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医薬品の製造・品質管理の高度化と国際化に対応した日本薬局方の改正のための研究
分担研究報告書

医薬品の名称、化学名及び構造式の改正に関する研究

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研究要旨

本研究は、日本薬局方（JP）収載医薬品など、我が国で承認されている医薬品の名称（日本名、英名、別名）、構造式、分子式、分子量、化学名、ケミカル･アブストラクツ・サービス(CAS)登録番号（CAS 番号）、および、基原の項に含まれる構造情報など医薬品の本質を規定する項目（以上を、名称関連事項と略す）について、医薬品の製造・品質管理の高度化と国際化に対応するために必要な検討事項を抽出し、今後の JP の改正作業に資することを目的とする。

今年度は、第 16 改正日本薬局方（JP16）に収載されている医薬品の名称、特に日本名別名について検討し、削除の必要性を明らかにした。これらの検討結果が、第 17 改正日本薬局方（JP17）作成の際に役立つことを期待する。

医薬品の名称関連事項は医薬品の本質を規定するものであり、その記載内容は、科学的に正しく、また、国際的にも調和している必要がある。

A．研究目的

日本薬局方（JP）には我が国で使用されている主要な医薬品が収載され、法律すなわち規格書としての役割を果たしている。加えて JP は、我が国の医薬品の規範書としての役割も負っている。JP 収載医薬品の医薬品各条は、医薬品の情報記載の規範を示しておりその波及効果は大きい。このような観点から、JP の記載内容は、

1）科学的に正しいこと、
2）整合性があること、
3）国際的に調和していること、
4）情報の電子化に対応していること、

などが必要要件となる。

本研究では、JP に収載されている医薬品を中心に我が国で承認されている医薬品の名称（日本名、英名、別名）、構造式、分子式、分子量、化学名、ケミカル･アブ

ストラクツ・サービス(CAS)登録番号（CAS番号)、および、基原の項に含まれる構造情報など、医薬品の本質を規定する項目（以上を、名称関連事項と略す）について、先に示した観点から記載内容を精査し、医薬品の製造・品質管理の高度化と国際化に対応した日本薬局方の改正に資するための調査研究を行う。

今年度は、第 16 改正日本薬局方(JP16)に収載された医薬品の名称、特に日本名別名について、局方全体の整合性の面から記載事項を検討し、修正の必要な項目を明らかにした。

B．研究方法

JP16 に収載されている医薬品（生薬を除く）721 品目の名称（日本名および別名）について、精査するとともに、JP 収載品目間での不整合を調べ、修正が必要な品目を調査した。

C．研究結果

1）JP16 収載医薬品の別名の数

JP16 収載の医薬品（生薬を除く）721 品目を調査した結果、710 品目（98.5%）に別名が存在した。また、105 品目（14.6%）が 2 個以上の別名を、23 品目（3.2%）が 3 個の別名を持つことがわかった。4 個以上の別名を持つ医薬品はなかった。721 品目の医薬品の別名の総数は、838 である。なお、別名がない 13 品目は、アヘン末、オレンジ油、カカオ脂、カルナウバロウ、牛脂などすべて化学構造式がない天然由来の医薬品であり、いわゆる一般的な化学薬品はすべて別名が付いている。

表 1　JP16 に収載された別名をもつ医薬品（生薬を除く）

	数	%
別名なし	13	1.8
別名 1 個	603	83.6
別名 2 個	82	11.4
別名 3 個	23	3.2
別名 4 個	0	0
計	721	100

2）JP16 収載医薬品の別名の分類

JP16 収載医薬品の全別名 838 について分類を行った。

1．従来からの別名
2．医薬品名の変更（日本語訳の変更を含む）に伴って生じた別名
3．科学的な理由で生じた別名
4．日本名の命名ルールの変更によって生じた別名

2-1）従来からある別名

今から 26 年前の昭和 61 年（1986 年）に告示された第 11 改正日本薬局方（JP11）の第一部には 585 品目の医薬品が収載されている。その中の約 20%（123 品目）に別名が掲載されている。それらはある種の必然性があって別名が掲載されていると考えられる。

それらのうち、JP16 にも残っている具体例としては、

「アクリノール」の別名「乳酸エタクリジン」
「アセトアミノフェン」の別名「パラセタモール」
「アスピリン」の別名「アセチルサリチル酸」
「アミノ安息香酸エチル」の別名「アネスタミン」と「ベンゾカイン」
「アンチピリン」の別名「フェナゾン」
「アンピシリン」の別名「アミノベンジルペニシリン」
「塩酸エチルモルヒネ」の別名「ジオニン」
「アスコルビン酸」の別名「ビタミン C」
「アセタゾラミド」の別名「アセタゾールアミド」
「イソプロピルアンチピリン」の別名「プロピフェナゾン」

「イソプロパノール」の別名「イソプロピルアルコール」
「エテンザミド」の別名「エトキシベンズアミド」
「エルゴカルシフェロール」の別名「カルシフェロール」と「ビタミンD」
「塩化ナトリウム」の別名「食塩」
「塩酸オキシブプロカイン」の別名「塩酸ベノキシネート」
「オキセサゼイン」の別名「オキセタカイン」
「d－カンフル」の別名「樟脳」
「キシリトール」の別名「キシリット」
「キタサマイシン」の別名「ロイコマイシン」
「グアイフェネシン」の別名「グアヤコールグリセリンエーテル」
「グリセリン」の別名「グリセロール」
「コレカルシフェロール」の別名「ビタミンD」
「サラゾスルファピリジン」の別名「スルファサラジン」
「酸化亜鉛」の別名「亜鉛華」
「三酸化ヒ素」の別名「亜ヒ酸」
「シアノコバラミン」の別名「ビタミンB_{12}」
「リン酸ジヒドロコデイン」の別名「リン酸ヒドロコデイン」
「塩酸ジブカイン」の別名「塩酸シンコカイン」
「次没食子酸ビスマス」の別名「デルマトール」
「スルファメトキサゾール」の別名「スルフイソメゾール」
「スルフイソキサゾール」の別名「スルファフラゾール」
「生理食塩水」の別名「等張食塩液」など
「D－ソルビトール」の別名「D-ソルビット」
「炭酸ナトリウム」の別名「重曹」と「重炭酸ナトリウム」
「タンニン酸アルブミン」の別名「タンナルビン」
「塩酸チアミン」の別名「ビタミンB_1」
「デキサメタゾン」の別名「デキサメサゾン」
「トコフェロール」の別名「dl-α-トコフェロール」と「ビタミンE」
「塩酸トリメトキノール」の別名「塩酸トレトキノール」
「L-トレオニン」の別名「L-スレオニン」
「二酸化炭素」の別名「炭酸ガス」
「ノスカピン」の別名「ナルコチン」
「パラアミノサリチル酸カルシウム」の別名「パスカルシウム」
「塩酸ピリドキシン」の別名「ビタミンB6」
「フィトナジオン」の別名「フィトメナジョン」と「ビタミンK」
「フェニトイン」の別名「ジフェニルヒダントイン」
「硫酸フラジオマイシン」の別名「硫酸ネオマイシン」
「ベタメタゾン」の別名「ベタメサゾン」
「塩酸ペチジン」の別名「オペリジン」
「塩酸ベラパミル」の別名「塩酸イプロベラトリル」
「ベンジルペニシリンカリウム」の別名「ペニシリンGカリウム」など
「マーキュロクロム」の別名「メルブロミン」
「D－マンニトール」の別名「D-マンニット」
「塩化メチルロザニリン」の別名「クリスタルバイオレット」
「塩酸リジン」の別名「塩酸L-リジン」
「リボフラビン」の別名「ビタミンB_2」
「酢酸レチノール」の別名「ビタミンA酢酸エステル」

などである。

これらの別名の中には、学術的な名称、化学的な名称、慣用名などが含まれている。多くは局方収載時に流通名として使用されていたため局方に別名として記載されるようになったと考えられる。歴史的に由緒のある名前も含まれていると思われるが、医薬品としての流通実態のない別名は

削除するのが望ましい。

2-2）最近になって医薬品名の変更あるいは日本語訳の変更に伴ってできた別名

国際一般名の変更、あるいは、国際調和の観点から、さらには、日本語訳の変更に伴って、最近、医薬品の名称が変更されたものがある。この場合にも、当然、旧日本名が別名として残されている。

これらの例として

「アドレナリン」の別名「エピネフリン」
「トドララジン塩酸塩水和物」の別名「塩酸エカラジン」
「ペントキシベリンクエン酸塩」の別名「クエン酸カルベタペンテン」
「ホルモテロールフマル酸塩」の別名「フマル酸フォルモテロール」
「エコチオパートヨウ化物」の別名「ヨウ化エコチオフェイト」
「ヒプメロースフタル酸エステル」の別名「ヒドロキシプロピルメチルセルロースフタル酸エステル」

がある。

これらは、２－１）よりも最近になって変更になった医薬品の別名であり、現在も流通している医薬品もあると思われる。しかし、２－１）と同様に医薬品としての流通実態を調査し、流通実態のない別名は削除されることが望ましい。

2-3）採用する科学名の変更に伴ってできた別名

化合物の名称は、種々の理由により時代とともに変化している。局方収載品目の日本名で化合物の名称がそのまま使われているような場合、どの化学名を日本名とするかの方針が変更になることがある。新しい名前も古い名前も科学的には正しい名前といえるが、局方が採用する名前が変更になると、それに伴って旧名称が別名として残る。また、経緯は不明であるが、これらの別名の中には、後に述べるような解説的な意味で最初から別名として加えられていたものもあると思われる。

これらの例として

有機化合物では（以下、下線部分が該当箇所を示す）

「L-リシン塩酸塩」の別名「塩酸L-リジン」
「アセチルシステイン」の別名「N-アセチル-L-システイン」
「エタノール」の別名「アルコール」
「グリシン」の別名「アミノ酢酸」
「フェノール」の別名「石炭酸」
などが、
また、

無機化合物では、

「三酸化ヒ素」の別名「三酸化二ヒ素」
「リン酸水素カルシウム水和物」の別名「第二リン酸カルシウム」
「リン酸二水素カルシウム水和物」の別名「第一リン酸カルシウム」
「亜硫酸水素ナトリウム」の別名「重亜硫酸ナトリウム」
「ピロ亜硫酸ナトリウム」の別名「メタ重亜硫酸ナトリウム」

などがある。

2-4）日本名の命名ルールの変更に伴ってできた別名

JP では、日本名が変更になった際には、当面の間、旧日本名を別名として残すルールとなっている。これは、流通している医薬品の名前が変更されたときに、医療の現場で混乱が起きるのを避けることが目的と聞いている。また、医薬品の製造販売者は、当面の間、医薬品名を変更することなく旧日本名のままで医薬品を販売することができる。「当面の間」が、具体的にどれだけの期間であるかはわからない。

JP 収載医薬品の日本名は、平成 18 年に JP15 が公布される際に多数が変更になった。これは、平成 14 年 12 月 27 日付け厚生労働省医薬局審査管理課事務連絡「第十

五改正日本薬局方原案作成要領」に従い、水和物、四級アンモニウム塩を含む塩類、エステル類などの日本名の命名ルールが変更されたことによる。以下に代表的なルール変更の例を挙げる。

１）水和物の場合には、「○○○水和物」のように、薬効本体名（○○○）に続けて「水和物」をつけて表記する。なお、二水和物、三水和物などの場合でも、水和物の数は示さない。

具体例：
アジスロマイシン水和物（旧日本名：アジスロマイシン）

２）アミンの酸塩の場合には、「○○○・・・酸塩」のように、薬効本体名（○○○）に続いて酸塩部を表記する。医薬品の薬効本体がアミン誘導体であり、局方収載の医薬品がアミン誘導体の無機酸塩又は有機酸塩である場合が該当する。

具体例：アクラルビシン塩酸塩（旧日本名：塩酸アクラルビシン）

３）四級アンモニウム塩の場合には、「○○○・・化物」のように、薬効本体名（○○○）を前に書き、続けて「・・化物」と表記する。医薬品の薬効本体が四級アンモニウムであり、その無機塩が医薬品である場合が該当する。

具体例：アンベノニウム塩化物（旧日本名：塩化アンベノニウム）

４）薬効本体がアルコールでそのエステル（プロドラッグ）が医薬品の場合には、「○○○・・・酸エステル」と、薬効本体名（○○○）の後にカルボン酸エステル（・・・酸エステル）を表記する。

具体例：ヒドロコルチゾン酪酸エステル（旧日本名：酪酸ヒドロコルチゾン）

５）薬効本体がカルボン酸で INN（医薬品国際一般名）を持ち、そのエステル（プロドラッグ）が医薬品の場合には、「○○○　・・・」と、薬効本体名（○○○）の後に１字あけ、アルコール部（・・・）を表記する。

具体例：セフテラム　ピボキシル（旧日本名：セフテラムピボキシル）

２－４－１）水和物の別名

JP15 公布時に実施された水和物の命名ルールの変更に伴い、JP15 では多くの収載医薬品の日本名が変更になり、旧日本名は別名として残った。

具体的には、前に示した「アジスロマイシン水和物」の別名「アジスロマイシン」のほかに、

アクリノール（「アクリノール水和物」の別名）
プロチレリン酒石酸塩（「プロチレリン酒石酸塩水和物」の別名）
ベルベリン塩化物（「ベルベリン塩化物水和物」の別名）

など、JP16 には「水和物」が付かない旧日本名が別名として８２個残っている。

２－４－２）アミンの酸塩の別名

JP15 公布時に実施されたアミンの酸塩の命名ルールの変更に伴い、JP15 では多くの収載医薬品の日本名が変更になり、旧日本名は別名として残った。

具体的には、前に示した「アクラルビシン塩酸塩」の別名「塩酸アクラルビシン」のほかに、

アジピン酸ピペラジン（「ピペラジンアジピン酸塩」の別名）

エナント酸フルフェナジン（「フルフェナジンエナント酸エステル」の別名）
クエン酸クロミフェン（「クロミフェンクエン酸塩」の別名）

など、JP16には「・・・酸○○○」のような旧日本名が別名として３７５個残っている。

２−４−３）四級アンモニウム塩の別名

JP15 公布時に実施された四級アンモニウム塩の命名ルールの変更に伴い、JP15ではいくつかの収載医薬品の日本名が変更になり、旧日本名は別名として残った。

具体的には、前に示した「アンベノニウム塩化物」の別名「塩化アンベノニウム」のほかに、

ヨウ化エコチオパート（「エコチオパートヨウ化物」の別名）
臭化ジスチグミン（「ジスチグミン臭化物」の別名）

など、JP16には「・・・化○○○」のような旧日本名が別名として３１個残っている。

２−４−４）薬効本体がアルコールでそのエステルが医薬品の別名

JP15 公布時に実施された薬効本体がアルコールでそのエステルが医薬品の場合の命名ルールの変更に伴い、JP15ではいくつかの収載医薬品の日本名が変更になり、旧日本名は別名として残った。

具体的には、前に示した「ヒドロコルチゾン酪酸エステル」の別名「酪酸ヒドロコルチゾン」のほかに、

エナント酸テストステロン（「テストステロンエナント酸エステル」の別名）
ニコチン酸トコフェロール（「トコフェロールニコチン酸エステル」の別名）
パルミチン酸レチノール（「レチノールパルミチン酸エステル」の別名）

など、JP16には「・・・酸○○○」のような旧日本名が別名として４６個残っている。

２−４−５）薬効本体がカルボン酸でそのエステルが医薬品の別名

JP15 公布時に実施された薬効本体がカルボン酸でINNを持ち、そのエステルが医薬品の場合の命名ルールの変更に伴い、JP15 ではいくつかの収載医薬品の日本名が変更になり、旧日本名は別名として残った。

具体的には、前述の「セフテラム　ピボキシル」の別名「セフテラムピボキシル」（スペースの有無に注意）のように、

セフポドキシムプロキセチル（「セフポドキシム　プロキセチル」の別名）
セフロキシムアキセチル（「セフロキシム　アキセチル」の別名）
セフジトレンピボキシル（「セフジトレンピボキシル」の別名）

など、JP16にはスペースのない旧日本名が別名として１２個残っている。

２−４−６）包接化合物が医薬品の別名

薬効本体が包摂されている医薬品の場合、薬効本体名（○○○）の後に１字あけ、包接体の名前（・・・）を表記することになった。これに伴い、従来スペースが入らなかった日本名にスペースが入り、スペースのない旧日本名は別名となった。

具体例として
「アルプロスタジル　アルファデクス」の別名「アルプロスタジル　アルファデクス」
「ジノスタチン　スチマラマー」の別名「ジノスタチンスチマラマー」
（共にスペースの有無に注意）

がある。

2-4-7）塩溶液が医薬品の別名

薬効本体を酸性あるいは塩基性の水溶液に溶かした塩溶液が医薬品の場合、医薬品の名前は薬効本体名で表記し、塩では表記しない。従来、塩で表記されていた旧日本名は別名となって残っている。

具体例として
「アドレナリン液」の別名「塩酸アドレナリン液」
「アドレナリン注射液」の別名「塩酸アドレナリン注射液」
「ノルアドレナリン注射液」の別名「塩酸ノルアドレナリン注射液」
「デヒドロコール酸注射液」の別名「デヒドロコール酸ナトリウム注射液」
がある。
なお、「アドレナリン液」、「アドレナリン注射液」、「ノルアドレナリン注射液」は薬効本体を塩酸酸性の水溶液に、「デヒドロコール酸注射液」はデヒドロコール酸を水酸化ナトリウム水溶液に溶かした医薬品である。

このように JP15 で実施された日本名の命名ルールの変更に伴って多くの別名が生まれた。これらの別名は、JP17 では削除されることが望ましい。

D．結論と考察

今回は、JP16 収載医薬品について日本名別名を調査した。JP16 収載医薬品 721 品目の 98%以上に別名が記載されており、別名の総数は 838 に達している。その多くは、JP15 交付時に行われた日本名命名ルールの改正によるものである。また、それ以外の別名は、さらに古くから別名として掲載されている。別名というシステムがはたしてきた一定の意義は認められるものの、医薬品の名称が「一物二名」というのは望ましいことではない。少なくとも米国薬局方や欧州薬局方にはこのようなシステムはないように思われる。別名は、手順を踏んで削除されることが望ましい。

E．参考文献

本調査研究を行うにあたって、JP11、JP16、米国薬局方（USP）、欧州薬局方（EP）、を参考資料として用いた。

F．知的所有権の取得状況

なし。

別紙４

研究成果の刊行に関する一覧表

雑誌

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Short Communication

Size separation and size determination of liposomes

We developed a method for separating liposomes by size and determining their average diameters. Liposomes with different average diameters were separated on a monolithic silica capillary column, and the size of the liposomes corresponding to each peak was determined online with a dynamic light scattering detector coupled to the capillary liquid chromatography system. The calculated diameters for the separated liposomes were similar to the diameter values measured in batch mode. We demonstrate that this combination of a monolithic capillary column and light scattering detection could be used for size separation of liposomes and could provide more details about average diameters than batch-mode analysis.

Keywords: Capillary liquid chromatography / Light scattering detection / Liposomes / Monolithic column
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1 Introduction

Advances in nanotechnology have contributed to the development of modern drug carrier systems, such as liposomes [1] and polymeric micelles [2, 3], that play an important role in the controlled delivery of pharmacological agents to their targets at a therapeutically optimal rate and dose [4]. Exact knowledge of the sizes of these nanoparticles is essential because size can substantially affect physicochemical and biopharmaceutical behavior. For example, variations in particle size can affect drug release kinetics, transport across biological barriers, and pharmacokinetics in the human body [5–7].

Among the methods for the characterization of macromolecules, flow-assisted techniques, such as size-exclusion chromatography (SEC) [8, 9], hydrodynamic chromatography [10, 11], field-flow fractionation [12–14], and capillary hydrodynamic fractionation [15],] are suitable for separation on the basis of differences in the physical size indexes of the analytes. Electrophoretic separation methods are also used for separation and characterization of colloids that are charged in buffered aqueous solutions [16–18].

SEC is the most commonly used fractionation method for particle sizing. Usually, SEC is performed on a column packed with polymer gel or porous silica microparticles with various pore-size distributions. Polymer samples are separated with such packed SEC columns [8], and nanoparticulate drug carriers, such as liposomes, are often separated from small solutes by means of SEC [19].

Recently, macromolecules, such as a polystyrene polymer, were separated on monolithic silica columns by SEC [20]. Monolithic columns have received much attention as a new technology for HPLC and capillary electrochromatography [21, 22]. These columns consist of a single piece of porous material (most often polymer- or silica-based) with a bimodal pore structure consisting of through-pores (pore size ~1.5–5 m) and mesopores in the skeleton (~10–25 nm) [23]. Because of the high porosity of the monolithic columns, they can be lengthened, thus leading to high separation efficiency.

Although the elution profile obtained by means of SEC provides insight into size distribution, it does not give information about absolute particle size. Dynamic light scattering (DLS), also known as photon correlation spectroscopy, is a non-invasive technique for measuring the size of molecules and particles, typically in the submicron region; and with the latest technology, particles sizes of <1 nm have been measured. DLS is routinely used for size and polydispersity measurements, along with aggregate quantification.

In this report, we describe a system that combines the high resolution of capillary LC with acquisition of absolute diameter data by means of DLS for the online size separation and size determination of liposomal formulations. Although a system using conventional LC coupled with DLS has already been reported [24], ours is the first system that uses capillary LC coupled with DLS.

2 Materials and methods

2.1 Liposome preparation

Liposome samples were prepared by an extrusion procedure. A lipid film containing dipalmitoylphosphatidylcholine,

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Abbreviations: DLS, dynamic light scattering; **SEC**, size-exclusion chromatography

cholesterol, and dipalmitoylphosphatidylglycerol (30:40:30, lipid content 20 mM) was suspended in 9.5% sucrose. The suspension was extruded through 200-, 100-, 80-, and 50-nm polycarbonate membranes in that order. Extrusions at every pore size were repeated. Extruded liposome samples were stored in the refrigerator. Samples were dissolved or dispersed in eluent and filtered through a 0.20-μm filter (Millex-LG, Millipore, Tokyo, Japan) prior to being applied to the columns. We used two liposomes with average diameters of 77 and 155 nm, as measured by batch-mode analysis in 10 mM sodium phosphate buffer (pH 7.2) containing methanol (5% v/v), which was also the solvent used as the chromatography eluent.

2.2 LC conditions

The schematics of the analytical system we developed are shown in Fig. 1. Separation was performed with a capillary LC system equipped with a capillary LC micro-flow pump (MP711V; GL Sciences, Tokyo, Japan), a four-port internal sample injector (fixed volume 10 nL; Valco Instrument, Houston, TX, USA), and a capillary ultraviolet–visible (UV–vis) detector (MU701; GL Sciences). Samples were analyzed on a MonoCap Amide column (500 mm × 0.2 mm id; mesopore size ~15 nm or ~20 nm; GL Sciences). The total porosity of the column was estimated using void times of hollow capillary column and monolithic capillary column, and total volume of the column. The eluent was 10 mM sodium phosphate buffer (pH 7.2) containing methanol (5% v/v). The eluent was delivered at a flow rate of 0.1 μL/min, and the column was kept at room temperature. The capillary UV–vis detector was operated at a wavelength of 210 nm. A sample volume of 10 nL was injected for each analysis. Downstream of the UV–vis detector, the same eluent was added by means of a semi-micro-flow pump (NanoSpace 3101 SI-2, Shiseido, Tokyo, Japan) through a T-joint to increase the flow rate. At the increased flow rate, the eluate from the UV–vis detector continuously flowed into the flow cell of a DLS detector via a reducing joint (2 mm, 1/16 inch, GL Sciences). Zetasizer Software was used to calculate the average diameters of the liposomes. A real-time parameter reading from the external device (the UV–vis detector in this case) can be also directly introduced into the optics unit and added to the light scattering sample record.

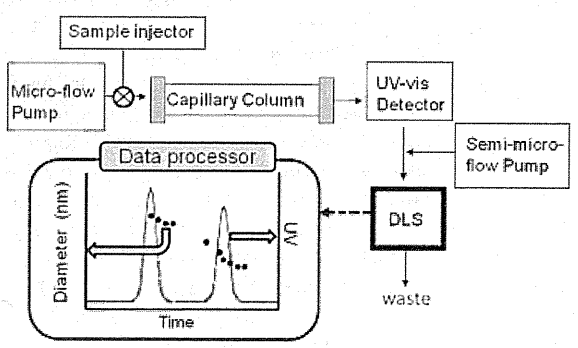

Figure 1. Schematic of analytical system for size separation of liposomes and determination of their average diameters. Samples were injected onto a monolithic capillary column and detected by a microLC UV–vis detector. The flow rate was increased downstream of the detector by means of a semi-micro-flow pump. At the increased rate, the eluate from the UV detector flowed continuously into the DLS detector.

3 Results and discussion

3.1 Separation of liposomes on the monolithic capillary column

In this report, we used the monolithic column which consisted of silica derivatized with an amide group, a neutral hydrophilic group that prohibits adsorption of the samples on the silica monolith by ion-exchange interactions and that permits the analysis of charged soft nanocarriers, such as liposomes derived from biomaterials. As the eluent, 10 mM sodium phosphate buffer (pH 7.2) containing methanol (5% v/v) was used. We attempted to separate liposomes with two different average diameters on a monolithic column with mesopore size of ~15 nm. However, this column did not effectively separate the liposomes (Fig. 2A). When we used a monolithic column with larger mesopores (average size ~20 nm), the resolution was improved (Fig. 2B). Because total porosity is also

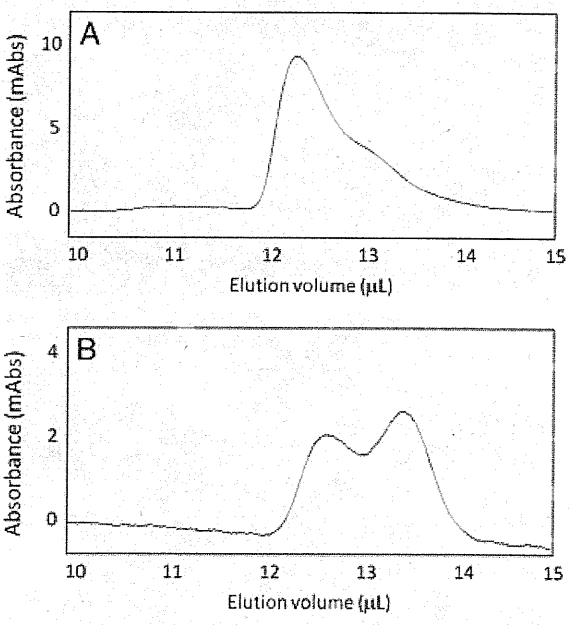

Figure 2. Effect of mesopore size on the separation of liposomes on monolithic columns with (A) ~15-nm mesopores and (B) ~20-nm mesopores. Column: capillary EX nano MonoCap Amide (500 mm × 0.2 mm id); eluent: 10 mM phosphate buffer (pH 7.2) containing 5% methanol; sample: mixture of liposomes with diameters of 155 and 77 nm, as determined by batch measurement; flow rate: 0.1 μL/min; detection wavelength: 210 nm.

increased from 88 to 95%, it is presumed that an increase in throughpore volumes also contributed to the improvement of resolution. SEM images of the latter monolithic column showed the presence of rough surfaces (micron and submicron ranges; Fig. 3). Typically, monolithic columns are more porous than conventional columns packed with spherical particles, and the higher porosity results in much lower column backpressure. Furthermore, the throughpore/skeleton size ratio of 2–4 for the monolithic column was much greater than the 0.25–0.4 ratio typical of columns packed with particles [25]. This higher ratio permits the use of a long column and thus high separation efficiency [26]. Therefore, we connected three 500-mm columns (total length 1500 mm) and then tried the separation again. As expected, the resolution was further improved (Fig. 4A), and the column pressure was only 1.5 MPa. In contrast, the same two liposomes could not be separated by batch-mode DLS analysis (Fig. 4B), and these results show the usefulness of the monolithic column. Also, there is no report that SEC using conventional LC can obtain such a high resolution of liposomes with two different average diameters within 100-nm range. We could not separate liposomes with two different average sizes (111 and 77 nm)

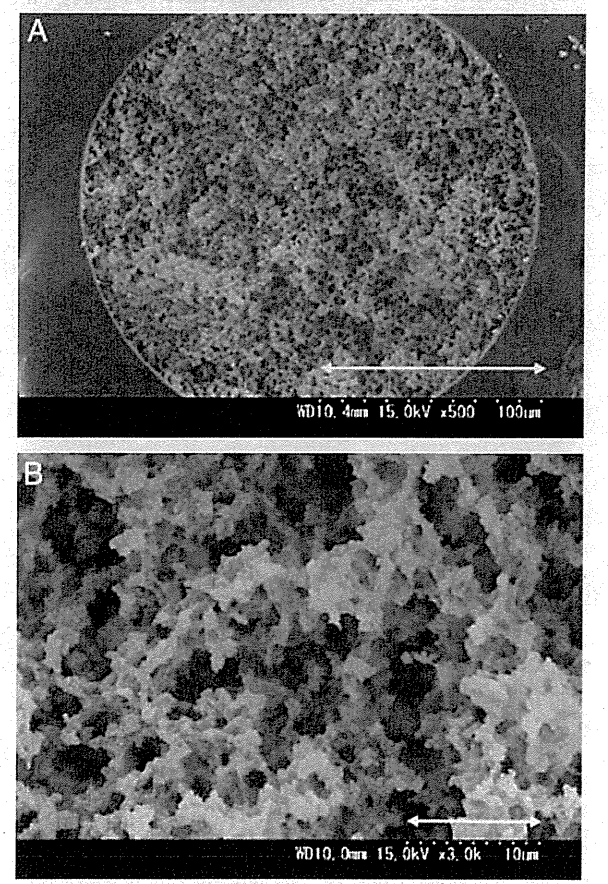

Figure 3. Scanning electron micrographs of monolithic capillary columns. Scale bars correspond to 100 μm for (A) and 10 μm for (B).

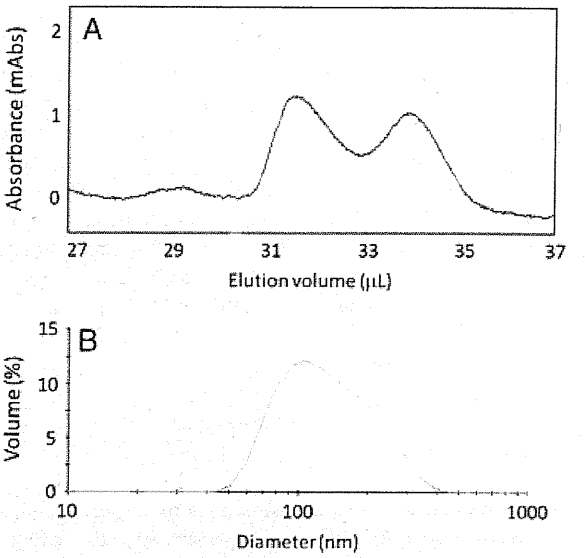

Figure 4. Analysis of liposomes in (A) flow-assisted mode and (B) batch mode. (A) Analysis in flow-assisted mode. Column: capillary EX nano MonoCap Amide (500 mm × 0.2 mm id) × 3; eluent: 10 mM phosphate buffer (pH 7.2) containing 5% methanol; sample: mixture of liposomes with sizes of 155 and 77 nm, as determined by batch measurement; flow rate: 0.1 μL/min; detection wavelength: 210 nm. (B) Analysis in batch mode with DLS detection. Sample: mixture of liposomes with diameters of 161 and 77 nm; dispersant: 10 mM phosphate buffer (pH 7.2) containing 5% methanol.

(data not shown). Therefore, the separation of 80 nm size difference is possible by this system.

3.2 Online data acquisition by DLS

Next, we evaluated a system that combined capillary LC with DLS detection. The DLS data were accumulated continuously and analyzed every 3 s, and the software recorded all correlation functions and intensity values. Because the volume of the DLS flow cell was 135 μL and the detection volume was 5 μL, and the flow rate of the capillary LC system was <1 μL/min, we increased the flow rate downstream of the UV–vis detector by adding the same eluent to the flow by means of a semi-micro-flow pump through a T-joint downstream of the detector. However, the flow was diluted by the additional eluent. Therefore, we examined the effect of the increased flow rate on the intensity of scattered light to make sure that DLS detection was still feasible at the higher flow rate. At flow rates ranging from 50 to 10 μL/min, DLS detection was possible, and diameters of the liposomes could be calculated. Figure 5A shows the average diameter and external UV input for monodisperse liposomes injected onto the monolithic column. The system was operated at a flow rate of 50 μL/min. From the single peak that was detected, we calculated an average diameter of 166 nm, which was close to the expected value (155 nm) for this liposome dispersion, as indicated by batch-mode

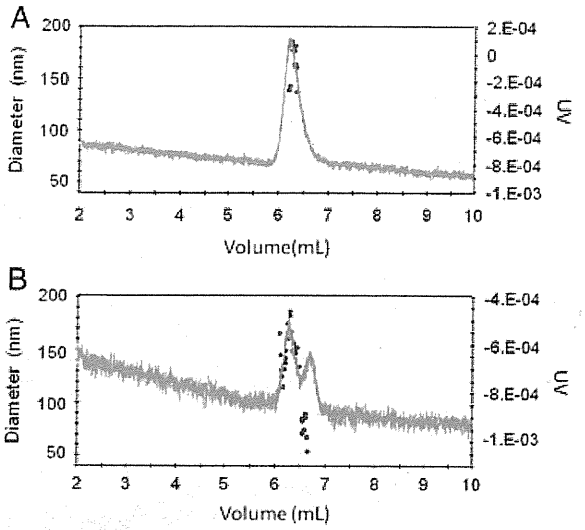

Figure 5. Plots of diameters and UV absorption versus elution volumes for liposomes injected onto a monolithic column. Column: capillary EX nano MonoCap Amide (500 mm × 0.2 mm id); eluent: 10 mM phosphate buffer (pH 7.2) containing 5% methanol; sample: liposomes with diameters of (A) 155 nm and (B) mixture of liposomes with diameters of 155 and 77 nm as determined by batch measurement. Capillary LC flow rate: 0.1 μL/min; semi-micro-flow pump flow rate: 50 μL/min; detection wavelength: 210 nm. AU: arbitrary unit.

analysis. We ascribed the similarity of the values to the low column backpressure, which did not affect the diameter of liposomes. The run-to-run repeatability of the calculated diameter for the eluted sample was determined to be 1.2% (RSD, $N = 3$). As far as I know, there have been no reports that the semi-micro-LC column or conventional column can separate liposomes with two different average diameters within 100-nm range. If we can use semi-micromonolithic LC column or conventional monolithic column long enough to separate liposomes with two different average diameters within 100-nm range, it is not required to dilute eluate from column before detector.

Next, we used the developed system to analyze a dispersion of liposomes with two different diameter ranges. Figure 5B shows the diameter and external UV input versus elution volumes for liposomes, separated onto a monolithic column. The size separation of the liposomes was good, and average diameters for the two detected peaks were calculated as 164 and 77 nm; these values were also similar to the values measured in batch mode, 155 and 77 nm, respectively.

4 Concluding remarks

We developed a system for simultaneous size separation and size determination of liposomes using a capillary LC system with DLS detection. By changing the mesopore size, we could improve the separation of liposomes with different average diameters. Because the column had a low backpressure, resolution could easily be increased by lengthening the column. After increasing the flow rate with a second pump, we used DLS detection to determine the diameters of the separated liposomes. Analysis with this system provided more-detailed information than conventional batch-mode analysis about the size of the liposomes, which affects their physicochemical and biopharmaceutical behavior.

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The authors have declared no conflict of interest.

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Stabilization of Liposomes in Frozen Solutions Through Control of Osmotic Flow and Internal Solution Freezing by Trehalose

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ABSTRACT: The purpose of this study was to elucidate the effect of trehalose distribution across the membrane on the freeze-related physical changes of liposome suspensions and their functional stability upon freeze–thawing. Cooling thermal analysis of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine liposome suspensions showed exotherm peaks of bulk (−15°C to −25°C) and intraliposomal (approx. −45°C) solution freezing initiated by heterogeneous and homogeneous ice nucleation, respectively. The extent of the intraliposomal solution freezing exotherm depended on liposome size, lipid composition, cosolutes, and thermal history, suggesting that osmotic dehydration occurred due to the increasing difference in solute concentrations across the membrane. A freeze–thawing study of carboxyfluorescein-encapsulated liposomes suggested that controlling the osmotic properties to avoid the freeze-induced intraliposomal solution loss either by rapid cooling of suspensions containing trehalose in both sides of the membrane (retention of the intraliposomal supercooled solution) or by cooling of suspensions containing trehalose in the extraliposomal media prior to freezing (e.g., osmotic shrinkage) led to higher retention of the water-soluble marker. Evaluation and control of the osmotically mediated freezing behavior by optimizing the formulation and process factors should be relevant to the cryopreservation and freeze-drying of liposomes. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:2935–2944, 2011

Keywords: liposomes; formulation; stabilization; thermal analysis; osmosis; calorimetry (DSC); excipients; freeze-drying

INTRODUCTION

The increase in the variety and clinical relevance of liposomal formulations has enhanced the importance of the freezing and freeze-drying processes for the distribution and long-term storage of the drug delivery systems that are not chemically and/or physically stable enough as aqueous suspensions.[1–4] These processes, however, expose the lipid systems to various stresses including ice growth, pH change, concentration of the surrounding solutes, and dehydration that often damage their structural integrity and pharmaceutical functions [e.g., release of active pharmaceutical ingredients (APIs)] of liposomes. Retaining water-soluble APIs is a particular challenge for development of liposome formulations.[1] Formulation and process design that are based on an understanding of the freeze-related stresses and required stabilization mechanisms should improve the stability of various liposome pharmaceuticals.[1–3]

Disaccharides (e.g., trehalose and sucrose) and some amino acids have been applied to protect the lipid systems from chemical and physical changes during freeze–thawing (cryoprotectants) and freeze-drying (lyoprotectants).[1,3] The stabilization of liposomes by disaccharides is explained mainly by three mechanisms. Some saccharides substitute the water molecules necessary to retain the supramolecular phospholipid assembly through molecular interactions with hydrophilic phospholipid head groups (water substitution).[5–7] The saccharides also form highly viscous amorphous freeze-concentrated phases and dried solids that prevent direct contact between liposome vesicles (bulking).[1,8,9] The reduced mobility of the surrounding molecules helps improve the chemical and physical stability of liposomes (vitrification). Use of the stabilizers is mostly dependent on empirical trial and error through analysis of the morphological (e.g., size) and functional (e.g., API or marker

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retention) traits of the resulting suspensions or dried solids.

Longstanding cryopreservation studies of living cells and microorganisms provide precepts valuable for the protection of liposomes against the freeze-induced stresses.[10–14] The cooling of cell and liposome suspensions induces the freezing of bulk solutions initiated by heterogeneous ice nucleation at the surface of containers or impurities (−5°C to −25°C) and the freezing of spatially restricted internal solutions initiated by homogeneous (spontaneous) ice nucleation (−25°C to −45°C).[15–21] The bulk solution freezing and the accompanying significant concentration of solutes surrounding the living cells and liposomes induce osmotic stress that removes the internal solution before they freeze, leading to morphological changes observable by microscopic methods (e.g., optical microscope and cryo-transmission electron microscopy).[21,22] Because the intracellular ice formation (IIF) is widely recognized to cause lethal damage through disordering of the complex membrane and intracellular structure (e.g., organelle), cryopreservation of the living cells and microorganisms is usually performed in two ways that prevent IIF, namely by slow cooling of suspensions containing extracellular solutes (cell dehydration) and by rapid cooling of the suspensions containing high-concentration membrane-permeating solutes [e.g., dimethyl sulfoxide (DMSO), cytoplasm vitrification].[12] On the contrary, only limited studies have been performed on the stabilization of liposomes taking various freezing-related physical changes into account.[17,23–25]

The purpose of this study was to elucidate the effect of intra- and extraliposomal trehalose on the freeze-related physical changes and functional stability of liposomes during freeze–thawing. The effect of saccharide distribution across the membrane on the stability of liposomes is of particular interest for formulation purposes because the liposome preparation methods significantly affect allocation of the nonpermeating solutes. Different solute concentrations across the membrane induce osmotic flow that shrinks or swells the liposomes in the aqueous suspensions.[4,22,26] Literature claims the requirement of disaccharides on both sides of the membrane to protect liposomes from freezing- and lyophilization-related stresses (e.g., addition before extrusion).[8] Recent reports suggested that the rational setting of different intra- and extraliposomal trehalose concentrations confers better stabilization.[1,27] Effect of trehalose on the freeze-related physical phenomena (e.g., freeze-induced dehydration and intraliposomal solution freezing) and functional stability of liposomes were studied mainly through thermal analysis and through the retention of encapsulated carboxyfluorescein (CF).[27,17]

MATERIALS AND METHODS

Materials

Chemicals obtained from the following sources were used without further purification: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (NOF Co., Tokyo, Japan); trehalose dihydrate, glucose, sucrose, and 5(6)-CF (Sigma–Aldrich Co., St. Louis, Missouri); DMSO, xylitol, and glycerol (Wako Pure Chemical Co., Osaka, Japan); and dextran 4000–6000 (Serva Electrophoresis GmbH, Heidelberg, Germany).

Preparation of Liposome Suspensions

Phospholipid films were obtained by drying their solution in a chloroform and methanol mixture (2:1) under vacuum at temperatures above the main transition temperature (T_m). Liposome suspensions were prepared by using a hand-held extruder (Avanti Polar Lipids, Alabaster, Alabama). The films hydrated by 10 mM Tris–HCl buffer (6%, w/w; pH 7.4) were extruded 12 times through a polycarbonate membrane filter (0.1–0.8 μm pore, 0.2 μm unless otherwise mentioned; Whatman, Maidstone, UK) while maintaining the apparatus at room temperature (POPC) or at temperatures 10 to 15°C higher than the T_m of the respective lipids. The DPPC liposomes extruded through the smaller pore membranes (0.1 and 0.2 μm) were reported to have a unilamellar structure, whereas those extruded through the larger pore membranes (0.4 and 0.8 μm) contained increasing ratios of multilamellar vesicles.[29,30] The term "0.2 μm liposome" will be used in the text given below to denote samples prepared by extrusion through the respective pore size membranes.

Some liposome suspensions containing the excipients predominantly in the extraliposomal media were prepared by adding the excipients approximately 30 min prior to the thermal analysis and freeze–thawing experiments. Those containing excipients in both the inside and outside of the membranes were prepared by the extrusion of lipids hydrated with the excipient-containing solutions. Some suspensions were eluted through Sephadex G-25 desalting columns (PD-10; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) equilibrated with the Tris–HCl buffer to obtain samples containing the excipient mainly in the intraliposomal solutions. The concentrations of DPPC in the column-eluted suspensions were obtained by phosphorous assay.[31] Measurement of the DPPC concentrations in the liposome suspensions indicated that approximately 90% of the liposomes passed through the Sephadex columns.

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Thermal Analysis

Thermal analysis of the frozen liposome suspensions was performed by using a differential scanning calorimeter (DSC Q10; TA Instruments, New Castle, Delaware) equipped with a refrigerating system and data processing software (Universal Analysis 2000, TA Instruments). Aliquots [10 μL, 4% (w/w) lipid] of suspensions in hermetic aluminum cells were cooled from 25°C to −70°C at varied speeds (1–10°C/min) and then heated to 25°C at a scanning rate of 5°C/min. The intensity of the intraliposomal solution freezing exotherm was shown as their ratio to the lipid content (J/g lipid). Some DPPC liposome suspensions were heat-treated at 45°C for 3 min before the cooling scan. The cooling scan of some suspensions were paused at certain temperatures (−10°C to −35°C) and maintained those temperatures for 30 or 60 min before further cooling to study the effect of low temperature storage on the physical changes. The column-eluted liposome suspensions were subjected to thermal analysis without the concentration adjustment. The homogeneous ice formation exotherms of these suspensions were calculated using the phosphate concentration data.

Measurement of Liposome Size by Dynamic Light Scattering

The size distribution of liposomes suspended in the Tris–HCl buffer (0.08% DPPC, 25°C) was determined using a dynamic light scattering (DLS) spectrophotometer (Photal DLS-7100SL; Otsuka Electronics Co., Osaka, Japan) with a He–Ne laser (632.8 nm) and a scattering angle (90°; 50 scans).

CF Retention Study

Dried DPPC films were hydrated with solutions containing 25 mM 5(6)-CF, 10 mM Tris–HCl buffer, and 0% or 12% trehalose, adjusted to pH 7.4 by NaOH. The CF-loaded vortexed multilamellar liposome suspensions (6% lipid, w/w) were prepared by extrusion through a 0.2-μm pore filter, and then eluted through the Sephadex G-25 column equilibrated with the buffer or trehalose-containing buffer. Freeze–thawing of the suspension was performed using the DSC system while the thermal profiles were simultaneously monitored. Aliquots of the liposome suspensions (10 μL, 4% DPPC, w/w) in unsealed aluminum pans were cooled to −35°C or −70°C at varied cooling speeds (1–10°C/min), and then heated to 25°C at 10°C/min on the DSC furnace. The freeze–thawed liposome suspensions were diluted by adding the Tris–HCl buffer or trehalose-containing buffer solutions (10 mL) in the glass tubes. The mildly agitated liposome suspensions underwent fluorescence measurement using a spectrometer (FP-6500; JASCO Corp., Tokyo, Japan). After the initial fluorescence measurements of the suspensions (2 mL) at 460 nm (excitation) and 550 nm (emission), those of the membrane-perturbed liposome suspensions were obtained by the addition of aliquots (20 μL) of Triton X-100. The CF leakage ratio was calculated using the following equation:

$$\%\,\text{Leakage} = \frac{\text{Initial fluorescence of treated sample} - \text{initial fluorescence of control}}{(\text{Final fluorescence of treated sample} - \text{initial fluorescence of control})} \times 100$$

RESULTS AND DISCUSSION

Freeze-Induced Changes in Liposome Suspensions

Figure 1 shows a thermogram of a frozen DPPC liposome suspension (4% in 10 mM Tris–HCl buffer, 0.2 μm) cooled at 5°C/min. The suspension showed a large exothermic peak of the freezing of the bulk solution (heterogeneous ice nucleation) at approximately −20°C and a smaller second exothermic peak of the freezing of the intraliposomal solution (homogeneous ice nucleation) at approximately −45°C.[15,17,32] The lower temperature exotherm disappeared by prior addition of a membrane-perturbing surfactant (1% Triton X-100), which supported the aforementioned definition of the peak rather than other interpretations (e.g., freezing of phosphatidylcholine headgroups) of the exotherm (data not shown).[33] The temperature

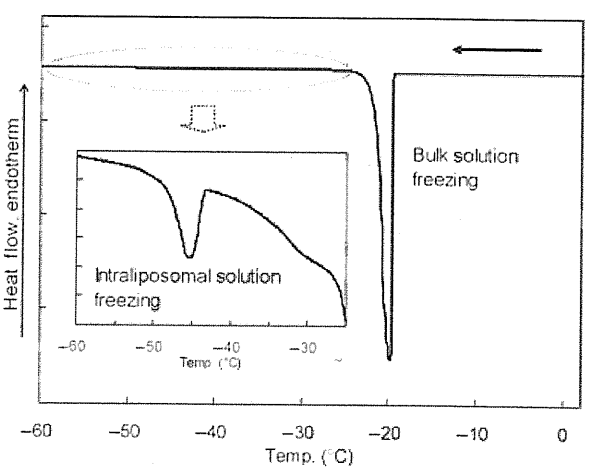

Figure 1. Cooling thermogram of a frozen DPPC liposome suspension. An aliquot (10 μL) of liposome suspension (4% lipid, w/w) in Tris–HCl buffer (10 mM, pH 7.4) was cooled from room temperature to −70°C at 5°C/min.

of the bulk solution freezing peak varied greatly between the scans. Some suspensions also showed a broad exotherm at approximately −30°C. The exotherm suggested the freezing of solutions released from the liposomes (dehydration) and/or freezing of the internal solutions initiated by external ice crystals that penetrated through the membrane.[12,16,34] The varied shape and overlapping of the peak with the large bulk solution freezing exotherm made further characterization difficult in this study. The frozen liposome suspensions showed only a gradual shift of the thermogram before the large ice melting endotherm during their heating scans (data not shown). DLS measurement of the DPPC liposome suspensions indicated a mean diameter of 203.9 ± 10.6 nm before the thermal analysis (three different preparations). Standard deviation of the liposome size obtained in each measurement was within 5% of the average value.

The effects of cooling speeds on the internal solution freezing exotherm of liposomes differing in size and lipid composition are shown in Figure 2. Slower cooling of the DPPC liposome suspension (0.2 μm) reduced the exotherm, indicating loss of the supercooled intraliposomal solution during the scan (Fig. 2a; 1 –2°C/min). The extraliposomal ice growth and concomitant concentration of solutes should generate osmotic forces that induce water evacuation from liposomes. Reported freeze-induced morphological rearrangement into multilamellar liposomes may also reduce the intraliposomal solution content.[4] The width of the bulk solution freezing peak got narrower in the slower cooling, suggesting a certain time required for the ice growth (data not shown). On the contrary, the limited effect of the cooling speed on the peak width of the intraliposomal solution freezing exotherm suggested independent ice formation in the individual liposomes. A certain amount of the intraliposomal solution interacting (hydrating) with the membrane lipid and/or solute molecules should remain unfrozen even below the intraliposomal solution freezing temperature.[11,11]

Reduction of the intraliposomal solution freezing exotherm was more apparent in the DPPC liposome suspensions temporarily (30 min) kept at temperatures between the bulk and the intraliposomal solution freezing during the cooling scan (Fig. 3). The finding that the intraliposomal solution freezing exotherm of a suspension held at −25°C was smaller than those of suspensions held at −30°C or −35°C suggested faster loss of the supercooled solutions in the temperature range just below the bulk solution freezing. Longer exposure to the temperature range should be one of the reasons for the reduction in the exotherms with the slower cooling. On the contrary, holding the suspension at a temperature above the bulk solution freezing temperature showed no apparent effect on the intraliposomal solution freezing

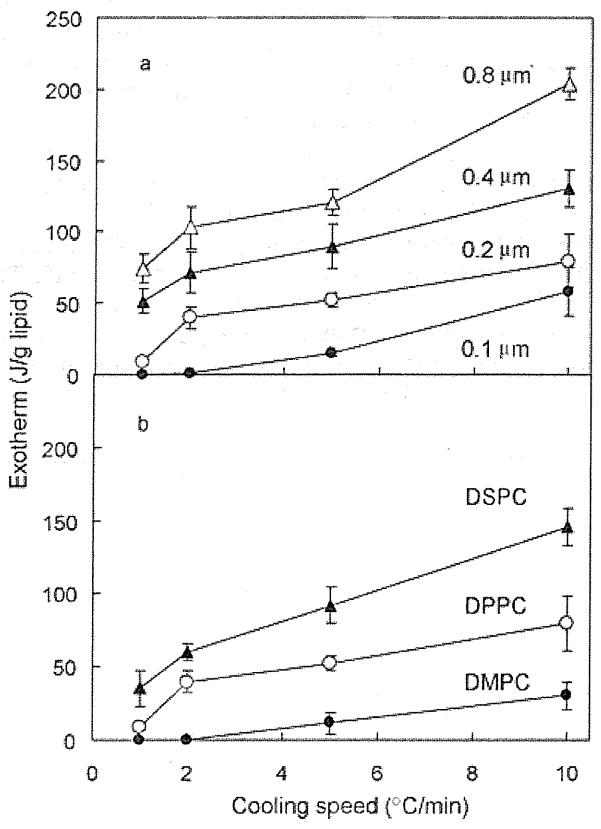

Figure 2. Effects of cooling speed on internal solution freezing exotherm of liposome suspensions with differing (a) extrusion membrane pore sizes (DPPC; 0.1 μm: •, 0.2 μm: ○, 0.4 μm: ▲, and 0.8μm: △) and (b) lipid compositions (0.2 μm; DMPC: •, DPPC: ○, and DSPC: ▲). Aliquots of liposome suspensions (10 μL, 4% lipid in 10 mM Tris–HCl buffer) were cooled at 1–10°C/min (average ± SD, $n = 3$).

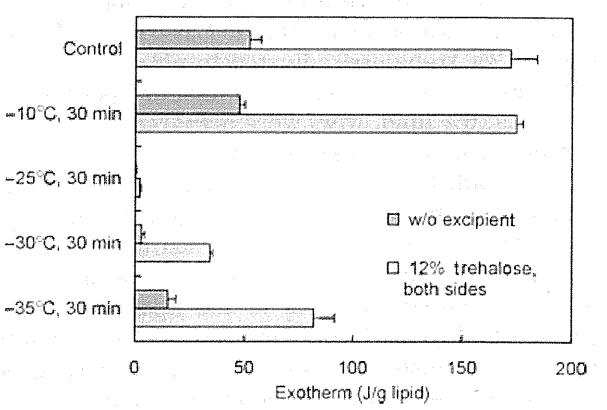

Figure 3. Effect of low temperature holding on internal solution freezing exotherms of DPPC liposome suspensions containing trehalose (0% or 12%, w/w) on both sides of the membrane (10 μL, 4% lipid in Tris–HCl buffer, 0.2 μm). The suspensions were held at different temperatures (−10°C to −35°C) for 30 min during cooling scans at 5°C/min.

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exotherm. The absence of apparent osmotic driving force may explain the limited effect of storage at above the bulk solution freezing temperature.

The DPPC liposome suspensions extruded through larger pore size filters (e.g., 0.4 and 0.8 μm) showed larger intraliposomal solution freezing exotherms (Fig. 2a). Factors including the possibility of higher initial solution contents per lipid weight, limited membrane disordering associated with the curvature, and slower dehydration due to the increase in multilamellar membranes would explain the large exotherms. Liposomes composed of phosphatidylcholines of different acyl chain lengths showed retention of the intraliposomal solution down to the homogeneous ice formation temperature in the order of DMPC < DPPC < DSPC (Fig. 2b). The intraliposomal solution freezing exotherm was not observed in the thermal analysis of POPC liposome suspensions (data not shown). All the liposome membranes are below their T_m (POPC: −5°C, DMPC: 24°C, DPPC: 41°C, DSPC: 54°C) at the bulk solution freezing temperature.[35] Possible differences in the membrane fluidity and rigidity would cause the freeze-induced dehydration to vary.

Effect of Trehalose Distribution on Freezing Profiles of Liposome Suspensions

The effects of intra- and extraliposomal trehalose on the freezing behavior of liposome suspensions were studied. The DPPC liposome suspensions containing trehalose on both sides of the membrane showed larger intraliposomal solution peaks that suggest reduced solution loss upon the bulk solution freezing. For example, cooling of the 0.2 μm DPPC suspensions at 10°C/min resulted in exotherms of approximately 80 and 200 J/g lipid, respectively, in the absence and presence of trehalose (Figs. 2 and 4). The addition of trehalose also lowered the peak temperature of the exotherm (approx. 3°C at 12% trehalose, w/w).[18,34] The trehalose-containing liposome lost a larger amount of the internal supercooled solution during the slower cooling of the suspensions. Temporary pausing of the cooling scan suggested a faster loss of the supercooled solution near the bulk solution freezing temperature (−25°C), also in the trehalose-containing liposome suspensions (Fig. 3). The addition of various low-molecular-weight saccharides and polyols to both sides of the lipid membrane increased the freezing exotherm of the intraliposomal solutions, suggesting that slower freeze-induced dehydration occurred due to the colligatively determined osmotic effect (Fig. 5). The limited effect of dextran on the exotherm could be explained by its lower molar concentration and its possible exclusion from the vicinity of the liposomes in the freeze-concentrated nonice phase.[36] The large (0.4 and 0.8 μm) or lower fluidity (DSPC) liposomes retained higher amounts of

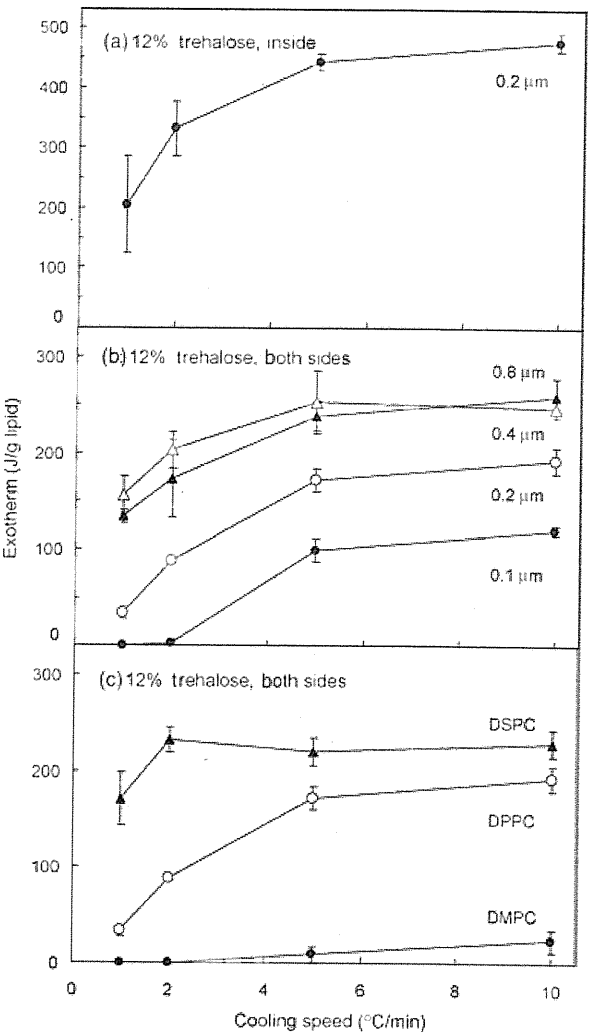

Figure 4. Internal solution freezing exotherms of liposome suspensions containing trehalose (12%, w/w) on (a) the inside and (b, c) both sides of liposomes. Aliquots of suspensions (10 μL, 4% lipid in 10 mM Tris–HCl buffer) containing liposomes with (b) differing extrusion membrane pore sizes (DPPC; 0.1 μm: •, 0.2 μm: ○, 0.4 μm: ▲, and 0.8 μm: △) and (c) lipid compositions (0.2 μm; DMPC: •, DPPC: ○, and DSPC: ▲) were cooled at 1–10°C/min (average ± SD, $n = 3$).

freezable intraliposomal solution in the presence of trehalose on both sides of the membrane (Figs. 4b and 4c).

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine liposomes containing trehalose on one side of the membrane showed different freezing behaviors. The addition of higher concentration trehalose to the extraliposomal media reduced the intraliposomal solution freezing exotherm (Fig. 6). The difference in the osmotic pressures across the membrane should induce solution flow that dehydrates the liposomes both prior to cooling and after the bulk solution freezing. The