even greater decrease in enthalpy due to the formation of more hydrogen bonds upon separation into phases. In other words the reason for the separation into phases is enthalpic and the separation becomes more energetically favourable as the concentration of the two polymers increases. Solutes added to this system can partition in either phase or in neither phase (float on the top or in the interface) depending on their own solvation properties. For example surfactants tend to partition more in the PEG layer, while carbohydrates tend to partition in the dextran layer.

The interactions between a particle and the solvent depend solely on the particle’s surface properties and thus it’s the surface properties of a particle alone that determine how the particle will partition in a two phase system (Walter et al 1990). Thus for a liposome, encapsulated materials have little or no effect on the partitioning, while substances adsorbed or conjugated to the surface, can affect the separation. For this reason two phase systems are suitable for observing differences in the surface characteristics between liposomes that have a polymer grafted on their surface and normal liposomes. A two phase system has been used before by Senior and Gregoriadis (1991) to compare the binding of plasma proteins to
PEGylated and non PEGylated liposomes. This system was a 5%PEG6000 and 5% Dextran T500.

A two phase system was prepared as described in section 2.6.3. Briefly appropriate volumes of water and stock solutions of sodium chloride, sodium phosphate pH 6.8, dextran T500 and PEG5000 were mixed together to give final concentrations of 5% PEG, 5% Dextran 0.16 M NaCl and 0.01 M sodium phosphate. Alternatively equal volumes of a 10% solution of PEG8000 and a 10% solution of dextran T500 in phosphate buffered saline (20 mM phosphate pH7.4) were mixed to give final concentrations of 5% (w/w) dextran and 5% (w/w) PEG. Phosphate salts have a tendency to partition in this system and hence impart a small electrical potential to the system (Donnan potential) (Walter et al 1990).

The partitioning of 22 kDa CA-liposomes in the pH 7.4, PEG8000 system is shown in table 7.1. The control non-polysialylated liposomes (DSPM) partition mostly in the bottom (dextran) layer and the interface, though a very small amount also partitions in the top (PEG8k) phase. The polysialylated liposomes on the other hand partition almost exclusively in the dextran layer and only a very small amount can be found in the interface. There is no significant partitioning in the PEG8000 layer.
The results clearly show that the polysialylated liposomes have quite different surface properties from the starting material (DSPM liposomes). The shift towards the bottom layer is consistent with the properties of colominic acid, which in the same system partitions exclusively to the dextran layer (data not shown). It is unlikely that simple adsorption of colominic acid will result in such a drastic shift, as the extensive dilution can drive forward the desorption of adsorbed substances.

Although this two phase system shows a change in the surface properties of the liposomes, it cannot be used to obtain information on the steric stabilisation capabilities of colominic acid. The analytical capabilities of two-phase systems can be further enhanced by the use of solutes that interact with the substances being analysed (Bordier 1981, Ekblad et al 1998). As separation in a two-phase system is based on enthalpic differences, this second affinity component can alter the partitioning behaviour of a given substance, since the differences in
the energy of solution may be counteracted by the energy of the interaction between the new
solute and the substance being analysed. For example triton X-114 has been used to change
the partitioning of proteins in such a two phase system (Bordier 1981). As proteins can form
stable complexes with Triton, the partitioning of proteins into the layer that triton is in,
increases. This is because the interaction between Triton and the proteins decreases the
enthalpy of solvation of the proteins in that layer, hence changing the equilibrium point. In
essence there is now an affinity component, which makes the presence of proteins in the
phase where triton partitions more energetically favourable. Another system exploits the
interaction between avidin and biotin to alter the partitioning of a biotinylated molecule
(Ekblad et al 1998). A system such as this is known as an affinity two-phase system. It is
obvious that the concentration of the affinity agent used is very important. Such a system has
been used by Senior et al (1991) to study the interaction of plasma proteins with liposomes
grafted with polyethylene glycol. The presence of an excess of plasma proteins, in the two
phase system, alters the distribution of the liposomes according to which proteins the
liposomes interact with. It was shown by Senior and co-workers that the presence of proteins
shifted the distribution of the liposomes towards the dextran layer and away from the
interface. A shift to the dextran layer was also observed for the PEG liposomes which
partitioned predominately in the PEG layer, showing that PEG liposomes still have some
affinity for proteins.

This plasma protein affinity two phase system was used in an attempt to determine if CA-
liposomes also have an affinity for plasma proteins. In addition a smaller PEG, of 5000
molecular weight was used to increase the partitioning of liposomes in the interface and the
PEG layer. Liposomes were mixed 1:1 (v/v) with plasma and samples were applied to the
two phase system after 1h, 2h and 4 hours. Liposomes diluted 1:1 (v/v) with PBS pH 7.4 were used as the negative control. The results are shown in the tables that follow.

<table>
<thead>
<tr>
<th>% partitioning of DSPM liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS, pH 7.4</td>
</tr>
<tr>
<td>Top</td>
</tr>
<tr>
<td>Interface</td>
</tr>
<tr>
<td>Bottom</td>
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</tbody>
</table>

**Table 7.2:** Partitioning of DSPM liposomes in a 5% dextran T500 and 5%PEG5000 two phase system. The liposomes were mixed with an equal volume of PBS and applied to the system directly or mixed with an equal volume of mouse plasma and incubated at 37°C for the indicated time before applying to the two phase system. The rest of the experiment was carried out as described in the legend of table 7.1.

<table>
<thead>
<tr>
<th>% partitioning of 22kCA-liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS, pH 7.4</td>
</tr>
<tr>
<td>Top</td>
</tr>
<tr>
<td>Interface</td>
</tr>
<tr>
<td>Bottom</td>
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</tbody>
</table>

**Table 7.3:** Partitioning of 22.7 kDa CA-liposomes in a 5% dextran T500 and 5%PEG5000 two-phase system. The experiment was carried out as described in the legend of table 7.2.
Table 7.4: Partitioning of 6 kDa CA-liposomes in a 5% dextran T500 and 5%PEG5000 two-phase system. The experiment was carried out as described in the legend of table 7.2

Table 7.2 shows that DSPM liposomes partition mostly in the bottom layer, but significant partitioning is also seen in the interface and the PEG layer. After incubation with plasma the distribution begins to shift towards the bottom layer. A steady state seems to be reached after 2 hours in plasma.

For the CA grafted liposomes however the addition of plasma seemed to have no effect on their partitioning behaviour. This result suggests either that CA-liposomes no longer have any affinity for plasma proteins, or that colominic acid dominates to such a degree that the enthalpic contribution from the binding of plasma proteins is negligible, or that the CA-liposomes can only interact with plasma proteins that partition exclusively in the bottom layer.

The integrity of the liposomes was monitored by measuring CF quenching, before and after the plasma incubation. It was found that the quenching of CF remained at >95% even after incubation for 4 hours with plasma at 37°C.
The two phase systems were useful in observing some of the physical differences between CA-liposomes and normal liposomes, hence providing further evidence in support of the presence of covalently bonded CA on the liposome surface. The limitations of two-phase systems however mean that they cannot provide adequate information on the binding of plasma proteins to the liposomes, which is necessary in order to determine the effectiveness of CA in shielding the liposomes. For this reason a different system was used to study in more detail the binding of plasma proteins to the liposomes as explained in the next section.

7.3 Analysis of plasma protein binding by SDS-PAGE

It has been shown before that many of the liposome binding proteins from plasma can be isolated by simply incubating liposomes with plasma and then purifying the liposomes from the mixture (Chonn and Cullis 1992, Johnston et al 2001). Any proteins that remain associated through the purification procedure can then be characterised by techniques such as SDS-PAGE (Johnston et al 2001) and Western Blotting (Chonn and Cullis 1992). Typically purification is accomplished by centrifugation, but this has many problems especially with proteins that associate weakly or transiently. Gel permeation chromatography has proven to be a more effective method (Chonn and Cullis 1992, Johnston et al 2001). For this reason, gel permeation chromatography on Sepharose CL-4B was used to isolate liposomes from the plasma incubation mixture. Typically a sample of liposomes was mixed with an equal volume of plasma and incubated at 37°C for 4 hours. The negative control was plasma mixed with an equal volume of PBS pH 7.4 and incubated at 37°C for 4 hours. Following the incubation the samples were passed through the gel permeation chromatography column and the void volume samples were collected and concentrated by ultrafiltration. The proteins that
associate in any way with the liposomes will be enriched in the void volume fractions, as even transient association with the liposomes will result in these proteins being carried by the liposomes through the matrix for a certain proportion of the time spent in the column. The very large plasma components such as plasma lipoproteins or the large multi-protein structures such as IgM, will also elute in the void volume fractions, but the comparison with the negative control can be used to account for them. After the chromatography step, the concentrated samples were analysed by SDS-PAGE. The results are shown in figure 7.1. It can be seen in figure 7.1 that conjugation of 22.7 kDa colominic acid (1.34 polydispersity) to liposomes has a significant effect on the binding of some proteins such as serum albumin, but other proteins seem to bind more easily. In addition a few new bands seem to be appearing. Conjugation of 6 kDa colominic acid on the other hand results in a drastic reduction in the binding of all protein that can be visualized by this technique. In fact the 6 kDa lane looks very similar to a negative control, with only a very few faint extra bands. As anticipated, the 6kDa colominic acid is much more effective at reducing plasma protein binding, presumably because of the greater degree of derivatisation. The larger colominic acid is expected to leave large patches of the liposome uncovered as the chains will electrostatically repel each other. The smaller colominic acid is likely to form a denser brush-like structure like low molecular weight PEG does. It is interesting that although 22.7 kDa colominic acid is very effective at reducing the binding of a protein that migrates near the 64 kDa marker and is the protein that binds the liposome the most, it is not so effective at reducing the binding of some higher molecular weight proteins, whose identities are unknown. This protein is likely to be albumin, not only because it migrates near the 64 kDa marker but also because of its well known tendency to adsorb to liposomes.
Figure 7.1: SDS-PAGE analysis of purified liposomes after plasma incubation. DSPM liposomes and DSPM liposomes polysialylated with 6 kDa or 22 kDa colominic acid were incubated with plasma at 37°C and then purified by gel permeation chromatography. The void volume fractions were concentrated by ultrafiltration. The negative control (PBS) was plasma mixed with phosphate buffered saline pH 7.4 to determine which plasma components elute in the void volume. The experiment was performed in duplicate. Unprocessed plasma was also run on the gel. It was diluted 1 in 20 and 10 µl loaded on the gel. The most intense band in the unprocessed lane is serum albumin. The markers are from top to bottom 250, 96, 64, 50, 36, 30 and 16 kDa.

It is expected that colominic acid would be a great deal more effective at preventing non-specific adsorption of proteins, rather than specific binding of relevant proteins, due to its charge and hydrophilicity. Johnstone et al 2001, used a similar system to look at the protein binding profiles of PEGylated liposomes, and found that the protein binding profiles of PEG2000 liposomes were very similar to control liposomes. (see section 1.4.2).
comparison with colominic acid there is a drastic decrease in protein binding suggesting that
colominic acid (both molecular weights) is much more effective than PEG2000 at preventing
the binding of plasma proteins.
The apolipoprotein apoE has been implicated in the clearance of small neutral liposomes by
the liver (Scherpof and Kamps 1998). It is expected that apoE will migrate close to the 36
kDa marker. A band near the 36 kDa marker is seen in all the lanes including the negative
control, suggesting it was a component of some of the large plasma components (e.g. HDL or
LDL). It was not determined however whether this band was indeed apoE.
The main conclusion that can be drawn from this experiment is that smaller colominic acids
are more effective at blocking protein binding onto liposomes than larger colominic acids.
Another interesting conclusion is that experiments that look at overall protein binding in
order to determine steric stabilisation effects can be misleading. A polymer that prevents
non-specific adsorption, would reduce overall protein binding, but may not significantly
reduce the binding of proteins that can cause opsonisation or compromise liposome integrity.
A more detailed analysis of the proteins that bind the liposome is important when steric
stabilisation effects are being investigated, in order to determine if proteins with known
opsonic activity also bind to polysialylated liposomes. For this reason a more specific
analysis method was used to specifically look at the binding of proteins that are more likely
to be involved in clearance mechanisms
7.4 Analysis of the binding of immunoglobulin and complement C3 to liposomes by
Western blotting

Immunoglobulin and complement C3 are two proteins that have been implicated in the
removal of liposomes from the circulation (Alving and Wassef 1992, Devine and Bradley
1998). Western blotting was used because it is a rapid and sensitive method with good
specificity, so the protein of interest can be detected despite the high protein background.
This technique has been used before to study the deposition of IgG and complement C3 on
GM1-containing liposomes after injection into mice and direct purification from the blood
(Chonn and Cullis 1992 - see section 1.4.1). Although apoE is thought to also play a
significant role in the clearance of neutral liposomes its affinity for the polysialylated
liposomes was not investigated. The Western blotting procedure is described in section 2.6.6.
Two duplicate SDS-PAGE gels were first prepared as described above in section 7.3. The
first gel was stained with coomassie blue as before, while the second gel was subjected to
an electroblotting procedure, where an electric current is used to transfer the protein bands
from the gel onto a PVDF membrane, making an exact replica of the gel on the membrane.
The membrane was then probed with an antibody against the protein of interest and that
antibody was detected using a secondary antibody that is covalently linked to horse radish
peroxidase (HRP). The membrane was exposed to a substrate for HRP that is converted into
an insoluble coloured product by the enzyme. Deposition of the insoluble product on the
filter, allows visualization of the secondary antibody and hence the protein of interest itself as
the secondary antibody is bound to the primary antibody, which is in turn bound to the target
protein. The results are shown in figure 7.2. The Coomassie blue stained duplicate gel is
shown on figure 7.1. The membrane was probed with a polyclonal anti-mouse
immunoglobulin antibody and with an anti-complement C3 antibody that binds the C3 proteins from many species, including mouse.

Figure 7.2: Western blot analysis of the binding of complement C3 and immunoglobulins to DSPM liposomes or DSPM liposomes polysialylated with 6 and 22 kDa colominic acid. The left half of the membrane (lanes 1-6) was probed with the anti-C3 antibody while the right half of the membrane (lanes 7-12) was probed with the polyclonal anti-mouse immunoglobulin serum. A Coomassie blue stained gel with the same samples is shown in figure 7.1. Unprocessed plasma was also run in both sides of the gel. It was diluted 1 in 20 and 10 μl loaded on the gel.

The Western blots are very informative. Immunoglobulins were chosen as a target protein since it is now known that many species have naturally occurring antibodies to the lipid components of liposomes, such as cholesterol. The binding of such antibodies to the liposome is a good example of an opsonisation process. Conjugation of 22kDa colominic acid reduces immunoglobulin attachment by effectively an order of magnitude, while 6kDa completely eliminates all detectable immunoglobulin binding. The 6kDa lane (lane 8) is the
same as the PBS negative control lane (lane 11). On the other hand DSPM liposomes (lanes 5 and 10) present a picture that is very similar to unprocessed plasma (lanes 1 and 12). These observations are in agreement with what was seen with the Coomassie blue stained gel. Comparing these results with those obtained for GM₁ liposomes by Chonn and Cullis (1992), shows that colominic acid is as effective as GM₁ is at preventing the binding of mouse IgG to liposomes.

The binding of C3 to the liposomes was investigated because C1q was shown to be directly activated by negatively charged PS-containing liposomes by Bradley et al (1999) and because the presence of colominic acid in the *E.coli* capsule is well known to be effective at preventing complement activation (Mushtaq et al 2005). It has in fact been shown that *E.coli* lacking colominic acid on their surface lose a great deal of their protection against complement attachment and phagocytosis (Mushtaq et al 2005). DSPM liposomes clearly show significant attachment of C3. The larger number of bands compared to the unprocessed plasma suggests complement activation and possibly attachment to proteins adsorbed onto the liposome. Both 6 kDa and 22 kDa CA-liposomes, bind C3 to a much smaller extent, showing that the presence of CA on the liposomal surface is very effective at inhibiting the activation and attachment of complement. There is no significant difference between the 6k and 22k liposomes. The result is similar to what is observed in bacteria with colominic acid on their capsule with respect to complement deposition: Colominic acid strongly decreases complement deposition.
7.5 Blood clearance of polysialylated liposomes

The in vitro experiments have confirmed that colominic acid is effective at reducing protein binding to liposomes even at low grafting density and it was found that colominic acid, like GM₃, strongly decreases binding of mouse immunoglobulins and complement C3 to the liposome. The partitioning of polysialylated liposomes to the dextran layer in the two phase system also suggests that colominic acid has a strongly bound hydration shell. This is expected to increase the repulsive hydration force around the liposome (see section 1.4) thus amplifying the forces that repel proteins and other surfaces away from it. To determine whether these properties of colominic acid can lead to increased plasma circulation times for CA-liposomes, the clearance of CA-liposomes from the blood was investigated after tail vein injection of the formulations in mice. Based on what was observed by Senior et al (1991) with PEGylated DSPC:cholesterol liposomes, a dose of 0.6μmol phospholipid was chosen as higher doses might saturate some removal mechanisms and obscure the differences in pharmacokinetics between polysialylated and non-polysialylated normal liposomes. Given the important differences in protein binding seen when using large or small colominic acid, the effect of the size of the colominic acid was studied first. The results are shown in figure 7.3. Liposomes grafted with 22.7 kDa or 6 kDa colominic acid, were used. The controls were DSPC:cholesterol 1:1 liposomes with or without 5mol% DSPM.
Blood clearance of polysialylated liposomes. The effect of CA molecular weight. (n = 4 ± StdDev)

100

OO

n

C

C

O

E

< Ü

E

2

D P S C

D S P M

22 kDa CA-liposomes

6 kDa CA-liposomes

- DPSC

- DSPM

- 22kDa CA-liposomes

- 6kDa CA-liposomes

Figure 7.3: Blood clearance of liposomes grafted with 22.7 kDa or 6 kDa colominic acid. 100 µl of liposomes (0.6 µmol of phospholipid) loaded with 0.2 M carboxyfluorescein were injected into the tail vein of 20-25 g balb/c female mice (4 per group). At each time point after the injection a 40 µl blood sample was taken from the tail and diluted to 500 µl with PBS, then CF was measured as described in section 2. The preparations injected were DSPC:cholesterol 1:1 (DSPC), DSPC:cholesterol:DSPM 1:1:0.1 (DSPM), DSPC:cholesterol:DSPM 1:1:0.1 grafted with 22.7 kDa colominic acid as described in section 6 and DSPC:cholesterol:DSPM 1:1:0.1 grafted with 6 kDa colominic acid as described in section 6. Statistical analysis was carried out using a Student's t-test, which showed that the difference between the DSPM and DSPC liposomes was not significant, while the difference between 22.7 kDa CA-liposomes and 6 kDa CA-liposomes as well as the difference between both CA-liposomes and the DSPM control were statistically significant. The t-test was only carried out for times points at which data from two or more formulations is available. A * indicates a statistically significant point between the control and 6 kDa liposomes. A † indicates a statistically significant point between the control and 22 kDa liposomes. A ‡ indicates a statistically significant point between 6 kDa and 22 kDa liposomes.

The results shown in figure 7.3, are surprising. Although 6kDa CA-liposomes outperformed 22.7kDa CA-liposomes as expected from the plasma protein adsorption work, both preparations were cleared faster than either of the controls. This result cannot be attributed to
an effect of the lipid composition, since DSPM containing liposomes are cleared at the about
the same rate as DSPC liposomes, which is not surprising since the negative charge of DSPM
is sterically hindered by the maleimide group. Comparing the results of this experiment with
the in vivo results obtained by Zhang (1999) using PC:cholesterol:PE liposomes with
0.1mol% grafted 18kDa colominic acid and injected at a dose of 1.6μmol of phospholipid per
mouse shows that these liposomes have a similar clearance profile to that of the 22.7kDaCA-
liposomes used here despite the 2.6-fold increased dose administered by Zhang (1999).
Zhang observed that ~40% of the liposomes remained in the circulation 1 hour after
injection, compared with ~48% after 74 minutes seen here. At 3 hours post injection 22% of
the liposomes were still found by Zhang in the circulation, compared with 17% after 4 hours
or ~25% after 3 hours observed here. The 6kDa liposomes outperform the polysialylated
liposomes synthesised by Zhang (60% after 1 hour for 6kDa liposomes).
To determine if the increased clearance is a consequence of a colominic acid mediated
interaction, an in vivo experiment was set up to determine the effect of pre-injecting the
animals with a relatively large amount of free colominic acid. The results are shown in figure
7.4. Without the CA pre-injection the 6kDa CA liposomes have a half-life of about 2 hours
compared with 220 minutes for the control at this particular dose. When the animals are
dosed with 5mg of CA, 2 minutes before injecting the liposomes the CA-liposomes have a
half-life in excess of 4 hours. These findings suggest that there is a specific interaction
between colominic acid and a component of the in-vivo environment that is promoting
clearance, as accelerated clearance is no longer observed when excess colominic acid is
injected before the 6kDa colominic acid liposomes.
Blood clearance of polysialylated liposomes. The effect of CA pre-injection (n = 4 ± StdDev)

Figure 7.4: The effect of free CA pre-injection on the circulation time of polysialylated liposomes. The experimental conditions are as described in the legend of figure 7.3, except that 14-17 g animals were used. For the CA pre-injection groups, the mice were injected via the tail vein with 50 μl of 100 mg/ml 22.7 kDa colominic acid 2 minutes prior to injection of the liposome formulation. The formulations tested were DSPC:Cholesterol:DSPM (1:1:0.1) liposomes with (DSPM) and without (6 kCA-liposomes) conjugated 6 kDa colominic acid. Statistical analysis with the student's t-test showed that pre-injection of CA does not have a significant impact on the circulation of DSPM liposomes. The differences seen with and without the CA pre-injection for CA-liposomes are statistically significant.

It was stipulated in section 1.5 that colominic acid conjugated liposomes may be recognised by a member of scavenger receptor family, as it has been demonstrated that this is the case for liposomes conjugated with polyacrylic acid, or with polyacetylated HSA, compounds which also have a large number of accessible negatively charged carboxyl groups (Fujiwara
et al 1996, Kamps et al 1997). A tantalizing piece of evidence has also been reported by Peiser et al (2000), who found that macrophage Class A scavenger receptors can mediate the phagocytosis of various *E.coli* strains, by CHO cells expressing the Class A type I or type II scavenger receptors (ScRI or ScRII). The crucial finding was that wild type CHO cells were found unable to take up K1 strain *E.coli*, while both CHO-ScRI and CHO-ScRII cells were able to take up the bacteria. The ScRII transfected cells bound the bacteria more strongly than the ScRI transfected cells. The association was inhibited both by polyinosinic acid, a known inhibitor of the scavenger receptor and by preopsonising the bacteria with antibodies against the K1 *E.coli* capsule. Colominic acid is an important component of the K1 *E.coli* capsule (Mushtaq et al 2005) and it is in fact commercially produced by extraction from a culture of this bacterial strain (Roth et al 1993). Therefore, this finding adds a great deal of support to the hypothesis that colominic acid-grafted liposomes are interacting with the scavenger receptor. The reason why this effect was not observed in the study by Zhang (1999) is that an eggPC:cholesterol based formulation was used, whose clearance is very fast compared that of DSPC:cholesterol liposomes. It is possible that colominic acid is blocking the faster clearance mechanisms so that despite the increased clearance through the scavenger receptor, overall there is an improvement. It is quite interesting that the 22.7 kDa CA-liposomes have a similar elimination profile compared with the CA-liposomes synthesised by Zhang, despite the differences in dose and liposomal formulation. Allen and Chonn (1987) observed a similar effect for asialoganglioside GM₁ (asGM₁) containing liposomes. PC:cholesterol based formulations were improved in terms of plasma half-life by incorporation of asGM₁, but the more stable SM:PC based formulations were not. Conclusive
proof of receptor involvement has not yet been obtained. Further work both in vitro and in vivo is required before such proof is obtained.

The implication of this finding with respect to the use of colominic acid as a steric stabilisation agent is that a dual targeting approach may be needed, where the scavenger receptor is temporarily blocked prior to injection of the liposomes. Such dual targeting approaches have been used successfully for other formulations. Since colominic acid itself is a patented excipient, its use for this purpose is quite feasible.

7.6 Conclusions and further work

In this chapter the work done on characterizing the properties of the polysialylated liposomes was described. Using a two phase system, it was shown that colominic acid can greatly increase the hydrophilicity of liposomes and create a very stable hydration shell around the vesicle. No significant affinity for plasma proteins was observed in this system for the polysialylated liposomes, but the control liposomes partitioned differently when plasma proteins were added to the system. The binding of plasma proteins was further analysed by purifying the liposomes after incubation with plasma and analysing the bound proteins using SDS-PAGE. It was found that the binding of proteins is greatly reduced by colominic acid and that smaller colominic acid (6 kDa) was a great deal more effective in this respect compared to larger colominic acid (22.7 kDa). Comparing these results with the work done with PEG liposomes, it can be concluded that colominic acid is much more effective at blocking protein binding, even at low grafting density. The polysialylated liposomes were found to have a faster plasma elimination rate compared to the control (non-polysialylated liposomes) with the 6kDa CA-liposomes having a longer plasma retention time compared to
the 22.7 kDa CA-liposomes. However when the animals were injected with 5 mg of colominic acid prior to injection of the liposomes, the clearance of 6 kDa CA-liposomes was slower compared to that of the control liposomes. It was concluded that although colominic acid is effective at blocking plasma protein-mediated clearance of the liposome, it introduces a new clearance mechanism most likely via the scavenger receptor. Use of colominic acid as a steric stabilisation agent is nevertheless quite feasible, but as the CA-liposomal constructs stand at present, it will most likely require temporary blocking of the scavenger receptor prior to administering the liposomal formulation. Colominic acid is a patented excipient and can conveniently be used for this purpose.

Overall it can be concluded that colominic acid can be an effective steric stabilisation agent for liposomes, but further work is needed before its full potential can be exploited. First of all the involvement of the scavenger receptor must be more carefully investigated both in vivo and in vitro. In vitro, the ability of different macrophage types and non-macrophage cell lines transfected with the scavenger receptor, to take up polysialylated liposomes needs to be investigated, as well as the effect that the presence colominic acid, polyanylated HSA, heparin and poly-inositol has on this uptake. In vivo, the free CA pre-injection experiments must be repeated with varying amounts of CA to demonstrate dose dependence. In addition, it must be determined if polyinositol and polyacetylated HSA have a similar effect on the half-life of polysialylated liposomes. More importantly the biodistribution of polysialylated liposomes needs to be investigated with and without CA pre-injection, paying particular attention to the uptake by the liver sinusoid endothelial cells, which are known to express the scavenger receptor and have a strong uptake of liposomes conjugated with polyacetylated HSA. Further work is also needed to develop a method for controlling the degree of
colominic acid grafting with greater precision. This may require creating a lipid-colominic acid conjugate, which can be directly incorporated into the liposome. Small molecular weight colominic acid may become soluble in a suitable organic solvent when conjugated with a lipid molecule.

Another reactive group which may be advantageous for attaching colominic acid to pre-formed liposomes is boronic acid. Boronic acids form a reversible co-ordinate bond with vicinal diols and thus, liposomes modified with boronic acid may be capable of forming a bond with the non-reducing end of unmodified colominic acid. It should be noted, that so far colominic acid has only been conjugated via the non-reducing end, while in bacteria colominic acid is attached to the surface at the reducing end. The impact of the colominic acid membrane orientation also needs to be investigated.
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