Polysialylated Liposomes: Preparation, Characterization and Stability Studies
In Vitro and In Vivo

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Abstract

Colominic acid (CA), a derivative of polysialic acid, with molecular weight at 15,000-18,000 and sialic acid (SA) were incorporated into liposomes for reducing the uptake of liposomes by reticuloendothelial system (RES) and enhancing the half-life of liposomes in the circulation. Colominic acid was initially coupled with phosphatidylethanolamine (PE) by the Schiff reaction to form a linear polymer (PE-OX-CA), and the yield of PE-OX-CA was approximately 22.8-34.1% depending on the reaction conditions. Conjugation of PE with CA in the presence of carbodiimide catalysts led to the formation of branched polymers (PE-CA). However, PE-OX-CA was difficult to be incorporated into lipid bilayers. Inclusion of PE-CA into liposomes composed of PC and equimolar cholesterol achieved 12.2-39.0%. Further animal results showed that the half-life of liposomes containing PE-CA did not improve significantly. The conjugation of colominic acid with the liposome surface (polysialylated liposomes) by the Schiff reaction was conducted. Results showed that the remaining of polysialylated liposomes (PC/Chol/PE 1:1.2:0.2, molar ratio) in the mouse circulation was improved to two-fold compared with native liposomes (PC/Chol/PE 1:1.2:0.2, molar ratio). The stability of polysialylated liposomes incubated in human serum albumin and mouse plasma was much better than native liposomes, and the surface charge of polysialylated liposomes was around -7.1mV to -11.1mV. Sialic acid coupled with PE (PE-SA) was incorporated into liposomes composed of PC and equimolar cholesterol. The half-life of PE-SA-containing liposomes in the mouse blood circulation was improved. In conclusion, polysialic acid stabilized liposomes and delayed the clearance of liposomes from the circulation.
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Abbreviations

CA  colominic acid
CF  5(6)-carboxyfluorescein
Chol  cholesterol
CMC  1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate
DCC  N, N’-dicyclohexylcarbodiimide
DCP  dicetylphosphate
DLPC  dilauroylphosphatidylcholine
DMAP  N,N’-dimethyl aminopyridine
DMF  N,N’-dimethyl formamide
DMPC  dimyristoylphosphatidylcholine
DMSO  dimethyl sulfoxide
DOPC  dioleoyl phosphatidylcholine
DOPE  L-α dioleoyl phosphatidylethanolamine
DOTAP  1,2-dioleoyl-3-trimethylammonium-propane
Dox  doxorubicin
DPPC  1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine
DPPE  1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine
DRV  dehydration-rehydration vesicles
DSPC  1,2-distearoyl-sn-glycero-3-phosphatidylcholine
DSPE  1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine
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<th>Abbreviation</th>
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<tr>
<td>DSPE-PEG</td>
<td>DSPE-polyethylene glycol</td>
</tr>
<tr>
<td>EDAC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GM₁</td>
<td>monosialoganglioside</td>
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<tr>
<td>HDL</td>
<td>high density lipoproteins</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>IC-mPEG</td>
<td>oxycarbonylimidazole- methoxy-polyethylene glycol</td>
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<td>LUV</td>
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PE-PEG phosphatidylethanolamine-polyethylene glycol
mPEG-SS monomethoxypolyethylene glycol succinimidyl succinate
PG phosphatidylglycerol
PI phosphatidylinositol
PS phosphatidylserine
RES reticuloendothelial system
SA sialic acid
SD standard deviation
SM sphingomyelin
SUV small unilamellar vesicles
TAP 1,2-diacyl-3-trimethylammonium-propane
TEA triethylamine
Tc high gel to liquid crystalline transition temperature
THF tetrahydrofuran
TTAN tetrabutyl ammonium
Chapter 1

Introduction
1.1 Liposomes

Liposomes as a potential drug delivery system have been developed for nearly 30 years (Gregoriadis, 1993a, 1993b, 1994, 1995). From the first time mention of the use of lipids in the formation of vesicles (Bangham et al., 1965) and the use of them later in the transfer of enzymes (Gregoriadis and Ryman, 1971, 1972a), the liposome carrier system has been investigated and applied in many fields such as gene therapy (Gregoriadis et al., 1996a; Gregoriadis, 1998), targeting of anti-cancer drugs (Gregoriadis, 1993c), as immunological adjuvants (Gregoriadis, 1996b) and antimicrobial therapy (Gregoriadis, 1995). The uses and characteristics of liposomes depended not only on the preparation process and lipid composition, but also on their stability in vivo (Gregoriadis, 1993c, 1995; Lasic, 1995a, 1995b). The pharmacokinetics of liposomes in the biological environment, the mechanisms of liposomes interaction with cells and proteins, and the factors related to the clearance of liposomes from blood have been recently summarised (Gregoriadis, G., 1988, 1992, 1994, 1995). Full understanding of liposome behaviour has certainly still a long way to go, but the encouraging clinical results of liposomal formulations carrying certain anticancer and antimicrobial drugs and antigens have demonstrated that liposomes are a very proving delivery system (Gregoriadis, 1995, 1998).

1.1.1 Lipids and other components of liposomes

Natural lipids extracted from animal tissues (eg. eggs or plants) are the mixtures consisting of different types and molecular weights. Lipids are purified to contain the same polar head group, but they still compose of acyl chains with different saturation and chain length. Egg PC, for instance, has different saturated acyl chains compared with PC obtained from
liver or brain. Semi or wholly synthetic lipids are prepared by chemical processes, which provide pure chemicals of a single molecular weight and chemical structure. A brief review on natural and synthetic lipids is shown in the Table 1.1. According to the molecular structure and the charge of a polar end group, lipids are also divided and named as follows: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidic acid (PA), and phosphatidylserine (PS) (Figure 1.1) (New, 1990). All lipids consist of a polar head and a non-polar tail (fatty acid chains) that make lipids preform as surfactants in water and to form an enclosed vesicle of size ranging from nanometers to tens of microns in diameter. Obviously, the lipid components of liposomes determine their final properties such as membrane fluidity, charge density, permeability, the efficiency of drug loading, the leaking of entrapped drug and vesicle stability in vivo (Gregoriadis, 1988, 1992, 1993a, 1993b, 1993c, 1994, 1995, 1998).

Besides phospholipids, cholesterol is another very important component of liposomes to appropriately alter the properties of lipid vesicles (Gregoriadis and Davis, 1979). A typical composition of liposomes is made of egg PC and cholesterol with an equal molar ratio. Other lipids including PG, PI, PA, PE and SA incorporated in liposomes are allowed to change their characteristics, mostly on surface charge and permeability. DSPC and DPPC, which have high gel to liquid crystalline transition temperature (Tc) form "rigid" vesicles and have been commonly used in many studies (Gregoriadis, 1988, 1992). New lipids with strong cationic end groups such as DODAP, DOTAP and DOTMA have been used in gene transfer (Gregoriadis, 1998; Liu, 1996a), and platelet-activation factor (PAF) (1-alkyl-2-acetoyl-sn-glycero-3-phosphatidylcholine) has been found to possess powerful
Table 1.1 Natural and synthetic lipids

<table>
<thead>
<tr>
<th>Natural lipids</th>
<th>Synthetic lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine (PC)</td>
<td>1,2-Distearoyl-sn-glycero-3-phosphatidylcholine (DSPC)</td>
</tr>
<tr>
<td></td>
<td>1,2-Dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC)</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (PE)</td>
<td>1,2-Distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE)</td>
</tr>
<tr>
<td></td>
<td>1,2-Dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE)</td>
</tr>
<tr>
<td>Phosphatidylglycerol (PG)</td>
<td>1,2-Distearoyl-sn-glycero-3-phosphatidylglycerol (DSPG)</td>
</tr>
<tr>
<td></td>
<td>1,2-Dipalmitoyl-sn-glycero-3-phosphatidylglycerol (DPPG)</td>
</tr>
<tr>
<td>Phosphatidic acid (PA)</td>
<td>L-α Dioleoyl phosphatidylethanolamine (DOPE)</td>
</tr>
<tr>
<td>Phosphatidylserine (PS)</td>
<td>1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP)</td>
</tr>
<tr>
<td>Phosphatidylinositol (PI)</td>
<td>1,2-Dioleoyl-3-dimethylammonium-propane (DODAP)</td>
</tr>
<tr>
<td>Cardiolipin (CA)</td>
<td></td>
</tr>
<tr>
<td>Lysophosphatides-PC, PE, PI, PS, PA</td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin (SM)</td>
<td></td>
</tr>
<tr>
<td>Ceramides</td>
<td></td>
</tr>
<tr>
<td>Cerebrosides</td>
<td></td>
</tr>
</tbody>
</table>

Date from the catalogues of Avanti Polar Lipids, Inc. 1995.

antihypertensive properties (Blank et al., 1979). Recently, liposomes incorporated PE or DSPE derivatives, PE-polyethylene glycol (PE-PEG) and DSPE-polyethylene glycol (DSPE-PEG) have attracted attention since they enhance the half-life of liposomes in vivo(Gregoriadis, 1995; Woodle, 1995; Lasic and Martin, 1995a). Conjugates of immunoglobulin with lipids for targeted drug delivery have been studied for a long time (Gregoriadis, 1993c, 1994), but have not fixed to exception mainly due to the recognition
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of immunoglobulin by the macrophages of the reticuloendothelial system (RES). Nevertheless, a great variety of lipids obtained naturally or synthetically offered much choice in the preparation of liposomes, depending on the requirements of the application.

![Diagram of lipids](image)

### 1.1.2 Liposome technology

Besides the lipid composition, methods of generating liposomes are also very important factors to affect the properties of and drug loading in liposome formulation. Generally,
liposomes are classified as multilamellar vesicles (MLV), small unilamellar vesicles (SUV) and large unilamellar vesicles (LUV). MLV contain two or more concentric lamellar arranged in an onion skin conformation and which can range in vesicle size from 0.2 to 10\(\mu\)m. LUV have a single bilayer and a size distribution which usually falls in the range 0.05-0.5\(\mu\)m. SUV are often referred to as limit size vesicles from 0.02-0.05\(\mu\)m in diameter (Figure 1.2) (Cullis et al., 1989).

The technology for the preparation liposomes includes freeze-drying (Mayer et al., 1985), microfluidization (Skalko et al., 1996), detergent removal (Mimms et al., 1981; Weder and Zumbuehl, 1984), dehydration / rehydration (Kirby and Gregoriadis, 1984; Gregoriadis et al., 1990), probe sonication (Mayhew et al., 1984), membrane extrusion (Hope et al., 1985; Mayer et al., 1986), reverse and evaporation phase (Szoka and papahadjopoulos, 1980), and solvent evaporation (Deamer, 1984). All methods applied in the making of liposomes rely on the character of drugs to be entrapped, the administration routes, the properties of the lipids and the final vesicle size (Table 1.2). In industry, large scale production is a major consideration (Gregoriadis, 1988, 1992).

1.1.3 Drug loading and delivery in vivo

Drug loading of the lipid bilayers or drug entrapment into the aqueous phase of vesicles depends on drug solubility, affinity to lipids, charge and molecular weight. If the drug is water soluble, most of it will be entrapped in the aqueous phase. Hydrophobic drugs will be loaded into the bilayer. The loading yield is determined by the preparation process and the vesicle structure. For example, drug loading in SUV (1-5%) is significantly lower than
**Table 1.2 Characterization of liposomes**

<table>
<thead>
<tr>
<th>Name</th>
<th>size (µm)</th>
<th>Preparation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small unilamellar vesicles (SUV)</td>
<td>0.02-0.05</td>
<td>Microfluidization, Ultra-sonication, Membrane extrusion, High pressure homogenization, French press</td>
</tr>
<tr>
<td>Multilamellar vesicles (MLV)</td>
<td>0.2-10</td>
<td>Vortexing or hand shaking of phospholipid dispersions, Dehydration-rehydration</td>
</tr>
<tr>
<td>Large unilamellar vesicles (LUV)</td>
<td>0.05-0.5</td>
<td>Reverse-phase evaporation, Detergent dialysis</td>
</tr>
</tbody>
</table>

**Fig. 1.2** Multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV)

that in MLV (27-54%) (Kirby and Gregoriadis, 1984; Mayer et al., 1985). The low incorporation efficiency of drugs in SUV is due to their small volume. With regards to MLV, even when of similar size, drug loading efficiency alters, depending on the method used. For instance, using mechanical mixing to make MLV with a size around 0.4-3.5µm, there is only 1-8.5% of the drug in liposomes. In contrast, adoption of the dehydration-
rehydration method leads to nearly 50% of the drug in liposomes (Kirby and Gregoriadis, 1984). On the other hand, if compounds have special affinity to lipids and are retained by liposomes, the loading efficiency is relatively high (Gregoriadis and Davis, 1979). Small hydrophilic molecules can rapidly leak out on contact with plasma or serum (Gregoriadis, 1988). High molecular water soluble drugs such as proteins and hydrophobic incorporated drugs into the bilayers can stay longer within the vesicles (Gregoriadis et al., 1971; Gregoriadis and Neerunjun, 1974, 1975).

At present, several liposome formulations have been approved for use in humans. They include Dauno Xome® and AmBisome® (Table 1.3) (Gregoriadis, 1995; Forssen, 1998; Hillery, 1997). Amphotericin B liposomes used in the treatment of serious fungal infections have been shown very high therapeutic indexes (Perez, et al., 1993). Doxorubicin liposome formulations have been reported to reduce the toxicity of the drug, reduce the dose and to concentrate the drug in the solid tumour (Rahman et al., 1993; Gabizon et al., 1992a, 1992b, 1993a, 1994). Other liposome-based formulations currently in clinical trial but not as yet approved incorporate drug such as prostaglandin E1, cis-platinum and annamycin (Gregoriadis, 1995). Hopefully, they will be approved and used in the medical treatment in the future.
Table 1.3 Partial listing of liposome-based products approved or under commercial development

<table>
<thead>
<tr>
<th>Product</th>
<th>Drug</th>
<th>Target disease</th>
<th>Company</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dauno Xome®</td>
<td>Daunorubicin</td>
<td>Kaposi’s sarcoma cancer (various types)</td>
<td>NeXstar</td>
<td>Approved Europe, US</td>
</tr>
<tr>
<td>AmBisome®</td>
<td>Amphotericin B</td>
<td>Fungal infections</td>
<td>NeXstar</td>
<td>Approved Europe</td>
</tr>
<tr>
<td>Mikasome®</td>
<td>Amikacin, Doxorubicin</td>
<td></td>
<td>NeXstar</td>
<td>Clinical trials-phase II</td>
</tr>
<tr>
<td>VincaXome®</td>
<td>Vincristine</td>
<td>Solid tumours</td>
<td>NeXstar</td>
<td>Under preclinical development</td>
</tr>
<tr>
<td>Doxil®</td>
<td>Doxorubicin</td>
<td>Kaposi’s sarcoma cancer (various types)</td>
<td>Sequus Pharm</td>
<td>Approved Europe, US</td>
</tr>
<tr>
<td>Amphocil®</td>
<td>Amphotericin B</td>
<td>Fungal infections</td>
<td>Sequus Pharm</td>
<td>Approved in the UK, Ireland</td>
</tr>
<tr>
<td>Ventus®</td>
<td>Prostaglandin E,</td>
<td>Systemic inflammatory disease</td>
<td>The Liposome Co.</td>
<td>Clinical trials-phase III</td>
</tr>
<tr>
<td>Nyotran®</td>
<td>Nystatin</td>
<td>Fungal infections</td>
<td>The Liposome Co.</td>
<td>Clinical trials-phase III</td>
</tr>
<tr>
<td>Atragen®</td>
<td>All trans-retionoic acid</td>
<td></td>
<td>Aronex Pharm</td>
<td>Clinical trials-phase III</td>
</tr>
<tr>
<td>Annamycin</td>
<td>Annamycin</td>
<td>Kaposi’s sarcoma cancer, Refractory breast cancer</td>
<td>Aronex Pharm</td>
<td>Clinical trials-phase II/III</td>
</tr>
<tr>
<td>SP1-77</td>
<td>Muramyltripeptide</td>
<td>Activation of tumouricidal macrophages</td>
<td>Aronex Pharm</td>
<td>Clinical trials-phase I/II</td>
</tr>
<tr>
<td></td>
<td>cis-platinum</td>
<td>Cancer</td>
<td>Ciba-Geigy</td>
<td>Clinical trials</td>
</tr>
<tr>
<td>E. coli 0157:H7 vaccine</td>
<td>E. coli 0157 infection</td>
<td></td>
<td>Novavax</td>
<td>Clinical trials-phase I</td>
</tr>
<tr>
<td>Hexapel™</td>
<td>Hepatitis B</td>
<td>Vaccine</td>
<td>Swiss Serum and Vaccine Institute</td>
<td>Clinical trials-phase I</td>
</tr>
</tbody>
</table>

Data from Gregoriadis, 1995; Forssen, 1998; Hillery, 1997
1.2 Stealth liposomes

1.2.1 Concept of sterically stabilised liposomes

Application of liposomes as drug delivery systems was predicted to have three major problems: scale-up, sterilisation and stability on storage. Moreover, when liposomes are administered in vivo, they are rapidly taken up by the fixed and free macrophages of the reticuloendothelial system (RES), primarily localized in the liver (Kupffer cells) and spleen (Gregoriadis et al., 1972a, 1972b). Liposomes composed of natural phospholipids and cholesterol were recognized and cleared from the circulation within a few minutes or few hours (Gregoriadis et al., 1972a, 1972b; Woodle and Papahajopoulos, 1989). Thus, in using liposomes in targeting delivery, drugs carried by such liposomes have not enough time to reach the target site. This is believed to be the main obstacle for liposomes in their use in clinical trials.

During the 70's and 80's, several methods that changed the pharmacokinetics of liposomes in the blood were reported (Greogriadis et al., 1974, 1975, 1979, 1980, 1982; Senior et al., 1983, 1985, 1987). Those endeavours aimed at physical modification of liposomes, including adjustment of vesicle size, surface charge, composition, bilayer rigidity and surface tension (Gregoriadis et al., 1979, 1980, 1984; Kirby et al., 1980a, 1980b, 1980c; Senior et al., 1983, 1985, 1987). The half-life of liposomes in the circulation was considerably improved by reducing the vesicle size, and by increasing the bilayer rigidity by addition of saturated lipids or cholesterol. Further improvement in the half-life of liposomes was achieved by incorporation of monosialoganglioside (GM₁) or PEG-
phosphalipid in such small, rigid liposomes (Senior et al., 1991; Allen et al., 1987, 1988, 1989, 1990; Gabizon et al., 1988a, 1988b, 1989, 1990; Woodle et al., 1990; Klibanov et al., 1990). Liposomes coated with PEG or GM₁ on the surface exhibited prolonged circulation from several hours to two days dependent on the lipid composition and vesicle size. This kind of liposome was named as a stealth liposome due to the ability to evade detecting by the RES (Lasic and Martin, 1995a) (Figure 1.3).

![Fig. 1.3 Stealth and other types of liposomes](image)

Different types of liposomes including conventional, stealth, targeted and cationic were developed to carry drugs, antigens and genes (Adapted from Lasic, 1997).

Lasic and his co-workers developed the first pharmaceutical formulation containing the anticancer drugs epirubicin and doxorubicin in sterically stabilised vesicles at Sequus Pharmaceuticals in 1989 (Lasic and Martin, 1995a). These liposomes circulated in humans with the half-life of days compared with dozens of minutes, or at most hours for conventional liposomes. Clinical trials on more than 1500 patients with Kaposi’s sarcoma
showed very encouraging therapeutic results (Norhfelt et al., 1995). In 1995 the liposomal formulation of doxorubicin in sterically stabilised liposomes became the first liposomal cancer therapeutic to be approved in the US, as Doxil. It became commercially available in December 1995.

Ongoing studies on sterically stabilized liposomes returned to the old problem: targeting. Work on targeting ligands, e.g., antibody, oligo-peptides and polysaccharides, which were chemically conjugated to the exterior surface of the sterically stabilized liposomes has been carried out by many workers (Mori et al., 1993a and 1993b; Ahmad et al., 1993; Allen et al., 1994a; Goren et al., 1996; Kirpotin et al., 1997). However, no successful clinical trial on the use of targeted stealth liposomes has been reported so far.

Generally, stealth liposomes provide a new approach and concept for the passive delivery of drugs to the target tissue with highly leaky vasculature, especially in the treatment of solid tumours in clinical trials (Allen, 1994b; Woodle, 1995; Harashima and Kiwada, 1996a). Obviously, other diseases, such as inflammations and infectious disease in which there is increased vascular permeability may be suitable for application of stealth liposomes as drug carriers.

1.2.2 Conventionally modified liposomes

1.2.2.1 Cholesterol

With the development of technical skills for the preparation of liposomes, more lipids were
introduced in making liposomes. It was found that liposomes incorporating cholesterol exhibited long half-life in vivo (Kirby et al., 1980a, 1980b, 1980c; Tumer et al., 1983) and also reduced the leaking of drugs. It appeared that cholesterol prevented the action of plasma high density lipoproteins (HDL) in removing lipid molecules from the vesicle bilayer (Gregoriadis and Davis, 1979; Krupp et al., 1976; Scherphof et al., 1978). Reports on cholesterol-rich liposomes showed vesicle stability not only with egg PC but also with DSPC. Gregoriadis and Davis (1979) reported results by using 5(6)-carboxyfluorescein as an indicator in the study of the effect of cholesterol on vesicle stability. They found that cholesterol-rich liposomes had longer half-life than cholesterol-free liposomes. Thirty minuets after injection there was 80% of PC/Chol/PA (7:7:0.87, molar ratio), 70% of PC/Chol/PA (7:5:0.6, molar ratio), 30% of PC/Chol/PA (7:2:0.25, molar ratio) and 20% of PC/PA (7:1, molar ratio) in the blood. And the leaking of CF from cholesterol-rich liposomes was relatively slower than cholesterol-free liposomes after incubation in the whole rat blood. Nevertheless, cholesterol plays a role in the stability of lipid bilayers as it restricts the mobility of phospholipid, and thus reduces the action of HDL.

Further work with PC/Chol liposomes with different charged lipids showed the same results (Gregoriadis and Senior, 1980). They made liposomes composed of PC with phosphatidic acid (negative) or stearylamine (positive) and concluded that liposomes with equal molar PC and cholesterol, regardless of surface charge, exhibited prolonged half-life in the blood compared with cholesterol-free liposomes. There was 40% left 5h after injection for cholesterol-rich liposomes, whereas cholesterol-free liposomes or liposomes with low ratio cholesterol (PC/Chol, 7:2, molar ratio) only 1% was in the circulation at the same time.
intervals. Similar conclusions were obtained by other research groups (Patel et al., 1983a; Dave and Patel, 1986; Moghimi and Patel, 1988, 1989). However, half-lives for cholesterol-free charged liposomes were always lower compared with those observed for cholesterol-rich charged vesicles.

The mechanism by which cholesterol changes the clearance rate of liposomes has been discussed. It is believed that cholesterol causes an increase of the rigidity of liposomes, which in turn impedes the adsorption or penetration of opsonic proteins on the liposome surface. Alternatively, the hydrophobicity produced by cholesterol on the liposome surface may be a more important parameter than rigidity since it prevents conditions on favourable of phagocytosis (Senior, 1987). Different degrees of hydrophobicity imposed by different amount of cholesterol appear to lead to the adsorption of different opsonic factors. For instance, cholesterol-poor or cholesterol-free liposomes were shown to bind opsonic factors that caused an increase in uptake by liver macrophages in vitro, whereas cholesterol-rich liposomes bound proteins which resulted in a strong increase in uptake by spleen cells (Moghimi and Patel, 1988, 1989). It was shown that cholesterol-poor liposomes during incubation with rat serum, adsorbed substantially higher quantities of serum proteins than cholesterol-rich liposomes. No obvious difference in the pattern of adsorbed proteins could be shown. Although many proteins are associated with opsonization eg. α-2 macroglobulin, fibronectin, apoE, apoA-1, and apo-A-1V (Green et al., 1980; Damen, et al., 1982; Juliano and Lin, 1980; Hoekstra et al., 1981), the type of proteins with affinity for cholesterol-rich or free liposomes are still not very clear. Interestingly, it has been reported by Liu (Liu et al., 1989) that cholesterol-free dioleoyl phosphatidylethanolamine
(DOPE) / oleic acid liposomes remain quite stable after incubation in human plasma. It thus appears that cholesterol is only one of many factors which determined the fate of liposomes.

1.2.2.2 Vesicle size

The half-life of liposomes has also been demonstrated to rely on their vesicle size (Gregoriadis and Senior, 1980; Senior et al., 1982, 1983). Once brought into body, liposomes firstly face an anatomical barrier. Large vesicles with size more than 300nm in diameters cannot pass through the tight packed of endothelial cells, and they were rapidly recognized by RES and mostly accumulated in the spleen (Liu et al., 1991). Otherwise, small vesicles with a diameter of around or below 60nm are greatly eliminated from the blood since they pass through the tissue with a fenestrated vascular endothelial lining such as liver (Liu et al., 1992). Besides the anatomical consideration, it was reported that macrophages are capable of endocytosing vesicles to very large size, with the large vesicles being taken up more easily than small ones (Roerdink et al., 1986). The rapid clearance of liposomes with increasing vesicle size has been reported by other studies (Juliano and Stamp, 1975; Abra and Hunt, 1981; Allen and Everest, 1983; Senior et al., 1985). As liposomes vary in size from about 30nm to several μm depending on the method of preparation, the choice of vesicle size will depend on the route of administration, the drug loaded they must carry as well as anatomical consideration. Thus, whereas large liposomes can carry high dose of drugs, they cannot access extravascular spaces. Small liposomes (diameter < 100nm), on the other hand, can localize extravascularly and be taken up by hepatic parenchymal cells, but they have quite low drug loading efficiency (Roerdink et al., 1986).
1981; Scherphof et al., 1983; Rahman et al., 1982). The ideal vesicle size for the long circulation of liposomes seems around 100nm in diameters. In addition, reports have indicated that a narrow size distribution of vesicles helps liposomes to remain in the circulation much longer (Liu et al., 1992).

1.2.2.3 Lipid Composition

Lipids involved in the formation of vesicles are described to have different functions in determining the fate of liposomes in vivo. Related parameters include degree of saturation, acyl chain length and polar head structure and charge. Generally, saturated phospholipids such as DSPC and DPPC which have relatively high transition temperatures (Tc) form liposomes with more rigid structure and are retained in the circulation much longer than the fluid PC liposomes (Gregoriadis et al., 1980, 1982; Hwang and Beaumier, 1980, 1988). For example, liposomes composed of cholesterol and phospholipids such as dilauroylphosphatidylcholine (DLPC), dioleoylphosphatidylcholine (DOPC), egg phosphatidylcholine (PC), dimyristoylphosphatidylcholine (DMPC) in a molar ratio of 0.5:1, exhibited the half-life of 0.1, 1, 2 and 6h, respectively (Gregoriadis and Senior, 1980). Moreover, incorporation of sphingomyelin (SM) in PC/Chol liposomes increased the circulation of liposomes significantly. Thus, increasing the molar ratio of SM/PC in the lipid composition (SM / PC, 0:100, 23:77, 47:53, 77:23, 100:0, molar ratios), the half-life of liposomes increased from 2h to 11h (2h, 4.5h, 7.0h, 11h, 11h, respectively) (Senior and Gregoriadis, 1982). The inhibition of SM on the uptake of liposomes by the RES has also been demonstrated in cultured mouse bone marrow macrophages (Allen et al., 1991a). Moreover, the inclusion of SM in liposomes resulting in inhibition of the uptake by both
Kupffer and splenic cells suggest that bilayer fluidity and hydrophobicity of liposomal membranes play an important role in attracting the opsonins which determine vesicle phagocytic fate (Moghimi and Patel, 1989). In contrast, PS was shown to promote the uptake of liposomes by the RES and drastically reduced the circulation time of liposomes (Klibanov et al., 1991). Actually, as lipid compositions and lipid molar ratios have varied widely in research reports, it is difficult to compare the results from different research groups. It is therefore not surprising that conflicting conclusions are often to be seen in the references.

1.2.2.4 Surface charge

In early studies, it was thought that neutral and positive charge on liposomes contributed to a longer circulation time than a negative charge (Greogriadis and Neerunjun, 1975). Thus, negative liposomes (PC/PS/Chol, 1:1:1, molar ratio) were cleared from the circulation more rapidly than positive (PC/SA/Chol, 18:2:10, molar ratio) and neutral (PC/Chol, 2:1, molar ratio) liposomes (Juliano and Stamp, 1975). In vitro study on the incubation of different charged liposomes in mouse peritoneal macrophages, the uptake order was found, negative (DPPC/Chol/PS, 1:1:0.1, molar ratio) > positive (DPPC/Chol/SA, 1:1:0.2, molar ratio) > neutral (DPPC/Chol, 1:1, molar ratio) (Hsu and Juliano, 1982). Yet, another study with small liposomes (33nm in diameters) showed the clearance of liposomes as follows: positive PC/Chol/SA (7:2:1, molar ratio) > negative PC/Chol/DCP (7:2:1, molar ratio) and neutral PC/Chol (8:2, molar ratio) (Souhami et al., 1981). Liu et al. (1996b) reported that liposomes with a neutral composition of PC and cholesterol exhibited relatively low liver uptake by using simple liver perfusion system of
mouse. And hepatic uptake of neutral or negatively charged liposomes did not involve serum components. In contrast, serum enhanced the liver uptake of positively charged liposomes. Such serum effect on liver uptake of the positively charged liposomes is likely due to liposome aggregation caused by serum proteins (Liu et al., 1996b). Obviously, the surface charge of liposomes may affect the half-life of liposomes, but how the surface charge plays a role in the reduction the uptake of liposomes by the RES is still confused.

### 1.2.2.5 Dose and species

Targeting of liposomes to liver cells had been studied in the early delivery research. It was found that there were at least two uptake pathways for liposomes, saturable and nonsaturable after intravenously injection (Beaumier et al., 1983; Abra and Hunt, 1981; Sato et al., 1986). Increasing the injected dose of liposomes (SM/Chol, 2:1, molar ratio), most of the liposomes accumulated in hepatic parenchymal cells other than Kupffer cells due to the saturation of Kupffer cells by initial high dose liposomes (Chow et al., 1989). The saturation kinetics of liposomes had been analysed based on the Michaelis-Menten equation (Beaumier et al., 1983; Harashima et al., 1993; Kume et al., 1991). By increasing the dose of liposomes, the clearance of liposomes decreased till the dose reached enough saturation.

The pharmacokinetics of liposomes also depends on the species. Whereas liposomes (HEPC/Chol/DCP, 5:4:1, molar ratio) were injected into mice, rats and rabbits at the dose of 1-100μmolHEPC/kg, the retention time of liposomes decreased with decrease the body weight (Harashima et al., 1996b). It means that circulation time was short in some small
animals. This performance had also been demonstrated in the study of doxorubicin liposomal formulation in the human (Lasic, 1997). The same formulation in mice only had a half-life of several hours, but in humans it was for several days (Huang et al., 1992a, 1992b; Northfelt et al., 1995; Uziely et al., 1995). The difference circulation time among species could not be explained by the higher density of Kupffer cells in smaller animals because immunohistochemical analysis revealed that there was no significant difference in the density of Kupffer cells among these species. Comparing rats and mice by perfused liver system, it is clear that the uptake of liposomes depended on the opsonin in rats, but was independent of opsinins in mice (Harashima et al., 1996b). Extrapolating the experimental results from small animals to human, the difference of species has to be considered.

1.2.3 Modification of liposomes by polyethylene glycol (PEG)

Among hydrophilic polymers, PEG has the advantage to be considered nontoxic and was approved by the Food and Drug Administration (FDA) for internal use in humans (Harris, 1985). The half-life of PEG in the blood extended from 18 min to one day as the PEG molecular weight increased from 6000 to 190,000 (Yamaoka et al., 1994). PEG with molecular weight of 2000-5000 does not have a long retention time in the circulation, but after covalently binding or coating to the surface of particles (Müller and Kissel, 1993; Illum and Davis, 1983; Moghimi et al., 1991; Porter et al., 1994), proteins (Sawhey et al., 1993) and liposomes (Allen et al., 1991b), it obviously reduced the adsorption of proteins and blood components, increased the biocompatibility, avoided the uptake by RES and formed long-circulating micelles. Proteins modified by PEG had reduced immunogenicity
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and antigenicity (Abuchowski et al., 1977).

1.2.3.1 Chemical conjugation of PEG to PE and DSPE

Conjugation of PEG with lipids has been realized by using different coupling reactions as described in recent review (Zalipsky, 1995a, 1995b) (Figure 1.4). Despite several methods used to conjugate PEG directly to lipids (Parr et al., 1994), most of the work has utilized mono-functional methoxy-PEG (mPEG), which is reactive at only one end and reacts with lipids containing an amino end group (PE or DSPE). The mPEG derivatives, mPEG-SS (monomethoxypolyethylene glycol succinimidyl succinate) reacted with PE or DSPE under the catalyst of triethylamine (TEA) were commonly used in many studies (Klibanov et al., 1990; Blume and Cevc, 1990; Zalipsky, 1993a). Other reactions including mPEG-oxycarbonylimidazole (IC-mPEG) (Allen et al., 1991; Woodle et al., 1992a), mPEG-dichlorotriazine (Blume and Cevc, 1990) and mPEG-tresylate (Senior et al., 1991;

\[
\text{PE-NH-CO-CH}_2-\text{CH}_2-\text{COO-mPEG}
\]

\[\uparrow\]

SS-mPEG

\[\text{mPEG-NH}_2-\text{PE}\]

\[\text{mPEG-tresylate}\]

\[\text{IC-mPEG or SC-mPEG}\]

\[\text{PE-NH}_2\]

\[\text{PE-NCO-mPEG}\]

\[\downarrow\]

\[\text{mPEG-O-CH}_2\text{COOH}\]

\[\text{mPEG-O-CH}_2\text{CO-NH-PE}\]

Fig.1.4 Coupling PEG with lipids

20
Tilcock et al., 1993) were also successful in the coupling PE with PEG. In spite of PE, PG has been used in the conjugation by firstly oxidized with NaIO₄, then coupled with mPEG-NH₂ (Zalipsky, 1995a). Senior et al. (1991) modified DSPC/Chol/DSPE liposome surface with tresylate ester of mPEGₙ₀₀₀. This reaction leads to formation of secondary amine-linked mPEG-DSPE conjugates from some of the DSPE on the exterior of the liposome bilayer and avoided the presence of mPEG residues inside liposomes. The combination of PE-mPEG with different chemical linkages into liposomes showed some differences that were explained by subtle differences in formulations (Woodle and Lasic, 1992b; Parr et al., 1994; Zalipsky et al., 1992). The advanced development of PEG-PE chemistry was attachment of ligands, antibody on the end of PEG to form Ab-PEG-PE. Several coupling methods have been reported (Herron et al., 1994; Tagawa et al., 1993; Blume et al., 1993b; Zalipsky et al., 1993b, 1994).

1.2.3.2 Effect of PEG on long circulating liposomes

Incorporation of PEG-PE into liposomes leads to prolonging the retention time of liposomes in the circulation. It was reported that 50% of PC/Chol/mPEGₙ₀₀₀-PE liposomes remained in the blood 5h after injection compared with 1% of remaining of PC/Chol liposomes (Klibanov et al., 1990). This conclusion was supported by other studies (Blume and Cevc, 1990; Senior et al., 1991; Allen et al., 1991). But the long circulation of PEG-liposomes was limited to small vesicle size. While the vesicle size was more than 300nm, the effect of PEG on enhancing the half-life of liposomes was not obvious (Litzinger and Huang, 1992), and the accumulation of PC/Chol/MPEGₙ₀₀₀-PE liposomes in the spleen reached 35% at 3h post-injection (Litzinger and Huang, 1992). On the other hand, the
saturation of mPEG-PE on the surface of liposomes depended on the polymer chain length. The thickness of mPEG on liposomes was estimated to be 6nm (Needham, 1992a). Increasing mPEG\textsubscript{750} -PE in the lipid composition to 10 mol% resulted in a linear increase of the half-life of liposomes in the blood, whereas the retention time of mPEG\textsubscript{2000}-PE and mPEG\textsubscript{5000}-PE liposomes showed a plateau at 5mol% of mPEG\textsubscript{2000}-PE and mPEG\textsubscript{5000}-PE (Mori et al., 1991).

Liposomes incorporated with mPEG\textsubscript{5000}-PE and different lipids, PC/Chol/mPEG\textsubscript{5000}-PE, PC/Chol/mPEG\textsubscript{5000}-PE/PG, PC/Chol/mPEG\textsubscript{5000}-PE/PS, PC/Chol/mPEG\textsubscript{5000}-PE/SM, DPPC/Chol/mPEG\textsubscript{5000}-PE and DSPC/Chol/mPEG\textsubscript{5000}-PE at molar ratio of 1:1:0.15:0.15, did not lead to significant differences in the mouse circulation (40-50% remaining 5h after injection) except PC/Chol/mPEG-PE/PS liposomes, which inhibited the action of mPEG. 0.65 % remaining of PC/Chol/mPEG-PE/PS liposomes was reported since PS had been well known for its activity in promoting liposomes uptake by the RES (Klibanov et al., 1991). Incorporation of 5.7 mol % of mPEG\textsubscript{1900}-DSPE in liposomes composed of SM/PC/Chol (1:1:1, molar ratio) had greatest ability to decrease RES uptake, and the half-life was in excess 20h (Allen et al., 1991). The total level of mPEG\textsubscript{1900}-DSPE liposomes in liver and spleen was below 15% of injected dose at 48h, and more than 50% of liposomes remained in the body (Allen et al., 1991).

The effect of PEG molecular chain length on the circulation time of liposomes in mice has been reported to follow the order: 12000=5000>2000>1000, as PEG was coupled with DOPE to form mPEG-DOPE and incorporated into PC/Chol liposomes (1:1, molar ratio)
with 6 mol % (vesicle size 200nm) (Maruyama et al., 1992). Similar conclusions were obtained in the study of DSPC/Chol/DSPE-mPEG liposomes (Maruyama et al., 1992) and PC/Chol/mPEG-PE liposomes (Mori et al., 1991; Woodle et al., 1992a). But another group found a similar circulation time for liposomes incorporating comparable concentrations of mPEG<sub>2000</sub> and mPEG<sub>5000</sub> in SM/PEG/Chol (1:1:1, molar ratio). The amounts of remaining of mPEG<sub>2000</sub> and mPEG<sub>5000</sub> liposomes were 35.1% and 28.3% 24h after injection, respectively (Allen et al., 1991).

Immunoliposomes incorporating mPEG<sub>750</sub>-PE, mPEG<sub>2000</sub>-PE and mPEG<sub>5000</sub>-PE were prepared with formulation of PC/Chol/ (1:0.5, molar ratio) and composed of rat Ig G<sub>2a</sub> antibody (34A) which specifically binds with a glycoprotein antigen gp112 and express high concentration in the lumenal surface of capillary endothelial cells of mouse lungs (Mori et al., 1991). The binding level (42-52%) of immunoliposomes with and without mPEG to the lung at 1h post-injection showed no significant difference except mPEG<sub>5000</sub>-PE, which only had 21% binding. mPEG<sub>5000</sub> interfered the combination of antibody on the antigen. Otherwise, immunoliposomes incorporated with mPEG have not improved the retention time of liposomes. 1h after injection, 6-10% remaining of mPEG-immunoliposomes in the blood was not extremely different from free liposomes (9% left at the same time) (Mori et al., 1991).

The entrapment of doxorubicin (DOX) in DSPC/Chol/mPEG<sub>1000</sub>-DSPE (1:1:0.06, molar ratio) with a size of 100nm was conducted by the transmenbrane pH gradient method, and the entrapment efficiency was more than 98% (Unezaki et al., 1995). While DOX-mPEG-
liposomes, DOX-free liposomes and free DOX were intravenously injected into Balb/c mice, which had been subcutaneously implanted with colon 26 carcinoma, results showed that DOX-mPEG-liposomes produced an approximately 3.6-10.5 folds increased DOX level in tumour (Unezaki et al., 1995). Administration of DOX-mPEG-liposomes at a dose of 10mg DOX/kg resulted in effective retardation of tumour growth and two fold prolongations of survival times compared with DOX-free-liposomes. Liposomal DOX effectively reduced the DOX concentration in the heart (Unezaki et al., 1995). The reason for DOX-mPEG-liposomes enhancing the therapeutic index was attributed to passive targeting because the long-circulation of liposomes in the blood gives more time for DOX to leak in the tumour. For DOX-mPEG-liposomes only 50% of injected dose remained 5h after injection and DOX-free liposomes only had 20% left at the same time (Unezaki et al., 1995). mPEG- liposomes carrying doxorubicin were also studied on beagle dogs (Gabizon et al., 1993a). Results showed that the clearance was significantly slower for doxorubicin encapsulated in mPEG\textsubscript{1900}-DSPE liposomes than in hydrogenated soybean phosphatidylinositol (HPI) liposomes with half-lives of 29h and 13h, respectively.

Another drug, adriamycin loaded in mPEG\textsubscript{2000}-PG/DMPC/DMPG/Chol was found to stay in the blood longer than free drug or drug loaded in the free liposomes (Sadzuka et al., 1995). Adriamycin concentration in the tumour was also improved two fold by the long-circulation of liposomes. The passive targeting was not associated with cardiotoxicity and the maximum levels of adriamycin in the heart was reduced to approximately 50% by mPEG-liposome formulation (Sadzuka et al., 1995).
1.2.4 Liposomes coated with monosialoganglioside (GM₁)

The use of glycolipids, such as GM₁, to reduce the uptake of liposomes by RES has been reviewed in detail by Allen (1992, 1993, 1994b). The structure of these glycolipids is shown in the Figure 1.5.

\[
\begin{align*}
\text{GM₁-ganglioside} & : \text{Galβ1-3GalNacβ1-4Galβ1-4Galβ1-1Ceramide} \\
& \quad | \quad \text{Neu5Acα2} \\
\text{GM₂-ganglioside} & : \text{GalNacβ1-4Galβ1-4Galβ1-1Ceramide} \\
& \quad | \quad \text{Neu5Acα2} \\
\text{GM₃-ganglioside} & : \text{Neu5Acα2-3Galβ1-4Galβ1-1Ceramide} \\
\text{Asialo-GM₁-ganglioside} & : \text{Galβ1-3GalNacβ1-4Galβ1-4Galβ1-1Ceramide} \\
\text{Asialo-GM₂-ganglioside} & : \text{GalNacβ1-4Galβ1-4Galβ1-1Ceramide} \\
\text{GT₁b-ganglioside} & : \text{Neu5Acα2-3Galβ1-3GalNacβ1-4Galβ1-4Galβ1-1Ceramide} \\
& \quad | \quad \text{Neu5Acα2-8Neu5Acα2} \\
\text{GD₁a-ganglioside} & : \text{Neu5Acα2-3Galβ1-3GalNacβ1-4Galβ1-4Galβ1-1Ceramide} \\
& \quad | \quad \text{Neu5Acα2} \\
\text{Globoside} & : \text{GalNacβ1-3Galα1-4Galβ1-4Galβ1-1Ceramide}
\end{align*}
\]

Neu5Ac: n-acetyl neuraminic acid (sialic acid); Gal: galactose; GalNac: n-acetyl galactosamine
Glc: glucose

Fig 1.5 Structure of glycolipids

In the early studies, incorporation of GM₁ and asialoganglioside (ASGM₁) into PC/Chol...
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Liposomes resulted in a two-fold increase in liver uptake of GM₁ or AS GM₁ liposomes as compared with neutral PC/Chol liposomes (Surolia and Bachhawat, 1977; Jonah et al., 1978). They attributed this increase to the binding of ganglioside terminal galactose residues to β-galactose receptors on liver parenchymal cells. However, the completely opposite result was reported by Allen in 1987. They found that incorporation of 5-7 mol% of GM₁ into liposomes (PC/Chol) not only reduced the susceptibility of these liposomes to lysis by the plasma components, but also increased their circulatory levels by three to ten fold (Allen and Chonn, 1985, 1987). For the same lipid composition the contrary conclusions of GM₁ were attributed to the purity of GM₁ and molar ratio of GM₁ in liposomes. As using a low ratio (4 mol% of lipids) and impure (85-90% purity) GM₁ to prepare liposomes, GM₁ cannot prolong the circulation time of liposomes (Allen and Chonn, 1987). When the lipid composition of liposomes was altered to more closely correspond to the composition of the outer red cell membrane by addition of sphingomyelin (SM), the liver uptake of liposomes was dramatically reduced in the presence of GM₁ and the level of liposomes circulating in the blood correspondingly increased (Allen and Chonn, 1987). The half-life of PC/Chol/SM/GM₁ (1:1:1:0.2, molar ratio, size at 100nm) reached about 16-24h, as comparing to 2h for normal PC/Chol liposomes. At 24h post-injection, the level of GM₁-liposomes and free liposomes in liver was 25% and 75%, respectively (Allen and Chonn, 1987). This conclusion was supported by other research results (Gabizon and Papahadjopoulos, 1988a; Maruyama et al., 1990; Liu et al., 1990, 1991). Allen had also examined the role of several other gangliosides and glycolipids, but none could substitute for GM₁ in their ability to prolong circulatory life (Allen et al., 1987, 1989b, 1991a). The increase in circulation time of liposomes appeared to be unique to GM₁. Shortening the four
carbohydrate chain of GM$_1$ to GM$_2$ and GM$_3$, it eliminated the effect on circulation times, as did removal of the sialic acid groups to give ASGM$_1$ (Allen et al., 1987, 1989a). However, the requirement on the purity of GM$_1$ prevented its further application since it is difficult to purify to drug standards (Allen, 1994b).

Liu et al. (1992) have reported that biodistribution of GM$_1$-liposomes highly depended on their size. As the diameter of GM$_1$-liposomes was less than 70nm, they were rapidly removed from the circulation and mainly accumulated in the liver parenchymal cells. These results were in agreement with an earlier observation by Surolia and Bachhawat (1977, 1978) who had shown that GM$_1$-liposomes was taken up by the liver parenchymal cells via direct interaction with hepatic asialoganglioside receptors. If the size of GM$_1$-liposomes was more than 300nm, most of them accumulated in the spleen (Liu et al., 1991). The activity of GM$_1$ in prolonging half-life of liposomes was limited to a relatively narrow size range, i.e., 70 to 200nm. This conclusion is similar to the effect of size on PEG-liposome circulation time.

Litzinger and Huang (1992) have shown that the inclusion of 5 mol% of GM$_1$ within PC/Chol liposomes containing 34A-antibody enhanced the binding of these liposomes to lung endothelial cells. GM$_1$ has also been used to prolong the relatively plasma-stable acid-sensitive liposomes in the circulation (Liu and Huang, 1990). This made these acid-sensitive liposomes potentially useful as a cytoplasmic delivery vehicle for drugs under in vivo conditions. Prolongation of thermosensitive liposomes composed of DPPC/DSPC at 9:1 molar ratio has also been reported after incorporation of GM$_1$ (Maruyama et al., 1993).
A strong correlation between the residence time of GM$_1$-liposomes in blood and their uptake by tumours was observed. GM$_1$-liposomes preferentially accumulated in the tumour as compared to the rest of the body (Gabizon and Papahadjopoulos, 1988a; Huang et al., 1992a, 1992b; Maruyama et al., 1993). The survival time of mice treated by DOX-GM$_1$-liposomes was longer than traditional DOX-liposomes (Gabizon et al., 1988a, 1992a; Huang et al., 1992a, 1992b). The reason has been attributed to the efficient extravasation of GM$_1$-liposomes from the leaky capillary vessel walls of tumours because they had sufficient circulation time. Cullis et al. (1989) reported that GM$_1$ is incapable of inhibiting the ability of doxorubicin-loaded liposomes to impair or block RES function. It is concluded, therefore, that liposomes exhibiting extended circulation life could induce RES blockage and did not avoid uptake by liver phagocytes (Parr et al., 1993).

1.2.5 Mechanism stealth liposome behaviour

A vast amount of research work has been devoted to a better understanding of the mechanism of stealth liposomes (Senior, 1992; Lasic et al., 1991, 1992; Chonn and Cullis, 1992a; Woodle et al., 1992b, 1992c; Needham et al., 1992a, 1992b; Torchilin et al., 1993, 1994; Jones, 1995). A generally assumed mechanism is based upon (1) creating a neutral surface charge and hydrophilic coating which avoids opsonization by plasma proteins (Gabizon and Papahadjopoulos, 1992b; Woodle and Lasic, 1992b; Blume and Cevc, 1993a; Tan et al., 1993); (2) the formation of a sterical hindrance to reduce the absorption of plasma proteins (Blume and Cevc, 1993a; Woodle and Lasic, 1992b); (3) adsorption of some special proteins which provide a deopsonization for liposomes (Moghimi and Patel, 1988, 1989; Moghimi, 1993, 1998); (4) interference with the binding of liposomes to
receptors on RES cells (Mori et al., 1991; Klibanov et al., 1991).

1.2.5.1 Hydrophilicity and surface charge

Hydrophilicity was considered a main requirement for stealth liposomes in the beginning study (Illum, 1986), but it turned out not to be a sufficient one. Indeed, liposomes were coated with a series of hydrophilic polymers, they were still removed from blood circulation very rapidly in mice (Blume and Cevc, 1993a). Dextran-coated liposomes circulated more briefly than PEG-coated ones (Pain et al., 1984), in spite of the more hydrophilic nature of dextran compared to PEG. The zeta-potential of liposomes incorporating 5-10 mol% pf PEG<sub>5000</sub>-DSPE at pH 7.2 was measured at -3 to -4mV in the absence of serum and approximately -5mV in the presence of serum (Blume and Cevc, 1993a). The neutral surface charge of PEG liposomes was suggested to reduce the adsorption of serum proteins, and thus prolong the half-life of pegylated liposomes. Experiments performed by Senior (Senior et al., 1991) using a phase partitioning method indicated that several hours were required for PEG coated liposomes to adsorb significant amounts of proteins which were otherwise adsorbed to the surface of conventional liposomes within 1min. This conclusion was supported by other studies (Chonn et al., 1992b). Liposomes incorporating PEG-COOH-DSPE, which contains a terminal carboxylic group and therefore a terminal negative charge increased the half-life of liposomes, but not as long as those observed for PEG-lipid lacking the terminal COOH group (Blume and Cevc, 1992, 1993a).

1.2.5.2 Sterical hindrance
Besides hydrophilicity and uncharged surface, the property of a flexible PEG chain forming a dense polymeric "cloud" over the liposome surface was believed to be another major feature for the coating polymers to provide long-circulating vesicles (Blume and Cevc, 1992, 1993a; Torchilin et al., 1993, 1994; Lasic et al., 1991, 1992). Due to the transient, flexible and rapidly changing structure of PEG, the immune system would have difficulties in modelling an antibody around it (Woodle and Lasic, 1992b). Otherwise, the presence of a hydrophilic, inert, highly flexible polymer extending out a sufficient distance (5-6nm) from the liposomes surface in aqueous solution would result in a low attachment of proteins to the liposome surface. Blume and Cevc (1992) have shown a reduction in total protein binding of DSPC liposomes from approximately 170g/mol lipid in the absence of PEG<sub>900</sub>-DSPE to approximately 55g/mol lipid, and although their numbers are roughly 3-fold higher, the percentage reduction in binding is comparable to that of Chonn et al. (1992b). In the study of interaction between liposome coated gangliosides and proteins, it is clear that gangliosides reduced the leakage of aqueous-space markers from liposomes (Allen et al., 1985). The effect, which was common to all gangliosides, not just GM<sub>1</sub>, was attributed to an inhibition of binding of high-density lipoproteins to the liposome surface, and slowing down the lipoprotein-mediated disintegration of the liposomes. 2mol% of GM<sub>1</sub> in liposomes was effective in reducing the exchange/transfer of lipid out of liposomes (Allen et al., 1989c). Increasing the ratio of GM<sub>1</sub> in liposomes from 2 to 10mol%, the adsorption of proteins was reduced significantly from 36.4 to 13.0 g protein/mol lipid (Chonn et al., 1992). The significant reductions in the binding of complements C1q, IgG, IgM and apoA1 in liposomes composed of SM/PC/Chol/GM<sub>1</sub> (1:1:1:0.2, molar ratio) was found, as well as general reduction in levels of total bound proteins (Allen, 1994b, Chonn
et al., 1992).

The conditions that lead to protein repulsion from surfaces to which PEG chain was attached to one chain end in a “blush” conformation were studied (Jeon et al., 1991a, 1991b). These authors elaborated a mathematical model taking into account the four types of interactions between a protein and a hydrophobic substrate (Figure 1.6). Let D be the distance between the anchorage and the substrate of two terminally attached PEG chains. In the case of small proteins (approximated as spheres with a radius of 2nm), D should be around 1nm, whereas for larger proteins (6-8nm), D should be around 1.5nm. This means

![Diagram of PEG protection mechanism](image)

**Fig.1.6** Mechanism of “cloud” protection of PEG

1: Hydrophobic attraction between the protein and the substrate; 2: Steric repulsion resulting from PEG chain constriction; 3: Van der Waals attraction between the protein and the substrate; 4: Van der Waals attraction between the protein and the PEG chains (adapted from Jeon et al., 1991a).
that water-soluble flexible polymer statistically exists as a distribution ('cloud') of probable conformations. The polymer flexibility correlates with its ability to occupy with high frequency many cells in solution, temporarily squeezing water molecules out of them (i.e., making them impermeable for other solution). To reach the liposome surface, protein molecules have to penetrate the whole cloud, forced by the liposome-attached polymer molecules. From the computer analysis it follows that a flexible polymer forms the conformational cloud with very high density in its central part, whereas a rigid polymer of the same length (its segment was conditionally assumed to be five times longer than for the flexible polymer) forms a broad, but loose and thus permeable, cloud. Thus a relatively small number of water-soluble and very flexible polymer molecules can create sufficient numbers of high-density conformational 'clouds' over the liposome surface, protecting the latter from being opsonized and recognized by RES cells. A rigid polymer of even good water solubility and hydrophilicity may not provide sufficient protection for the liposome surface. The number of possible conformations for such polymers is lower; besides, the conformational transition proceeds with a slower rate than those of flexible polymers. It means that the density of the conformational 'cloud' for a rigid polymer will be very uneven during a single collision act, and the number of water molecular disturbed much smaller.

In terms of the 'cellular' model, it appears that there should exist a sufficient water space through which the minimal diffusion of plasma proteins towards the liposome surface is still possible. Thus, to protect liposomes one has to bind a much larger number of rigid polymer molecules on the liposome surface. This probably can explain the difference of PEG with other hydrophilic polymers such as dextra in sterically stabilizing liposomes. Dextran with insufficiently mobile properties cannot transfer from one conformation to
another, and that prevents it achieving the optimum action to increase the retention time of liposomes to a large degree.

1.2.5.3 Dysopsonins

From biological considerations, it has been suggested that both the reduction in the adsorption of opsonins and the selective adsorption of certain plasma components (dysopsonins) prevent the recognition and uptake of stealth liposomes by macrophages; the competition between these two mechanisms is believed to be the key in controlling the vesicle uptake by macrophages, and hence their biodistribution (Moghimi et al, 1988, 1989, 1993, 1998). Senior et al. (1991, 1992) suggested that two serum components (one with a molecular weight below 30,000Da and the other with a molecular weight higher than 100,000 Da) are the principal factors which result in a dysopsonic action. Park and co-workers (1992) reported liposomes having aliphatic dicarboxylic acids coupled to DOPE at their surfaces presented a strong dependence of circulation time on the chain length of the DOPE derivatives. They speculated that one possible mechanism for the observed effects for the intermediate length chains would be the adsorption of dysopsonins to the liposome surface which might require that terminal carboxylic groups be located at a specific distance from the liposome surface. Park and Huang (1993) chemically modified GM₁ and synthesized various neoglycolipid analogs of GM₁ in order to study the effect of carbohydrate chains of GM₁ on the circulatory time of liposomes. They found that the negatively charged carboxyl group of GM₁ is not critical to prolong the circulation time of liposomes and the polyhydroxyl side chain of the sialic acid is much more important than the carboxyl group. While GM₁ analogs lost the binding activity for cholera toxin,
it was incapable of prolonging the circulatory life of liposomes. It appears that GM₃-
specific protein(s) might bind to GM₃ in manner similar to that of cholera toxin. Binding
of these GM₃-specific proteins to liposomes should lead to a reduced level of opsonization
and / or inactivation of bound opsonin(s), and resulted in a reduced uptake by the RES.
Generally, the interactions between injected vesicles and blood components are complex.
These compounds may reversibly or irreversibly adsorb on the surface of the liposomes,
and may be replaced by others. The liver-perfusion study has revealed that the different
clearance rates for liposomes with and without blood components (Liu et al.,1995, 1996b).
Although it is not known which protein plays a key role, the interaction between vesicles
and blood proteins is confirmed. The liposome behaviour in the circulation changes with
the environment inside body.

1.2.5.4 Receptors

PEG or GM₃ liposomes may change the binding behaviour of liposomes to receptors on the
RES. The receptors involved in liposome binding include the C3 receptor (alternative
complement pathway), the Fc receptor (classical complement pathway) and the scavenger
receptor (poly-anions, negatively charged phospholipids?) (Allen, 1994b). The removal of
PEG liposomes from circulation may occur by the same mechanism as the removal of
conventional liposomes, as a result of the gradual removal of PEG from the liposome
surface and their subsequent opsonization (Allen, 1992, 1994b). Mori et al. (1991) and
Klibanov et al. (1991) showed that PEG₅₀₀₀ containing liposomes, also containing biotin-
cap-PE, could not recognize their “receptor” strepavidin. As PEG is gradually lost from the
liposome surface, the binding would become less hindered. Blume and Cevc (1993a)
revealed that PEG liposomes need several hours to accumulate proteins on the surface. Once this happens, uptake of PEG liposomes by RES is triggered. GM₁ and PEG can overcome complement activation and rapid clearance by RES for liposomes containing the negatively charged lipids PG and PA, but not for liposomes with PS since the high affinity of PS to “receptor” is not hidden by GM₁ or PEG (Woodle et al., 1992a; Lasic et al., 1991; Allen et al., 1991a). The study of cell surface receptors involved in stealth liposomes is just in the beginning. The complex interrelationships between liposomes and these receptor are not very clear.

1.3 Liposomes coated with polysaccharides

In 1968, Ashell and Morell (1974) demonstrated for the first time that survival of the glycoprotein in the circulation depends on the terminal sugar of the oligosaccharide chain of the protein. The specific receptor exists on the hepatocyte surface for glycoproteins with a β-linked terminal galactose or N-acetylgalactosamine oligosaccharide. Another sugar-specific mechanism identified the presence of mannose receptors on the cells of the RES, including the liver sinusoidal (Kupffer) cells and alveolar macrophages (Stahl and Schlesinger, 1980). Therefore, incorporation of various glycosides onto the liposome surface probably permits the targeting of liposomes towards different types of liver cell. (Bachhawat, 1974; Surolia et al., 1975, 1978). A recent review about the carbohydrate-mediated liposomal targeting was reported by Jones (1994).

Initial studies on GM₁-liposomes interaction with the galactose-binding Ricinus Communis Lectin (RCA₁) showed that the combination was markedly affected by the
surface density of the sugar residues and lengths of the surface-bound oligosaccharide chains. The binding of liposome-associated GM₁ to RCA₁ was 100 to 1000 times higher than those of free galactose and p-nitro-β-D galactopyranoside (Surolia and Bachhawat, 1977). Furthermore different glycoside residues on the surface of liposomes and on their uptake by various organs was investigated (Ghosh et al., 1980, 1981, 1982), eg. asialoganglioside, galactoyl cerebrosides, p-aminophenyl derivatives of β-galactoside, α-mannoside, and β-N-acetylglucosaminides. It was found that both β-galactosyl- and α-mannosyl-bearing liposomes were removed rapidly from the circulation and taken up by the liver. The uptake of liposomes containing β-galactoside on the surface by hepatocytes was three-fold higher than that by nonparenchymal cells, whereas the uptake by nonparenchymal cells was seven-fold higher from liposomes having α-mannoside on their surface. The observations on galactose- and mannose-specific uptake of liposomes by the hepatocytes and nonparenchymal cells, respectively, have since been confirmed in a number of laboratories both in vivo (Szoka et al., 1983; Gregoriadis and Senior, 1984; Dragsten et al., 1987) and in vitro (Barratt et al., 1986; Haensler and Schuber, 1988; Muller and Schuber, 1989).

Upon application polysaccharides as ligand to target to liver cells, some of the studies revealed that carbohydrates attached to the liposome surface could increase the circulation time (Szoka and Mayhew, 1983; Spanjer and Scherphof, 1983; Gregoriadis and Senior, 1984). Till now, dextran was used mostly to prolong circulation time of liposomes. Attachment of dextran to drugs and enzymes has shown that not only does it confer chemical and biological stability but also increases their circulatory lifetimes (Marshall et
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al., 1977; Yasuda et al., 1990). However, the stability, biological properties, and circulation times of these conjugates highly depended on the size and charge of dextran and the mode of conjugation (Fujita et al., 1991). Conjugation of dextran onto the surface of PE-liposomes by the cyanogen bromide activation method (Pain et al., 1984) significantly prolonged their circulation time and decreased levels of liposomal accumulation in the liver. The rate of clearance of dextran-coated liposomes from the circulation was found to be dependent on the density of dextran molecules on the surface. At 18 mol% dextran on the surface, 50% of the injected dose remained in the circulation, which is 2.5-fold higher than the liposomal formulation without dextran. The function of dextran on liposomes was not very clear. It may result from increasing hydrophilicity on the surface of liposomes and reducing binding of plasma proteins, which are responsible for rapid removal of liposomes from the circulation.

Other polysaccharides such as o-palmitoylamylopectin, o-palmitoyl mannan (Takada et al., 1980), and o-palmitoyl pullulan (Sunamoto et al., 1986, 1987) have not enhanced the circulation time of liposomes. But o-palmitoyl pullulan for binding of cell-specific antibody on the liposomes resulted in effective transport of drug to specific cell.

1.4 Polysialic acid and sialic acid

Sialic acid (5-amino-3,5-dideoxy-2-nonulosonic acid) (Figure 1.7) was found in many glycoproteins and glycolipids. It is interesting that the sialic acid is often at the terminal of polysaccharides and oligo-saccharides, which may contribute to some special functions. Actually, from the affinity of sialic acid to viruses, it is revealed that sialic acid is a very
important regulator of cellular and molecular interactions in binding of both pathogens and nonpathological cellular to host cells (Schauer, 1995, 1997).

![Structure of sialic acid](image)

**Fig. 1.7 Structure of sialic acid**

The functions of sialic acid are summarised as following: (1) Sialic acid contributes significantly to the overall negative charge of cell surfaces, and glycoproteins, which may be one of the reasons of antiadhesion. The electrical repulsion stabilizes cells and also protects proteins from uptake by RES (Morell et al., 1971; Schauer et al., 1984a, 1984b, 1990, 1995, 1997; Patel et al., 1995); (2) Sialic acid masks recognition sites which was demonstrated in many studies, e.g. desialylated erythrocytes are rapidly cleared from the bloodstream (Jancik et al., 1974, 1975), as well as lymphocytes (Kaufman et al., 1981). Further experiments showed the binding of desialylated erythrocytes to macrophages in liver and spleen (Jancik et al., 1978); (3) Sialic acid residues on the cell surface are considered to be the primary receptors for influenza viruses (Paulson, 1985; Paul et al., 1989). An indirect role for sialic acid in helping sialoglycoproteins to attain a critical structure has been proposed (Suzuki et al., 1985, 1994).

Polysialic acid is a structurally diverse family of linear carbohydrate chains that consist of N-acetylneuraminic acid or N-glycolylneuraminic acid residues, usually joined internally
by α 2,8-, α 2,9-, or alternating α 2,8-/α 2,9-ketosidic linkages (Figure 1.8). Summaries of the occurrence and structure of polysialic acid containing glycoconjugates have been published (Tory, 1990, 1992; Cho and Tory, 1994). The existence of polysialic acid in bacterial capsular (McGuire and Binkley, 1964; Rohr and Tory, 1980), fish eggs (Inoue and Iwasaki, 1978), embryonic neural membrane and extraneural cells and tissues (Bock et al., 1980; Roth et al., 1987), and tumours (Glick et al., 1991; Tory et al., 1992) has been found. The polysialic acid capsule on *Escherichia coli K1* and *Neisseria meningitidis* group *B* and *C* is a neurovirulent determinant associated with neonatal meningitis in humans (Tory, 1979). Expression of the polysialic acid capsulate appears to help these neuropathogens to penetrate the blood-brain barrier and colonize the meninges of neonates (Silver and Vimir, 1990) since there may be specific polysialic acid receptors in the brain (Kim et al., 1992). The polysialic acid chains may also enhance the virulence of encapsulated cells by masking the somatic O-antigen and preventing it from detection by the immune system (Tory, 1995). Accordingly, polysialic acid on neuroinvasive bacteria and tumour cells probably is an elaborate survival mechanism that evolved to trick the human immune detection. α 2,8- but not α 2,9- polysialic acid is a poor immunogen because of its immune tolerance which results in difficulties in the development of an effective vaccine to protect against *E. coli K1* and *N. meningitidis* serogroup *B* (Finne et al., 1983). The study of polysialic acid on fish egg such as lake trout eggs, rainbow trout eggs revealed that these molecules related to the function of cellular processes, including species-specific cell-cell recognition during fertilization and early embryogenesis (Inoue, 1993), and in providing the egg with a protective barrier (Inoue et al., 1991). α 2,8-
Chapter 1 Introduction

Structure of the meningococcal group B capsular polysaccharide (PSB) depicting the terminal acid residues, (2-8)-linkage N-neuraminic acid, average unit n=199, from N. meningitidis or E. coli K1.

Seroogroup C capsular polysialic acid (PSC) from N. meningitidis C 2-9 linkage, average unit n=74

Polysialic acid (PSK92) from E. coli K92, heteropolymer of alternate (2-8), (2-9) linkage, average unit=78

Fig. 1.8 Structure of polysialic acid
polysialic acids of different chain lengths have been demonstrated to cap N-linked oligosaccharides on neural cell adhesion molecules (N-CAMs). The function of polysialic acid was associated with normal morphogenesis and neural development (Rutishauser, 1989). The removal of polysialic acid from the surface of a neuroblastoma/sensory neuron cell hybrid increased cell-substrate adhesion (Rutishauser, 1989). The polyanionic shield provided by polysialic acid may increase the intercellular space between cells (Yang et al., 1992). The first human tumours shown to express α2,8-polysialylated NCAMs were neuroblastomas (Livingston et al., 1988) and nephroblastomas (Roth et al., 1988). The role of polysialic acid on tumours could be analogous to neutral cell adhesion molecules which contain longer or shorter polymers of sialic acid, respectively. Possibly polysialic acid on the surface of malignant tumours alters normal cell to cell interactions and processes, and may raise the invasive potential (Livingston et al., 1988). The observation of long chain polysialic acid on human neuroblastomas suggested that polysialic acid was a codevelopmental antigen in brain, since the embryonic form of N-CAMs was less frequently expressed on adult brain tissue (Glick et al., 1991). Polysialylated N-CAMs may be used as a molecular marker for assessing the metastatic potential of adult head and neck tumours (Scott et al., 1994).

Gregoriadis et al. (1993d) indicated that polysialic acid B is very stable with a half-life up to 40h. The retention time of polysialic acid depends on the chain length. Fernandes and Gregoriadis (1996, 1997) demonstrated the action of polysialic acid on enhancing enzyme stability in the blood. Actually, a lot of functions of sialic acid and polysialic acid are still not very clear.
1.5 Experiment of design

These studies are designed to use polysialic acid as a kind of new polymers for modifying liposomes and enhancing the half-life of liposomes in the circulation. The reasons for choosing polysialic acid are based on following views: Firstly, polysialic acid B has been demonstrated to have a long retention time in the mouse circulation from our previous studies (Gregoriadis et al., 1993d). It is also believed that sialic acid may impart a special protection on the cell surface (Schauer, 1995). When sialic acid was removed from the surface of erythrocytes, or from liposomes containing GM₁, the cells and liposomes were rapidly recognized by RES and removed from the circulation. Thus, the presence of surface sialic acid is a clear determining factor in the avoidance of RES uptake (Allen and Chonn, 1987). Otherwise, polysialic acid B was found to have poor immunogenity for humans (Wyle et al., 1972) and polysialic acid A and C are relatively poor immunogens in very young infants (Gold et al., 1977). Those conclusions suggest that polysialic acid may help liposomes to avoid recognition by the RES when it is conjugated onto the liposome surface. On the other hand, the cell membrane is always shielded by different types of carbohydrate. Although we still do not exactly know the action of carbohydrates on the cell surface, it is assumed that carbohydrates provide some protection and reduce the adhesion among cells. However, the data on stealth liposomes coated with either PEG or GM₁ all confirm the importance of sterical stabilization. The formation of a “cloud” or “brush” on the liposome surface by coating with a flexible polymer seems to be an efficient approach to prevent the uptake of liposomes by macrophages. Therefore, incorporation of polysialic acid into liposomes is supposed to be more advantageous since it may provide both sterical protection and special biofunctions.
A derivative of polysialic acid B, named colominic acid (CA) with molecular weight around 15,000-18,000 was employed in this study because it is commercially available from the Sigma company. Polysaccharides are usually believed to be safe, to have excellent biocompatibility and be biodegradable, but data related to the behaviour of colominic acid in vivo have not been found. We firstly investigated the interaction between colominic acid and blood cells and the rapid toxicity of colominic acid to mice during intravenous injection. The stability of colominic acid under heating and different pHs was studied as well.

For inhibiting the rapid uptake of liposomes by RES, colominic acid must be incorporated into the lipid bilayer and be stable on the exterior of vesicles. Colominic acid itself cannot be inserted into lipids by the usual technology because of its high hydrophilicity. Thus, we previously tried to couple lipids with colominic acid, and then introduced the conjugates into liposomes. Another method used was directly connecting colominic acid to the surface of liposomes by covalent conjugation. Few reference has been published on the chemical modification of polysialic acid. Jennings and co-workers (1981) indicated that oxidized polysialic acid can be conjugated with proteins by the Schiff base. Mattews and Petrak (1993) described the conjugation of DPPE with colominic acid by a thiol bond. In these studies, two chemical conjugations were used depending on the structure of colominic acid and lipids. Colominic acid was oxidized to form the -CHO group at a non-reducing end, and thus it reacted with PE-NH₂. The resulting products were linear polymers. Colominic acid was also coupled with PE in the presence of carbodiimide to form branched polymers. The effects of reaction conditions on the yield and the purification process were
evaluated. In the investigation of the action of colominic acid on liposomes, a series of studies in vitro such as stability study, releasing of entrapped materials and surface charge were conducted. Liposomes containing colominic acid (polysialylated liposomes) were injected into T.O. mice and the retention of liposomes in the circulation was detected at time intervals.

Since we tried to understand why and how colominic acid affects the half-life of liposomes, sialic acid, a monosaccharide, was introduced in the study. Sialic acid was conjugated with PE, and then incorporated into liposomes. The effects of sialic acid on the half-life of liposomes in vivo were investigated. The results of sialylated liposomes were compared with those of polysialylated liposomes.

The behaviour of polysialylated liposomes exposed to the medium of biology are relatively complicated. From this research we will only understand some of the characteristics of colominic acid. The results of liposomes coated with colominic acid may offer some information, which will help us to look forward to the role of polysialic acid action on the liposome surface, and subsequently infer further applications of polysialic acid.
Chapter 2

Materials and Methods

2.1 Materials

Sodium borohydride ($\text{NaBH}_4$) ($\text{H}^3$) (5mCi, 13.7 GBq/mmol) and sodium borohydride ($\text{H}^3$) (25mCi, 37 GBq/mmol) were obtained from Du Pont NEN (London, UK). Acetic anhydride ($\text{C}^{14}$) (100μCi) was obtained from Sigma (London, UK). Colominic acid (CA) and sialic acid (SA) were obtained from Sigma (London, UK). Oxidized colominic acid (OX-CA) and hydrolysed colominic acid were prepared in the laboratory. Neuraminidase (5units) was purchased from Sigma (London, UK). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were obtained from Lipid Products. 1,2-Distearoyl-sn-glycero-3-phosphatidylcholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine (DSPE) were from Sygena Ltd (London, UK). Cholesterol (Chol) was obtained from Sigma (London, UK). HCl was obtained from Merck Ltd. (London, UK). 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC) was from Aldrich (London, UK). Tissue solubilizer was from Amersham (London, UK). The following additional chemicals were obtained from Sigma (London, UK): cupric sulfate ($\text{CuSO}_4$); sodium periodate ($\text{NaIO}_4$); 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC); N,N'-dimethyl aminopyridine (DMAP); 1,4-dioxane; tetrahydrofuran (THF); N, N'-dimethyl formamide (DMF); dimethyl sulfoxide (DMSO); hydrogen peroxide ($\text{H}_2\text{O}_2$); fluorescein isothiocyanate (FITC); 5(6)-carboxyfluorescein (CF); sodium cyanoborohydride ($\text{NaBH}_3\text{CN}$); human serum albumin (HSA); resorcinol; NaHCO$_3$; sodium borohydride ($\text{NaBH}_4$); toluene; monosodium phosphate (NaH$_2$PO$_4$); disodium phosphate(Na$_2$HPO$_4$); chloroform (CHCl$_3$); methanol (MeOH). N, N'-dicyclohexylcarbodiimide (DCC); tetrabutyl ammonium (TTAN); molybdenum blue; glycine; fluorescamine; sucrose.
Filtration gels including Sephadex G-10, G-25, Sepharose CL-4B and CL-2B were products of Pharmacia (London, UK). Dialysis tubing (8000 molecular weight cut-off) was obtained from Merck Ltd. (London, UK). Silica acid (particle size 230-400 mesh) and thin layer chromatography (TLC) plates (silica gel) were from Sigma (London, UK).

2.2 Methods

2.2.1 Radiolabelling of colominic acid

2.2.1.1 Tritiation of colominic acid with sodium borohydride (NaBH₄) (H³)

100µg of colominic acid or oxidized colominic acid in 100µl of distilled water were treated with 10µl of NaBH₄ (H³) (2.92µCi/µl in 0.1M NaOH) in a water bath (30 °C) for 24h. After the reaction had been finished, 50µl of acetic acid (0.1M) were added into the mixture to destroy the unreacted NaBH₄ (H³). The solution was then transferred into a dialysis tube and dialysed against distilled water for 24h. For detecting the purity of samples, 10µl of tritiated colominic acid were passed through a Sephadex G-25 column (1cm x 20cm) and washed with distilled water or phosphate buffer saline (PBS) (pH 7.4). The fractions (100µl) were counted in 4ml of scintillation liquid on a Liquid Scintillation Counter (Wallac 1409).

2.2.1.2 Tritiation of reduced colominic acid with NaBH₄ (H³)

200mg of colominic acid were reacted with NaBH₄ (0.1M, 2ml) at room temperature (20 °C) for 2h to form an opening ring at the reducing end. After that, free NaBH₄ was removed
Chapter 2 Materials and Methods

from the mixture solution by passing through a Sephadex G-25 column (2.5cm x 30cm). Reduced colominic acid was then lyophilized by freeze-drying.

Reduced colominic acid in 1ml of PBS was mixed with NaIO₄ (0.1M, 2ml). The reaction was carried out at room temperature in the dark for 15min. Free NaIO₄ was immediately removed from the mixture by passing it through a Sephadex G-25 column (2.5cm x 30cm). The oxidized colominic acid (CHO-CA-CHO) was collected and freeze-dried overnight.

100mg of CHO-CA-CHO in 1ml of PBS were reacted with NaBH₄ (H³) (50μl, 1.8μCi/10μl) at room temperature for 2h. Unreacted NaBH₄ (H³) was separated by dialysing against distilled water for 24h, and the solution was concentrated by freeze-drying. Further purification of radiolabelled CHO-CA-CHO was carried out by passing through a Sephadex G-25 column (2.5cm x 30cm). The fractions of the void volume were collected and lyophilized.

2.2.1.3 Radiolabelling of colominic acid with acetic anhydride (Ac₂O) (C¹⁴)

2.2.1.3.a Radiolabelling of colominic acid by the O-acetylation reaction

20mg of colominic acid were suspended in 50ml of anhydrous toluene. Then, 500μl of acetic anhydride (C¹⁴) (500μl, 25μCi) were added dropwise into the mixture. The reaction was conducted at room temperature for 12h, and subsequently refluxed for 0.5h. The solvent was removed by rotary evaporation. The colominic acid powder obtained was redissolved in 5ml of water. The mixture was firstly purified by dialysis, then passed
through a Sephadex G-10 column (1 x 20cm).

2.2.1.3.b Radiolabelling of hydrolysed colominic acid with acetic anhydride (Ac₂O)

(\(^{14}\text{C}\))

50mg of colominic acid were dissolved in 20ml of 4M NaOH solution, and the mixture was incubated at room temperature for 24h. After the reaction had been completed, the colominic acid solution was dialysed till no NaOH was detected. The samples were freeze-dried. 10mg of hydrolysed colominic acid were added into radiolabelled acetic anhydride (25\(\mu\)Ci) dissolved in 20ml of anhydrous toluene. The reaction was conducted at 115 °C for 0.5h. The solvent was then removed by rotary evaporation and the residue was redissolved in 1ml of distilled water. The free amino group of radiolabelled hydrolysed colominic acid was acetylated by mixing with 100\(\mu\)l of free acetic anhydride and reacting at room temperature for 24h.

2.2.1.3.c Radiolabelling of hydrolysed colominic acid in NaHCO₃ solution

Hydrolysed colominic acid (2.5mg) in 1ml of NaHCO₃ (5%w/v) was mixed with acetic anhydride (\(^{14}\text{C}\)) (50\(\mu\)Ci) which was dissolved in 1ml of methanol. The reaction was carried out at room temperature for 48h. Then, unreacted acetic anhydride was removed from the mixture by dialysis against distilled water.

2.2.2 Fluorescence marking of colominic acid

10mg of colominic acid dissolved in 1ml of dimethylsphoxide (DMSO) were mixed with 0.1mg of tetrabutyl ammonium (TTAN) and 0.2mg of fluorescein isothiocyanate (FITC).
The mixture was stirred at room temperature for 24h. After the reaction had been completed, uncoupled FITC was removed by passing through a Sephadex G-25 column. FITC-colominic acid was collected and freeze-dried.

2.2.3 Quantitation of colominic acid

2.2.3.1 Assay of colominic acid concentration

Resorcinol reagents: 0.1 M resorcinol in water and 0.1M CuSO$_4$ in water were prepared firstly. Then, 20ml of resorcinol solution were mixed with 80ml of concentrated hydrochloric acid and 250μl of CuSO$_4$ solution. The reagent was stored in a brown bottle.

4-30μg of colominic acid dissolved in distilled water solution were mixed with 500μl of resorcinol reagent, and the final volume was adjusted to 1ml by addition of distilled water. The mixture was placed in a 100 °C water bath for 30min, then cooled to room temperature. The solution was purple in colour. Adsorption measurements at 570nm were carried out in an UV-visible spectrophotometer (Wallac).

2.2.3.2 Quantitation of amino groups

Quantitation of amino groups of the hydrolysed colominic acid was carried out by a fluorescence assay using glycine as a standard. 30mg of glycine in 1ml distilled water and 7.2mg of fluorescamine in 24ml dioxan were prepared previously. 10-200μl of the glycine solution were then mixed with 500μl of the fluorescamine solution and 1.75ml PBS. Fluorescence at 390nm (excitation wavelength) and 475nm (emission wavelength) was
measured immediately. A calibration curve of glycine was used as a standard for the estimation of the amino groups of hydrolysed colominic acid (Figure 2.1). 0.1-0.5ml of hydrolysed colominic acid solution (10mg/ml) were mixed with 500μl of the fluorescamine solution and 1.75ml of PBS and measured by the same method.

\[
y = 1.948x + 2.146 \quad r^2=0.9957
\]

![Figure 2.1 Standard curve of glycine in 500μl of fluorescamine](excitation wavelength (Ex) set at 390nm, emission wavelength (Em) set at 475nm)

2.2.4 Stability of colominic acid

2.2.4.1 Degradation of colominic acid by neuraminidase

10mg of colominic acid and 10μl of tritiated colominic acid were incubated with neuraminidase (2 units) in sodium acetic buffer (pH 5.5) at 37 °C for 2h. The solution was then detected by passing through a Sephadex G-25 column (1cm x 20cm) and compared with the behaviour of N-acetyl neuraminic acid by gel filtration as above.
2.2.4.2 Degradation of colominic acid under different pH conditions

10mg of colominic acid mixed with 10μl of tritiated colominic acid were dissolved in 1ml of PBS (pH 7.4), 0.1M NaH₂PO₄ (pH 4.7), 0.1M Na₂HPO₄ (pH 9.0) and 0.1M acetic acid and incubated at 37°C for 24h and 7 days. Depolymerization of colominic acid was detected by passing through a Sephadex G-25 column (1cm x 20cm).

2.2.4.3 Degradation of colominic acid by mouse plasma

100μg of colominic acid and 10μl of tritiated colominic acid were mixed with 100μl of fresh mouse plasma and incubated at 37°C for 24h. The degradation of colominic acid was detected by passing through a Sephadex G-25 column (1cm x 20cm).

2.2.4.4 Degradation of colominic acid under heating

10mg of colominic acid in 1ml of distilled water were heated at 100°C for 1, 3 and 9h. Then, 100μg of hydrolysed colominic acid were labelled with NaBH₄ (H³). The radioactivity per μg of colominic acid was assayed by passing through a Sepharose CL-4B column (1cm x 20cm).

2.2.4.5 Toxicity of colominic acid to blood cells

10-2500μg of colominic acid in 990μl saline were mixed with 10μl of fresh mouse whole blood. The morphology of blood cells was observed by light microscopy. 2ml of mouse blood were centrifuged at 3000rpm for 10min to spin down the blood cells. The blood cells were then suspended in 5ml of saline. 200μl samples of blood cells were mixed with 10-5000μg of colominic acid, and the final volume was adjusted to 500μl with 0.9% NaCl.
(saline). After incubation at 37°C for 1 and 3h respectively, the haemolysis of blood cells was evaluated by measuring the supernatant in a visible spectrophotometer at 576nm. In control experiments the same volume of blood cells in saline was incubated in the absence of colominic acid. Total release of haemoglobin from red cells was achieved by addition of 10μl of triton X-100 (10%) into 200μl of blood cell suspension.

2.2.4.6 Blood clearance and tissue distribution of colominic acid

5mg (20,000dpm) or 10mg (32,000dpm) of tritiated colominic acid in 200μl of PBS were intravenously injected into the tail vein of TO mice (three mice in each group). 50μl blood samples were taken at time intervals and suspended in 0.5ml of PBS. The plasma was isolated with blood cells by centrifugation at 2500rpm for 5min, and then counted in 4ml of scintillation liquid. Colominic acid remaining in the circulation was calculated from the following equation.

\[
\text{% of injected dose} = \frac{R_{50\mu l}}{R_{\text{total}}} \times \frac{1}{V_{\text{blood}}} \times 50 \times 100\%
\]

[2-1]

\(R_{50\mu l}\): Radioactivity of 50μl blood sample.
\(R_{\text{total}}\): Radioactivity of total injected colominic acid.
\(V_{\text{blood}}\): Total blood volume of each mouse (μl) (7.5% of body weight).

The mice were killed by dislocation of the neck 3h after injection. The organs, including liver, spleen, heart, lung and kidney were taken out, washed with saline, homogenized in 2 ml of saline and digested by the addition of 200μl of tissue solubilizer. The radioactivity
of colominic acid in the tissue was counted and calculated as a percentage of the injected dose.

2.2.5 Chemical modification of colominic acid and sialic acid

2.2.5.1 Conjugation of PE with colominic acid by the Schiff reaction

2.2.5.1.a Oxidation of colominic acid with sodium periodate

10mg of colominic acid were mixed with 1ml of 0.1M sodium periodate (NaIO₄), and then kept in the dark at room temperature for 15min. Subsequently, 0.1 ml of ethylene glycol were added into the mixture to stop the reaction. The mixture was passed through a Sephadex G-25 column (1 x 20cm), and the fractions of the void volume were collected. Oxidized colominic acid (OX-CA) was freeze-dried overnight.

2.2.5.1.b Radiolabelling of oxidized colominic acid (OX-CA) with sodium borohydride (H³)

100μg of OX-CA in 100μl distilled water were mixed with 20μl of tritiated NaBH₄ (2.92μCi/μl in 0.1M NaOH). The mixture was left at 30 °C for 12h, and then diluted with distilled water to 1ml. 10μl samples were passed through a Sepharose CL-4B column (1 x 20cm). The fractions were measured in a scintillation counter. The ratio of CHO to colominic acid was calculated as follows:
\[
\frac{R_{OX}}{R_{CA}} \quad [2-2]
\]

- \( R_{OX} \): Radioactivity of OX-CA / \( \mu g \)
- \( R_{CA} \): Radioactivity of CA / \( \mu g \)

### 2.2.5.1. Conjugation of colominic acid with PE by the Schiff reaction (PE-OX-CA)

10mg of OX-CA in 1ml of 0.1M \( \text{Na}_2\text{HPO}_4 \) solution were mixed with 2.5mg PE and trace amount of PE-C\(^{14} \) as indicator dissolved in 2ml of THF. The reaction was kept at the room temperature for 1-3 days under the protection of nitrogen gas. Then, 1ml of 0.1M \( \text{NaBH}_3\text{CN} \) was added into the mixture and the reaction was carried out at room temperature for 12h. Subsequently, THF was removed from the mixture by rotary evaporation, and the solution was lyophilized. The residue was redissolved in 1ml of water. After separation of the precipitate by centrifugation (50000rpm(170300g)/30min), 20\( \mu l \) of supernatant were passed through a Sephadex G-25 column (1 x 20cm). The lipid (PE) precipitate was washed with water three times and dried again. Both lipid precipitate and water soluble substances were analysed by thin layer chromatography (TLC) plates using CHCl\(_3\)/MeOH (7:3, v/v) as the elution solvent and molybdenum blue or iodine as indicator. Further analysis of the conjugation products by NMR and UV-visible spectrometer was conducted. The conjugation ratio between the lipids and colominic acid was calculated by the following equation:

\[
\frac{C_w}{C_t} \times 100\% \quad [2-3]
\]

- \( C_w \): \( C^{14} \) in water solution
- \( C_t \): Total \( C^{14} \) in the reaction
Conjugation of colominic acid (\(\%\)) = \(\frac{W_{PE}}{W_{CA}} \times 100\%\)  \[2-4\]

\(W_{PE}\): Conjugated PE (\(\mu\)mol)
\(W_{CA}\): Total colominic acid (\(\mu\)mol)

### 2.2.5.2 Conjugation of colominic acid with PE in the presence of DCC catalyst (PE-CA)

17.7 mg of colominic acid (56.6 \(\mu\)mol of COOH) in 1 ml of water and 20 mg of PE (28.3 \(\mu\)mol of \(\text{NH}_2\)) in 2 ml of THF were mixed together under magnetic stirring. 42.9 \(\mu\)mol of catalyst DCC (or CMC, EDAC) and 1 mg of DMAP in 1 ml of THF were added into the mixture. The reaction was carried out at room temperature for 24 h under the protection of nitrogen gas. Then, the mixture was left at 4°C overnight. The precipitate produced in the solution was filtered out. THF was evaporated by rotary evaporation. The residues were freeze-dried overnight, and then redissolved in 5 ml of anhydrous chloroform to produce two fractions: precipitate (A) and solution (B). The precipitate (A), which consisted of free colominic acid, less conjugated colominic acid and catalysts as using water soluble catalysts, eg. CMC or EDAC were isolated from chloroform solution (B) by filtration. In the organic phase (B), there were CA-PE conjugates, free DCC and free PE. Addition of 1 ml of water into A led to the precipitation of the DCC derivatives from the mixture. Solution A was centrifuged at 3000 rpm for 10 min to separate the clear supernatant. 100 \(\mu\)l of supernatant(A) were counted by a \(\beta\)-counter to measure the unreacted colominic acid. The chloroform solution (B) was dried again and dissolved in 5 ml of anhydrous chloroform. 100 \(\mu\)l of B were counted in 4 ml scintillation liquid. Solution B was also
analysed by thin layer chromatography (TLC) plates (silica gel) using CHCl₃/MeOH (7:3, v/v) as mobile phase and molybdenum blue or iodine as indicator. Separation of colominic acid conjugates from free PE and catalyst was achieved by passing through a silica acid column (1x10cm) (silica acid particle size 230-400 mesh) with gradient washing (CHCl₃/MeOH 8:0, 8:0.5, 8:1, 8:1.5, 7:3, 5:5, 2:8, 0:8 v/v).

2.2.5.3 Chemical conjugation of PE with sialic acid (PE-SA)

2.2.5.3.a Synthesis of sialic acid-PE conjugated products and purification

20mg of PE (26.9µmol) dissolved in 2ml of THF and 11.5mg of sialic acid (SA) (36.9µmol) in 1ml of distilled water were mixed together, then maintained in a small sealed bottle under the protection of nitrogen gas. 7.6mg of DCC (36.9µmol) and 1mg of DMAP were added into the mixture, and the reaction was carried out under stirring for 2h at room temperature. After the reaction had been completed, the solvent was removed by rotary evaporation. The residues were dissolved in 5ml of acetone to produce a precipitate and a soluble substance in acetone. The precipitate including free sialic acid, unreacted PE and side products of DCC were fileted out. The acetone solution was subsequently purified by passing through silica gel (1x10cm) (silica acid particle size 230-400 mesh) with gradient washing (CHCl₃/MeOH 8:0, 8:0.5, 8:1, 8:1.5, 8:2, and 7:3, v/v).

2.2.5.3.b Reaction rates

20 mg of PE (26.9µmol) and 11.5 mg of sialic acid (36.9µmol) were mixed together and left at 30 °C in a water bath. The unreacted sialic acid in the mixture was detected by the
resorcinol method at 2, 6 and 12h since conjugated sialic acid loses its reducing property and can not react with the resorcinol reagent. The conjugated sialic acid in the mixture was calculated as follows:

\[
\text{Conjugated sialic acid (\%) = } \frac{W_t - W_u}{W_t} \times 100\% \quad [2-5]
\]

\(W_t\): Total sialic acid  
\(W_u\): Unreacted sialic acid

2.2.5.3.c Analysis of the conjugated product

PE-SA conjugated products were identified by NMR and UV. During the reaction, trace amounts of \(^{14}\text{C}\)-labelled sialic acid (50\(\mu\)l, 1687900dpm) or \(^{14}\text{C}\)-labelled PE (200\(\mu\)l, 129490dpm) were added into the mixture. After the samples were subjected to thin layer chromatography (TLC) and eluded with CHCl\(_3\)/MeOH (7:3 v/v), the spot which turned out to be blue by spraying with molybdenum blue was removed from the TLC plate, placed into a scintillation tube and counted in 4ml scintillation liquid.

2.2.5.4 Chemical conjugation of colominic acid with the liposome surface

Small unilamellar liposomes (SUV) composed of PC, PE and cholesterol (1:0.05:1.05, 1:0.1:1.1, 1:0.2:1.2, 1:0.3:1.3, molar ratios) (32\(\mu\)mol of PC) in 1ml of water solution were mixed with 10-20mg of OX-CA (or 10mg of hydrolysed OX-CA, Mn=7800) and trace amount of FITC-OX-CA or tritiated OX-CA in 1ml of 0.1M phosphate buffer (pH 9.0). The mixture was stirred under the protection of nitrogen at 30 \(^\circ\)C for 24-48h. Then, 1ml of 0.1M NaBH\(_3\)CN was added into the mixture and the reaction was carried out at room
temperature for 12h. Liposomes coupled with OX-CA were separated from unreacted OX-CA by centrifugation (50000rpm(170300g)/50min) (Sorvall COMBI PLUS) and washed three times by the addition of 2ml of PBS. Conjugation of OX-CA on the liposome surface was calculated by the measurement of tritium or fluorescence in the supernatant and pellets.

2.2.6 Preparation of liposomes

2.2.6.1 Preparation of PC/PE/Chol SUV liposomes

PC, PE and cholesterol (64μmol of PC) (1:0.1:1.1; 1:0.2:1.2; 1:0.3:1.3, molar ratios) mixed with tritiated PC were dissolved in 2ml chloroform, then dried to form a thin film on the wall of a round bottom flask by rotary evaporation. The lipid film was rehydrated in 2ml of distilled water and the suspension was sonicated in a Ultra-sonic sonicator (Soniprep 150 (MSE) Sanyo) till the vesicle size was around 100nm-200nm. Large particles were removed from the suspension by centrifugation at 20000rpm (27300g) for 10min (Sorvall COMBI PLUS).

2.2.6.2 Incorporation of PE-OX-CA into MLV and SUV liposomes

Water-soluble PE-OX-CA linear products (1-10mg) mixed with C\(^{14}\)-PE-OX-CA or tritiated PE-OX-CA were dissolved in 2ml of distilled water. PC (32μmol) with equimolar cholesterol was dried by rotary evaporation to form a thin film on the flask wall. Afterwards, the PE-OX-CA solution was added into the flask. The lipid film was rehydrated by vortexing and kept at room temperature for 0.5h to form multilamellar vesicles (MLV). MLV liposomes were sonicated to produce SUV liposomes.
liposomes were isolated from free PE-OX-CA by ultracentrifugation at 50000rpm (170300g) for 50min. The radioactivity of PE-C\(^{14}\) in the liposome pellets was counted and calculated as follows:

\[
\text{PE-OX-CA (\%) = } \frac{C}{C_t} \times 100\% \quad [2-6]
\]

C: Radioactivity of PE-OX-CA in pellet  
C\(_t\): Radioactivity of total PE-OX-CA

### 2.2.6.3 Incorporation of PE-OX-CA into DRV liposomes

PC (32\(\mu\)mol of PC) and equimolar cholesterol dissolved in CHCl\(_3\) were dried by rotary evaporation to form a thin film on a round bottom flask. The lipid film was rehydrated as above to produce MLV liposomes, which were then sonicated till the particle size was around 100nm. 10mg of PE-OX-CA were added into the SUV liposomes, and the mixture was left at room temperature for 0.5h. The mixture was then freeze-dried overnight. The dry powder was rehydrated with 0.1ml of water allowed to stay at room temperature for 30min and 0.9ml PBS were added into the mixture to form DRV liposomes. After separation of DRV liposomes from free PE-OX-CA by centrifugation as above, the incorporation of PE-OX-CA in liposomes was measured and calculated using equation [2-6].

### 2.2.6.4 Incorporation of PE-CA into liposomes

1-4mg of PE-CA which were doubly labelled with tritium on the CA and C\(^{14}\) on the PE
were dissolved in chloroform and mixed with PC (32μmol) and equimolar cholesterol. The methods used in the preparation of MLV, DRV and SUV liposomes were those used in 2.2.6.1 and 2.2.6.3. The incorporation of PE-CA in liposomes was calculated as follows:

\[
\text{Incorporation of PE-CA ( \%) } = \frac{R_{CA}}{R_{total}} \times 100\% \quad [2-7]
\]

\(R_{CA}\): Radioactivity of tritiated PE-CA in liposomes.
\(R_{total}\): Radioactivity of total PE-CA added in the preparation.

**2.2.6.5 Incorporation of PE-SA into liposomes**

PE-SA in chloroform was mixed with 32 μmol PC and cholesterol in molar ratios of 0.05:1:1.05, 0.1:1:1.1, 0.2:1:1.2 and 0.3:1:1.3. The preparation of SUV liposomes was the same method as in 2.2.6.1. The incorporation ratios of PE-SA in liposomes were calculated by counting PE-SA (which was labelled with C\(^{14}\)) in both supernatant and pellets after centrifugation at 50000rpm (170300g) for 50min.

\[
\% \text{ of incorporation } = \frac{R_p}{R_p + R_s} \times 100\% \quad [2-8]
\]

\(R_p\): Radioactivity in pellet
\(R_s\): Radioactivity in supernatant

**2.2.7 Characterization and stability of polysialylated liposomes**

**2.2.7.1 Vesicle size**
Vesicle size of liposomes was measured by photon correlation spectroscopy (PCS) (Malvern Autosizer 2) when the size of liposomes was smaller than 1μm. For MLV and DRV liposomes, the vesicle size was measured in Mastersizer x (Malvern).

2.2.7.2 Surface charge

The surface charge (zeta-potential) of liposomes and polysialylated liposomes was measured in MALVERN zetasizer 3000 after they were suspended in phosphate buffer (0.05M, ionic strength 50mM) (pH 7.4).

2.2.7.3 Stability of liposomes incorporating colominic acid on their surface in albumin solution and mouse plasma

Liposomes composed of 8μmol PC, PE and cholesterol (molar ratios: 1:0.1:1.1, 1:0.2:1.2, 1:0.3:1.3) incorporating colominic acid (in 0.5ml of PBS, pH 7.4) were incubated with 100μl of 0.5-5% (w/v) human serum albumin (HSA) solution or 5-50μl of fresh mouse plasma at 37 °C for 2h. The vesicle size of liposomes before and after incubation was measured by photon correlation spectroscopy in a Malvern Autosizer 2.

2.2.7.4 Adsorption of mouse plasma on the vesicle surface

Liposomes composed of 8μmol PC, PE and cholesterol (molar ratios: 1:0.1:1.1, 1:0.2:1.2, 1:0.3:1.3) incorporating colominic acid in 0.5ml of PBS were mixed with 50μl of mouse plasma and 5μl of I\textsuperscript{125} labelled mouse plasma, and incubated at 37 °C for 2h. After centrifugation at 50000rpm (170300g) for 50min, the absorption of plasma on the vesicles in the pellets was counted in minigamma counter (WALLAC minigamma counter 1275).
and calculated as follows:

\[
\text{% of adsorption of protein} = \frac{C}{C_t} \times 100\% \quad [2-9]
\]

C: Radioactivity of proteins in the pellet after centrifugation. 
C\(_t\): Radioactivity of total proteins in 50μl of mouse plasma.

2.2.7.5 Release of 5(6)-carboxyfluorescein (CF) from liposomes

Liposomes composed of PC, PE and cholesterol (molar ratios: 1:0.05:1.05, 1:0.1:1.1, 1:0.2:1.2, 1:0.3:1.3) and entrapped CF were prepared as before (Gregoriadis, 1988). Briefly, lipids in CHCl\(_3\) were firstly dried to form a thin film. Then, 2ml of 0.2M CF solution were added into lipids which were rehydrated to obtain MLV liposomes. MLV liposomes were then probe sonicated to produce SUV liposomes. The SUV liposomes were dialysed against distilled water for 24h, then centrifuged at 50000rpm (170300g) /50min and washed with water by centrifugation three times. The efficiency of loading CF in liposomes was assayed by fluorescence spectrometry (PERKIN-ELMER LS-3) after addition of 10μl of Triton X-100 (10%) into the SUV liposomes to release total CF. The concentration of CF in liposomes was calculated using standard CF curve (Figure 2.2).

SUV liposomes entrapped with CF were then resuspended in 2ml of PBS. 1ml of resuspended liposomes was mixed with 10mg of OX-CA, then kept at 37 °C for 24h and finally centrifuged at 50000rpm (170300g) for 50min to remove the unreacted OX-CA and released CF. The entrapment efficiency of CF in the polysialylated liposomes was assayed again.
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0.1ml of PC/PE/Chol liposomes loaded with CF and 0.1ml of polysialylated liposomes loaded with CF were incubated in 0.5ml of PBS or 50μl of mouse plasma mixed with 450μl of PBS for 48h at 37 °C. 10μl samples were then mixed with 3ml of PBS at time intervals. The release of CF from liposomes was directly measured by fluorescence spectrometry (PERKIN-ELMER LS-3) and calculated as follows:

\[
\% \text{ of CF release} = \frac{C_t - C_0}{C - C_0} \times 100\% \quad [2-10]
\]

\(C_0\): The initial CF amount in the supernatant before incubation.
\(C\): The total CF amount entrapped in liposomes.
\(C_t\): The CF amount in the sample supernatant at time \(t\).
2.2.8 Animal studies with polysialylated liposomes

2.2.8.1 Blood clearance of polysialylated liposomes

Polysialylated liposomes composed of 3.2µmol PC, PE, cholesterol (1:0.1:1.1, 1:0.2:1.2, molar ratios) and conjugated 0.1 mg colominic acid on the surface were marked by addition of tritiated PC in the preparation. 0.2ml of polysialylated liposomes were intravenously injected into TO mice (3-4 mice in each group, 20-30g body weight for each) by the tail vein. The colominic acid-free liposomes with the same composition and dose were used as control. 50µl of blood were taken at time intervals, mixed with 500µl of PBS and centrifuged at 3000rpm for 5min. 450µl of plasma supernatant were then counted in 4ml of scintillation liquid in a WALLAC 1409 liquid scintillation counter. The proportion of liposomes in the circulating blood was calculated as follows:

\[
\text{R}_{450\mu l \text{ blood}} \times 550 = \frac{\text{450}}{\text{R}_{\text{total}}} \times 100\% \quad [2-11]
\]

\[
\text{R}_{\text{total}} = \frac{\text{R}}{V} \times 50\mu l \quad [2-12]
\]

\text{R}_{450\mu l \text{ blood}}: \text{Radioactivity in 450µl of supernatant samples.}
\text{R}_{\text{total}}: \text{Radioactivity of injected dose in 50µl of blood}
\text{R}: \text{Radioactivity of 200µl of liposomes (injected dose)}
\text{V}: \text{Total blood volume of each mouse (7.5% of body weight).}

Polysialylated liposomes composed of 3.2µmol PC, 3.2µmol cholesterol and 0.24mg PE-
Chapter 2 Materials and Methods

2.2.8.2 Tissue distribution of polysialylated liposomes

0.2ml of polysialylated liposomes composed of 1.6μmol PC, PE and cholesterol (1:0.1:1.1, 1:0.2:1.2, molar ratios) were intravenously injected into mice. 3h after injection, the mice were killed by dislocation of the neck. The liver, spleen, heart, lung and kidney were taken out, washed with saline, homogenized in 5 ml of saline and digested by the addition of 100μl of Triton X-100 (10%) or 100μl of tissue solubilizer and 100μl of H2O2. 0.5ml of tissue solution samples were counted in the scintillation counter. The tissue distribution of polysialylated liposomes was calculated as follows:

\[
\% \text{ of injected dose in tissue} = \frac{R_{0.5} \times 10}{R} \times 100\% \quad [2-13]
\]

\(R_{0.5}\): Radioactivity in 0.5ml of tissue samples.
\(R\): Radioactivity in injected dose.

2.2.8.3 Blood clearance of sialylated liposomes (PE-SA)

Liposomes composed of 3.2μmol PC, PE-SA and cholesterol (1:0.1:1.1, 1:0.2:1.2, molar ratios) with a trace amount of H\(^3\) labelled PC were injected intravenously into the tail vein of T.O. male mice. 50μl blood samples were taken at time intervals and diluted in 500μl PBS. The blood samples were centrifuged at 3000rpm for 5min (Jouan B3.11), and then 450μl supernatant were separated and counted for tritium in 4ml of scintillation liquid. The clearance of sialylated liposomes from the mouse blood circulation was calculated as mentioned in 2-11 and 2-12.
2.2.8.4 Tissue distribution of sialylated liposomes (PE-SA)

Sialylated liposomes composed of 3.2μmol PC, PE-SA and cholesterol (1:0.1:1.1, 1:0.2:1.2, molar ratios) were injected intravenously into the tail vein of T.O. mice. The mice were killed by dislocation of the neck 3h after injection. Liver, spleen, kidney, heart and lung were removed, washed in saline, homogenized in 2ml PBS and digested by the addition of 100μl of Triton X-100 (10%) or 100μl of tissue solubilizer and 100μl of H₂O₂. The distribution of PE-SA liposomes was assayed in a scintillation counter and calculated as 2-13.
Chapter 3

Radiolabelling of Colominic Acid with Sodium Borohydride ($H^3$) and Acetic Anhydride ($C^{14}$)
3.1 Introduction

Isotopic labelling of polysialic acid has been achieved by chemical or biological modification to introduce tritium (H$^3$) or carbon-14 (C$^{14}$) into its molecular structure. Up to now, the reported methods included the use of NaBH$_4$ (H$^3$) (Lifely et al., 1986; Finne and Makela, 1985; Halberstadt et al., 1993), acetic anhydride (C$^{14}$) (Jennings et al., 1985) and directly labelling of polysialic acid in biological culture (Tory et al., 1982). From a review of those processes, the most optimal method is the preparation of radioactive polysialic acid in a growing culture of _E. coli K-235_ by addition of glucose (C$^{14}$) (Tory et al., 1982). The tritiation of polysialic acid with NaBH$_4$ (H$^3$) by reducing its terminal group is more convenient than covalent coupling of acetic anhydride (C$^{14}$) to its amine group, but detail discussion on this chemical labelling has not yet been reported. Considering the detection and quantitation of colominic acid in the conjugates, we radiolabelled colominic acid with H$^3$ and C$^{14}$, respectively. We also tried to improve the labelling process and evaluated the factors determining the final labelling efficiency.

3.2 Labelling of colominic acid with sodium borohydride (NaBH$_4$) (H$^3$)

3.2.1 Tritiation of colominic acid

In the structure of the polysaccharide, the ring of the terminal unit with a free OH group at the anomeric carbon can be opened as it reacts with reducing agents such as NaBH$_4$ (H$^3$). The carbonyl group forms a primary or secondary hydroxyl. This end of the polysaccharide is usually named as the reducing end. For other units, the rings cannot
be opened by the reducing reagent since the anomeric carbon -OH is connected. If free -OH is replaced by other groups such as methyl, the end unit will lose its reduction activity. Colominic acid is a linear homopolysaccharide with 2→8 linkage of N-acetylneuraminic acid. When colominic acid is treated with NaBH$_4$ (H$^3$), the -C=O group is reduced to -CHOH (H$^3$) (Figure 3.1). From the structure of colominic acid, it is clear that one mole of colominic acid can only interact with one mole of H$^3$. Actually, the calculation of molar ratios between NaBH$_4$ (H$^3$) and colominic acid shows that 4 moles of colominic acid react with 1 mole of NaBH$_4$ (H$^3$) because water participates in the reaction. The scheme of labelling of colominic acid is shown in Figure 3.2.

![Fig. 3.1 Scheme of colominic acid radiolabelling with NaBH$_4$ (H$^3$)](image-url)
\[
4 \text{R-C-R'} + \text{NaBH}_4 + 3\text{H}_2\text{O} \rightarrow 4\text{R-CH-R'} + \text{NaH}_2\text{BO}_3
\]

Fig. 3.2 Scheme of NaBH\(_4\)(H\(^3\)) reduction

3.2.2 Purification of tritiated colominic acid

In the method of tritiation of colominic acid with NaBH\(_4\) (H\(^3\)) reported by Lifely (1986), free NaBH\(_4\) (H\(^3\)) was hidden by addition of methanol- 4M HCl. Adopting the same method in this study, we found this could not completely eliminate the free NaBH\(_4\) (H\(^3\)). However, by dialyzing the samples against distilled water (pH7.4), most of the free NaBH\(_4\) (H\(^3\)) moved out the dialysis tube within 24h (Figure 3.3), and the purity of samples was quite high (Figure 3.4). The desalting process was also carried out by gel filtration, using Sephadex G-10, G-25 and Sepharose CL-4B columns (1x20cm), respectively (Figure 3.5). Colominic acid moved down with the void volume in Sephadex G-10, G-25 and Sepharose CL-4B columns. The separation of colominic acid with Sepharose CL-4B preformed much better than with Sephadex G-10 and G-25. Isolation of colominic acid in a Sepharose CL-2B column showed that colominic acid could be retained (Figure 3.6), which offered a useful method for the further separation of liposomes from free colominic acid in the case of direct polysialylation of the liposome surface. Actually, the purity of tritiated colominic acid extremely influenced the calculation of its molecular weight and estimation of its ratio in the conjugates. Thus, all tritiated colominic acid samples used in this study were purified by firstly dialyzing against distilled water, and then passing through a Sephadex G-25 column.
Fig. 3.3 Dialysis of tritiated colominic acid against distilled water

Colominic acid labelled with NaBH₄ (H³) using different volumes (2-15μl) of NaBH₄ was left in a dialysis tube for 48h under stirring. The free NaBH₄ in water was counted in a scintillation counter at time intervals. Almost all free NaBH₄ were removed out from the dialysis tube within 24h.
Tritiated colominic acid was passed through a Sephadex G-25 column (1x20cm) to detect its purity using free NaBH₄ as control. No significant amount of free NaBH₄ was found in the colominic acid solution.
Fig. 3.5 Separation of colominic acid from free NaBH₄ by passing through Sephadex G-10, G-25 and Sepharose CL-4B gel filtration columns

10μl of colominic acid solution (10μg/10μl) treated by NaBH₄ (H³) were separated with different gels (Sephadex G-10, Sephadex G-25, Sepharose CL-4B) (1x20cm). The first fraction was colominic acid, and the second one was free NaBH₄. Results showed that Sepharose CL-4B was much more efficient than other gels.
Fig. 3.6 Separation of colominic acid from SUV liposomes with Sepharose CL-2B

PC/PE/Chol (molar ratio 1:0.3:1.3) SUV liposomes marked with tritiated PC and tritiated colominic acid were passed through a Sepharose CL-2B column (1x20cm) individually. Liposomes appeared in the first peak and colominic acid eluted out as a second peak.
3.2.3 Effect of the amount of NaBH₄(H³) on the radioactivity of colominic acid

100µg of colominic acid in 100µl of distilled water were mixed with 2-20µl NaBH₄ (H³) (2.92µCi/µl in 0.1M NaOH), and then kept in a water bath (30 °C) for 24h. The radioactivity per µg of colominic acid labelled with 2µl of NaBH₄ (H³) was lower than with the higher volume of NaBH₄ (H³). Products with similar radioactivity were achieved even when the volume of NaBH₄ (H³) was increased to 20µl (Table 3.1). Thus, 4µl (2.92µCi/µl) of NaBH₄ (H³) is enough to label 100µg of colominic acid. However, the volume of NaBH₄ (H³) for completely reducing other derivatives of polysialic acid still depended on their chain length. The lower molecular weight of polysialic acid, the higher amounts of NaBH₄ (H³) consumed in the reaction because oligo-sialic acids have more terminal groups to participate in the reduction.

Table 3.1 Effect of the NaBH₄ volume on the CA radiolabelling efficiency

<table>
<thead>
<tr>
<th>Volume of NaBH₄ (µl)</th>
<th>Radioactivity of CA (H³ cpm/µg) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1153.1 ± 156.4</td>
</tr>
<tr>
<td>4</td>
<td>3650.6 ± 87.8</td>
</tr>
<tr>
<td>6</td>
<td>3677.4 ± 150.1</td>
</tr>
<tr>
<td>10</td>
<td>3830.4 ± 82.3</td>
</tr>
<tr>
<td>15</td>
<td>3344.3 ± 150.5</td>
</tr>
<tr>
<td>20</td>
<td>3650.8 ± 95.7</td>
</tr>
</tbody>
</table>
3.2.4 Effect of the reaction time on the radiolabelling of colominic acid

100µg of colominic acid were mixed with 10µl of NaBH₄ (H³⁻) (2.92uCi/µl in 0.1M NaOH) and left at 30 °C. At the time intervals, 10µl of the mixture solution were passed through a Sephadex G-10 column (1x20cm), and the fractions were counted in 4ml scintillation solution. Results showed that the reducing reaction was rapidly finished during 2h. The radioactivity per µg of colominic acid did not increase with further increasing reaction time (Table 3.2).

Since neither improvement of the reaction time nor increasing the volume of NaBH₄ changed the labelling efficiency, it is assumed that the -C=O at the reducing end of colominic acid was totally reduced by tritium. Calculation of the molar ratio of tritium to colominic acid can reveal the average molecular weight of colominic acid. Otherwise, the tritium coupled to colominic acid was quite stable. Storage of tritiated colominic acid at 4 °C for three months did not result in loss of tritium (Figure 3.7).

Table 3.2 Effect of reaction time on the CA radiolabelling efficiency

<table>
<thead>
<tr>
<th>Reaction time (h)</th>
<th>Radioactivity of CA (H³ cpm/µg) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3733.0 ± 18.4</td>
</tr>
<tr>
<td>4</td>
<td>3315.5 ± 9.19</td>
</tr>
<tr>
<td>14</td>
<td>3332.8 ± 76.4</td>
</tr>
<tr>
<td>24</td>
<td>3427.2 ± 43.1</td>
</tr>
</tbody>
</table>
Fig. 3.7 Stability of radiolabelled colominic acid after storage

Tritiated colominic acid stored at 4 °C for three months was passed through a Sephadex G-25 column. Results showed that colominic acid before and after storage was eluted in the same fractions.
3.2.5 Radiolabelling of oxidized colominic acid (OX-CA)

From the reaction mechanism (Figure 3.1), it is clear that labelling efficiency depends not only on the labelling process but also the polymer chain length. Polysaccharides with high molecular weight and few terminal groups cannot achieve high radioactivity since there is only one hydrogen at the reducing end to be labelled in the reaction. With the intent of increasing labelling efficiency per µg samples, we employed OX-CA in the labelling reaction. Details of the scheme are shown in Figure 3.8.

Fig. 3.8 Scheme of tritiation of oxidized colominic acid
Briefly, colominic acid is firstly oxidized by addition of $\text{NaIO}_4$ to form an aldehyde group at the non-reducing end. While OX-CA is labelled with $\text{NaBH}_4 (\text{H}^3)$, both reducing end and non-reducing end of OX-CA are tritiated by $\text{NaBH}_4 (\text{H}^3)$. The radioactivity per $\mu$g of colominic acid must therefore be enhanced twice. 100$\mu$g of OX-CA mixed with 4-20$\mu$l of $\text{NaBH}_4 (\text{H}^3) (2.92 \mu\text{Ci}/\mu\text{l in 0.1M NaOH})$ were reacted at 30°C for 24h. The free $\text{NaBH}_4 (\text{H}^3)$ was removed by passing through a Sephadex G-10 column (1x20cm). Results showed that the radioactivity per $\mu$g of OX-CA was enhanced as two-fold (Table 3.3). When the volume of $\text{NaBH}_4 (\text{H}^3)$ was increased to more than 10$\mu$l, the radioactivity of OX-CA did not change significantly (Table 3.3).

Table 3.3 Comparison of tritiated CA and tritiated OX-CA

<table>
<thead>
<tr>
<th>NaBH$_4 (\text{H}^3) (\mu$l</th>
<th>Colominic acid (CA) (cpm/µg)</th>
<th>Oxidized colominic acid (OX-CA) (cpm/µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3700.3</td>
<td>3766.2</td>
</tr>
<tr>
<td>6</td>
<td>3676.5</td>
<td>5516.5</td>
</tr>
<tr>
<td>10</td>
<td>3775.0</td>
<td>6773.4</td>
</tr>
<tr>
<td>15</td>
<td>3344.8</td>
<td>7273.1</td>
</tr>
<tr>
<td>20</td>
<td>3650.0</td>
<td>7267.3</td>
</tr>
</tbody>
</table>
3.3 Radiolabelling of colominic acid with acetic anhydride (C\textsuperscript{14})

3.3.1 Scheme of the labelling reaction

Radiolabelling of colominic acid with acetic anhydride (H\textsuperscript{3}) has been mentioned by Jennings et al. (1985). In brief, the colominic acid was firstly deacetylated under the treatment of strong basic solution, and then reacted with acetic anhydride (H\textsuperscript{3}) to form a N-acetylate replacement (Figure 3.9). On completion of deacetylation, each N-neuraminic acid unit of colominic acid was labelled by this approach. Hence, the radioactivity of colominic acid was quite high. In the paper of Jennings et al. (1985), they obtained 100,000cpm/µg products, but in labelling colominic acid with NaBH\textsubscript{4} (H\textsuperscript{3}), the radioactivity per µg of colominic acid was only 4000cpm/µg achieved. However, the most important reason for developing the acetic anhydride method was based on the fact that the isotope coupled to colominic acid is not affected by oxidation with NaIO\textsubscript{4}. It can thus be used as an indicator of the conjugation of oxidized colominic acid with PE by the Schiff reaction. In contrast, oxidation of colominic acid labelled at its reducing end with NaBH\textsubscript{4} (H\textsuperscript{3}) leads to total destruction of the tritiated terminal conformation (Figure 3.10). While the non-reducing end is oxidized by NaIO\textsubscript{4}, the C6→C7 and C7→C8 linkage bonds at the reducing end are broken as well, and the tritium on C6 disappears. The problem of the acetic anhydride method is the depolymerization of the molecular chain during the deacetylation. Some reports have demonstrated that the colominic acid is not stable under heating (Lifely et. al., 1986). If the deacetylated processes were carried out in a hot strong NaOH solution, it is believed that most of the colominic acid would be digested. Therefore, mild hydrolysis was
introduced in our study for the purpose of reducing the destruction of colominic acid.

Fig. 3.9 Scheme of labelling hydrolysed colominic acid with acetic anhydride (C\textsuperscript{14})
Tritiated colominic acid was treated with NaIO₄ in the dark for 15min, and then passed through a Sephadex G-25 column. Results showed that peaks of colominic acid before and after oxidation appeared in different positions.
In fact, the degree of deacetylation is very difficult to control. In this study we also tried to prepare O-acetylated colominic acid by directly conjugating acetic anhydride to the hydroxyl group. The scheme for the reaction is shown in Figure 3.11.

![Diagram of the reaction scheme](image)

**Fig.3.11 Labelling of colominic acid by O-acetylation**

### 3.3.2 Labelling of O-acetylated colominic acid

The O-acetylated colominic acid was directly prepared by mixing colominic acid with acetic anhydride (C\(^{14}\)) under anhydrous conditions, and purified by dialyzing against distilled water. Results showed that free acetic anhydride was removed within 24h (Table 3.4). When the labelled colominic acid and free acetic anhydride were passed through a Sephadex G-10 column (1x20cm) individually, they were eluted in different peaks (Figure 3.12). Results (Figure 3.12) showed that colominic acid was conjugated
with the acetic anhydride successfully. The radioactivity per μg colominic acid was around 300-900 cpm/μg. We did not get the same high radiolabelling of colominic acid as Jennings et al. (1985) because we used C\(^{14}\) as the isotope and the yield of directly labelling the hydroxyl groups was not very high.

Table 3.4 Dialysis of colominic acid against 0.1M NaHCO\(_3\)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>C(^{14}) (dpm) in dialysis water (500μl)</th>
<th>C(^{14}) (dpm) in colominic acid solution (50μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.2</td>
<td>11810.5</td>
</tr>
<tr>
<td>12</td>
<td>212.5</td>
<td>6870.7</td>
</tr>
<tr>
<td>24</td>
<td>368.0</td>
<td>5973.2</td>
</tr>
<tr>
<td>48</td>
<td>24.6</td>
<td>4648.2</td>
</tr>
</tbody>
</table>

3.3.3 Labelling of hydrolysed colominic acid

Incubation of colominic acid in 4M NaOH solution resulted in deacetylation of the -CO-NH- structure, which led to the production of hydrolysed colominic acid with free amine groups. Compared with colominic acid in which deacetylation degree was less than 5%, hydrolysed colominic acid had more than 30% of deacetylation degree depending on the incubation time and temperature (Table 3.5). On leaving colominic acid in boiling 4M NaOH solution for 4h, the deacetylation reached nearly 100% (Table 3.5). We had tried to label deacetylated colominic acid with acetic anhydride (C\(^{14}\)) in 0.1M NaHCO\(_3\) solution. However, the resulting radioactivity of colominic acid was only 200-
300cpm/μg. This low labelling efficiency was attributed to decomposition of acetic anhydride (C\textsuperscript{14}) in alkali solution.

![Graph showing radioactivity and fractions](image)

**Fig. 3.12** Detection and separation of C\textsuperscript{14}-labelled colominic acid

Colominic acid labelled with acetic anhydride (C\textsuperscript{14}) was passed through a Sephadex G-10 column. Labelled colominic acid appeared in a different peak compared with free acetic anhydride.
Table 3.5 Hydrolysis of colominic acid by 4M NaOH

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Fluorescence</th>
<th>NH$_2$ / colominic acid (umol/umol %) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.5</td>
<td>3.18 ± 0.01</td>
</tr>
<tr>
<td>24*</td>
<td>163.7</td>
<td>38.2 ± 0.8</td>
</tr>
<tr>
<td>4**</td>
<td>424.7</td>
<td>100.0 ± 1.4</td>
</tr>
</tbody>
</table>

Concentration of colominic acid: 2mg/2.25ml;
24*: sample was left in 4M NaOH at room temperature for 24h
4**: sample was left in 4M NaOH at 100 °C for 4h

3.3.4 Oxidization of C$^{14}$-labelled colominic acid

C$^{14}$-labelled Colominic acid was oxidized by addition of 0.1 M NaIO$_4$ and passed through a Sephadex G-25 column. As shown in Figure 3.13, the peak of labelled colominic acid treated with NaIO$_4$ was eluted in the same fractions as normal colominic acid, suggesting that C$^{14}$ bonding on colominic acid was not affected by oxidation.
Chapter 3 Radiolabelling of Colominic Acid with Sodium Borohydride (H\textsuperscript{1}) and Acetic Anhydride (C\textsuperscript{14})

Fig. 3.13 Oxidation of C\textsuperscript{14}-labelled colominic acid with NaIO\textsubscript{4}

C\textsuperscript{14}-labelled CA was treated with NaIO\textsubscript{4} in the dark for 15 min and passed through a Sephadex G-25 column. No significant changes in elution before and after treatment were found.
3.4 Conclusions

Radiolabelling of colominic acid with two chemical modifications achieved different labelling efficiency. Generally, tritiation of colominic acid with NaBH₄ (H³) presented 3000-4000cpm/µg depending on the radioactivity per mmol of NaBH₄ (H³). On the other hand, labelling of colominic acid with acetic anhydride (C¹⁴) only resulted in 300-900cpm/µg since O-acetylation reaction did not reach a high level replacement. Considering the further application of labelled colominic acid; H³-colominic acid and C¹⁴-colominic acid are suitable for different coupling reactions. The tritiated colominic acid is quite stable and can be used as an indicator in many studies except oxidation because H³ is removed by NaIO₄. Labelling of colominic acid with C¹⁴ changes the structure of colominic acid, which is unfavourable for biological studies. Otherwise, data of labelling OX-CA with NaBH₄ (H³) which provided two-fold higher radioactivity than non-oxidized colominic acid confirmed that: 1) the reduction did not affect the structure of colominic acid except the reducing end; 2) the tritiation reaction was almost completed; calculation of the molar ratio of H³ to the number of polysaccharides would indicate the molecular weight of colominic acid and its derivatives as described by Lifely et al. (1986).
Chapter 4

Conjugation of Lipids with Colominic Acid
4.1 Introduction

Polysialylation of liposomes can be realized either by directly conjugating colominic acid to the vesicle surfaces or by firstly coupling polysaccharides to the lipids, then incorporating them into liposomes. The chemical reactions adopted to couple colominic acid with lipids are determined by the stability of lipids and polysialic acids, and the solvent used to dissolve both polysaccharides and lipids. These greatly influence the yield of final products. Actually, the solubility of colominic acid and lipids is completely different. Phosphatidylethanolamine (PE) can dissolve in chloroform, ethanol, methanol and tetrahydrofuran (THF), but it is insoluble in acetone, water and DMSO. 1,2-dipalmitoyl-sn-glycero-3-phosphatidylethanolamine (DPPE) and 1,2-dimyristoyl-sn-glycero-3-phosphatidylethanolamine (DSPE) are soluble in a mixture of chloroform with acetic acid (1: 0.1, v/v) or chloroform with methanol (1:1, v/v) and in ether. Like all types of N-acetyl-neuraminic acid polymers, colominic acid is water soluble. It also dissolves in DMSO and tetrabutyl ammonium (Gregoriadis et al., 1993). In order to conduct the reaction in a homogeneous medium, cosolvents for dissolving both lipids and colominic acid were initially studied.

Besides solubility, another important factor in the coupling reaction is the stability of lipids and polysaccharides. It is known that two types of chemical degradation reaction affect the integrity of the phosphalipid bilayer (New, 1990): 1) hydrolysis of the ester bonds; 2) peroxidation of the unsaturated chains. In the process of the four ester bonds of the lipid, the two acylester bonds are more liable to hydrolysis than the glycerophosphate and the phosphatidylcholine ester bonds. Factors influencing the
Chapter 4 Conjugation of Lipids with Colominic Acid

hydrolysis rate include the temperature, pH, bilayer rigidity and buffer. The optimum pH for a stable lipid is around pH 6.5. The temperature dependence of the hydrolysis rate constants is expressed by a linear relationship between the log hydrolysis constant and 1/T. Therefore, the conjugation of lipids with colominic acid should be carried out under mild pH conditions to reduce the destruction of lipids. At the same time, the reaction must be maintained at low temperature because both lipids and colominic acid are sensitive to heating with later depolymerizing at high temperature. The conditions of chemical conjugation are strictly limited by the properties of materials.

From the structure of colominic acid, it is known that hydroxyl (-OH) and carboxyl (-COOH) can be involved in the covalent chemical linkage with lipids (Figure 4.1).

From the structure of colominic acid, it is known that hydroxyl (-OH) and carboxyl (-COOH) can be involved in the covalent chemical linkage with lipids (Figure 4.1).

The molecular weight of colominic acid is around 15,000-18,000. The non-reducing end can be oxidized to form -CHO group. The ring at the reducing end will be opened as colominic acid reacts with NaBH₄. -COOH can combine with -NH₂ in the presence of catalyst to form an amide linkage.

The acetylated degree of colominic acid is more than 95% (Chapter 3, Table 3.5), and unless colominic acid is deacetylated, not enough free amine groups are available for the
conjugation. Alternatively, the -CH₂OH·CH₃OH structure at the non-reducing end of colominic acid can be rapidly oxidized to form an aldehyde group (Figure 4.1).

Phospholipids used in the conjugation all possess amine groups (e.g., PE, DPPE and DSPE). The site of colominic acid that can be coupled to PE depends on the coupling reaction adopted. It could be at the terminal group of colominic acid (-CHO) to form a linear polymer (PE-OX-CA) or in the middle of the colominic acid chain (-COOH) to form a grafted polymer (PE-CA) (Figure 4.2). Linear conjugates appear more suitable than branched ones since with equimolar coupling the structure of the final compound is easier to be determined due to no more than one substitution per molecule of colominic acid. PE-OX-CA has only one conformation on conjugation. On using the same reaction to modify the surface of liposomes, polysaccharides will only be found on the exterior of vesicles to form a hydrophilic, brush like layer.

\[
\text{CHO} + \text{PE-NH₂} \rightarrow \text{CH=N-PE} \\
\text{COOH COOH} + \text{PE-NH₂} \rightarrow \text{CONHPE} \text{CONHPE} \\
\text{COOH} \quad \text{CONHPE} \\
\text{CONHPE} \quad \text{CONHPE}
\]

Fig. 4.2 Linear and branched colominic acid conjugates

PE couples to the non-reducing end of colominic acid to form a linear polymer. PE couples to the -COOH group of colominic acid to form a branched polymer.

As already mentioned, conjugation of the amine group of lipids with the carboxyl groups of colominic acid provides a branched polymer (Figure 4.2). Regardless of the way
COOH is coupled to NH$_2$ in the presence of DCC or application of other kinds of reaction, the conjugation of PE-NH$_2$ with CA-COOH cannot be totally completed. The alternative grafting makes polymers present different properties. The possibility of incorporation of this kind of conjugate into liposomes will be discussed in a further study. In this chapter, we have studied the effect of catalyst, solvents and pH on the reaction yield.

4.2 Conjugation of colominic acid with PE by the Schiff reaction

4.2.1 Oxidation of colominic acid with NaIO$_4$

Oxidation of colominic acid to form an aldehyde group at the non-reducing end has been known from the previous studies (Jennings and Lugowski, 1981) to occur quickly. The report of Jennings (1981) also indicated that the oxidation time was very important in the determination of the oxidation position. When the reaction was stopped after a short time, the oxidation would mainly occur at the non-reducing end. Long oxidation time was claimed to break the chain of polysaccharides, but no data were given to support this claim. If there were more than one aldehyde group produced in one molecule of colominic acid, the final PE-OX-CA coupling products will be of the branched conformation and as PE-OX-CA is incorporated into lipid bilayer, it may influence the formation of liposomes. So we firstly studied the effect of oxidation time and amount of NaIO$_4$ on the oxidation process. The oxidation degree was estimated by the radiolabelling method, in which the aldehyde -CHO is reduced to be the hydroxyl -OH (H$_2$O) under the treatment of NaBH$_4$ (H$_3$) (Figure 3.8).
Colominic acid was oxidized by the addition of NaIO$_4$ and samples were kept in the dark for 15min, 30min, 1h and 3h, respectively. Free NaIO$_4$ was removed by passing through Sephadex G-25 columns. The yield of oxidized colominic acid (OX-CA) was around 82-92% (Table 4.1). On labelling 100µg OX-CA with NaBH$_4$ ($\text{H}^3$), results showed that the radioactivity per µg of OX-CA oxidized for 15min was the same as one per µg of OX-CA oxidized for 2h (Table 4.2). The molar ratio between $-\text{CHO}$ and colominic acid was approximately 1:1 (Table 4.2). It assumed that the colominic acid chain was not affected by oxidation since no more aldehyde groups were produced.

Unlike other types of polysialic acid, which have 7-8 and 8-9 adjacent CHOH-CHOH, colominic acid is of the 2-8 carbon linkage type. Oxidation along the chain of colominic acid would be less likely to occur. On the other hand, it also confirms that oxidation did not open the ring of the polysaccharide. The equal molar ratio of CHO to colominic acid indicated that almost all reducing ends of colominic acid were oxidized. Moreover, when colominic acid was treated with 0.2, 0.5, 1 and 2ml of NaIO$_4$ in the dark for 15min, the radioactivity per µg of OX-CA did not increase with increasing amount of NaIO$_4$ in the mixture (Table 4.3 and 4.4).

Normally, the structure of $-\text{CHOH}--\text{CHOH}$ or $-\text{CHOH}--\text{CHNH}_2$, $-\text{CHOH}--\text{CO}$, which contains two adjacent carbon atoms is the conformation that is subject to oxidation. One mole of periodate is consumed for each mole of carbon-carbon bond oxidized with a dialdehyde derivative of the sugar being formed. From our data, it is concluded that neither the polysaccharide chain nor the sialic acid ring was changed during the oxidation process. One mole of $-\text{CHO}$ produced in one mole colominic acid leads to
equimolar conjugation of PE with OX-CA. The coupling product will be of the linear type.

Table 4.1 Oxidation of colominic acid under different reaction times

<table>
<thead>
<tr>
<th>Oxidative time</th>
<th>Colominic acid / NaIO₄ (mol/mol)</th>
<th>Yield (%) (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15min</td>
<td>6.7 x 10⁻³</td>
<td>92</td>
</tr>
<tr>
<td>30min</td>
<td>6.7 x 10⁻³</td>
<td>87</td>
</tr>
<tr>
<td>1h</td>
<td>6.7 x 10⁻³</td>
<td>82</td>
</tr>
<tr>
<td>2h</td>
<td>6.7 x 10⁻³</td>
<td>92</td>
</tr>
</tbody>
</table>

Table 4.2 Effect of oxidation time on the oxidation of colominic acid

<table>
<thead>
<tr>
<th>Oxidation time</th>
<th>Radioactivity (cpm/µg) (mean ± SD)</th>
<th>CHO (µmol) / colominic acid (µmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15min</td>
<td>7977 ± 50.2</td>
<td>1.22 : 1</td>
</tr>
<tr>
<td>30min</td>
<td>6574 ± 210.5</td>
<td>1.00 : 1</td>
</tr>
<tr>
<td>1h</td>
<td>6753 ± 134.2</td>
<td>1.03 : 1</td>
</tr>
<tr>
<td>2h</td>
<td>6837 ± 87.9</td>
<td>1.04 : 1</td>
</tr>
</tbody>
</table>

Radioactivity of colominic acid (cpm/µg): 3278;
Average molecular weight of colominic acid (Mn): 15242.
Chapter 4 Conjugation of Lipids with Colominic Acid

Table 4.3 Oxidation of colominic acid with different amounts of NaIO₄

<table>
<thead>
<tr>
<th>Oxidative time (min)</th>
<th>Colominic acid / NaIO₄ (mol/mol)</th>
<th>Yield (%) (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1.34 x 10⁻³</td>
<td>87</td>
</tr>
<tr>
<td>15</td>
<td>3.35 x 10⁻³</td>
<td>93</td>
</tr>
<tr>
<td>15</td>
<td>6.70 x 10⁻³</td>
<td>85</td>
</tr>
<tr>
<td>15</td>
<td>13.4 x 10⁻³</td>
<td>95</td>
</tr>
</tbody>
</table>

Table 4.4 Effect of the amount of NaIO₄ on the oxidation of colominic acid

<table>
<thead>
<tr>
<th>NaIO₄ (0.1M) (ml)</th>
<th>Oxidized colominic acid (cpm/μg) (mean ± SD)</th>
<th>CHO (μmol) / colominic acid (μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>7598 ± 40.3</td>
<td>1.16 : 1</td>
</tr>
<tr>
<td>0.5</td>
<td>6372 ± 79.2</td>
<td>0.97 : 1</td>
</tr>
<tr>
<td>1</td>
<td>6551 ± 112.7</td>
<td>1.00 : 1</td>
</tr>
<tr>
<td>2</td>
<td>6556 ± 29.6</td>
<td>1.00 : 1</td>
</tr>
</tbody>
</table>

Radioactivity of colominic acid (cpm/μg): 3278; Average molecular weight of colominic acid (Mn): 15242.

4.2.2 Conjugation of PE with OX-CA (PE-OX-CA)

4.2.2.1 Purification and identification of PE-OX-CA

10mg OX-CA in 1ml 0.1M Na₂HPO₄ solution were mixed with 2.5mg PE and trace amounts of PE-C¹⁴ which were dissolved in 2ml THF. The reaction was kept at room temperature for 1-3 days under the protection of nitrogen gas. After THF was removed
by rotary evaporation, precipitates appeared in the mixture, possibly representing the unreacted PE because of its insolubility in water. The precipitate (A) and solution (B) were separated by ultracentrifugation at 50000rpm (170300g) for 30min, and then freeze-dried. On redissolving component A in 1ml of chloroform and B in 1ml of water and detecting the radioactivity, a great amount of PE-C\textsuperscript{14} was found in the water solution (Table 4.5). However, PE itself is poorly water-soluble. It would therefore appear that the conjugate of PE with OX-CA is a hydrophilic product which can dissolve in water because of the significant difference in molecular weight between OX-CA (Mn 15,000-18,000) and PE (Mw 744). A control test was carried out to mix the same amount of colominic acid with PE and PE-C\textsuperscript{14} (331040 dpm). After samples were freeze-dried and redissolved in 1ml water, PE-C\textsuperscript{14} (32.4dpm) was absent in the supernatant (Table 4.5). This implies that the PE in the experiment was chemically connected with OX-CA rather than non-covalently absorption.

Table 4.5 Detection of PE-C\textsuperscript{14} in water before and after conjugation

<table>
<thead>
<tr>
<th>Sample</th>
<th>After reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>100μl PE-C\textsuperscript{14} (331040dpm) + CA</td>
<td>32.4 dpm</td>
</tr>
<tr>
<td>100μl PE-C\textsuperscript{14} (331040dpm) + OX-CA</td>
<td>26000 dpm</td>
</tr>
</tbody>
</table>

OX-CA,10mg (0.67μmol); PE, 2.5mg (3.36μmol).

Further detection of PE-OX-CA by passing through a Sephadex G-25 column showed that the conjugates were eluted in the same peak as did intact colominic acid (Figure 4.3). 90.2% of PE-OX-CA was washed out without retention. In contrast, with PE-C\textsuperscript{14}
suspended in 1ml of water or dissolved in 1ml THF and passed through the same column, only 0.6% (400dpm) of the total radioactivity PE (66208dpm) was eluted out. Most of the PE-C\textsuperscript{14} was retained on the column. Moreover, examination of lipids in an organic phase by the resorcinol reaction did not produce any purple colour. This indicated that most of the conjugates was dissolved in a water phase.

![Graph showing Sephadex G-25 chromatography of PE-OX-CA](image)

**Fig. 4.3** Sephadex G-25 chromatography of PE-OX-CA

Conjugates of PE (C\textsuperscript{14}) with OX-CA (PE-OX-CA) was passed through a Sephadex G-25 column (1x20cm). Tritiated colominic acid used as control.

Analysis of PE-OX-CA on a TLC plate showed a new spot (Rf = 0), which contained lipids since it turned to blue after spaying with molybdenum blue (Figure 4.4). The spot of free PE had a Rf value of 0.64 (Figure 4.4). This demonstrated the formation of PE-
OX-CA in the reaction. Although PE-OX-CA was more hydrophilic than PE, PE-OX-CA with a higher molecular weight did not move on the TLC plate with the mobile phase of CHCl₃/MeOH (7:3, v/v). The coupling product was also assayed by ¹H-NMR. On comparing the NMR spectra of OX-CA and PE-OX-CA with PE, it was confirmed that PE was coupled with OX-CA (Figure 4.5).

![Detection of PE-OX-CA by TLC](image)

**Fig. 4.4 Detection of PE-OX-CA by thin layer chromatography (TLC)**

TLC plate: silica gel; eluent: CHCl₃/MeOH 7:3 (v/v); Indicator: molybdenum blue

### 4.2.2.2 Reaction mechanism

Coupling of colominic acid by the Schiff base is a common reaction widely used in the modification of proteins. Polysaccharides (polysialic acid A and C) have been connected with tetanus toxoid to produce water soluble products, which presented high levels of polysaccharide specific antibodies in rabbits and mice (Jennings and Lugowski, 1981). It was hoped that conjugates of polysialic A and C could be used as potential human vaccines. The main advantage of the Schiff reaction is the mildness of the processes without side products. The reaction can be carried out in the water.
Fig. 4.5 $^1$H-NMR spectra of PE, colominic acid and PE-OX-CA
Normally, resulting products are coupled in equimolar terms. Kinetic studies on the formation of the Schiff base indicated that the yield depended on the pH, ionic strength and concentration of reacted materials. The reaction rate in the Schiff coupling increased while the solution was kept at high pH. However, the ester bonds of lipids are sensitive to the pH change. A strong basic or acid solution will destroy the lipid. It has been reported that colominic acid was deacetylated in the presence of a strongly basic solution (Lifely et al., 1986). Thus, all reactions in this study were maintained at 20-40 °C and pH 5-9 phosphate buffer.

Unlike the reaction with the small molecules, the Schiff reaction in coupling polymers is also affected by the conformation of the polymer chain and steric hindrance (Figure 4.6). When polysialic acids were mixed with proteins at 40 °C for two weeks, only 0.4 - 2.5 moles of polysialic acids were coupled to one mole of protein (Jennings and Lugowski, 1981). Fernandes and Gregoriadis (1996, 1997) have added 50 moles of colominic acid into one mole of catalase, and 3.8 moles of colominic acid in one mole of enzyme was obtained. Actually, the Schiff reaction was conducted slowly, and the yield was not high. We have prolonged the reaction time to one week, and conjugation of oxidized colominic acid to PE did not improve significantly. Thus, for reducing the hydrolysis of PE during the reaction, the conjugation time was shortened to 1-3 days. The reacted OX-CA was around 22.8-34.1% depending on the amounts of OX-CA added in the reaction.
4.2.2.3 Effect of pH on the coupling reaction

Conjugation of OX-CA to PE under different pH conditions was investigated by dissolving 10mg OX-CA in 1ml 0.1M Na₂HPO₄ (pH 9.0), 0.1M NaH₂PO₄ (pH 4.7) or 0.1M PBS (pH=7.4), and mixing with 2.5mg PE in 1ml EtOH. The yield of PE-OX-CA in 0.1M PBS (pH 7.4) and 0.1M Na₂HPO₄ (pH 9.0) was higher than one in 0.1M NaH₂PO₄ (pH 4.7) (Table 4.6). According to the theory of the Schiff reaction, the
aldehyde undergoes a condensation reaction with a primary amine to give a product containing an azomethine (-C=) linkage and water. The reaction is reversible and catalysed by the cation. The rate determining step is pH dependent. Therefore, the conjugation rate was accelerated by keeping the reaction in a basic solution. From Table 4.6, it was found that the coupling yield in PBS was higher than in other buffer solutions. The reason was not very clear, but we assumed it related to the stability of PE during the reaction. On the other hand, 34.5 μmol% of OX-CA coupled to PE in PBS implied that the reaction can be carried out at a mild condition.

Table 4.6 Effect of pH on the conjugation of PE with oxidized colominic acid (OX-CA)

<table>
<thead>
<tr>
<th>pH</th>
<th>Radioactivity (C¹⁴) of PE-OX-CA (dpm)</th>
<th>Reacted PE (μmol%)</th>
<th>Reacted OX-CA (μmol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1M NaH₂PO₄ (pH 4.7)</td>
<td>1660</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>PBS (pH 7.4)</td>
<td>21750</td>
<td>7.0</td>
<td>34.5</td>
</tr>
<tr>
<td>0.1M Na₃HPO₄ (pH 9.0)</td>
<td>6350</td>
<td>2.1</td>
<td>10.3</td>
</tr>
<tr>
<td>Control (pH 7.4)</td>
<td>676</td>
<td>0.2</td>
<td>0.99</td>
</tr>
</tbody>
</table>

OX-CA 11.4mg; PE 2.5mg; OX-CA/PE (μmol/μmol), 0.67:3.3; Total radioactivity of PE-C¹⁴ in the mixture, 307724dpm; The control was made by mixing colominic acid with PE in PBS.

4.2.2.4 Effect of solvents on the coupling reaction

Conjugation of hydrophobic PE to hydrophilic OX-CA was extremely affected by the solvent used in the coupling reaction. When PE was suspended in water containing OX-CA, the coupling yield could not achieve a high value. Thus, for dissolving both PE and
OX-CA in a homogeneous medium, cosolvents including DMSO/water, DMF/water, THF/water, ethanol/water and methanol/water in different volume ratios were investigated in this study. Finally, it is found that THF/water with a ratio of 2 to 1 (v/v) and ethanol/water with a ratio of 1 to 1 (v/v) were able to dissolve either PE or OX-CA. The yield of coupling OX-CA was around 43.3-62.2 μmol% (Table 4.7).

Table 4.7 Effect of solvent on the conjugation of PE with oxidized colominic acid (OX-CA)

<table>
<thead>
<tr>
<th>Solvent (v/v)</th>
<th>Reacted CA (μmol%)</th>
<th>Reacted PE (μmol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH / water 1:1</td>
<td>43.3</td>
<td>3.9</td>
</tr>
<tr>
<td>THF / water 2:1</td>
<td>62.2</td>
<td>5.6</td>
</tr>
<tr>
<td>THF / water 5:3</td>
<td>44.4</td>
<td>4.0</td>
</tr>
</tbody>
</table>

OX-CA 15.3mg; PE 7.4mg; OX-CA/PE, 0.09:1 (μmol/μmol)

4.2.2.5 Effect of the molar ratio of OX-CA to PE on the reaction

Increasing the molar ratio of OX-CA to PE in the mixture, the yield of conjugates was improved since the reversible conjugation of lipids with OX-CA was accelerated by increasing the concentration of either OX-CA or lipid. Comparing addition of 14.5mg OX-CA in the reaction with 40mg OX-CA, coupled PE was increased from 3.3 μmol% to 13.5 μmol% (Table 4.8). However, the molecular weight of OX-CA (Mn=15,000-18,000) is much higher than PE (MW=744), which leads to one mole of PE (744mg) reacting with 15000-18000mg of OX-CA in the case of a molar ratio of OX-CA to PE of 1 to 1. Therefore, in the reaction the amount of PE added was always in 2-5 fold that of OX-CA. Otherwise, hydrophobic PE was easy to separate from the mixture.
Chapter 4 Conjugation of Lipids with Colominic Acid

Table 4.8 Effect of molar ratio of oxidized colominic acid and PE on the conjugation yield

<table>
<thead>
<tr>
<th>OX-CA</th>
<th>Reacted PE (μmol%)</th>
<th>Reacted CA (μmol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.5mg (0.97μmol)</td>
<td>3.3</td>
<td>22.8</td>
</tr>
<tr>
<td>20mg (1.33μmol)</td>
<td>4.9</td>
<td>24.8</td>
</tr>
<tr>
<td>40mg (2.67μmol)</td>
<td>13.5</td>
<td>34.1</td>
</tr>
</tbody>
</table>

PE 5mg (6.72μmol)

4.3 Conjugation of colominic acid with PE in the presence of carbodiimide catalyst

4.3.1 Reaction mechanism

The formation of amide bonds in the presence of carbodiimide catalysts is widely used in the synthesis of proteins, especially after the water-soluble carbodiimide catalysts (CMC, EDAC) were found. The catalysis mechanism of carbodiimide is shown in Figure 4.7. The reaction yield depends on: i) the type of carbodiimide used; ii) the strength of the acid and the nucleophilicity of its anion; iii) the type of solvent; iv) the presence in the reaction medium of other active compounds. Linear colominic acid is grafted randomly with PE to form a branched conformation (Figure 4.2). The coupling ratio of PE to colominic acid is related not only to the reaction conditions but also to the polymer steric conformation. Moreover, it determines the characteristics of the final products.
4.3.2 Assay of the conjugated product

Coupling of PE to CA in the presence of DCC occurred rapidly at room temperature. The produced conjugates have been detected on TLC plates with an eluant of CHCl₃/MeOH (8:2, v/v) and an indicator of molybdenum blue, and several new compounds were found (Figure 4.8). In the reaction, PE labelled with C¹⁴ and colominic acid labelled with H³ were used as an indicator to assay the conjugated products. After the spots were removed from the TLC plates, dissolved in CHCl₃ and counted in a scintillation counter, double labelled PE-CA by C¹⁴ related to PE-C¹⁴ and H³ related to CA-H³ appeared at the same spot (Table 4.9). It indicated that PE and CA were combined together (Table 4.9). Free CA (Rf 0) on TLC plate was kept at the original position because of its high molecular weight. Three new PE-CA conjugates with
Chapter 4 Conjugation of Lipids with Colominic Acid

Fig. 4.8 Detection of PE-CA conjugates with thin layer chromatography (TLC)

TLC plate: silica gel; eluent: CHCl₃/MeOH 8:2 (v/v); Indicator: molybdenum blue

Table 4.9 Radioactivity of spots on the TLC plate

<table>
<thead>
<tr>
<th>Samples</th>
<th>Rf</th>
<th>H³ (dpm)</th>
<th>C¹⁴ (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>colominic acid</td>
<td>0.00</td>
<td>1112</td>
<td>10</td>
</tr>
<tr>
<td>PE-CA</td>
<td>0.05</td>
<td>289</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>200</td>
<td>388</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>66</td>
<td>198</td>
</tr>
<tr>
<td>PE</td>
<td>0.67</td>
<td>-----</td>
<td>1361</td>
</tr>
</tbody>
</table>

TLC plate: silica gel; eluent: CHCl₃/MeOH 7:3 (v/v)

Different Rf were attributed to the different degrees of PE grafting on CA. PE-CA products with high molar ratios of PE remained in the original spot (Rf 0.05). Decreasing the molar ratio of PE in the conjugates, increased the polarity of PE-CA. Thus, PE-CA (Rf 0.50) moved faster than PE-CA (0.05) on TLC plates. The products
Chapter 4 Conjugation of Lipids with Colominic Acid

(CHCl₃/MeOH 8:0.5, 8:1, 8:1.5, 8:2, 7:3, 5:5, 2:8, 0:8 v/v), DCC moved out as the first peak, then colominic acid conjugation products with high polarity and low molecular weight were eluted. The final one was PE-CA conjugate of higher CA molecular weight (Table 4.10). Generally, PE-CA products were a mixture of alternative grafting polymers since there are many carboxyl groups in each colominic acid chain to couple with PE. Some of the PE-CA can dissolve in water, and another PE-CA can dissolve in methanol and CHCl₃.

Table 4.10 Chromatography of PE-CA on silica acid gel

<table>
<thead>
<tr>
<th>CHCl₃/MeOH</th>
<th>H³ (dpm)</th>
<th>C¹⁴ (dpm)</th>
<th>CA (%)/PE(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:0.5</td>
<td>27.3</td>
<td>13.3</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>8.9</td>
<td>17.6</td>
<td>----</td>
</tr>
<tr>
<td>8:1.0</td>
<td>49.6</td>
<td>12.2</td>
<td>----</td>
</tr>
<tr>
<td></td>
<td>211.3</td>
<td>581.0</td>
<td>0.99/4.3</td>
</tr>
<tr>
<td>8:1.5</td>
<td>1250.6</td>
<td>2587.8</td>
<td>5.8/19.0</td>
</tr>
<tr>
<td></td>
<td>1477.3</td>
<td>1938.3</td>
<td>6.9/14.2</td>
</tr>
<tr>
<td>8:2.0</td>
<td>1324.0</td>
<td>508.4</td>
<td>6.2/3.7</td>
</tr>
<tr>
<td></td>
<td>2727.7</td>
<td>248.8</td>
<td>12.7/1.8</td>
</tr>
<tr>
<td>7:3.0</td>
<td>680.0</td>
<td>191.7</td>
<td>3.2/1.4</td>
</tr>
<tr>
<td></td>
<td>1338.1</td>
<td>357.9</td>
<td>6.3/2.6</td>
</tr>
<tr>
<td>5:5</td>
<td>3804.1</td>
<td>2627.8</td>
<td>17.8/19.3</td>
</tr>
<tr>
<td></td>
<td>3364.3</td>
<td>2320.0</td>
<td>15.7/17.0</td>
</tr>
<tr>
<td>0:1</td>
<td>3132.9</td>
<td>1841.2</td>
<td>14.6/13.5</td>
</tr>
<tr>
<td></td>
<td>1203.7</td>
<td>300.8</td>
<td>5.6/2.2</td>
</tr>
<tr>
<td></td>
<td>890.6</td>
<td>114.9</td>
<td>4.2/0.8</td>
</tr>
</tbody>
</table>
4.3.3 Effect of solvent on the coupling reaction

Solvents used in the reaction depended on the solubility of lipid, colominic acid and catalyst. Cosolvents THF/water (2:1, v/v) and dioxan/water (1:1, v/v) could dissolve all PE, CA and catalyst, and the yield of conjugation was improved significantly (Table 4.11). The yield of reaction carried out in dioxan was much higher than in THF. It was thought that the catalyst efficiency and reactivity of PE and CA related to the solvents. When the ratio of THF to water was increased from 1 to 5, the solution became turbid. Dioxan itself is not a good solvent for PE, but PE in 250μl of THF exhibited good solubility in 1ml of dioxan.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Conjugates (%) THF/water (2:1, v/v)</th>
<th>Conjugates (%) CHCl/water (1:1, v/v)</th>
<th>Conjugates (%) Dioxan/water (1:1, v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDC</td>
<td>7.1</td>
<td>7.8</td>
<td>-----</td>
</tr>
<tr>
<td>DCC</td>
<td>11.4</td>
<td>29.6</td>
<td>47.3</td>
</tr>
<tr>
<td>CMC</td>
<td>22.5</td>
<td>30.1</td>
<td>75.2</td>
</tr>
<tr>
<td>Control</td>
<td>2.5</td>
<td>-----</td>
<td>-----</td>
</tr>
</tbody>
</table>

PE 5mg; CA 1mg; CA-H 200μl, (50μl 27594.1cpm, 55695.7dpm), N,N'-dimethyl aminopyridine (DMAP) 0.813mg; Conjugates are expressed as μmol% of conjugated CA. The control was made by mixing PE and CA without catalysts.

4.3.4 Effect of the catalyst on the coupling reaction

CMC and EDAC are common water-soluble carbodiimide chemicals for catalysing the formation of amide bonds. In spite of water, CMC still dissolves in DMF. EDC is
insoluble in THF and dioxan, which may be the reason of low reaction efficiency of catalysis by EDC. DCC is soluble in many organic solvents such as chloroform, benzene, THF, DMF and dioxan. Although DMSO can dissolve both colominic acid and DCC, it interferes with the carbodiimide catalysts since DMSO joins the reaction. The yield of reaction in the presence of CMC was higher than in presence of DCC (Table 4.12).

Table 4.12 Effect of the catalyst on the reaction of PE with colominic acid

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Yield (μmol% of CA) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCC</td>
<td>12.5 ± 1.4</td>
</tr>
<tr>
<td>CMC</td>
<td>29.7 ± 1.1</td>
</tr>
<tr>
<td>EDC</td>
<td>10.8 ± 1.8</td>
</tr>
<tr>
<td>Control</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

PE 2.5mg, CA 100μg, solvent, THF/water (2:1, v/v); control was made by mixing PE and CA without catalysts and kept at the same conditions.

4.4 Conclusions

Conjugation of PE with OX-CA produced a linear hydrophilic polymer. The reaction rate was determined by pH, solvents, temperature and the reactivity of PE and OX-CA. The yield of coupled OX-CA was approximately 10.3-62.2% depending on the amount of OX-CA added in the mixture. Although the molar ratio of PE to OX-CA was increased from 6.72/0.97 to 6.72/2.67, the yield of coupled PE only increased from 3.3% to 13.5%. The reactivity of OX-CA in the Schiff reaction may be inhibited by its molecular conformation. Conjugation of PE with CA in the presence of DCC catalyst
led to the formation of branched PE-CA products which had different solubility. The conjugates with low molar ratios of PE were more hydrophilic than those with high molar ratios of PE. Thus, PE-CA can dissolve in water, methanol and chloroform depending on the grafting degree of PE on colominic acid. In the report of Mattews and Petrak (1993), they coupled DPPE with colominic acid in the same conformation, but did not describe the properties of final products. Conjugation of PE with CA by formation of amide bonds was very easy to perform at room temperature and the yield was around 7.1-75.2% depending on the catalysts and solvents. DCC and CMC exhibited higher catalyst efficiency than EDC.
Chapter 5

Incorporation of PE-OX-CA and PE-CA into Liposomes
5.1 Introduction

Polysialic acids coupling with the phospholipid moiety at the reducing end was found to form hydrophobic aggregates (Lifely et al., 1988). The phospholipid moiety exists commonly in *N. Meningitidis* group B and C polysaccharides. It has also been found in *E. Coli* polysaccharides. Biological studies have confirmed that sialic acid is related to the recognition and adhesion of cells (Schauer et al., 1984a, 1984b, 1990, 1995, 1997; Patel et al., 1995). In previous studies, we have coupled PE with colominic acid by directly connecting PE to the non-reducing end of the colominic acid, to form linear products (PE-OX-CA), or to the carboxyl group to form grafting polymers (PE-CA). Since the chain length of colominic acid (Mn=15,000-18,000) is much longer than the lipids (PE, MW 744), the linear PE-OX-CA is water soluble. In contrast, PE-CA dissolves in chloroform, methanol and also water depending on the grafting degree of PE on polysaccharides. The higher the ratios of PE to CA in the conjugates, the greater their solubility in chloroform. The possibility of using those two kinds of conjugated products in the preparation of liposomes is discussed in this chapter.

5.2 Incorporation of PE-OX-CA into liposomes

Incorporation of PE-OX-CA into liposomes was achieved by addition of PE-OX-CA, dissolved in 2ml of distilled water, into PC/Chol (1:1, molar ratio) lipid film and rehydration lipids to form MLV liposomes. For measuring incorporation of PE-OX-CA in MLV liposomes, PE-OX-CA has been tritiated with NaBH₄(H³) to form PE-OX-CA-H³. Another labelling method is introducing PE-C¹⁴ into the PE-OX-CA conjugates during the preparation. After separation of free PE-OX-CA from the pellets by centrifugation...
(35krpm (83500g)/30min), the radioactivity of PE-OX-CA in both supernatant and pellets was counted. It was found that the incorporation rate of PE-OX-CA was less than 5\% (Table 5.1). This would be due to the water solubility of PE-OX-CA leading to its entrapment in the aqueous phase rather than in the lipid phase. When PE-OX-CA was incorporated into liposomes by the DRV method, the incorporation efficiency was improved to 14.5\% (Table 5.2). However, the size of liposomes prepared by both the MLV and DRV methods was too large to be intravenously injected into mice. We reduced the vesicle size by sonication and found it resulted in decreasing the incorporation of PE-OX-CA in liposomes, particularly PE-OX-CA in liposomes was nearly 0\% (Table 5.3). The reason was attributed to the hydrophilicity and the large molecular weight of PE-OX-CA. Water soluble PE-OX-CA is difficult to be combined with lipid bilayer, and that caused low incorporated efficiency. On the other hand, PE-OX-CA may be affected by the strong ultrasonic shaking and disappeared from the liposome surface.

Table 5.1 Incorporation of PE-OX-CA into MLV liposomes

<table>
<thead>
<tr>
<th>PE-OX-CA (mg)</th>
<th>Radioactivity (dpm) before centrifugation</th>
<th>Radioactivity in pellets (dpm)</th>
<th>Incorporation (%)</th>
<th>Vesicle size (\mu m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>931.0</td>
<td>45.1</td>
<td>4.8</td>
<td>5.30</td>
</tr>
<tr>
<td>20</td>
<td>1524.5</td>
<td>60.6</td>
<td>4.0</td>
<td>8.45</td>
</tr>
</tbody>
</table>

PC/Chol (1:1, molar ratio, 8\mu moles of PC)
Chapter 5 Incorporation of PE-OX-CA and PE-CA into Liposomes

Table 5.2 Incorporation of PE-OX-CA into DRV liposomes

<table>
<thead>
<tr>
<th>PE-OX-CA (mg)</th>
<th>Radioactivity (dpm) before centrifugation</th>
<th>Radioactivity in pellets (dpm)</th>
<th>Incorporation (%)</th>
<th>Vesicle size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>931.0</td>
<td>135.4</td>
<td>14.5</td>
<td>7.95</td>
</tr>
<tr>
<td>20</td>
<td>1524.5</td>
<td>211.2</td>
<td>13.9</td>
<td>8.24</td>
</tr>
</tbody>
</table>

PC/Chol (1:1, molar ratio, 8 μmols of PC)

Table 5.3 Incorporation of PE-OX-CA into SUV liposomes

<table>
<thead>
<tr>
<th>PE-OX-CA (mg)</th>
<th>Radioactivity (dpm) before centrifugation</th>
<th>Radioactivity (dpm) in pellets</th>
<th>Incorporation (%)</th>
<th>Vesicle size (nm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.7</td>
<td>3933.9</td>
<td>28.6</td>
<td>0</td>
<td>204.0 ± 8.1</td>
</tr>
<tr>
<td>8.1</td>
<td>17110.0</td>
<td>43.7</td>
<td>0</td>
<td>192.4 ± 4.0</td>
</tr>
</tbody>
</table>

PC/Chol (1:1, molar ratio, 16μmols of PC)

5.3 Stability of liposomes incorporating PE-OX-CA in albumin solution

Incorporation of PE-OX-CA into small vesicles by the sonication method resulted in low incorporation efficiency. When those liposomes were incubated in the human serum albumin (HSA) solution at 37 °C for 2h, the vesicle size and polydispersity significantly increased with increasing the concentration of albumin (Figures 5.1a and 5.1b). It implied that the density of colominic acid on the liposome surface was too low to inhibit the adsorption of proteins.

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Chapter 5 Incorporation of PE-OX-CA and PE-CA into Liposomes

Fig. 5.1a Stability of PE-OX-CA liposomes after incubation in albumin solution

5mg or 10mg of PE-OX-CA were incorporated into liposomes composed of equimolar PC (16μmol) and cholesterol. Liposomes were incubated in different concentration of albumin solution at 37 °C for 2h. The vesicle size was measured by PCS. The size of liposomes changed with increasing concentration of HSA.
Fig. 5.1b Polydispersity of PE-OX-CA liposomes after incubation in albumin solution

5mg or 10mg of PE-OX-CA were incorporated into liposomes composed of equimolar PC (16μmol) and cholesterol. Liposomes were incubated in different concentration of albumin solution at 37 °C for 2h. Polydispersity of PE-OX-CA liposomes was measured by PCS.
5.4 Incorporation of PE-CA into liposomes

PE-CA synthesized in the presence of DCC catalyst formed several conjugates which had different solubility in organic solvents. With increasing molar ratio of PE to CA in the PE-CA conjugates, the solubility of PE-CA in chloroform was increased. PE-CA was separated by passing through a silica acid column, and collected as hydrophobic and hydrophilic fractions by gradient elution (Table 4.10). The fraction of PE-CA which moved down the column by washing with a low volume ratio of methanol in the solvent mixture was of high hydrophobicity. In contrast, increasing the volume ratio of methanol in the eluent, the fractions eluting from the column were of high hydrophilic properties, which can be found from the ratio of H³ (which related to colominic) to C¹⁴ (which related to PE) (Table 4.10). Incorporation of both hydrophobic and hydrophilic PE-CA into liposomes was carried out. There was around 39.0% of hydrophobic PE-CA in MLV liposomes and 23.4% of hydrophobic PE-CA in SUV liposomes (Table 5.4). The hydrophilic PE-CA in incorporation was about 12.2-15.3% in SUV liposomes (Table 5.5). After introducing hydrophilic PE-CA into DRV liposomes and adding 1 ml of sucrose (10%) to reduce the vesicle size, the incorporation of hydrophilic PE-CA increased to 24.9-27.0% as shown in Table 5.6. When PE-CA liposomes were passed through a Sepharose 2B column, they eluted in the same fractions as normal liposomes (Figure 5.2). Further study on the hydrolysis of surface colominic acid from PE-CA liposomes by treatment with neuraminidases showed that some of the colominic acid existed on the liposome surface (Figure 5.3). However, due to the branched structure of PE-CA, the arrangement of PE-CA on the liposome bilayers was complicated. Some of the PE-CA on the liposome surface might have a long tail composed of both colominic acid and PE.
Table 5.4 Incorporation of hydrophobic PE-CA into liposomes

<table>
<thead>
<tr>
<th>PE-CA (200µl)</th>
<th>Radioactivity (H^3 dpm) before centrifugation</th>
<th>Radioactivity in the pellets (H^3 dpm)</th>
<th>Incorporation (%)</th>
<th>Vesicle size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLV</td>
<td>45792</td>
<td>17864</td>
<td>39.0</td>
<td>4.98</td>
</tr>
<tr>
<td>SUV</td>
<td>45792</td>
<td>10720</td>
<td>23.4</td>
<td>0.251</td>
</tr>
</tbody>
</table>

PC/Chol (1:1, molar ratio, 16µmols of PC); PE-CA 2mg/200µl.

Table 5.5 Incorporation of hydrophilic PE-CA into SUV liposomes

<table>
<thead>
<tr>
<th>PE-CA (µl)</th>
<th>Radioactivity in SUV (H^3 dpm) before centrifugation</th>
<th>Radioactivity in the pellets (H^3 dpm)</th>
<th>Incorporation (%)</th>
<th>Vesicle size (nm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>22986.2</td>
<td>3528.0</td>
<td>15.3</td>
<td>198.4 ± 11.4</td>
</tr>
<tr>
<td>300</td>
<td>68958.4</td>
<td>8436.2</td>
<td>12.2</td>
<td>163.0 ± 5.7</td>
</tr>
<tr>
<td>400</td>
<td>91944.0</td>
<td>11768.3</td>
<td>12.8</td>
<td>150.5 ± 2.0</td>
</tr>
</tbody>
</table>

PC/Chol (1:1, molar ratio, 32µmols of PC); PE-CA 1mg/100µl

Table 5.6 Incorporation of hydrophilic PE-CA into DRV liposomes

<table>
<thead>
<tr>
<th>PE-CA (µl)</th>
<th>Radioactivity in DRV (H^3 dpm) before centrifugation</th>
<th>Radioactivity in pellets (H^3 dpm)</th>
<th>Incorporation (%)</th>
<th>Vesicle size (µm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>3528.0</td>
<td>952.6</td>
<td>27.0</td>
<td>378.3 ± 38.8</td>
</tr>
<tr>
<td>300</td>
<td>8436.2</td>
<td>2176.5</td>
<td>25.8</td>
<td>277.4 ± 0.1</td>
</tr>
<tr>
<td>400</td>
<td>11768.3</td>
<td>2930.3</td>
<td>24.9</td>
<td>279.8 ± 27.7</td>
</tr>
</tbody>
</table>

PC/Chol (1:1, molar ratio, 32µmols of PC); PE-CA 1mg/100µl
Liposomes incorporated with PE-CA were passed through a Sepharose 2B column and the fractions were counted in a scintillation counter. Since PE-CA was double labelled by $^3$H, which related to colominic acid, and $^{14}$C, which related to PE, liposomes with $^3$H and $^{14}$C at the same fractions indicated that PE-CA was incorporated into lipid bilayers. PE-CA liposomes moved down the column at the same position as normal liposomes (PC/Chol/PE 1:1.2:0.2, molar ratio).
Chapter 5 Incorporation of PE-OX-CA and PE-CA into Liposomes

Fig. 5.3 Enzyme hydrolysis of PE-CA liposomes

PE-CA liposomes were incubated with neuraminidases in sodium acetic buffer (pH 5.5) at 37°C for 2h and passed through a Sephadex G-25 column. The loss of radioactivity from liposomes indicated that some of the PE-CA existed on the surface of liposomes.
5.5 Surface charge of PE-CA liposomes

Liposomes incorporating PE-CA showed different zeta-potentials compared with native liposomes. Native liposomes gave the zeta-potential of -16.8mV to -35.4mV in the phosphate buffer (pH 7.4, ionic strength 50mM). The zeta-potential of PE-CA liposomes was around -41.5mV to -53.8 mV (Figure 5.4). The low zeta-potential of PE-CA liposomes was attributed to the arrangement of PE-CA on the lipid bilayers, which influenced the surface properties of liposomes. According to other studies (Woodle and Lasic, 1992b; Torchilin and Papisov, 1994), the optimum surface charge of stealth liposomes is near
neutral like PEG-liposomes. It is interesting that incorporation of negatively charged GM₁ into liposomes prolonged the circulation time of liposomes (Allen and Chonn, 1987). Avoidance of liposome uptake by the RES was believed to have two pathways; opsonins and dysoponins (Moghimi and Patel, 1988, 1989). Sometimes charged liposomes still remained in the circulation for a long time if they could adsorb some special proteins such as IgA (Moghimi and Patel, 1998). Inclusion of PE-CA into liposomes created different surface charge, which may change the absorption of proteins on the liposome surface.

5.6 Stability of PE-CA liposomes in albumin solution and mouse plasma

Incubation of liposomes incorporating PE-CA in human serum albumin (HSA) solution for 2h did not result in increasing their vesicle size (Figure 5.5a and 5.5b). When liposomes were incubated in mouse plasma, the PE-CA (20%)-containing liposomes coagulated and their vesicle size increased with extension of incubation time (Figure 5.6a and 5.6b). However, the vesicle size of PE-CA (10%)-containing liposomes did not change significantly with increasing the incubation time. Generally, the increasing vesicle size might cause liposomes to be recognized by macrophages since large liposomes were eliminated from blood circulation more rapidly than small ones (Gregoriadis and Senior, 1980; Liu et al., 1991, 1992). Although the dysoponin mechanism showed that adsorption of special proteins enhanced the half-life of liposomes (Moghimi and Patel, 1988), it is not clear how liposomes combined with these kinds of proteins. Most of the reports indicated that adsorption of proteins to particles accelerated the clearance process (Senior, 1987; Senior et al., 1991). Incorporation of PE-CA into lipid bilayers may reduce the absorption of proteins to the liposome surface depending on the ratio of PE-CA in vesicles, suggesting
that PE-CA incorporated in liposomes changed the liposome surface properties.

Fig. 5.5a Stability of PE-CA liposomes after incubation in albumin solution

Liposomes incorporating 10% and 20% of PE-CA were incubated in albumin solution at 37 °C for 2h, and the vesicle size was measured by PCS. Results showed that the vesicle size of PE-CA liposomes did not change significantly with increasing the concentration of albumin solution.
Liposomes incorporating 10% and 20% of PE-CA were incubated in albumin solution at 37°C for 2h, and polydispersity was measured by PCS. Polydispersity of PE-CA liposomes did not increase significantly with increasing the concentration of albumin solution.
Fig. 5.6a Stability of PE-CA liposomes after incubation in mouse plasma

Liposomes incorporating 10% and 20% of PE-CA were incubated in mouse plasma and PBS, respectively, at 37 °C for 24h. The particle size was measured at the time intervals. Results showed the particle size increased with prolonging the incubation time.
Chapter 5 Incorporation of PE-OX-CA and PE-CA into Liposomes

10% of PE-CA liposomes incubated in mouse plasma
20% of PE-CA liposomes incubated in mouse plasma
10% of PE-CA liposomes incubated in PBS
20% of PE-CA liposomes incubated in PBS

Fig. 5.6b Polydispersity of PE-CA liposomes after incubation in mouse plasma

PE-CA liposomes were incubated in mouse plasma at 37 °C for 24h. The polydispersity changed with increasing the incubation time.
Chapter 5 Incorporation of PE-OX-CA and PE-CA into Liposomes

5.7 Blood clearance of PE-CA liposomes

Two fractions of PE-CA eluted from a silica acid column with mixed solvents of CHCl₃/MeOH at the volume ratio of 5:5 (intact PE-CA 5:5) and 8:1.5 (intact PE-CA 8:1.5) were incorporated into PC/Chol (1:1, molar ratio) liposomes, respectively. Since the molecular weight of PE-CA was difficult to measure, the incorporation of PE-CA was calculated by the weight. 2.4 mg PE-CA (10% of weight of PC) mixed with 24.4 mg of PC and 12.6 mg of cholesterol was sonicated to form small vesicles with the diameter around 145.4 - 164.2 nm (Table 5.7). The total incorporation of PE-CA (5:5) and PE-CA (8:1.5) in liposomes was approximately 0.29 mg and 0.60 mg.

Table 5.7 Vesicle size of PE-CA liposomes

<table>
<thead>
<tr>
<th>Liposomes</th>
<th>Vesicle size (nm)(mean ± SD)</th>
<th>Polydispersity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC/Chol/PE (20%)*</td>
<td>164.2 ± 1.2</td>
<td>0.019 ± 0.010</td>
</tr>
<tr>
<td>PC/Chol/PE-CA (5:5)**</td>
<td>151.0 ± 6.2</td>
<td>0.173 ± 0.40</td>
</tr>
<tr>
<td>PC/Chol/PE-CA(8:1.5)***</td>
<td>145.4 ± 0.8</td>
<td>0.261 ± 0.005</td>
</tr>
</tbody>
</table>

*PC/Chol/PE (1:1.2:0.2, molar ratio) intact PC/Chol/PE (20%); ** Liposomes (PC/Chol 1:1, molar ratio) were incorporated with PE-CA (5:5) eluted from a silica column with CHCl₃/MeOH (5:5, v/v); ***Liposomes(PC/Chol 1:1, molar ratio) were incorporated with PE-CA (8:1.5) eluted from a silica column with CHCl₃/MeOH (8:1.5, v/v).

Both of the PE-SA liposomes were dispersed in 2 ml of PBS. 200 μl of suspension was intravenously injected into T.O. male mice by tail vein (three mice in each group). The animal results showed no significant improvement of liposome retention time in the
circulation (Figure 5.7). On the other hand, liposomes contained PE-CA (5:5) were removed from the circulation more rapidly than native liposomes (PC/Chol/PE 1:1.2:0.2, molar ratio). The suspected reason was that incorporation of PE-CA grafting polymers on the liposome surface made both colominic acid and PE became exposed outside of vesicles at the same time. The free lipids on the liposome surface have not been completely protected, and that resulted in the uptake of liposomes by the RES.

5.8 Conclusions

Incorporation of PE-OX-CA and PE-CA into liposomes has not achieved the purpose of protection of liposomes with polysaccharides. Neither PE-OX-CA nor PE-CA can efficiently cover the surface of liposomes and enhance the half-life of liposomes significantly. We think it is due to the low proportion of colominic acid on the liposome surface. Hydrophilic PE-OX-CA of high molecular weight is very difficult to incorporate into the lipid bilayer, which certainly cannot provide any shielding on liposomes. Otherwise, PE-CA grafting polymers may form different arrangement on the lipid membrane, although their incorporation reaches 12.8-39.0%. Mattews and Petrak (1993) have reported the grafting CA-DPPE polymers with similar conformation and indicated that they probably changed the fate of liposomes in the circulation after inclusion into lipid vesicles, but they have not reported any animal results. From this study, it is demonstrated that branched PE-CA polymers cannot form optimum vesicles. Linear hydrophilic lipid-polysaccharide conjugates (PE-OX-CA) need a special technology to be incorporated into liposomes. The density of polymers on the surface of liposomes is a very important factor to prolong the retention time of liposomes in the circulation (Mori et al., 1991).
PE-CA (5:5) and PE-CA (8:1.5) collected from a silica column as fractions with two ratio CHCl₃/MeOH solvent 5:5 and 8:1.5, individually. PE-CA was incorporated into PC/Chol (1:1, molar ratio) liposomes as the ratio of 10% (w/w) of PC. Then liposomes (3.2μmol of PC) which marked with H³-PC in 200μl of PBS were intravenously injected into T.O. mice by tail vein. PC/Chol/PE (1:1.2:0.2, molar ratio) (intact PC/Chol/PE 20%) was as control. Data were represented as mean ± standard deviation (SD) of three mice. PC/Chol/PE-CA (5:5) (p*=0.017) is significantly different with control; PC/Chol/PE-CA (8:1.5) (p=0.334) showed no significant difference compared with control.

Fig. 5.7 Blood clearance of liposomes containing PE-CA
Chapter 6

Conjugation of Colominic Acid with the Liposome Surface and Vesicle Stability In Vitro and In Vivo
6.1 Introduction

The optimum polysialylated liposomes have conjugation of colominic acid only around the exterior of the liposomes so that it can form a hydrophilic stearic hindrance to reduce the uptake of liposomes by RES. It is believed that the action of colominic acid is significantly influenced by its proportion and position on the liposome surface. Commonly, two methods are employed in the preparation of liposomes containing colominic acid. One is synthesis of lipid-colominic acid (PE-CA) conjugated products and incorporation of PE-CA into vesicle bilayers as mentioned in previous studies (Chapter 4 and 5). Another approach is the preparation of liposome vesicles at first, and then colominic acid is conjugated to the surface of liposomes by chemical binding. We have tried to use the first method for modification of liposomes and found it could not control the arrangement of colominic acid on the lipid membrane. Since lipids and PE-CA conjugates were mixed to form multiple bilayers, colominic acid could be inside or outside of liposome vesicles. However, some of the colominic acid was entrapped into the lipid bilayers. Further animal results showed that the circulation time of PE-CA liposomes has not been significantly improved. We studied the factors which determinated the fate of liposomes in the circulation, and supposed that changing the preparation process by directly coupling colominic acid with liposome surfaces might increase the density of colominic acid on the liposome surface and improve the arrangement of colominic acid on lipid bilayers (Figure 6.1). The conjugation of lipids with colominic acid by the Schiff base to form linear polymers has been confirmed in the chapter 4. In this chapter two kinds of liposomes, (PC/Chol/PE) and (DSPC/Chol/DSPE), were modified with oxidized colominic acid (OX-CA), and their stabilities in vivo and in vitro were evaluated.
Chapter 6 Conjugation of Colominic Acid with the Liposome Surface and Vesicle Stability In Vitro and In Vivo

Fig. 6.1 Modification of the liposome surface with oxidized colominic acid (OX-CA)
6.2 Conjugation of OX-CA with the liposome surface

In chapter 4, we have particularly discussed the synthesis of PE-OX-CA conjugated products by the Schiff reaction (Figure 4.3). Data showed that the connection between the lipid and the oxidized colominic acid was very easily carried out in PBS. As introducing the same reaction process for modification of liposome surfaces, the key point was concentrated on the identification and quantitation of colominic acid on the vesicles. Therefore, two methods were used to assay colominic acid. First one is conjugation of colominic acid with fluorescein isothiocyanate (FITC) to form FITC-CA, and then FITC-CA was oxidized with NaIO₄ to form FITC-OX-CA. Since FITC was coupled on the free amino groups of colominic acid, the fluorescence was not affected by the oxidation. The detail of this reaction approach has been described by Gregoriadis, et al. (1993). When 10mg of colominic acid were mixed with a different amount of FITC, the latency of FITC per µg of colominic acid achieved almost the same value as shown in Table 6.1.

Table 6.1 Marking of colominic acid with fluorescein isothiocyanate (FITC)

<table>
<thead>
<tr>
<th>FITC (mg)</th>
<th>Yield (%)</th>
<th>fluorescence/µg of CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>87</td>
<td>90.8</td>
</tr>
<tr>
<td>10</td>
<td>95</td>
<td>127.6</td>
</tr>
<tr>
<td>20</td>
<td>92</td>
<td>120.8</td>
</tr>
</tbody>
</table>

Colominic acid: 10mg

Increasing FITC amounts up to 20mg, the fluorescence per µg of colominic acid did not increase. As introducing FITC-OX-CA and OX-CA in the coupling reaction and
Chapter 6 Conjugation of Colominic Acid with the Liposome Surface and Vesicle Stability In Vitro and In Vivo

subsequently detecting the fluorescence in both pellets and supernatant after separation of SUV by centrifugation, it was found that fluorescence remained in pellets. Compared to the control in which PC/Chol/PE liposomes only mixed with FITC-CA, fluorescence had not been measured in the pellets. This means that OX-CA was successfully conjugated to the liposome surface without loss during the washing process. DSPC/Chol/DSPE (1:1.6:0.6, molar ratio, 32μmol of DSPC) had been mixed with 2.5mg of FITC-OX-CA and reacted at room temperature for 24h. After the reaction was completed, there was 2.28mg (89.1%) of FITC-OX-CA on the liposomes. Modification of PC/Chol/PE (1:1.2:0.2, molar ratio, 12.8μmol of PC) with 10.2mg of OX-CA and 0.8mg of FITC-OX-CA had been carried out under the same conditions. Resulting data showed that 1.06mg (9%) of total OX-CA was coupled to the surface of liposomes.

Another method for quantitative analysis of colominic acid is tritiation of oxidized colominic acid. Unlike the previous labelling process (chapter 3), colominic acid was firstly reduced with normal NaBH₄, and then oxidized with NaIO₄ to form two -CHO groups at both of the polymer ends as shown in Figure 6.2. The quantitative radiolabelling of -CHO group by addition of NaBH₄(H³) was realized by controlling the amount of NaBH₄(H³) in the reaction mixture. The detail studies on the radiolabelling efficiency related on the amount of NaBH₄(H³) have been reported in the chapter 3. When labelling 100mg of OX-CA (67μmol) with 50μl of NaBH₄(H³)(9μCi, 0.2 μmol), the radioactivity for 667μg (0.044μmol) of OX-CA is 369 dpm. -CHO was partially reduced to form -OH (H³) at one end of OX-CA. The structure of labelled OX-CA could be as follows:

unreacted OX-CA: CHO-CA-CHO
Fig. 6.2 Scheme of radiolabelling of OX-CA with NaBH₄ (H³)
labelled OX-CA with -CHO groups: CH₂OH(H^3)-CA-CHO

labelled OX-CA without -CHO groups: CH₂OH(H^3)-CA- OH(H^3)CH₂

Actually, using the mixture of labelled OX-CA in the coupling reaction, the calculation of the amount of colominic acid on the liposome surface was not affected by labelled OX-CA at both ends since it did not join the reaction. The calculation is as follows:

\[ C_i = C + C_o + C_u \]  
\[ \% \text{ of conjugation} = \frac{C}{C_i} \times 100\% \]

- **C** is the total moles of OX-CA
- **C** is the moles of reacted OX-CA
- **C₀** is the moles of OX-CA with double labelling ends
- **C_u** is the moles of unreacted OX-CA

Table 6.2 showed the conjugation ratio of OX-CA to PC/PE/Chol liposomes. After purification, 19.7-30.5% of colominic acid remained in the pellets. This result suggested that OX-CA was conjugated with PE exposed on the surface of liposomes. The conjugation ratio of CA to PE was not very high (CA/PE molar ratios: 0.085-0.13μmol / 0.46-2.76μmol). However, increasing the molar ratio of PE in the liposome composition from 5 to 30μmol%, the amount of coupling colominic acid was not significantly increased. This is probably due to several reasons. First, the particle size of liposomes which joined in the coupling reaction had been reduced to 100-200nm by sonication. The conjugation might be saturated by the limited surface areas. Otherwise, polysaccharides with negative charge and non-linear conformation in the solution would produce the steric repulsive force
Table 6.2 Conjugation of OX-CA with the surface of liposomes

<table>
<thead>
<tr>
<th>PE*</th>
<th>Radioactivity after reaction (dpm)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Pellet</td>
</tr>
<tr>
<td>5%</td>
<td>1251</td>
<td>1095</td>
</tr>
<tr>
<td>10%</td>
<td>1268</td>
<td>1007</td>
</tr>
<tr>
<td>20%</td>
<td>1131</td>
<td>1128</td>
</tr>
<tr>
<td>30%</td>
<td>1616</td>
<td>728</td>
</tr>
<tr>
<td><strong>DSPE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>2859</td>
<td>846</td>
</tr>
<tr>
<td>60%</td>
<td>1601</td>
<td>928</td>
</tr>
</tbody>
</table>

*PC/Chol/PE (1:1.05:0.05; 1:1.1:0.1; 1:1.2:0.2; 1:1.1:0.3, molar ratios) (intact 5%, 10%, 20% and 30% of PE) (9.2 μmol of PC) liposomes were mixed with 100μl of labelled OX-CA (3694dpm) (6.67mg).

**DSPC/Chol/DSPE (1:1.2:0.2; 1:1.6:0.6, molar ratios) (intact 20% and 60% of DSPE) (8.8μmol of DSPC) liposomes were mixed 100μl of labelled OX-CA (3694dpm) (6.67mg).

between two close colominic acid polymer chains, which prohibited the coupling of OX-CA reaching a high level. Moreover, considering the arrangement of lipids as double molecular layers in vesicles, even liposome vesicles were SUV structure, there was only 50% of PE towards water phase, which could join the coupling reaction. Another 50% of PE enclosed inside the vesicles had no opportunity to react with colominic acid. However, 50% of PE on the liposome surface may be hidden by the multiple lipid bilayers if the vesicles were not absolutely simple SUV. On the other hand, the amino groups of PE on the liposome surface might be trapped between cholesterol and PC, and thus they cannot react with OX-CA. Therefore, the amount of PE involved in the reaction is less than 50%
of the amounts added. We had tried to make the liposomes with molar ratio of PE to PC more than 1:1, and found that lipids could not form a stable vesicle formulation. After sonication, all lipids coagulated together and precipitated. The same performance had also been found in the preparation of DSPC/Chol/DSPE liposomes as the ratio of DSPC/DSPE was more than 1:0.8. In this study, the maximum molar ratio of PC/Chol/PE was 1:1.3:0.3. The conjugation of colominic acid was 4.4mg on PC/Chol/PE liposomes (PC/Chol/PE 1:1.3:0.3, molar ratio, 32μmol of PC).

The control study had been made by mixing tritiated colominic acid (1.2mg, 50372dpm) with PC/Chol/PE liposomes under the same conditions. Results showed that less than 1% of colominic acid was adsorbed to the liposome surface (Table 6.3). The similar conclusion had been obtained in the previous coating study, in which liposomes were incubated in colominic acid solution and almost no specific adsorption was found. However, for the OX-CA coupling reaction, 19.7-30.5% of OX-CA, at least, was conjugated on the liposome surface depending on the reaction conditions.

Table 6.3 Control study on the adsorption of CA on liposomes

<table>
<thead>
<tr>
<th>PC/Chol/PE* (molar ratio)</th>
<th>Radioactivity (dpm)</th>
<th>CA on the surface (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Pellet</td>
</tr>
<tr>
<td>1:1.1:0.1</td>
<td>41121</td>
<td>31</td>
</tr>
<tr>
<td>1:1.2:0.2</td>
<td>35572</td>
<td>61</td>
</tr>
</tbody>
</table>

Radioactivity of CA (1.2mg): 50372dpm; * PC/Chol/PE liposomes (8μmol of PC)
Nevertheless, the density of polymers on the liposome surface extremely influences the half-life of liposomes in the blood and the stability of lipid bilayers. The best coupling rate of PEG into liposomes was suggested around 5-10mol% of relatively short chains (n 40-130) unlike the theory stated that the higher grafting density of long chains would result in better stabilization (Mori et al., 1991). It is believed that membrane inclusion of PEG-lipids showed saturation behaviour. The overall pressure above the bilayer could destabilize liposomes by creating hydrophobic defects which then acted as sites of attachment for opsonins (Klibanov et al., 1991). For polysialylated liposomes, the maximum coupling of colominic acid was limited by the conjugation method. Besides the increasing vesicle size with coupling OX-CA to the liposome surface (Table 6.4), the destruction of vesicles has not been found. The reason of changing vesicle size was attributed to not only the coupling OX-CA to increase vesicle size but also some crosslinking among vesicles. Since colominic acids consist of different molecular weights, the double oxidation of oligo-sialic acids would result in the crosslinking, and thus the average vesicle size was changed.

**Table 6.4 Effect of conjugation on vesicle size**

<table>
<thead>
<tr>
<th>PC/Chol/PE (molar ratio)*</th>
<th>Size before conjugation (nm) (mean ± SD)</th>
<th>Size after conjugation (nm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1:1:0.1</td>
<td>115.6 ± 1.6</td>
<td>149.1 ± 3.3</td>
</tr>
<tr>
<td>1:1.2:0.2</td>
<td>88.1 ± 0.5</td>
<td>134.3 ± 2.5</td>
</tr>
<tr>
<td>1:1.3:0.3</td>
<td>141.7 ± 13.5</td>
<td>172.7 ± 6.0</td>
</tr>
</tbody>
</table>

* Liposomes (9.2 μmol of PC) were mixed with labelled OX-CA (3694dpm)(6.67mg).
6.3 Stability of OX-CA on the surface of liposomes

Liposomes coupled with OX-CA were suspended in mouse plasma at 37 °C for 2h. After separation by centrifugation, the radioactivity in both pellets and supernatant was counted. It was found that about 45-75% of OX-CA remained on the liposome surface. The connection between OX-CA and PE was quite stable towards the plasma proteins. Although the linear conjugate product PE-OX-CA had high water solubility, the remaining of PE-OX-CA on the lipid bilayers was confirmed.

![Graph](image)

Fig. 6.3 Stability of OX-CA on the liposome surface

PC/Chol/PE (1:1.05:0.05; 1:1.1:0.1; 1:1.2:0.2; 1:1.3:0.3, molar ratios) liposomes coupled with tritiated OX-CA on the surface were incubated in 100μl of mouse plasma at 37 °C for 2h. Results showed that OX-CA was stable on liposomes.
6.4 Surface charge of polysialylated liposomes

Zeta-potentials of liposomes before and after conjugation with colominic acid were found to be significantly different. Data showed that zeta-potentials of non-polysialylated liposomes was of -16.8mV to -35.4mV, but zeta-potentials of liposomes coated with colominic acid were of -7.1mV to -11.1mV (Figure 6.4). This suggested that some polysaccharides were coupled on the liposome surface, and changed the surface charge of the liposomes. The control study was carried out by addition of 2.5mg CA to native liposomes and incubation at room temperature for 24h. Compared with native liposomes, the zeta-potential of CA and liposome mixture had a similar surface charge (-15.4mV to -34.1mV) (Figure 6.4). This may suggest that the adsorption of CA on liposomes was very low, and the different surface charge of polysialylated liposomes from non-polysialylated liposomes was caused by the conjugation of colominic acid to the liposome surface. Blume and Cevc (1993a) indicated that uncharged vesicles were more stable in the blood than charged ones since surface charge of vesicles may cause the adsorption of plasma proteins, and thus promotes the recognition of liposomes by macrophages. However, Moghimi et al. (1988, 1989) reported that the selective adsorption of certain plasma components (dysopsonins) prevented the uptake of liposomes. The competition between opsonins and dysopsonins was the key in controlling the vesicle uptake by macrophages (Moghimi et al., 1988, 1989). It is not clear which surface charge is optimum for stealth liposomes. Polysialylated liposomes with neutral surface charge may result in prolonging their circulation time.

The confusing result of the zeta-potential measurement was that non-polysialylated
liposomes composed of positively charged PE had more negative zeta-potentials than liposomes coupled with negatively charged CA on the surface. On increasing the molar ratio of PE in the lipid composition, the zeta-potential of native liposomes decreased. Oppositely, the zeta-potential of liposomes coupling CA did not change significantly with
increasing the molar ratio of PE (Figure 6.4). It is assumed that the zeta-potential of native liposomes was greatly determined by the cholesterol component since the amount of cholesterol in the lipid composition was equal with moles of total lipids (moles of PC plus PE) rather than moles of PC. In other words, the moles of cholesterol increased with increasing the molar ratio of PE in the preparation, e.g. vesicles composed of PC/Chol/PE (1:1.1:0.1) consist in 1.1 moles of lipids and 1.1 moles of cholesterol. Accordingly, the zeta-potential of liposomes containing cholesterol did not regularly change with the increasing of PE in the composition (Figure 6.5). If liposomes were prepared without cholesterol, their zeta-potentials were of -8.1mV to -19.9mV which was more positive than liposomes with cholesterol (-16.8mV to -35.4mV) (Figure 6.5). Inclusion of cholesterol into lipid bilayers changed the surface charge of liposomes. The early report also indicated that the cholesterol increased the fluidity or micro viscosity of liposome bilayers, reduced the permeability of the membrane structure and stabilized liposomes in vivo and in vitro (Kieby and Gregoriadis, 1980a, 1980b, 1980c). Otherwise, it has been reported that liposomes with positive charge were more stable than ones with negative charge in the circulation (Gregoriadis and Neerunjun, 1975; Juliano and Stamp, 1975). In fact, from zeta potential studies, it is easy to understand that liposomes composed of positively charged PE were still with negatively charged surfaces due to the effect of cholesterol. The long half-life of liposomes with positively charged lipids may be not because they contain positively charged surfaces, but because the arrangement of lipids and cholesterol is different.
Fig. 6.5 Zeta-potential of PC/PE and PC/Chol/PE liposomes

PC/PE (1:0.05; 1:0.1; 1:0.2; 1:0.3; 1:0.5, molar ratios) and PC/Chol/PE (1:1.05:0.05; 1:1.1:0.1; 1:1.2:0.2; 1:1.3:0.3; 1:1.5:0.5, molar ratios) liposomes were suspended in 0.05M phosphate buffer (ionic strength 50mM, pH 7.4). Results showed that cholesterol significantly affected the liposome surface charge.
Chapter 6 Conjugation of Colominic Acid with the Liposome Surface and Vesicle Stability In Vitro and In Vivo

6.5 Stability of polysialylated liposomes in albumin solution and mouse plasma

Conjugation of colominic acid with the liposome surface improved the stability of vesicles in either PBS buffer or protein solution. Normally, PC/Chol/PE liposomes cannot be stored for a long time because of the coagulation of vesicles. When PC/PE/Chol liposomes were stored at 4 °C for four weeks, vesicles precipitated and the size changed form 84-101nm to 1.06-11.9μm (Figure 6.6). The polydispersity increased from 0.1 to 1.0. The same behaviour had been reported by Plessis et al. (1996). However, polysialylated liposomes can be stored in the solution for, at least, one month without producing any precipitation. The size and polydispersity of polysialylated liposomes before and after storage did not change significantly (Figure 6.7 and 6.8). This suggests that the colominic acid inhibited the aggregation of vesicles. The linear polymer may act as steric barrier or change the surface charge and hydrophilicity, which make liposomes more stable. Further study on suspensions of polysialylated liposomes in human serum albumin (HSA) solution showed similar results (Figure 6.9 and 6.10). Increasing the protein concentration from 0.1 to 5%, non-polysialylated liposomes aggregated. Their size increased from 122.7nm to 1002.6nm and the polydispersity increased from 0.18 to 1.0 because of adsorption of proteins. Polysialylated liposomes did not significantly increase in size with increasing the protein concentration. For example, polysialylated PC/Chol/PE (1:1.1:0.1, molar ratio) with diameter around 155.1nm was still at 153.0nm after incubation in albumin solution for 2h. Instead of albumin, fresh mouse plasma was added into polysialylated liposomes. The non-polysialylated and polysialylated liposomes were assayed after incubation in mouse blood plasma. Results showed that the size and polydispersity of polysialylated liposomes did not greatly increase on increasing the concentration of mouse plasma (Figure 6.11 and 6.12).
This may result from increasing hydrophilic properties and formation of a steric barrier on the liposome surface by coating colominic acid to reduce the affinity of liposomes to proteins. Many studies indicated that the half-life of liposomes in the circulation depended on their size and the ability to avoid adsorption plasma proteins (Gregoriadis and Senior, 1980; Blume and Cevc, 1993a) Liposomes with large size were removed from the circulation more rapidly than small ones. Particularly, as vesicle size is more than 300nm, most of the liposomes were filtered out and accumulated in the spleen in a short time (Liu and Liu, 1991). Since the adsorbed proteins cause the opsonization of macrophage, the clearance of intact liposomes from circulation was enhanced with increasing adsorption of plasma proteins (Roerdink et al., 1986). Thus, the stabilization of polysialylated liposomes in the protein solution and mouse plasma may result in the low uptake of liposomes by the RES.
Fig. 6.6 Vesicle size of PC/Chol/PE liposomes before and after storage

PC/Chol/PE (1:1.05:0.05; 1:1.1:0.1; 1:1.2:0.2; 1:1.3:0.3; 1:1.5:0.5, molar ratios) liposomes in PBS were left at 4 °C for four weeks. Some precipitates appeared in the suspension and the vesicle size increased from around 100nm to several micrometers.
Fig. 6.7 Stability of polysialylated liposomes after storage at 4 °C for four weeks

PC/Chol/PE (1:1.05:0.05; 1:1.1:0.1; 1:1.2:0.2; 1:1.3:0.3; 1:1.5:0.5, molar ratios) liposomes containing OX-CA in PBS were kept at 4 °C for four weeks. The size change before and after storage was measured by PCS. No precipitates were produced in the suspension.
Fig. 6.8 Polydispersity of polysialylated liposomes after storage

PC/Chol/PE (1:1.05:0.05; 1:1.1:0.1; 1:1.2:0.2; 1:1.3:0.3; 1:1.5:0.5, molar ratios) liposomes coated with OX-CA were measured the polydispersity in PBS after storage for 4 weeks.
Fig. 6.9 Stability of polysialylated and non-polysialylated liposomes in albumin solution

PC/Chol/PE liposomes with and without OX-CA were suspended in 0-5% human serum albumin solution (HSA) at 37 °C for 2h. The vesicle size was measured by PCS. Results showed that polysialylated liposomes did not change the size with increasing the concentration of HSA.
Fig. 6.10 Polydispersity of polysialylated liposomes after incubation in albumin solution

Polysialylated and non-polysialylated liposomes were incubated in 0-5% of HSA solution at 37 °C for 2h. The polydispersity of non-polysialylated vesicles significantly changed with increasing the concentration of albumin solution.
Chapter 6 Conjugation of Colominic Acid with the Liposome Surface and Vesicle Stability In Vitro and In Vivo

Fig. 6.11 Stability of polysialylated liposomes after incubation in mouse plasma

Polysialylated and non-polysialylated liposomes incubated in 5, 10 and 50μl of mouse plasma at 37 °C for 2h. The vesicle size was measured by PCS. The size of polysialylated liposomes did not increase significantly with increasing the concentration of plasma.
Fig. 6.12 Polydispersity of polysialylated liposomes after incubation in mouse plasma

Polysialylated and non-polysialylated liposomes were incubated in 5-50μl of mouse plasma at 37 °C for 2h. The polydispersity of vesicles was measured by PCS. The polydispersity of polysialylated liposomes did not change significantly with increasing the concentration of plasma.
6.6 Adsorption of proteins on polysialylated liposomes

Adsorption of proteins onto the liposome surface was significantly reduced by coupling colominic acid (Figure 6.13). For the vesicles with similar lipid composition, non-polysialylated liposomes adsorbed 21.7-46.5% of plasma proteins, and polysialylated liposomes only absorbed 6.1-19.3% of plasma proteins. Obviously, liposomes under the protection of colominic acid are more stable in the mouse plasma. Considering the effect of vesicle size, liposomes (64μmol of PC) were firstly prepared and suspended in 2ml of distilled water. 1ml of liposomes was modified with OX-CA. Another 1ml of liposomes was used as control. The action of plasma proteins on promoting the clearance of liposomes has been demonstrated larger molecular mass proteins on the surface of vesicles result in more rapidly clearance (Senior et al., 1991). It was assumed that the predominant mechanisms of liposome clearance from blood were opsonization by immune and non-immune components in blood, such as immunoglobulins, complement factor proteins, and fibronectin, which trigger subsequent macrophage uptake (Moghimi and Patel, 1998; Harashima et al., 1998). The reaction with plasma (lipoproteins), which can be expressed as liposome attached with lipoproteins caused the recognition by the receptor on macrophages, and was uptake by macrophages (Moghimi and Patel, 1998). The forces to make a macromolecule bind to the surface of liposomes probably include electrostatic, hydrophobic, Van der Waals, hydrogen bonding, and specific interactions such as antibodies (Lasic, 1993). Clearly, liposomes uncharged and coated with polymers are more stable because any strong ionic and hydrophobic binding are absent, which prevents the penetration of a segment of the macro-molecules into bilayers (Woodle, 1995). Polysialylated liposomes which reduced the absorption of proteins may contribute to the
long circulation time of vesicles.

Fig. 6.13 Adsorption of mouse plasma proteins on polysialylated liposomes after incubation for 2h

0.5ml of PC/Chol/PE (1:1.05:0.05; 1:1.1:0.1; 1:1.2:0.2; 1:1.3:0.3, molar ratios) polysialylated and non-polysialylated liposomes (8μmol of PC) were mixed with 50μl of mouse plasma which was labelled by I\(^{125}\), and incubated at 37 °C for 2h. The adsorption of proteins onto polysialylated liposomes was significantly less than onto non-polysialylated liposomes.
6.7 Release of 5(6) carboxyfluorescein (CF) from polysialylated liposomes

The leaking of CF from polysialylated liposomes was found to depend on the medium used. As liposomes were incubated in PBS for 24h, the release of CF from coupled and native liposomes was around 3.2 - 11.3% (Figure 6.14). This indicated that conjugation of OX-CA to the liposome surface did not destroy the lipid bilayers. After modification of liposomes with OX-CA, the lipid vesicles were still enclosed and could effectively entrap small molecular substances. Liposomes composed of PC/Chol/PE (1:1.05:0.05, molar ratio) in PBS released CF more quickly than polysialylated liposomes composed of PC/Chol/PE (1:1.05:0.05, molar ratio) and PC/Chol (1:1, molar ratio). In contrast, the release of CF from polysialylated liposomes composed of PC/Chol/PE (1:1.2:0.2, molar ratio) was quicker than from native PC/Chol/PE liposomes (1:1.2:0.2, molar ratio) (Figure 6.14). When polysialylated and non-polysialylated liposomes were incubated in fresh mouse plasma, the release of CF from coupled liposomes was quicker than from native liposomes 5h after incubation (Figure 6.15). 24h post-incubation, 23.3-33.2% of the CF was released from polysialylated liposomes. The reason for the rapidly leaking of the CF from polysialylated liposomes may cause by the removing of PE-OX-CA from vesicles. However, the effect of plasma and whole blood on the release of CF from liposomes has been reported (Kirby et al., 1980; Gregoriadis and Davis, 1979). It was found that the release of CF from cholesterol-rich liposomes (PC/Chol 7:7, molar ratio) was significantly slower than from cholesterol-poor liposomes (PC/Chol 7:2, molar ratio) during 2h incubation in mouse plasma. Latency of CF entrapped in cholesterol-poor liposomes was rapidly decreased to 13% 1.5h after incubation. This suggests that the properties of lipid bilayers were determined by their components. Conjugation of OX-CA to the liposome surface reduced
the adsorption of plasma proteins, but with increasing incubation time, the hydrolysis of OX-CA may lead to the leaking CF from vesicles.

![Graph showing release of CF from polysialylated liposomes after incubation in PBS](image)

**Fig. 6.14 Release of CF from polysialylated liposomes after incubation in PBS**

0.1ml of polysialylated and non-polysialylated liposomes entrapped with CF were added 0.5ml PBS and incubated at 37 °C. 10µl of samples was taken at the time intervals and mixed with 3ml of PBS to measure the fluorescein. The release of CF from both polysialylated and non-polysialylated liposomes was not very rapid.
Fig. 6.15 Release of CF from polysialylated liposomes after incubation in mouse plasma

0.1ml of polysialylated and non-polysialylated liposomes entrapped with CF were mixed with 50μl mouse plasma and 450μl of PBS and incubated at 37 °C. 10μl of samples was taken at the time intervals and diluted with 3ml of PBS. The fluorescein was measured. It is clear that the polysialylated liposomes released CF faster than non-polysialylated liposomes as the samples were left in mouse plasma more than 5h.
6.8 Blood clearance of polysialylated liposomes

The clearance of polysialylated liposomes composed of PC/Chol/PE (1:1.2:0.2, molar ratio) from the mouse circulation was much slower than non-polysialylated liposomes (PC/Chol/PE, 1:1.2:0.2, molar ratio). Up to 35.1% of conjugated liposomes (injected dose: 3.2µmol of PC) remained in the mouse blood 6h after injection while only 10.0% of non-polysialylated liposomes were in the circulation at the same bleeding time (Figure 6.16). At 24h post-injection, 19.6% of polysialylated liposomes were still in the circulation compared with 5.8% remaining of non-polysialylated liposomes. In the study, we also used low molecular weight colominic acid (CUT-OX-CA, Mn 7,800) to couple with the surface of liposomes, and the same conclusion was obtained. Liposomes with CUT-OX-CA showed long circulation, 6h after injection, about 33.7% of CUT-OX-CA liposomes remained in the mouse blood. 24h after injection, there still were 9.8% of CUT-OX-CA liposomes in the circulation. The vesicle size of polysialylated liposomes was shown in Table 6.5. Although non-polysialylated liposomes were smaller than coupled liposomes, they were cleared out from the circulation more rapidly than polysialylated liposomes. Another study on polysialylated PC/Chol/PE (1:1.1:0.1; 1:1.2:0.2, molar ratios) (intact PE-OX-CA 10% and PE-OX-CA 20%) liposomes gave similar results even when the injected dose was reduced to 1.6µmol of PC (Figure 6.17). Liposomes with colominic acid on the surface were removed from the circulation more slowly than non-polysialylated liposomes. 3h after injection, 22.8% of modified liposomes (PE-OX-CA 20%) and 20.9% of modified liposomes (PE-OX-CA 10%) remained in the blood compared with 9.2% remaining of non-polysialylated liposomes. The vesicle size was shown in the table 6.6.
Chapter 6 Conjugation of Colominic Acid with the Liposome Surface and Vesicle Stability In Vitro and In Vivo

Table 6.5 Vesicle size of injected liposomes (injected dose: 3.2μmol of PC)

<table>
<thead>
<tr>
<th>Liposomes*</th>
<th>Vesicle size (nm) (mean ± SD)</th>
<th>Polydispersity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE 20%</td>
<td>86.0 ± 3.1</td>
<td>0.260 ± 0.060</td>
</tr>
<tr>
<td>PE-OX-CA 20%</td>
<td>138.0 ± 5.3</td>
<td>0.163 ± 0.063</td>
</tr>
<tr>
<td>PE-CUT-OX-CA 20%</td>
<td>98.0 ± 1.8</td>
<td>0.100 ± 0.029</td>
</tr>
</tbody>
</table>

*PC/Chol/PE (1:1.2:0.2, molar ratio) liposomes were coupled with OX-CA (intact PE-OX-CA 20%) or short chain OX-CA (intact PE-CUT-OX-CA 20%); PC/Chol/PE (1:1.2:0.2, molar ratio) (intact PE 20%) was used as control.

Table 6.6 Vesicle size of injected liposomes (injected dose: 1.6μmol of PC)

<table>
<thead>
<tr>
<th>Liposomes*</th>
<th>Vesicle size (nm) (mean ± SD)</th>
<th>Polydispersity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE 10%</td>
<td>66.5 ± 3.6</td>
<td>0.236 ± 0.007</td>
</tr>
<tr>
<td>PE-OX-CA 10%</td>
<td>143.0 ± 2.0</td>
<td>0.145 ± 0.034</td>
</tr>
<tr>
<td>PE-OX-CA 20%</td>
<td>77.5 ± 2.8</td>
<td>0.219 ± 0.016</td>
</tr>
</tbody>
</table>

*PC/Chol/PE (1:1.1:0.1;1:1.2:0.2, molar ratios) liposomes were coupled with OX-CA (intact PE-OX-CA 10% or PE-OX-CA 20%); PC/Chol/PE (1:1.1:0.1, molar ratio) (intact PE 10%) was used as control.

However, the half-life of polysialylated liposomes has not achieved to 40h like polysialic acid B (Gregoriadis et al., 1993). The effect of colominic acid on prolonging the circulation time of liposomes is less than for PEG liposomes (Woodle et al., 1990, 1995). It was reported that 50% of PC/Chol/mPEG<sub>5000</sub>-PE remained in the circulation 5h after injection (Klibanov et al., 1990). Compared with dextran (Pain et al., 1984), colominic acid enhanced the half-life of liposomes two folds. This infers that colominic acid may play a role to deter
the uptake of liposomes by the RES, although the action of colominic acid was not fully understood. The half-life of polysialylated liposomes may relate to many characteristics such as the density of colominic acid on the surface, the conformation of colominic acid towards the medium and the biocompatibility. Woodle (1992) and Torchilin (1994) indicated that the formation of steric hindrance was the main mechanism of long circulating PEG liposomes. Colominic acid to avoid the recognition by macrophages was believed to have the similar action. From in vitro studies, we had demonstrated the effect of colominic acid on stabilization of liposomes in plasma and reduction of absorption of proteins, which all contributed to the enhancing the half-life of liposomes. Allen and other studies reported that enzymatic removal of sialic acid from circulating cells, or from monosialogangliosides results in their rapid uptake into Kupffer cells of liver (Allen and Chonn, 1987; Schauer, 1982a, 1982b). The long circulation time of polysialylated liposomes may relate to both biological and physical hindrance providing by colominic acid on the surface of liposomes.

6.9 Tissue distribution of polysialylated liposomes

The distribution of liposomes in organs (heart, kidney and lung) was quite low compared with those in the liver and blood (Figure 6.18). It indicated that the clearance of polysialylated liposomes by the liver was still a main route.
Fig. 6.16 Blood clearance of polysialylated liposomes (injected dose: 3.2μmol)

PC/Chol/PE (1:1.2:0.2, molar ratio) liposomes were coupled with OX-CA (intact PE-OX-CA 20%) or short chain OX-CA (intact PE-CUT-OX-CA 20%). 200μl of samples containing 3.2μl of PC was intravenously injected into TO mice by tail vein (3-4 mice in each group). Results showed the significant difference between polysialylated liposomes and non-polysialylated liposomes (PE-OX-CA 20%, p**=0.0087) (PE-CUT-OX-CA 20%, p*=0.020). p<0.05 means that date has significant difference compared with control; Results are expressed as the mean ± standard deviation (SD) of 3-4 mice.
100μl (1.6μmol of PC) of PC/Chol/PE (1:1.1:0.1; 1:1.2:0.2, molar ratios) liposomes coupled OX-CA on the surface were injected into TO mice, respectively. Comparing polysialylated liposomes (intact PE-OX-CA 10% or PE-OX-CA 20%) with non-polysialylated liposomes (intact PE 10%), results showed significant difference (PE-OX-CA 10%, p**=0.0089), (PE-OX-CA 20%, p*=0.013). p<0.05 means that data has significant difference compared with control; Results are expressed as the mean ± SD of three mice.

Fig. 6.17 Blood clearance of polysialylated liposomes (injected dose: 1.6μmol)
Fig. 6.18 Tissue distribution of polysialylated liposomes in mice 3h after injection

100μl (1.6μmol of PC) PC/Chol/PE (1:1.1:0.1; 1:1.2:0.2, molar ratios) liposomes coupled OX-CA on the surface were intravenously injected into TO mice. 3h after injection, the mice were killed and the organs were removed, washed with saline, homogenized and counted. It is clear that most of the polysialylated and non-polysialylated liposomes accumulated in the liver. Results are expressed as the mean ± SD of three mice.
6.10 Conclusions

Conjugation of colominic acid to the liposome surface improved the stability of liposomes in albumin solution, reduced the adsorption of mouse plasma to vesicles, and enhanced the circulation time of liposomes in mouse circulation. The retention time of polysialylated liposomes in the mouse circulation was improved two-fold compared with non-polysialylated liposomes. In addition, the surface charge of liposomes coated with colominic acid was near neutral. Compared with animal results of incorporation of PE-CA or PE-OX-CA into liposomes (chapter 5), conjugation of colominic acid with the liposome surface effectively formed a steric hindrance on the liposome surface to reduce the uptake of liposomes by the RES. Thus, colominic acid and other types of polysialic acid may be interesting polysaccharides for preparation of stealth liposomes.
Chapter 7

Modification of Liposomes with Sialic Acid and its Stability In Vitro and In Vivo
7.1 Introduction

Sialic acid structured as 5-amino-3,5-dideoxy-2-nonulosonic acid has been found as the main terminal group in many glycoproteins and glycolipids. In this special position, sialic acid is supposed to significantly contribute to molecular properties in either masking recognition sites or serving as recognition determinates (Kelm and Schauer, 1997). From the affinity of sialic acid to the virus, it is revealed that sialic acid is a very important regulator of cellular and molecular interactions in binding of both pathogens and nonpathological cellular to host cells (Schauer, 1982a, 1982b, 1985). The clearance of sialic acid from the cell surface will cause the response of macrophage to take up the cells (Schauer et al., 1984a, 1984b, 1990, 1995; Allain et al., 1996). Other studies show that the concentration of sialic acid in the tumour patients is significantly higher than in normal people (Patel et al., 1995; Klapan et al., 1993). The assay of blood level of sialic acid has been used as a tumour mark (Lagana et al., 1995). Derivatives of sialic acid can also prevent the flu virus (Ryan et al., 1995). Park and Huang (1993) had intended to identify the function of sialic acid on GM₁ liposomes by the chemical modification of final sugar groups. It is interesting that for oxidized GM₁ liposomes only 4.9% is left in the circulation 3h after injection compared with the 58.0% of native GM₁ liposomes under the same conditions. Other modifications on sialic acid of GM₁ such as methylation, reduction and conjugation with DOPE all changed the stability of GM₁ liposomes in the circulation, but they believed the protection of GM₁ to liposomes was associated with the whole structure of GM₁ rather than the simple sialic acid residues. Yamauchi and his colleagues (1993) have conjugated sialic acid with 2-(2-palmitoylamido-1-ethyl)-5-acetoamine and incorporated it into liposomes. Resulting data showed that the half-life of liposomes
incorporating sialic acid conjugates was enhanced significantly in the rat. In our previous studies, we have revealed that colominic acid on the liposome surface not only improved the stability of liposomes in contact with mouse plasma but also prolonged the half-life of liposomes in the mouse circulation (chapter 6). The reason may be attributed to the special function of sialic acid or the formation of a sterical shield on the vesicle surface. In understanding which factors result in the long circulation of polysialylated liposomes, sialic acid was conjugated with PE to produce a PE-SA product, and then incorporated into PC/Chol liposomes. The clearance of liposomes incorporating a different molar ratio of PE-SA from the blood circulation and their stabilities in vitro were investigated in this chapter.

7.2 Conjugation of sialic acid with PE (PE-SA)

Conjugation of sialic acid with PE in the presence of carbodiimide catalysts (DCC) rapidly occurred at room temperature. A precipitate of DCC derivatives appeared in the mixture solution after reaction for 2h. When a trace amount of C\textsuperscript{14} labelled sialic acids was used as indicator in the reaction and PE was dyed with molybdenum blue, the spots of PE-SA conjugates and PE control were easily visible. Sialic acid was counted after removing the spots into scintillation tubes. A new lipid product which moved faster than PE on a TLC plate was found (Figure 7.1), and the conjugated sialic acid and the conjugated PE appeared at same position (Table 7.1). The mechanism of reaction was shown in Figure 7.2. The carboxyl (-COOH) group of sialic acid reacted with the amine (-NH\textsubscript{2}) of PE to form amide bonds under the DCC catalysts. The polymerization of the -COOH of SA with the -OH of SA to form polysialic acid may interfere to the conjugation of PE with SA, but
oligo-sialic acid was isolated from the final mixture because of its water solubility. Another by-product was the conjugate of PE with the oligomer of sialic acid, which was removed from the mixture by passing through a silica gel column according to the different polarity between PE-SA and PE-oligo-SA.

Table 7.1 Detection of PE-SA conjugated product with thin layer chromatography (TLC)

<table>
<thead>
<tr>
<th>Rf</th>
<th>PE control</th>
<th>PE-SA conjugate (dpm)</th>
<th>SA control(dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>14.5</td>
<td>321.6</td>
</tr>
<tr>
<td>0.18</td>
<td></td>
<td>+ 59.7</td>
<td></td>
</tr>
<tr>
<td>0.24</td>
<td>+</td>
<td></td>
<td>156.7</td>
</tr>
<tr>
<td>0.64</td>
<td>+</td>
<td>+ 58.7</td>
<td></td>
</tr>
<tr>
<td>0.82</td>
<td></td>
<td>+ 123.0</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CHCl₃/MeOH 7:3 (v/v); ‘+’ means spots showed blue colour after spraying molybdenum blue solution

Unlike insolubility of PE in acetone, PE-SA could easily dissolve in acetone. After THF and water were removed from the mixture, 5ml of acetone was added into residues. The precipitates including free sialic acid, DCC derivatives, free PE and some of the DCC were filtered out. Using this method to purify the product, the purity of the PE-SA was detected by TLC again. Almost no PE was left in the solution. The yield was nearly 72-90%. Further purification of PE-SA by passing through silica acid gel (1cm x 10cm column) and washing with gradient mixture solvent (CHCl₃ : MeOH) was carried out to remove all unreacted DCC and PE-SA- oligomer (Table 7.2). During the study, PE was also coupled
Fig. 7.1 Detection of PE-SA and PE with thin layer chromatography

TLC plate: silica gel; eluent: CHCl₃/MeOH 7:3 (v/v); Indicator: molybdenum blue

Fig. 7.2 Scheme of conjugation of PE with sialic acid
with SA in the presence of CMC. The yield (50-65%) of reaction catalysed by CMC was comparatively lower than by DCC (70-90%), but CMC precipitated in acetone which was quite convenient to remove it from the solution. The yield of lipid conjugates was not significantly improved with increasing the reaction time to 24h.

Table 7.2 Purification of PE-SA by gradient elution

<table>
<thead>
<tr>
<th>CHCl₃ / MeOH (v/v)</th>
<th>Fractions (4ml each)</th>
<th>Radioactivity (Ci¹⁴)(dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:0.5</td>
<td>1</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>144.2</td>
</tr>
<tr>
<td>8:1.0</td>
<td>3</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>247.7</td>
</tr>
<tr>
<td>8:1.5</td>
<td>5</td>
<td>352.3</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>364.7</td>
</tr>
<tr>
<td>8:2.0</td>
<td>7</td>
<td>894.4</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4327.0</td>
</tr>
<tr>
<td>7:3.0</td>
<td>9</td>
<td>1341.7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>2312.3</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>923.6</td>
</tr>
</tbody>
</table>

PE-SA conjugates were identified by ¹H-NMR spectra and UV scanning. PE-SA and PE showed different UV spectra. The absorption peak of PE shifts from 242 nm to 377 nm after coupling with SA. PE-SA ¹H-NMR spectra: δ0.87 (2CH₃, 6H), δ1.25 (CH₂, 54H), δ1.67 (CH, 10H), δ1.96 (CH-OH, 8H), δ2.08 (CH, 6H), δ3.82 (CH, 10H), δ5.1 (NH, 1H), δ5.35 (NH, 2H).
However, for measuring PE-SA in liposomes, radiolabelled PE-SA was prepared by two methods. First, C\textsuperscript{14} labelled sialic acid was used as an indicator and coupled with PE in the conjugation reaction. Second, PE-SA was labelled with tritiated sodium borohydride. Both of the labelling results were shown in Table 7.3.

Table 7.3 Radiolabelling of PE-SA conjugated product

<table>
<thead>
<tr>
<th>Methods</th>
<th>Radioactivity (C\textsuperscript{14})</th>
<th>Radioactivity (H\textsuperscript{3})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C\textsuperscript{14} labelling SA</td>
<td>3270.2 dpm/20μl</td>
<td></td>
</tr>
<tr>
<td>H\textsuperscript{3} labelling SA</td>
<td></td>
<td>116.6dpm/10μl</td>
</tr>
</tbody>
</table>

7.3 Reaction rate

PE-SA conjugates cannot be detected by resorcinol reagent since sialic acid in PE-SA loses its reducing property. In previous study of PE-CA conjugated products, the same behaviour has been found when we tried to measure colominic acid in PE-CA. Usually, reducing sugars are unstable in strong alkali, which causes rearrangements and breakdown of the molecules. When hexoses were heated in a strong acid solution, it produced a derivative of furfural, which can react with phenols to produce coloured compounds. If the free -OH group of anomeric carbon is unavailable at the reducing end of polysaccharides, eg. glycoside, the terminal ring cannot be opened to lose the colour response. We assumed that coupling PE to the carboxyl group of sialic acid inhibited the formation of furfural configuration as well, with which the combination of phenol with PE-SA is impossible. Using the resorcinol method to determine the concentration of sialic acid during the
reaction process at the time intervals, only free sialic acid in the solution could be detected. The conjugated sialic acid was calculated by measurement of the free sialic acid. Results showed that 60.3% conversion of sialic acid was completed within 2h. After 24h, the conversion reached 91.4% (Table 7.4).

Table 7.4 Reaction rate of sialic acid conjugation with PE

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Yield (%) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>54.2 ± 1.2</td>
</tr>
<tr>
<td>3</td>
<td>60.3 ± 0.09</td>
</tr>
<tr>
<td>20</td>
<td>91.4 ± 0.7</td>
</tr>
</tbody>
</table>

PE/SA molar ratio was 26.9 :37.2; The reaction was kept at 30 °C and the solvent used was THF/water 2:1(v/v).

7.4 Incorporation of PE-SA into liposomes

PE-SA was incorporated into liposomes by probe-sonication method, and the vesicle size was reduced to around 100nm through sonication. The incorporation efficiency of PE-SA in MLV liposomes was around 45.7-55.3% since the solubility of PE-SA in water is poor (Table 7.5). However, the incorporation of PE-SA in lipid bilayers was decreased to 8.8-19.8% on reducing the vesicle size (Table 7.6). Some of the PE-SA was lost from lipid vesicles under the strong ultra-sonic treatment due to the arrangement of lipid bilayers or was released from the vesicles, or entrapped in the liposomes. The free PE-SA was separated from suspension by centrifugation, and then liposomes incorporating PE-SA were detected by passing through a Sephadex G-25 column. Nearly 93.6% of PE-SA
Table 7.5 Incorporation of PE-SA into MLV liposomes

<table>
<thead>
<tr>
<th>PC/PE-SA/Chol (molar ratio) (32 μmol of PC)</th>
<th>Incorporation of PE-SA in MLV (%) (mean ± SD)</th>
<th>Vesicle size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0.05:1.05</td>
<td>45.7 ± 3.0</td>
<td>9.48</td>
</tr>
<tr>
<td>1:0.1:1.1</td>
<td>47.1 ± 0.9</td>
<td>6.96</td>
</tr>
<tr>
<td>1:0.2:1.2</td>
<td>49.0 ± 2.1</td>
<td>9.3</td>
</tr>
<tr>
<td>1:0.3:1.3</td>
<td>48.0 ± 2.5</td>
<td>7.67</td>
</tr>
<tr>
<td>1:0.5:1.5</td>
<td>55.3 ± 4.5</td>
<td>11.1</td>
</tr>
</tbody>
</table>

Table 7.6 Incorporation of PE-SA into SUV liposomes

<table>
<thead>
<tr>
<th>PC/PE-SA/Chol (molar ratio) (32 μmol of PC)</th>
<th>Incorporation of PE-SA in SUV (%) (mean ± SD)</th>
<th>Vesicle size (nm) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0.05:1.05</td>
<td>19.8 ± 0.3</td>
<td>141.7 ± 3.6</td>
</tr>
<tr>
<td>1:0.1:1.1</td>
<td>17.5 ± 2.7</td>
<td>143.5 ± 13.8</td>
</tr>
<tr>
<td>1:0.2:1.2</td>
<td>10.3 ± 1.1</td>
<td>101.1 ± 0.3</td>
</tr>
<tr>
<td>1:0.3:1.3</td>
<td>8.6 ± 0.6</td>
<td>88.0 ± 0.4</td>
</tr>
<tr>
<td>1:0.5:1.5</td>
<td>8.8 ± 0.4</td>
<td>115.2 ± 17.3</td>
</tr>
</tbody>
</table>

moved down the gel filtration column in the void volume. It demonstrated that PE-SA was incorporated into liposomes (Figure 7.3). Certainly, PE-SA distributed in both inside and outside of the bilayer membrane since the present technique cannot control the arrangement of PE-SA just towards the outside of vesicles. We have tried to monitor the ratio of sialic
acid on the liposome surface using neuraminidase hydrolysis or NaOH(4M) hydrolysis. Data were confused because liposomes were destroyed in NaOH solution. The neuraminidase could not release sialic acid from lipid conjugates.

Fig. 7.3 Incorporation of PE-SA into PC/Chol liposomes

10 mol% PE-SA (mol% of PC) was incorporated into PC/Chol (1:1, molar ratio) liposomes. Compared with sialic acid, PE-SA liposomes passed through a Sephadex G-25 column in void volume.
7.5 Stability of PE-SA liposomes in albumin solution

The ability of PE-SA conjugates to protect liposomes against the adsorption of proteins on the surface of liposomes and their stability in the protein solution were investigated by detection of the stability of PE-SA liposomes in human serum albumin (HSA) solution. Normal liposomes composed of PC, PE and cholesterol precipitated in the HSA solution. The size changed with increasing the concentration of HSA (Figure 6.9), suggesting that some of the proteins were absorbed on the liposome surface that caused the lipid vesicles to flocculate. Liposomes in 1% of HSA precipitated more quickly than in 2% and 0.5% of HSA since high concentration proteins gave the solution a high viscosity. Increasing the protein concentration, the size of normal liposomes increased from 122.7nm to 1002.6nm and the polydispersity was poor (Figure 6.9). Liposomes incorporating PE-SA were more stable after incubation in protein solution. The size of PE-SA liposomes (PE-SA 30%) only changed from 153.4 nm to 164.9 nm, also the size of PE-SA (10%) liposomes changed from 138.0nm to 129.6nm(Figure 7.4 ). Obviously, sialic acid on the surface of liposomes improved their hydrophilicity which resulted in the good biocompatibility between sialylated liposomes and proteins. Another reason related to the low absorption of proteins to the sialylated liposome surface may be attributed to different surface charge of PE-SA liposomes. Although sialic acid cannot form a steric shield, the function of sialic acid reducing the adhesion among cells may provide same biological protection for liposomes. Thus, sialylated liposomes may remain in the circulation much longer than non-sialylated liposomes.
Fig. 7.4 Stability of PE-SA liposomes after incubation in albumin solution

5-30mol% (mol% of PC) of PE-SA was incorporated into PC/Chol (1:1, molar ratio) liposomes. The size change was measured 2h after incubation in human serum albumin (HSA) solution.
7.6 Surface charge of PE-SA liposomes

The zeta-potentials of liposomes incorporating PE-SA were -12.5mV to -16.5mV, but zeta-potentials of native liposomes were -16.8mV to -35.4mV (Figure 7.5). The existence of sialic acid on the liposome surface influences the surface charge of liposomes. Hitoshi Yamauchi (1993) has incorporated the conjugates of sialic acid with 2-(2-palmitoylamidol-1-ethyl)-5-acetoamine in DPPC liposomes, and reported that the zeta-potentials of those liposomes were -10mV. Negatively charged surfaces have been postulated as necessary for long circulating liposomes because it may produce electrical repulsion and reduce the adhesion between liposomes and other membrane surfaces (Allen, 1994b). However, the early experimental results revealed that positively charged liposomes remained in the circulation much longer than negatively charged liposomes (Gregoriadis and Neerunjun, 1975). For liposomes composed of DSPC/PEG-DSPE, zeta-potentials at pH 7.2 with 5-10 mol% PEG in the bilayers were measured at -3 to -4mV (Blume and Cevc, 1993). Increasing surface negative charge by incorporation of PEG-COOH-PE into liposomes has not resulted in a long half-life of liposomes in the circulation (Park et al., 1992). This suggests that a terminal negative charge is incompatible with prolonged circulation times, but even better results are obtained when the terminal is uncharged (Allen, 1994b). PE-SA on the liposome surface may provide much better uncharged surface electrophoretic mobility than native liposomes, and substantially change the degree of hydration and absorption force of PE-SA liposomes.
Fig. 7.5 Zeta-potential of PE-SA liposomes

PC/Chol/PE and PC/Chol/PE-SA (1:1.05:0.05; 1:1.1:0.1; 1:1.2:0.2; 1:1.3:0.3, molar ratios) liposomes were suspended in 0.05M phosphate buffer (ionic strength 50mM, pH 7.4).
7.7 Release of CF from PE-SA liposomes

CF was entrapped into PE-SA liposomes with the method mentioned in chapter 2. Adding 2ml of 0.2M CF into the lipid mixture, the loading of CF in liposomes (PC/Chol/PE-SA, 32μmol of PC) with different molar ratios of PE-SA was around 0.0245-0.0437% (Table 7.7). The structure of vesicles was relatively stable, even when the molar ratio of PC to PE-SA was increased to 1:0.3. When PE-SA liposomes were incubated in PBS and mouse plasma at 37 °C, the release of CF from PE-SA liposomes was increased on increasing the incubation time (Figure 7.6a, 7.6b and 7.7a, 7.7b). However, the release of CF from PE-SA liposomes in both PBS and mouse plasma was more rapid than from normal liposomes. After incubation of liposomes in PBS for 24h, 11.7-21.8% of CF leaked from PE-SA liposomes compared with 4.3-7.5% of CF leaking from native liposomes. On incubation in mouse plasma for 24h, the release of CF from PE-SA liposomes was 20.5-35.0%, and native liposomes only released 5.4-8.9% of entrapped CF. The rapid release of entrapped CF from PE-SA liposomes may be attributed to the incorporation of PE-SA in lipid

<table>
<thead>
<tr>
<th>PC/PE/Chol liposomes (molar ratio) (32μmol of PC)</th>
<th>Total entrapment of CF in liposomes (µg)</th>
<th>CF loading (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:0.05:1.05</td>
<td>44.4</td>
<td>0.0296</td>
</tr>
<tr>
<td>1:0.1:1.1</td>
<td>36.8</td>
<td>0.0245</td>
</tr>
<tr>
<td>1:0.2:1.2</td>
<td>65.6</td>
<td>0.0437</td>
</tr>
<tr>
<td>1:0.3:1.3</td>
<td>31.0</td>
<td>0.0207</td>
</tr>
</tbody>
</table>

2ml of 0.2M CF was added into liposomes
bilayers, resulting in the increase of permeability of the membrane.

Fig. 7.6a Release of CF from PE-SA liposomes after incubation in PBS

0.1ml of PC/Chol/PE and PC/Chol/PE-SA (1:1.05:0.05; 1:1.1:0.1, molar ratios), intact PE 5% and PE10% or PE-SA 5% and PE-SA 10% mixed with 0.5ml PBS were incubated at 37 °C for 48h, and 10μl samples were taken at the time intervals and diluted in 3ml PBS. The release of CF from liposomes was measured and calculated.
Fig. 7.6b Release of CF from PE-SA liposomes after incubation in PBS

0.1ml of PC/Chol/PE and PC/Chol/PE-SA (1:1.2:0.2; 1:1.3:0.3, molar ratios), intact PE 20% and PE 30% or PE-SA 20% and PE-SA 30% mixed with 0.5ml of PBS were incubated at 37 °C for 48h, and 10μl samples were taken at the time intervals and diluted in 3ml PBS. The release of CF from liposomes was measured and calculated.
Fig. 7.7a Release of CF from PE-SA liposomes after incubation in mouse plasma

0.1ml of PC/Chol/PE and PC/Chol/PE-SA (1:1:0.05; 1:1.1:0.1, molar ratios) liposomes mixed with 50μl of mouse plasma and 450μl of PBS were incubated at 37 °C for 48h. 10μl of samples were taken out at the time intervals and diluted in 3ml of PBS. The release of CF from liposomes was measured and calculated.
0.1ml of PC/Chol/PE and PC/Chol/PE-SA (1:1.2:0.2; 1:1.3:0.3, molar ratios) liposomes mixed with 50µl of mouse plasma and 450µl of PBS were incubated at 37 °C for 48h. 10µl of samples were taken out at the time intervals and diluted in 3ml of PBS. The release of CF from liposomes was measured and calculated.
7.8 Blood clearance of PE-SA-liposomes

The clearance of PC/PE-SA/Chol liposomes from mouse circulation was slower than PC/PE/Chol liposomes. 6h after injection, 20.5% of PE-SA (10%) liposomes and 11.0% of PE-SA (20%) liposomes remained in the mouse circulation compared with 6.1% remaining of native liposomes (PC/Chol/PE 1:1.2:0.2, molar ratio) (intact PE 20%) (Figure 7.8). On the other hand, 13.2% of PE-SA (10%) liposomes and 10.1% of PE-SA (20%) liposomes remained in the circulation 10h post-injection (Figure 7.8). Meanwhile, native liposomes only had 1.57% of injected dose left in the circulation at the same time. It is clear that sialic acid might act as some protection preventing liposomes from being rapidly removed by RES. The vesicle size of PE-SA liposomes was shown in Table 7.8.

Table 7.8 Vesicle size of injected PE-SA liposomes

<table>
<thead>
<tr>
<th>Liposomes (molar ratio)</th>
<th>Vesicle size (nm) (mean ± SD)</th>
<th>Polydispersity (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC/Chol/PE (1:1.2:0.2)</td>
<td>128.8 ± 3.1</td>
<td>0.146 ± 0.027</td>
</tr>
<tr>
<td>PC/Chol/PE-SA(1:1.1:0.1)</td>
<td>153.8 ± 2.3</td>
<td>0.145 ± 0.027</td>
</tr>
<tr>
<td>PC/Chol/PE-SA(1:1.2:0.2)</td>
<td>145.6 ± 3.6</td>
<td>0.414 ± 0.012</td>
</tr>
</tbody>
</table>

The mechanism of PE-SA prolonging circulation is not explained by this study. Incorporation of PE-SA into liposomes certainly changed the physical properties of vesicles such as the surface tension, surface charge and hydrophilicity. These physical characteristics may potentially stabilized liposomes in the blood circulation. On the other
Fig. 7.8 Blood clearance of PE-SA liposomes

10mol% and 20mol% (mol% of PC) PE-SA were incorporated into PC/Chol(1:1, molar ratio) liposomes, respectively. 200μl of samples (3.2μmol of PC) was intravenously injected into TO mice by tail vein. Compared with native liposomes, PE-SA liposomes (PE-SA 10%, p**=0.0003; PE-SA 20%, p***=0.0097) prolonged the circulation time significantly. p<0.05 means that data have significant difference compared with control; Results are expressed as the mean ± standard deviation (SD) of three mice.
hand, the biological actions of sialic acid on the stability of liposomes are assumed. It has been demonstrated that glycoproteins without sialic acid residues were promptly removed from the circulation and recovered from the liver (Morell et al., 1971). The same function of sialic acid for the survival of erythrocytes were also confirmed by Durocher et al. (1975). Hitoshi Yamauchi and co-workers (1993) had incorporated the sialic acid derivative, Neu5Acβ-PA into liposomes composed of DPPC and cholesterol and injected them into rats and tumour bearing mice. Results showed that liposomes remained longer in the circulation in both mice and rats after intravenous injection and decreased accumulation in the liver. Other studies on GM₁-liposomes indicated that the negatively charged sialic acid on the GM₁ residues probably shielded the surface of liposomes and played an important role in preventing opsonization (Allen and Chonn, 1987; Allen, 1988; Allen and Mdhra, 1989a). Sialic acid also was described to avoid the adhesion among cells (Schauer and Kamerling, 1997). Therefore, sialic acid on the liposome surface may have similar action which delays the recognition of liposomes by RES. However, the half-life of PE-SA liposomes is not as long as PE-OX-CA liposomes. The reason is attributed to the fact that sialic acid cannot provide steric hindrance to reduce the uptake of particles by RES.

7.9 Tissue distribution of PE-SA liposomes

PE-SA liposomes distributed in the mouse body 3h after injection was shown in figure 7.9. The accumulation of free and PE-SA liposomes in the liver and the spleen suggests that the uptake of PE-SA liposomes by macrophages was still a main route to remove liposomes from in vivo. The vesicle size of injected PE-SA liposomes was shown in Table 7.9.
7.10 Conclusions

Inclusion of sialic acid into liposomes enhanced the stability of vesicles in the protein solution and prolonged the half-life of liposomes in mouse circulation. Although the survival of PE-SA liposomes is shorter than PE-OX-CA liposomes, the action of sialic acid on the surface of liposomes has been found. This conclusion supported the previous study results (Yamauchi et al. 1993; Allen, 1994b). Reviewing the structure of sialic acid on natural cell membrane, it could be seen that sialic acid is always connected with other oligo or long chain polysaccharides (Schauer et al., 1995; Schauer and Kamerling, 1997). The protection of sialic acid may relate to its steric conformation. However, the long-circulation liposomes are created by many factors. Upon the hydrophilic and neutrally charged surface, the formation of shielded protection by polymers is more important in preventing the uptake by RES (Gabizon and Papahadjopoulos, 1992b; Torchilin and Papisov, 1994). Sialic acid and polysaccharides with sialic acid as terminal groups may be useful polymers to stabilize liposomes.
Fig. 7.9 Tissue distribution of PE-SA liposomes in mice 3h after injection

200μl (3.2μmol of PC) of PE-SA liposomes (PC/Chol/PE-SA 1:1.1:0.1; 1:1.2:0.2, molar ratios) was intravenously injected into TO mice. The mice were killed at 3h post-injection and the radioactivity in organs was measured and calculated. Results are expressed as the mean ± SD of three mice.
Chapter 8

Evaluation of the Properties of Colominic Acid
8.1 Introduction

Using colominic acid as a steric barrier to protect liposomes from uptake by RES, the properties of polysaccharides such as the stability in vivo and in vitro, the toxicity to tissue and blood cells and the metabolism from in vivo are quite important in determining the fate of liposomes in the circulation. It has been known that N-acetyl neuraminic acid is a unit of colominic acid and widely exists on the glycoproteins and glycolipids of human cells (Schauer, 1982a, 1982b). The toxicity of N-acetyl neuraminic acid polymers was assumed to be very low, but no data have been reported to support this assumption. Jennings and his colleagues (1981) have modified tetanus toxoid vaccine with polysialic acid to improve their immunological ability. Gregoriadis et al. (1993) considered the possibility of polysialic acid B as a drug carrier since the half-life of polysialic acid B in mouse circulation extended up to 40h. Fernandes and Gregoriadis (1996, 1997) have coupled polysialic acid to the catalases and found that the coated enzyme had a long circulation time in the mouse blood. However, cationic polysaccharides such as chitason have been demonstrated to cause the aggregation of blood cells when they were used as drug delivery carriers (Carreno-Gomez et al., 1997; Richardson et al., 1996). The interaction between colominic acid and blood cells was observed under microscopy in this chapter. The biodegradation and stability of colominic acid under different pHs and temperature were investigated.

Colominic acid purchased from Sigma company is a hydrolysed product of polysialic acid B (From *E.coli*). Its molecular weight is not known. However, polymer chain length is very important for determining the retention time of polysialic acid in the
circulation (Gregoriadis et al., 1993). The short polysialic acid is eliminated from the blood much more quickly than large polymers. 50% of polysialic acid B (Mn= 61,000) can stay for 40h in the circulation after intravenous injection. The half-life of polysialic acid C (Mn= 22,000) is shorten at 1h (Gregoriadis et al., 1993). In this chapter we measured the molecular weight of colominic acid by a radiolabelling method.

8.2 Stability of colominic acid

8.2.1 Degradation of colominic acid by neuraminidase

Hydrolysis of colominic acid by neuraminidase to produce a low molecular weight components was confirmed. Colominic acid treated with 2units of neuraminidase was detected by passing through a Sephadex G-25 column (1cm x 20cm). A new polymer, which moved down the column behind normal colominic acid was found (Figure 8.1). Using a monosaccharide, sialic acid as a control to pass through the same column, the peak of sialic acid appeared at the same fractions (Rt) as degraded colominic acid, suggesting that the molecular weight of colominic acid digested by enzyme was much lower than free colominic acid. The 2→8 linkage colominic acid was almost totally depolymerized. We have applied enzyme hydrolysis methods to measure the incorporation of PE-CA in lipid bilayers (Figure 5.3). The release of tritiated colominic acid from the liposome surface showed the existence of colominic acid. Jennings et al. (1981) reported the assay of polysialic acid in the conjugates of proteins with treatment of neuraminidase. On the other hand, the degradation of colominic acid by neuraminidase was used in preparation of the oligo-sialic acid.
10mg of colominic acid and trace amounts of tritiated colominic acid were incubated with neuraminidase (2 units) in pH 5.5 sodium acetic buffer at 37 °C for 2h. The solution was passed through a Sephadex G-25 column and compared with colominic acid. Colominic acid did not diffuse into Sephadex G-25 gel and passed through the column in void volume. Colominic acid hydrolysed by neuraminidase moved down the column behind the free colominic acid due to its small molecular weight.

**8.2.2 Stability of the colominic acid under heating**

Colominic acid was reported to be unstable at a high temperature (Lifely et al., 1986). In this study, the molecular weight of depolymerized colominic acid was assayed by labelling it with NaBH₄ (H³). When colominic acid was heated at 100 °C for 1, 3 and 9h, respectively, and then labelled with NaBH₄ (H³), the radioactivity per µg of
colominic acid increased with decreasing the polymer chain length since there were more reducing ends in short oligomers to react with NaBH₄ (Figure 8.2). The average molecular weight of degraded colominic acid relied on the heating time (Table 8.2). For example, the molecular weight of colominic acid was shortened to 3947 after heating for 9h. However, further detection of digested colominic acid by passing through a Sepharose 2B column resulted in multiple peaks as shown in Figure 8.3. This confirmed the depolymerization of colominic acid at high temperature. Colominic acid was stable at room temperature and during storage at 4 °C. In the case of incubation of colominic acid at 37 °C for 24h, the stability of colominic acid did not change significantly (Figure 8.4).

8.2.3 Stability of colominic acid under different pH conditions

Incubation of colominic acid under different pH conditions was carried out to evaluate the effect of pH on the stability of colominic acid. It is interesting that colominic acid was not influenced by pH conditions as it was incubated for a short time (24h) at 37 °C (Figure 8.5). Results showed that the peak of colominic acid passing through a Sephadex G-25 column appeared as the same fractions although it had been treated at pH 7.4, 4.7 or 9.0. It means colominic acid did not depolymerize by acid and base. However, when the mixture was kept at the same pH for one week, colominic acid incubated in acetic acid and pH 4.7 produced low molecular weight polymers which moved down from a Sephadex G-25 column behind the normal colominic acid (Figure 8.6). Colominic acid in neutral and pH 9.0 buffer solution did not change with increasing the incubation time (Figure 8.6). It is concluded that the effect of acid buffer
on the stability of colominic acid is much greater than neutral buffer. The reason for the reduced stability of colominic acid by acid is not very clear. Actually, colominic acid can be stored in a neutral buffer at 4 °C for three months without producing oligo-sialic acid (Figure 8.7).

![Graph showing the stability of colominic acid under heating](image)

**Fig. 8.2 Stability of colominic acid under heating**

Colominic acid was boiled at 100 °C for 1, 3 and 9h, respectively and labelled with NaBH$_4$ (H$^3$). 10μl of solution was passed through a Sepharose 4B column. The radioactivity per μg of colominic acid increased with increasing the heating time.
Colominic acid was boiled for 3h and passed through a Sepharose CL-2B column. Multiple peaks on gel filtration indicated that polysaccharides have different molecular weight, which moved down the column at different elution volume.
Fig. 8.4 Stability of colominic acid after incubation at 37 °C for 24h

Colominic acid incubated at 37 °C for 24h was passed through a Sephadex G-25 column. No significant difference before and after incubation was found.
Fig. 8.5 Stability of colominic acid after incubation in different pHs for 24h

Colominic acid was incubated in PBS (pH 7.4), 0.1M NaH₂PO₄ (pH 4.7) and 0.1M Na₂HPO₄ (pH 9.0) at 37 °C for 24h, and then passed through a Sephadex G-25 column, respectively. No significant difference among three samples was found.
Chapter 8 Evaluation of the properties of Colominic Acid

Colominic acid was incubated in PBS (pH 7.4), 0.1M NaH$_2$PO$_4$ (pH 4.7), 0.1M Na$_2$HPO$_4$ (pH 9.0) and 0.1M acetic acid at 37 °C for one week. Colominic acid was stable in neutral and pH 9.0 solution, but it was depolymerized after incubation in acid solution.

Fig. 8.6 Stability of colominic acid after incubation in different pHs for one week
Colominic acid stored at 4 °C for three months was passed through a Sephadex G-25 column. The behaviour of colominic acid on gel filtration before and after storage did not show significant difference.

Figure 8.7 Stability of colominic acid after storage for three months
8.2.4 Stability of colominic acid in mouse plasma

Biodegradability of colominic acid in the mouse blood directly influences the fate of polymers in the circulation. Due to the rapid clearance of colominic acid from the blood, it is difficult to measure the stability of colominic acid in vivo. We treated colominic acid with fresh mouse plasma at 37 °C for 24h, and then detected the colominic acid by passing through a Sephadex G-25 column. Compared with colominic acid treated with neuraminidase, which was depolymerized to produce a new peak on gel filtration, colominic acid incubated in mouse plasma did not change its behaviour on the column (Figure 8.8). It is supposed that colominic acid was not hydrolysed by enzyme since the breakdown products of colominic acid were not found after incubation. This may result from absence of this kind of enzyme in mouse plasma to destroy colominic acid.

8.2.5 Toxicity of colominic acid to normal blood cells

The toxicity of colominic acid to blood cells might significantly determine its use as a liposome surface barrier. Usually, positively charged polymers such as chitosan and polylysine have been proved to strongly cause haemolysis and aggregation of blood cells (Carreno-Gomez et al., 1997; Richardson et al., 1996). Although colominic acid contains carboxyl groups which make it a negatively charged polysaccharide, and it is supposed to present much better biocompatibility than cationic polymers, data on toxicity of colominic acid have not been reported in previous studies. Colominic acid was mixed with fresh mouse whole blood, and the morphology of blood cells is shown in Figure 8.9. The blood cells kept their original shape when the concentration of colominic acid was increased to 2.5mg/ml. Further investigation on the interaction...
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Fig. 8.8 Stability of colominic acid after incubation in mouse plasma

Colominic acid mixed with fresh mouse plasma and incubated at 37 °C for 24h. The mixture was detected by passing through a Sephadex G-25 column and counting in 4ml scintillation liquid. Compared with oligo-colominic acid, which was digested by neuraminidase, colominic acid in mouse plasma was not destroyed significantly.
Fig. 8.9 Effect of colominic acid on blood cells

Fresh mouse blood cells incubated in colominic acid solution and observed under the microscopy. A: normal blood cells; B: 100μg/ml of colominic acid; C: 1mg/ml of colominic acid; D: 2.5mg/ml of colominic acid.
between colominic acid and blood cells was carried out by incubation of different concentrations of colominic acid with mouse blood cells for 1 and 3h. The release of haemoglobin from broken red cells was assayed by measuring the supernatant in a UV-visible spectrophotometer. Results showed that the stability of red blood cells did not significantly change with prolonging the incubation time and increasing the concentration of colominic acid (Figure 8.10). The concentration of colominic acid was increased to 10μg/μl, the lysis of blood cells was less than the lysis of blood cells by addition of Triton X-100, in which blood cells were entirely destroyed and haemoglobins were released. Unlike other polysaccharides, eg. chitosan which damaged the cell membrane and cause lysis of blood red cells (Carreno-Gomez et al., 1997), colominic acid preformed much better biocompatibility with blood cells. In further application of colominic acid as drug carriers and as liposome surface steric barrier, it may be more advantageous than other polysaccharides.
0.01-5mg of colominic acid mixed with 0.2ml suspension of fresh mouse blood cells were adjusted to a final volume of 0.5ml and incubated at 37 °C for 1 and 3h. The absorption of released haemoglobin from red cells was measured at 576nm in an UV-visible spectrometer. The control was prepared by addition of 10μl Triton X-100 (10%) into 0.2ml blood samples. No haemolysis of mouse blood cells was found with increasing the concentration of colominic acid.
8.2.6 Blood clearance and tissue distribution of colominic acid

In the experiment, two-injected doses (5mg and 10mg) were adopted and intravenously injected into TO mice. No rapid toxic response was found from altering injected doses. We also measured the retention time of colominic acid in the mouse circulation by bleeding from a tail vein at 5min, 30min, 1h and 3h. Unlike polysialic acid B, which has been demonstrated to remain in the circulation up to 40h (Gregoriadis, et al., 1993), colominic acid was eliminated from mouse circulation very quickly. 93.4% of colominic acid (injected dose: 10mg) and 95.4% of colominic acid (injected dose: 5mg) was removed from blood within 0.5h. The remaining colominic acid (injected dose: 10mg) decreased to 1.1% 3h after injection (Figure 8.11). The repaid clearance of colominic acid may result from its water solubility and its molecular weight (Mn=15,000), which is much lower than polysialic acid B (Mn=60,000). On the other hand, the clearance rate reduced with increasing the injected dose. At the same bleeding time, colominic acid with 10mg injected dose was cleared slowly compared with 5mg injected dose (Figure 8.11). However, the detail metabolism of colominic acid in vivo was not clear. As shown in Figure 8.12, most of the colominic acid accumulated in the kidney rather than in the liver and the spleen 3h after injection, suggesting that colominic acid was mainly cleared from urine.

From the study of PEG liposomes, it has been demonstrated that the retention time of liposomes coated with hydrophilic polymers was not related to the half-life of polymers (Yamaoka et al., 1994). 50% of PEG (Mn 1900) can only remain in the circulation for about 15 min, but it prolonged the half-life of liposomes to several hours depending on
the composition of liposomes. Therefore, the half-life of materials seems not to
determine their further behaviour after being conjugated with the liposome surface.

Fig. 8.11 Clearance of colominic acid from the blood circulation
5mg and 10mg of tritiated colominic acid in 200μl PBS were intravenously injected into T.O. mice (male, 25-30g) (three mice in each group) by tail vein, respectively. Results are expressed as the mean ± SD of three mice.
5mg and 10mg of tritiated colominic acid were intravenously injected into T.O. mice (three mice in each group) by tail vein, respectively. 3h post-injection the mice were killed and the organs were collected and detected. The data of muscle means % of injected doses per g of muscle. Other data mean % of injected doses in whole organs. Results are expressed as the mean ± SD of three mice.
8.3 Quantitative analysis of the molecular weight of colominic acid

Many methods have been used to measure the molecular weight of polysaccharides, for example, gel electrolysis, light-scattering, ultracentrifugation and chemical method. When a polymer molecule contains a particular functional group, the molecular weight can be determined by analysis of the functional group. If the polymer has a group at the end of the polymer chain which can be assayed, the molecular weights are said to be determined by end-group assay, which counts the number of molecules in solution. A number-average molecular weight ($M_n$) is obtained for polydisperse samples as expressed in the following equation:

$$M_n = \frac{n_1M_1 + n_2M_2 + n_3M_3 + \ldots \ldots + n_M}{n_1 + n_2 + n_3 + \ldots \ldots + n_M}$$

A radiolabelling method used for the measurement of colominic acid chain length has been mentioned by Lifely (1986). Yet, the detail description for this method has not been reported. The analysis method based on the theory that one H at the reducing end of colominic acid can be replaced by H$^3$ while colominic acid reacts with NaBH$_4$ (H$^3$). Measuring the radioactivity per mole of colominic acid, the numbers of the end groups in one mole colominic acid will be known. The average molecular weight of colominic acid can be calculated as follows:

The radioactivity per mole of NaBH$_4$ is given by the company

Radioactivity of NaBH$_4$ / mmol = 13.3 GBq / mmol

$$= 13.3 \times 10^6 \text{ KBq / mmol}$$

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So radioactivity per each hydrogen in NaBH₄ = radioactivity of NaBH₄ / 4

\[ = 3.325 \times 10^6 \text{KBq} / \text{mmol.} \]
\[ = 3.325 \times 10^3 \text{KBq} / \mu\text{mol} \quad [8-3] \]

Radioactivity per µg of labelled colominic acid is measured by counting the labelled sample.

Radioactivity of colominic acid / µg = 3734 cpm/µg

\[ = 0.124 \text{KBq} / \mu\text{g} \quad [8-4] \]

Since the reduction is carried on as 2mol of H³ to produce 4mol of products, the final molecular weight must be divided by 2.

\[
\begin{align*}
4 \text{R-C-R'} + \text{NaBH}_4 + 3\text{H}_2\text{O} & \rightarrow 4\text{R-CH-R'} + \text{NaH}_2\text{BO}_3 \\
\end{align*}
\]

\[
\text{Mn of colominic acid} = \frac{R_h}{R_c} \times \frac{1}{2} \quad [8-5]
\]

R_h: Radioactivity of hydrogen/µmol.
R_c: Radioactivity of colominic acid/µg.

The molecular weight of colominic acid which was labelled under different radiolabelling times was calculated and shown in Table 8.1. Comparing with the results obtained from Gel Permeation Chromatography (Mn = 17,800) (Matthews and Petrak, 1994), we obtained a similar molecular weight (15,072-16,267). Certainly, the molecular weight calculated from different analysis methods is not exactly same. Using the tritiation method to measure colominic acid, the final results still depended on the purity of tritiated colominic acid, data of NaBH₄(H³) and separation process. Dialysis of
Tritiated colominic acid was found to lose some low molecular components. Purification by gel filtration can remove most of the free NaBH₄(H⁺), and colominic acid was retained. Moreover, depolymerized colominic acid was assayed with the same method, and results showed that the chain length of colominic acid was decreased with increasing heating time (Table 8.2).

### Table 8.1 Calculation of the molecular weight of colominic acid

<table>
<thead>
<tr>
<th>Labelling time (h)</th>
<th>Radioactivity* (cpm/μg)</th>
<th>Radioactivity (KBq/μg)</th>
<th>Molecular weight (Mn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3734</td>
<td>0.1245</td>
<td>13353</td>
</tr>
<tr>
<td>4</td>
<td>3309</td>
<td>0.1103</td>
<td>15072</td>
</tr>
<tr>
<td>8</td>
<td>3066</td>
<td>0.1022</td>
<td>16267</td>
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<tr>
<td>14</td>
<td>3278</td>
<td>0.1093</td>
<td>15210</td>
</tr>
<tr>
<td>24</td>
<td>3458</td>
<td>0.1153</td>
<td>14419</td>
</tr>
</tbody>
</table>

* NaBH₄(H⁺) 13.3 GBq / mmol; Radioactivity of H⁺: 3.325 x 10⁵ KBq / μmol

### Table 8.2 Calculation of the molecular weight of degraded colominic acid**

<table>
<thead>
<tr>
<th>Heating time (h)</th>
<th>Radioactivity* (dpm/μg)</th>
<th>Radioactivity (KBq/μg)</th>
<th>Molecular weight (Mn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>18419</td>
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<tr>
<td>1</td>
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<tr>
<td>3</td>
<td>44363</td>
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<tr>
<td>9</td>
<td>70293</td>
<td>1.1716</td>
<td>3947</td>
</tr>
</tbody>
</table>

* NaBH₄(H⁺) (37.0 GBq / mmol); Radioactivity of H⁺: 9.25 x 10⁶ KBq / μmol
** Colominic acid was heated at 100 °C.
Chapter 8 Evaluation of the properties of Colominic Acid

8.4 Conclusions

Colominic acid is quite stable after incubation in a neutral buffer and in mouse plasma for 24h, as well as storage at 4 °C for three months. However, the chain length of colominic acid was decreased with increasing the heating time. When colominic acid was heated in a boiling water bath for 9h, its average molecular weight was significantly reduced from 18,419 to 3,947. Colominic acid was totally depolymerized under the treatment of neuraminidase.

Unlike positively charged polysaccharides such as chitosan (Carreno-Gomez et al., 1997; Richardson et al., 1996), the negatively charged colominic acid presented good biocompatibility with blood cells. Haemolysis of blood cells incubated in colominic acid solution has not been found, even if the concentration of colominic acid was increased to 10mg/ml. The half-life of colominic acid in the mouse circulation was much shorter than polysialic acid B due to its low molecular weight. 93.4% of colominic acid (injected dose: 10mg) and 95.4% of colominic acid (injected dose: 5mg) was eliminated from the mouse circulation within 0.5h, and most of the colominic acid was removed from urine. Gregoriadis et al. (1993) has demonstrated that the retention time of polysialic acid depended on its chain length. 50% polysialic acid B (Mn 61000) remained at 40h post-injection, compared with less than 1% remaining at 3h post-injection. The rapid clearance of colominic acid from blood may be associated with its low toxicity. It can be concluded that colominic acid is safe to be used as drug carriers.
General discussion and further work

Stealth liposomes have been prepared by incorporation of different hydrophilic polymers into lipid bilayers, and their half-life was believed to relate to properties and density of polymers on the vesicle surface. Modification of liposomes with either PEG or GM₃ significantly increased the half-life of liposomes in the circulation and improved the delivery of anticancer drugs into solid tumours by passive targeting (Lasic, 1997; Woodle, 1995b). Like PEG and GM₃, other polymers such as polyvinylpyrrolidone, poloxamers and poloxamines were reported to have a similar action to enhance the circulation time of liposomes (Moghimi, 1995; Allen, 1994b). Polysaccharides, as kind of natural biodegradable materials, have been supposed to increase the half-life of liposomes more significantly than synthetic polymers since natural cell membranes were covered with many carbohydrates (Jones, 1994). However, the animal result of liposomes coating with dextran showed the circulation time of liposomes was only improved two fold (Pain et al., 1984). The reason was attributed to the inflexible polysaccharide chain and low density “cloud” of dextran on the liposome surface which cannot totally shield the liposome to avoid the uptake by the RES (Blume and Cevc, 1992, 1993a). In this study, we coupled colominic acid with lipid vesicles and the circulation time of liposomes was enhanced, but it is not as long as we predicted (Figure 6.17 and 6.18). Torchilin et al. (1993) indicated that flexible polymer chains with sufficient mobility are required in providing a “flexible brush” on the outside of liposomes, and which result in a “dense cloud of possible conformations”. The most important effect of PEG on the vesicle surface is the mobility of the polar chain leading to steric dynamic forces arising from the polar chain headgroups sweeping the interfacial region between the hydrophobic membrane interior and the
surrounding protein solution (Blume and Cevc, 1993a). Comparing polysaccharides with PEG, the flexibility of polymer chain may be one of the reasons which determine the half-life of liposomes in the circulation.

The argued point is that natural cells which were shielded with polysaccharides can remain in the blood much longer and were not cleared out rapidly. Polysaccharides on the cell membrane seem to provide efficient protection. Thus, we think the density of polysaccharides on the liposome surface may be more important in determining the fate of liposomes. The density of polymers can be expressed as the incorporation efficiency of polymers into bilayers and the polymer chain length which associated to the thick of polymer “cloud”. The presence of PEG extending out a sufficient distance (5-6nm) from the liposome surface would, in aqueous solution, result in a large number of possible conformations and a high transition rate from one conformation to another (Torchilin et al., 1993). The short PEG chain with molecular weight 750 cannot prolong the circulation time of liposomes (Maruyama et al, 1991). While GM₁ was shortened to GM₂ and GM₃, it lost the ability to avoid the uptake of liposomes by the RES (Allen et al., 1987, 1989a). Otherwise, the maximum amount of PEG-DSPE which can be accommodated in phospholipid bilayers composed of PC/SM/Chol (1:1:1, molar ratio) decreased with increasing PEG molecular weight and was as high as 15 mol% for PEG120, decreasing to 5-7 mol% for PEG2000 and PEG 5000 (Allen et al., 1991b). Increasing the molar ratio of PEG in liposomes will lead to decreasing encapsulation efficiency and increasing leakage (Blume and Cevc, 1993a). Moreover, for reducing the clearance of liposomes by the RES, the molar ratio of GM₁ in lipids must be more than 5 mol% (Allen and Chonn, 1987).
suggests that the density of polymers on the vesicle surface greatly influences the characteristics of liposomes. Pain et al. (1984) conjugated dextran (average molecular weight 70,000) to the surface of liposomes and found that the survival time of liposomes was prolonged two-fold. It is not clear that the low efficiency of dextran is associated with the low incorporation of dextran on the liposome surface or the high molecular weight of dextran used in the study. Both of the reasons can reduce the density of dextran on the vesicle surface, and thus it cannot prevent the uptake of liposomes by the RES. In addition, the conjugation of polysaccharides with lipids and the incorporation of polysaccharides into lipid bilayer are not easily carried out. From the study of polysialylated liposomes, we demonstrated that incorporating efficiency of colominic acid into liposomes considerably influenced the circulation time of liposomes. When conjugates of colominic acid and PE (PE-OX-CA) were incorporated into liposomes, the incorporation efficiency of PE-OX-CA in lipid bilayers was very low because of its high molecular weight and hydrophilicity (Table 5.3), and thus the low density of PE-OX-CA on the surface of lipid membrane cannot prevent the uptake of liposomes by the RES. On the other hand, the retention time of coated liposomes was increased as colominic acid was directly conjugated with the liposome surface (Figure 6.16 and 6.17), suggesting the importance of the position and proportion of colominic acid on the liposome surface. Therefore, the poor circulation time of liposomes coating with some polysaccharides may relate to not only the properties of materials themselves, but also the conjugation method and preparation process. That may be another reason for the low efficiency of polysaccharides in reducing liposome clearance by macrophages.

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Incorporation of colominic acid into liposomes changes the surface hydrophilicity and the surface charge. Both of the polysialylated liposomes and sialylated liposomes were found to have zeta-potentials nearly neutral (-7.1mV to -11.1mV) compared with native liposomes which have zeta-potentials around -16.8mV to -35.4mV. In the studies of the stability of liposomes coated polymers, it is found that liposomes with neutral surface charge have much longer circulation times (Allen, 1994b). For liposomes composed of DSPC/PEG-DSPE, the zeta-potential was measured at -3 to -4mV in the absence of serum and approximately -5mV in the presence of serum (Blume and Cevc, 1993a). The reduced mobility in PEG-containing liposomes is due to hydrodynamic drag produced by the steric effects of the extended polymers (Woodle et al., 1992c). Park and co-workers (1992) have investigated the effect of negatively charged PEG-DOPE on the circulation time of liposomes. The half-life of liposomes incorporating negative PEG-COOH-DOPE was enhanced, but the best of the derivatives resulted in blood levels of only approximately 50% of those seen for GM₁ (Allen, 1994b). It is concluded that a terminal negative charge is incompatible with prolonged circulation times. However, the positively and negatively charged surface probably relates to the adsorption of plasma proteins after intravenous injection. Moghimi and Patel (1998) indicated the binding of dysopsonins inhibits the uptake of vesicles by the RES. While for charged liposomes combined with some special plasma components such as γ-globulins and small molecular proteins, the uptake of liposomes by the RES was suppressed. If charged liposomes adsorb other plasma proteins, they promote the clearance processes. Surface charge of liposomes was one of the factors associated with the opsonization and deopsonization. However, it is not very clear which range of surface charge will make liposomes remain in the circulation much longer.
Polysialylated liposomes with long circulation time may result from their neutral surface.

Actually, the reduction of the total amount of opsonins binding to liposomes was thought to be very important in prolonging the circulation time of liposomes. The ability of liposomes binding plasma complements depends on the lipid composition (Senior, 1987). GM₁ liposomes were reported to reduce the binding of high-density lipoproteins to the liposome surface (Allen, 1989b). As the amount of GM₁ incorporated in liposomes was increased from 2 to 10 mol%, the combination of proteins on liposomes was significantly reduced from 36.4 to 13.0 g protein/mol lipid (Chonn et al., 1992). Senior et al. (1991) indicated that liposomes coated with PEG took several hours to absorb significant amounts of proteins while native liposomes absorbed plasma proteins within one minute. The total binding by DSPC liposomes reduced from 170 g protein/mol lipid in the absence of PEG-DSPE to 55 g protein/mol lipid (Blume and Cevc, 1993a). Also, the low adsorption of plasma proteins to polysialylated liposomes suggested that colominic acid on the liposome surface may inhibit opsonin action (Figure 6.9 and 6.11). Polysialylated liposomes with long circulation times may be a consequence of their reduction to bind plasma proteins, which decreases in recognition by the RES.

In spite of the steric "cloud" and hydrophilic surface provided by colominic acid, the function of sialic acid which reduces the adhesion between cells and recognition by the RES may contribute to prolonging the circulation time of polysialylated liposomes. Comparing sialylated liposomes with polysialylated liposomes, the effect of sialic acid on reducing the recognition of liposomes by the RES was confirmed. Unlike polymers which
can form a steric hindrance, sialic acid covers the liposome surface with only single molecular layers, but it delays the clearance of liposomes from the blood (Figure 7.8). The reason may be attributed to the special function of sialic acid in masking the recognition site (Schauer, 1995, 1997). Park and Huang (1993) have modified sialic acid of GM$_1$-lipid derivatives. They found that the oxidation of GM$_1$ resulted in the loss of its activity and the half-life of oxidized GM$_1$ liposomes was short. This suggested that a very special sialic acid structure of GM$_1$ is required for its ability to reduce the RES uptake of liposomes. Colominic acid on the surface of liposomes may play both biological action and steric hindrance to avoid the uptake of liposomes by the RES.

The mechanistic study of the stealth liposomes revealed the formation of a stearic barrier on the surface of liposomes by coating polymers, which has been an accepted hypothesis to explain the in vivo observations (Woodle and Lasic, 1992). However, liposome interaction with serum and plasma proteins concerns the potential for the acquisition of opsonic components, that is the adsorption of protein ligands capable of interacting with one or more receptors on the macrophage cell surface (Patel, 1992). Liposomes can be intentionally opsonized with non-specific antibodies (Chonn et al., 1991; Alving and Wassef, 1992) as well as antibodies against phospholipids and cholesterol, fibronectin, tuftsin derivatives, pentraxins, etc. to enhance their recognition (Liu, 1996b; Hu and Liu, 1996). It is not clear to what extent the opsonic molecules in blood can play in promoting recognition and phagocytosis of the intravenously injected non-opsonized vesicles by phagocytic cells located in the liver, spleen and bone marrow (Moghimi, 1998). The clearance process of polysialylated liposomes as a natural, biodegradable carrier system is
not understood. Further investigations on the proteins involved in promoting recognition have to be conducted.

In addition, the retention time of liposomes in the circulation is not the only factor determining the administration of liposome carrier systems (Woodle, 1998). Many other characteristics such as the entrapment efficiency, the possibility of liposomes crossing the endothelial barrier to gain access to extravascular tissue and the ability to release the drug once reaching the tumour surface are all relatively important in influencing the clinical therapeutic index and safety (Storm et al., 1994). Further study on accumulation of polysialylated liposomes loading anticancer drugs in the tumour should be continued. Sometimes long circulating PEG-liposomes result in unexpected toxicity for the skin on administration to humans (Gabizon, 1994). The toxicity of polysialylated liposomes loading anticancer drugs have to be investigated. The studies in some large animals such as rats should be carried out since many reports indicated that the circulation time of liposomes increased with increasing the size of species (Harashima et al., 1996).

Generally, liposomes coated with polysaccharides provide a potential route to prepare stealth liposomes. The chain length and conformation of polysaccharides, as well as the conjugation methods all determine the fate of liposomes in vivo. Till now, dextran and polysialic acid used in the modification of liposomes are all linear polymers. The oligo-branched polysaccharides like most of the carbohydrates on cell membranes may be more advantageous for protecting liposomes.
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