SCIENTIFIC WRITING: RESEARCH REPORT AND MANUSCRIPT WRITING

Professor Dr. Narong Sarisuta

Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University

Definition of Research

- Research is everywhere
- Research is a format, planned, systematic, intensive process of carrying on the scientific method of analysis results in some sort of format record
- Research is a method of acquiring knowledge

(National Science Foundation-
Theetranont C, Kumnuanta J, Hiebert G. 1992. Resource book for researchers. Chiang Mai University)

What is Research?

Research is a format, planned, and systematic process of carrying the method of acquiring fact of knowledge with a well-defined objective.

Research is composed of 3 characteristics:

1. Being a method of acquiring knowledge.
2. Being a format, planned, and systematic process following the scientific method.
3. Having a well-defined objective.

(National Research Council of Thailand-NRCT)

Two Criteria for Justified Research Project :

RELEVANCE and MERIT

RELEVANCE

Your research project should be relevant to needs of public community or program goals of the funding agency.

To understand, to predict, and to control problems arised in your community.

MERIT

Your research project may have merit because of --

- Cost-effectiveness
- Ingenuity (cleverness) of the approach
- Quality of the research opportunity (occasion)
- Credentials of the investigators
- Unique assess to experimental material
- Special research facilities
- Other reasons

Justified research project will have both project relevance and intrinsic merit.

Research Report

There must always be a written record or report for any research project, otherwise it will not be considered as research.

Research = body + voice + mind

การวิจัย = กาย + วาจา + ใจ

Components of the Research Report:

1. TITLE AND AUTHOR'S NAME
2. ABSTRACT
3. ACKNOWLEDGEMENTS
4. INTRODUCTION
5. EXPERIMENTAL
   - MATERIALS
   - METHODS
6. RESULTS
7. DISCUSSION
8. CONCLUSION
9. REFERENCES
10. APPENDIX

1. TITLE

- Titles are of great importance for current awareness and for information retrieval.
- The wording of titles should be chosen carefully to provide information on the contents and to function as **“point of entry”** for information retrieval.
- The title of a research paper should provide specific words of identification.
- A clearly expressed title will control your writing and keep you on course.
- Consider the following strategies for writing a title.

1. Name a general subject, followed by a colon, and followed by a phrase that renames the subject.

Poor title: Saving the Software

Better title: Computer Control: Software Safeguards and Computer Theft

Key words: computer theft, software safeguards

2. Name a general subject and narrow it with prepositional phrase.

Poor title: Effects of Temperature and Humidity

Better title: Effects of Temperature and Humidity on the Physical Properties of Piroxicam Tablets

Key words: temperature and humidity, piroxicam

Effects of Temperature and Humidity on the Physical Properties of Piroxicam Tablets

Narong Sarisuta,* Tassanee Thamsakdakorn, and Somboon Jateleela

PHOTODISC, INC.

The authors studied piroxicam tablets containing various proportions of filler (lactose or mannitol) after storage for 12 weeks at 40 °C and 52% relative humidity (RH) and at 40 °C and 96% RH. The tablets were prepared by wet granulation using povidone as a binder. They were compressed at forces of 500–600 kg for hardness values of 6–7 kg and stored in a controlled environmental chamber. Physical properties of the tablets such as weight, thickness, hardness, loss on drying, disintegration, and dissolution were assessed every 2 weeks. This article discusses the methods used to conduct the studies and the results.

Narong Sarisuta, PhD, *is an associate professor,* ***Tassanee Thamsakdakorn*** *is a graduate student, and* ***Somboon Jateleela, PhD,*** *is an assistant professor, all in the Department of Manufacturing Pharmacy, Mahidol University, 447 Sri-Ayudhya Road, Bangkok 10400, Thailand, tel. +66 2 644 8680, fax +66 2 247 4696, e-mail (pynst@mahidol.ac.th).*

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Pharmaceutical scientists generally recognize that aging of tablets can result in chemical and physical changes, creating problems with stability and bioavailability. Extensive studies of the effects of storage time, temperature, and humidity on tablet properties such as hardness, disintegration, and dissolution have been conducted with various drugs, including naproxen, prednisolone, norfloxacin, enalapril maleate, furosemide, glybenclamide, and acetaminophen (1–8). The literature offers limited information on the relationships between such alterations in physical properties and aging, types of formulations, and drug–excipient ratios in tablets. Very few studies of tablets containing mixed diluents have been reported (9,10). The authors therefore investigated the effects of storage under dry and humid conditions on the physical properties of tablets containing piroxicam (a slightly soluble drug) and either lactose or mannitol (soluble fillers) at various drug–filler ratios.

MATERIALS

The piroxicam USP used in this study was obtained from Resfar (Italy). Lactose BP and cornstarch USP were purchased from DMV (Veghel, The Netherlands). Povidone (Kollidon K30) was obtained from BASF (Germany). Mannitol and magnesium stearate BP were purchased from BDH Chemicals (England) and Farco Fine Chemicals (Italy), respectively.

3. Name a general subject and cite a specific work that will illuminate the topic.

Poor title: Active Entrapment of Liposomes

Better title: Active Entrapment of Amphotericin B Liposomes Utilizing Lipid Composition, Surface Charge of Bilayers and pH of Hydration Medium

Key words: amphotericin B, liposomes, surface charge

4. Name a general subject, followed by a colon, and followed by a phrase that describes the type of study

Poor title: Dissolution Kinetics of Three-Component Compressed Solid Mixtures

Better title: Dissolution Kinetics of Three-Component Compressed Solid Mixtures: Flaking Spheres with Largely Different Solubilities

Key words: three-component dissolution kinetics, largely different solubility solid dissolution

Dissolution Kinetics of Three-Component Compressed Solid Mixtures with Largely Different Solubilities: Flaking Spheres

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Received 31 December 1999; revised 3 May 2000; accepted 15 May 2000

Published online 10 August 2000

ABSTRACT: The dissolution kinetics of three-component compressed solid spheres of various compositions of mannitol (MAN), sulfamethoxazole (SMX), and trimethoprim (TMP) were studied. Because the solubility of MAN was ~300 times that of SMX and TMP, the flaking phenomenon during dissolution process was observed. The critical flaking line was drawn between two critical flaking points in the triangular composition diagram of MAN–SMX–TMP mixtures (i.e., the compositions of 0.5022 MAN and 0.4978 SMX mass fractions, and 0.4906 MAN and 0.5094 TMP mass fractions), thus dividing the system into two distinct regions; namely, nonflaking and flaking regions. In the case of nonflaking region, only three dissolution behaviors for MAN–SMX–TMP three-component mixtures were proposed. The calculated dissolution rates of all components by using the multicomponent dissolution model previously proposed were found to satisfactorily approximate the observed values for both two- and three-component solid mixtures. In the case of flaking region, the observed dissolution rate of MAN was found to be the exponential function of the mass fraction ratio of either SMX or TMP to MAN for both two- and three-component mixtures. The slope of the semi-logarithmic plot of the observed MAN dissolution rate versus the mass fraction ratio of either SMX or TMP to MAN was defined as retarding coefficients of SMX or TMP, r'_{SMX} or r'_{TMP}, respectively. The erosion rate of either SMX or TMP was the product of mass fraction ratio of drug to MAN and the exponential function of such mass fraction ratio. The mathematical model was developed on the basis of simultaneous erosion followed by dissolution of eroded particles to predict the dissolution rate of each drug from MAN–SMX–TMP compressed solid spheres within the flaking region. © 2000 Wiley-Liss, Inc. and the American Pharmaceutical Association J Pharm Sci 89:1196–1211, 2000

Keywords: three-component dissolution kinetics; dissolution of compressed solid mixtures; largely-different-solubility solid dissolution; sulfamethoxazole; trimethoprim; mannitol

INTRODUCTION

The kinetic models of dissolution for pure compounds have received considerable attention for many decades, but much less attention has been given to those of multicomponent systems despite the fact that the simultaneous dissolution processes of more than one phase are often taking place in practice.[1] Early pharmaceutical studies of the dissolution of two-component systems were reported for mixtures of weak acids–tribasic sodium phosphate[2] and salicylic acid–polyvinylpyrrolidone.[3] Diffusion models have also been proposed to depict the dissolution of nondisintegrating polyphase mixtures containing two noninteracting components and complexing components.[4] This approach has also been applied to mixtures of polymorphs,[5] as well as to noninteracting compressed mixtures of aspirin–salicylic acid, aspir-

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Journal of Pharmaceutical Sciences, Vol. 89, 1196–1211 (2000)

5. Name a general subject, followed by a colon, and followed by a question

Poor title: Nanoparticles

Better title: Nanoparticles: Will the Dream Come True?

Key words: nanoparticles, future trends

6. Establish a specific comparison

Poor title: A Comparison of Butt and Peel Adhesive Strength of Film-Coated Tablets

Better title: The Influence of Drug-Polymer Interactions on Butt and Peel Adhesive Strength of Film-Coated Tablets

Key words: butt adhesive strength, peel adhesive strength, drug-polymer interactions

Drug Development and Industrial Pharmacy, 32 463–471, 2006

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DOI: 10.1080/03639040500528962

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The Influence of Drug-Excipient and Drug-Polymer Interactions on Butt Adhesive Strength of Ranitidine Hydrochloride Film-Coated Tablets

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Krisana Srikummoon
Production Division, Government Pharmaceutical Organization, Rama 6 Road, Bangkok 10400, Thailand

ABSTRACT The influence of fillers and polymeric films on adhesive strength of hydroxypropyl methylcellulose (HPMC) and Eudragit E100® films coated on ranitidine HCl tablets containing either spray-dried rice starch (SDRS) or lactose monohydrate as fillers after storage at 45°C/75% RH for four weeks was investigated by the use of butt adhesion technique. The adhesive strength of film-coated tablets of fillers without drug was found to slightly decrease after storage. In contrast, the adhesive strength of drug-containing film-coated tablets significantly reduced, the degree of which was higher for Eudragit E100® than HPMC. Physicochemical characterization by employing differential scanning calorimetry (DSC) and diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) revealed that the drug was obviously incompatible with lactose and possibly mild interaction with Eudragit E100® was suggested. The results indicated that the adhesive strength of film-coated tablets would be affected not only by the drug-excipient interaction, but also by the drug-polymeric film interaction.

KEYWORDS Film-coated tablets, drug-excipient interaction, drug-polymer interaction, adhesive strength, ranitidine HCl, hydroxypropyl methylcellulose, Eudragit E100®

2. ABSTRACT

- The abstract should briefly (80-100 words) present in one paragraph, the problem and experimental approach and state the major findings and conclusions.
- It should be self-explanatory and suitable for reproduction without rewriting.
- Footnotes or unidentified abbreviations may not be used.
- If a reference must be cited, complete publication data must be given.
- The abstract should be written in past tense.

Journal of Liposome Research, 2011; 21(1): 28–37

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RESEARCH ARTICLE

Preparation of dry reconstituted liposomal powder by freeze-drying at room temperature

Ruthairat Benjakul[1], Busaba Panyarachun[2], and Narong Sarisuta[1]

[1]*Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand, and*
[2]*Department of Anatomy, Faculty of Medicine, Srinakharinvirot University, Bangkok, Thailand*

Abstract

The aim of this study was to develop a novel, one-step method of liposome preparation by freeze-drying at room temperature as well as to investigate the physicochemical properties of dry reconstituted liposomal powder that was prepared. The method was based on utilizing sublimation of a volatile solid inert carrier, that is, chlorobutanol hemihydrate (CBN), instead of ice, which was less sophisticated and simpler than the conventional freeze-drying process. The optimum conditions used in the sublimation process of CBN were a temperature of 25–30°C and a pressure of 1.5–2.0 mBar for 8 hours. The influence of various parameters, such as type, particle size, and ratio of sugar lyoprotectant (i.e., mannitol or sucrose) and CBN to lipid on reconstitution time, liposome size, zeta potential, vesicle type, and lamella structure of reconstituted liposomes, were studied. The results revealed that the obtained liposomes were oligolamellar vesicles with particle sizes ranging from 400 to 1,000 nm. Type and ratio of sugar and CBN to lipid were found to significantly affect the reconstitution time. On the other hand, liposome size was independent of type of sugar and ratio of CBN to lipid, yet became smaller at higher sugar-to-lipid ratio and smaller sugar and CBN size. In all cases, traces of residual solvents were definitely below the acceptable limit.

Keywords: *Freeze-drying; liposomes; chlorobutanol; sublimation; dry reconstitute vesicles*

PROBLEM (OBJECTIVE)

Abstract

The aim of this study was to develop a novel, one-step method of liposome preparation by freeze-drying at room temperature as well as to investigate the physicochemical properties of dry reconstituted liposomal powder that was prepared. The method was based on utilizing sublimation of a volatile solid inert carrier, that is, chlorobutanol hemihydrate (CBN), instead of ice, which was less sophisticated and simpler than the conventional freeze-drying process. The optimum conditions used in the sublimation process of CBN were a temperature of 25–30°C and a pressure of 1.5–2.0 mBar for 8 hours. The influence of various parameters, such as type, particle size, and ratio of sugar lyoprotectant (i.e., mannitol or sucrose) and CBN to lipid on reconstitution time, liposome size, zeta potential, vesicle type, and lamella structure of reconstituted liposomes, were studied. The results revealed that the obtained liposomes were oligolamellar vesicles with particle sizes ranging from 400 to 1,000 nm. Type and ratio of sugar and CBN to lipid were found to significantly affect the reconstitution time. On the other hand, liposome size was independent of type of sugar and ratio of CBN to lipid, yet became smaller at higher sugar-to-lipid ratio and smaller sugar and CBN size. In all cases, traces of residual solvents were definitely below the acceptable limit.

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EXPERIMENTAL APPROACH

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MAJOR FINDINGS

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CONCLUSIONS

Keywords: *Freeze-drying; liposomes; chlorobutanol; sublimation; dry reconstitute vesicles*

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RESEARCH ARTICLE

Phospholipid vesicle–bound lysozyme to enhance permeability in human intestinal cells

Wasu Witoonsaridsilp[1], Busaba Panyarachun[2], Montree Jaturanpinyo[1], and Narong Sarisuta[1]

[1]*Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand and*
[2]*Department of Anatomy, Faculty of Medicine, Srinakharinviroj University, Bangkok, Thailand*

Abstract

Background: Oral peptide and protein drug delivery still remain the area of challenges for pharmaceutical scientists due to their low stability and permeability in gastrointestinal (GI) tract. In this study phospholipid vesicle–bound lysozyme were prepared and assessed for their physicochemical properties, secondary structure, and permeation across Caco-2 cells.

Results: Lysozyme was found to be substantially bound onto negatively charged vesicles via electrostatic interaction as evidenced by zeta potential measurements regardless of cholesterol content. In contrast, the size of phospholipid vesicle–bound lysozyme became larger with the increasing cholesterol content. The secondary structure of vesicle-bound lysozyme examined by FTIR was unchanged compared to that in buffer solution. The apparent permeability of vesicle-bound lysozyme across Caco-2 cells monolayer was significantly enhanced with a size dependent manner compared to that of solution.

Conclusion: The permeation across Caco-2 cell monolayers of phospholipid vesicle–bound lysozyme was demonstrated to be significantly enhanced with a size-dependent manner.

Keywords: Lysozyme, phospholipid vesicles, secondary structure, permeation, Caco-2 cells

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RESEARCH ARTICLE

Development of delayed-release proliposomes tablets for oral protein drug delivery

Charintra Tantisripreecha[1], Montree Jaturanpinyo[1], Busaba Panyarachun[2], and Narong Sarisuta[1]

[1]*Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand, and*
[2]*Department of Anatomy, Faculty of Medicine, Srinakharinvirot University, Bangkok, Thailand*

Abstract

Context: One among many attempts to improve oral protein drug delivery was utilizing the colloidal drug carriers particularly liposomes.

Objective: The purpose was to develop proliposomes of bovine serum albumin (BSA) in the form of granules and delayed-release tablets by using simple tablet manufacturing process.

Materials and methods: BSA proliposomes granules were prepared by spraying 7:3 (w/w) – lecithin:cholesterol solution mixture onto BSA-mannitol granules rotating in a glass coating pan. BSA proliposomes granules were directly compressed into tablets and subsequently coated with Eudragit® L100 film. The physical properties and stability in gastrointestinal fluids of delayed-release BSA proliposomes tablets as well as reconstituted liposomes were assessed.

Results: The BSA proliposomes tablets disintegrated readily and the obtained reconstituted BSA liposomes exhibited multilamellar vesicles, the size and entrapment efficiency of which were around 2–3 μm and 10–14%, respectively. The delayed-release BSA proliposomes tablets were found to be relatively stable in United States Pharmacopoeia (USP) simulated gastric and intestinal fluids. Increase in amount of BSA in granules resulted in the increase in entrapment efficiency and loading capacity.

Discussion: The Fourier transform infrared spectroscopy (FTIR) results indicated increase in α-helix structure of BSA entrapped in liposomes. ^{31}P phosphorous nuclear magnetic resonance spectroscopy (^{31}P-NMR) spectrum indicated interaction between BSA molecules and phosphoric acid polar groups of bilayers membrane.

Conclusion: The delayed-release BSA proliposomes tablets developed could completely be reconstituted into liposomes with sufficient resistance to the hostile environment in gastrointestinal tract.

Keywords: Proliposomes, bovine serum albumin, liposomes, delayed-release tablets, gastrointestinal stability

3. ACKNOWLEDGEMENTS

- This section should acknowledge financial support, technical assistance, advice from colleagues, gifts, etc.
- Permission must be received from persons whose contribution to the work is acknowledged in the manuscript.

Acknowledgements

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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4. INTRODUCTION

Use the first few paragraphs to establish the nature of your study, but keep the introduction short and directed toward the issues.

Subject. **Identify your specific topic, and then define, limit, and narrow it to one issue.**

Drug Development and Industrial Pharmacy, 2011, 1–10, Early Online

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[2]*Department of Anatomy, Faculty of Medicine, Srinakharinvirot University, Bangkok, Thailand*

Abstract

Context: One among many attempts to improve oral protein drug delivery was utilizing the colloidal drug carriers particularly liposomes.

Objective: The purpose was to develop proliposomes of bovine serum albumin (BSA) in the form of granules and delayed-release tablets by using simple tablet manufacturing process.

Materials and methods: BSA proliposomes granules were prepared by spraying 7:3 (w/w) – lecithin:cholesterol solution mixture onto BSA-mannitol granules rotating in a glass coating pan. BSA proliposomes granules were directly compressed into tablets and subsequently coated with Eudragit® L100 film. The physical properties and stability in gastrointestinal fluids of delayed-release BSA proliposomes tablets as well as reconstituted liposomes were assessed.

Results: The BSA proliposomes tablets disintegrated readily and the obtained reconstituted BSA liposomes exhibited multilamellar vesicles, the size and entrapment efficiency of which were around 2–3 μm and 10–14%, respectively. The delayed-release BSA proliposomes tablets were found to be relatively stable in United States Pharmacopoeia (USP) simulated gastric and intestinal fluids. Increase in amount of BSA in granules resulted in the increase in entrapment efficiency and loading capacity.

Discussion: The Fourier transform infrared spectroscopy (FTIR) results indicated increase in α-helix structure of BSA entrapped in liposomes. ^{31}P phosphorous nuclear magnetic resonance spectroscopy (^{31}P-NMR) spectrum indicated interaction between BSA molecules and phosphoric acid polar groups of bilayers membrane.

Conclusion: The delayed-release BSA proliposomes tablets developed could completely be reconstituted into liposomes with sufficient resistance to the hostile environment in gastrointestinal tract.

Keywords: Proliposomes, bovine serum albumin, liposomes, delayed-release tablets, gastrointestinal stability

Introduction

Delivery systems of peptide and protein drugs by using other non-invasive routes of administration have notably been the challenge for many decades, especially oral drug delivery systems. Numerous limitations in development of oral peptide drug delivery include intestinal membrane permeability, size limitations, intestinal and hepatic metabolism, enzymatic degradation in alimentary canal, and in some cases, solubility limitations[1]. Many attempts have been made to overcome these barriers, one of which was utilizing the colloidal drug carriers particularly liposomes for oral delivery of peptide and protein drugs[2–10]. However, aqueous dispersion forms of liposomes suffer from serious stability problems associated with the aggregation, fusion, and phospholipid hydrolysis that could limit their shelf-lives. Although freeze drying is most frequently used today as a possible tool in improving the stability of liposomes, it still exhibits some difficulties in terms of residual water content and chemical stability problems caused by the lyoprotectants used[11]. Furthermore, this technique itself consumes a larger amount of energy in the production process with considerably high cost due to the lyophilization step as compared to other techniques. For large scale production

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Introduction

SUBJECT

Delivery systems of peptide and protein drugs by using other non-invasive routes of administration have notably been the challenge for many decades, especially oral drug delivery systems. Numerous limitations in development of oral peptide drug delivery include intestinal membrane permeability, size limitations, intestinal and hepatic metabolism, enzymatic degradation in alimentary canal, and in some cases, solubility limitations[1]. Many attempts have been made to overcome these barriers, one of which was utilizing the colloidal drug carriers particularly liposomes for oral delivery of peptide and protein drugs[2-10]. However, aqueous dispersion forms of liposomes suffer from serious stability problems associated with the aggregation, fusion, and phospholipid hydrolysis that could limit their shelf-lives. Although freeze drying is most frequently used today as a possible tool in improving the stability of liposomes, it still exhibits some difficulties in terms of residual water content and chemical stability problems caused by the lyoprotectants used[11]. Furthermore, this technique itself consumes a larger amount of energy in the production process with considerably high cost due to the lyophilization step as compared to other techniques. For large scale production of liposomes, not only the desired product properties should be focused on, but efficiency and feasibility of the production process should be recognized as well[12].

Dry liposomal formulations in the form of proliposomes appear to be the promising candidate with respect to its simplicity and practicality as compared to the freeze-dried products. Proliposomes are definitely dry free-flowing particles composed of drug, phospholipid, and water soluble porous powder, which upon addition of water are hydrated to form a liposomal dispersion[13]. Proliposomes could be fabricated into various dosage forms including tablets/capsules, transdermal delivery systems[14], and those for vaginal administration[15]. Due to their advantages on specific site-targeting ability as well as stability, proliposomes have recently been investigated as oral drug delivery systems for peptides and protein drugs. Although various methods for producing proliposomes have been developed including fluidized-bed method[16], film-deposition on carriers method[7], spray drying method[17], and crystal-film method[18], there are still some limitations with respect to both stable formulations in gastrointestinal tract and manufacturing processes in industrial scale.

The purpose of this investigation was to develop proliposomes of bovine serum albumin (BSA) in the form of granules and delayed-release tablets by using conventional tablet manufacturing process. The physicochemical properties and *in vitro* stability of delayed-release BSA proliposomes tablets and reconstituted liposomes in USP simulated gastric and intestinal fluids were thoroughly evaluated. The conformational change of BSA entrapped in liposomes as well as its interaction with bilayer membrane was also investigated.

4. INTRODUCTION

Use the first few paragraphs to establish the nature of your study, but keep the introduction short and directed toward the issues.

Subject. **Identify your specific topic, and then define, limit, and narrow it to one issue.**

Background. **Provide relevant historical data. Discuss a few key sources that touch on your specific issue. If writing about a major figure, give relevant biographical facts, but not an encyclopedia-type survey.**

Introduction

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BACKGROUND

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Problem. **The point of a research paper is to explore or resolve a problem, so identify and explain the complications that you see.**

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PROBLEM

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Problem. **The point of a research paper is to explore or resolve a problem, so identify and explain the complications that you see.**

Thesis sentence. **Within the first few paragraphs, establish the direction of the study and point toward your eventual conclusions.**

Introduction

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THESIS SENTENCE

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5. EXPERIMENTAL SECTION

- The experimental procedures should be described in sufficient detail to enable others to repeat the experiments.
- Names of products and manufacturers (with city, state address) should be included only if alternate sources are deemed (considered) unsatisfactory.
- Brand names may be used only once in the manuscript. For subsequent designation, use “formulation A”, “product B”, etc.

- Novel experimental procedures should be described in detail, but published procedures should merely be referred to by literature citation of both the original and any published modifications.
- The purity of key compounds and description(s) of the method(s) used to determine purity should be included in this section. (eg. USP, BP or AR grades)
- Identification of and precautions for handling hazardous chemicals and dangerous procedures should be placed at the beginning of this section.

EXPERIMENTAL SECTION

Materials

Sulfamethoxazole (Siris Limited, Hyderabad, India); trimethoprim (Lupin Chemicals, Samutprakarn, Thailand); mannitol, benzoic acid, and hydrochloric acid (May and Baker, Dagenham, England); and *n*-hexane (E. Merck, Darmstadt, Germany) were used as received. All chemicals used were of USP, BP, or reagent grades.

Preparation of the Compressed Spheres

The amount of each component [sulfamethoxazole (SMX), trimethoprim (TMP), and mannitol (MAN)] was accurately weighed according to the required composition in mass fraction, as shown in Figure 1, for a mixture of 5 g and geometrically blended in a glass mortar and pestle for 12 min. By means of a single-punch tablet press (Diaf, model TM 206, Copenhagen, Denmark) instrumented with a strain gauge on the upper punch holder and connected to a dynamic strain amplifier (Kyowa, model DPM-612A, Tokyo, Japan), preamplifiers (Gould, model 210-310031-1, Cleveland, OH, U.S.A.), and an oscillographic recorder (Gould, model SC 274-111122, U.S.A.), the

Materials

BSA, cholesterol, and Triton-X® were obtained from Merck, Darmstadt, Germany. Lecithin was from Union Chemical, Bangkok, Thailand. Stearylamine was purchased from Sigma, St. Louis, Missouri, USA. Mannitol was from Roquette, Lestrem Cedex, France. Polyvinylpyrrolidone (PVP K30) was from BASF, Germany. Dichloromethane, acetonitrile, and propylene glycol were from Labscan, Stillorgan, Co Dublin, Ireland. Microcrystalline cellulose (CEOLUS® PH101) was obtained from Asahikasei Chemical Corporation, Tokyo, Japan. Sodium starch glycolate (Explotab®) was from Penwest, Lestrem, France. Magnesium stearate was from Peter Greven, Netherland. Polymethacrylates (Eudragit® L100) was a gift from Degussa, Darmstadt, Germany. Bradford protein assay reagent (Bio-Rad®) was obtained from Bio-Rad, Hercules, California, USA.

Methods

Preparation of BSA proliposomes granules

BSA and mannitol powders at various ratios by weight of 0.5:9.5, 1:9, 2:8, 3:7, and 5:5 for total weight of 10 g were mixed together in a dry mixer for 5 min, which were subsequently granulated with 5 mL of 10%w/w PVP K30 solution and dried at 50°C for 2 h in hot air oven (KSL Engineering, Bangkok, Thailand). The lipid mixtures of lecithin:cholesterol (w/w) of 7:3 and lecithin:cholesterol:stearylamine (w/w) of 6:3:1 for 2.0 g were dissolved in 50 mL dichloromethane and subsequently sprayed onto 10 g of BSA-mannitol granules rotating in a glass coating pan (KSL Engineering, Bangkok, Thailand) using pneumatic air brush gun (H&HS Single Action, Paas Air Brush, Illinois, USA).

Preparation of delayed-release BSA proliposomes tablets

An amount of 10 g of BSA proliposomes granules were mixed together with 7 g sodium starch glycolate and 17.5 g microcrystalline cellulose in a dry mixer for 10 min, after which 0.35 g magnesium stearate was added and further mixed for 3 min. The blends were then compressed into tablets at a target weight of 350 mg and hardness of 4–5 kg using a single punch tablet machine (Diaf® TM 206, Salgelse, Denmark) and a 3/8 inch punch and die set.

The prepared BSA proliposomes tablets were subsequently coated with enteric film coating solution containing 7% w/w Eudragit® L100 and 0.5% w/w propylene glycol in 95% ethanol. The coating solution was sprayed continuously onto 35 g of BSA proliposomes tablets rotating in a glass coating pan (KSL Engineering, Bangkok, Thailand) until the desired coating weight was reached. The pan speed was 15 rpm, spray rate was 4 mL/min, atomizing air pressure was 8 psig, and inlet air temperature was 25°C.

6. RESULTS

- The results should be presented concisely.
- Tables and figures should be designed to maximize the presentation and comprehension of the experimental data.
- Attention should be paid to the matter of significant figures (usually no more than three).
- The same data should not be presented in more than one figure or in both a figure and a table.
- As a rule, interpretation of the results should be reserved for the discussion section of an Article, but under some circumstances it may be desirable to combine results and discussion in a single section.

7. DISCUSSION

- The purpose of the discussion is
  - to interpret the results and
  - to relate them to existing knowledge in the field in as clear and a brief fashion as possible.
- Information given elsewhere in the manuscript should not be repeated in the discussion.
- Extensive reviews of the literature should be avoided.

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RESEARCH ARTICLE

Phospholipid vesicle–bound lysozyme to enhance permeability in human intestinal cells

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[2]*Department of Anatomy, Faculty of Medicine, Srinakharinviroj University, Bangkok, Thailand*

Abstract

Background: Oral peptide and protein drug delivery still remain the area of challenges for pharmaceutical scientists due to their low stability and permeability in gastrointestinal (GI) tract. In this study phospholipid vesicle–bound lysozyme were prepared and assessed for their physicochemical properties, secondary structure, and permeation across Caco-2 cells.

Results: Lysozyme was found to be substantially bound onto negatively charged vesicles via electrostatic interaction as evidenced by zeta potential measurements regardless of cholesterol content. In contrast, the size of phospholipid vesicle–bound lysozyme became larger with the increasing cholesterol content. The secondary structure of vesicle-bound lysozyme examined by FTIR was unchanged compared to that in buffer solution. The apparent permeability of vesicle-bound lysozyme across Caco-2 cells monolayer was significantly enhanced with a size dependent manner compared to that of solution.

Conclusion: The permeation across Caco-2 cell monolayers of phospholipid vesicle–bound lysozyme was demonstrated to be significantly enhanced with a size-dependent manner.

Keywords: Lysozyme, phospholipid vesicles, secondary structure, permeation, Caco-2 cells

Introduction

Rapid advancements in biotechnology have posed new challenges for pharmaceutical research scientists to develop delivery systems for peptide (>3 amino acids) and protein drugs. To date pharmacologically active macromolecules have been limited to be administered orally since they have several limitations owing to poor intestinal membrane permeability, size limitation, intestinal metabolism, hepatic metabolism, and in some cases solubility limitations.[1] Since most macromolecules being proved as therapeutic agents are hydrophilic in nature, no passive diffusion via transcellular pathway can take place. Unfortunately, they are also restricted to paracellular transport because of their large size. Molecular mass of drugs will not affect the bioavailability when it ranges from 500–700 Da. Increasing however beyond this range such as protein or peptide, bioavailability would decrease abruptly. Another obstacle of oral absorption of protein or peptide drugs apart from the hepatic first pass metabolism is proteolytic degradation, which would occur not only in the intestinal lumen but also at the brush border membrane, in the mucosal cell.[2,3]

Two main categories to modify the epithelial permeability are enhancing transcellular or paracellular transports by coadministration of absorption enhancers such as cell membrane permeabilization (e.g. bile salts, surfactants) or tight junction openers (e.g. zolunar occludin toxins, sodium caprate, chitosan derivatives).[4] These approaches have some limitations since once tight junctions are opened or membranes are permeabilized, transport is enhanced not only of the drugs but also of undesirable substances. The next strategy to improve the permeability is using formulation approaches composing the particulate

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The calibration curve in logarithmic coordinate plot of lysozyme in incubation medium HBSS pH 7.4 with concentrations ranging from 1.0–30.0 μg/mL was constructed with the r^2 of 0.9993. The retention time was about 14 min and the runtime was 45 min. The accuracy of HPLC analytical method in terms of mean values of % recovery ($n = 3$) at 30.0, 10.0, 2.5, and 1.0 μg/mL were 101.10 ± 1.33, 100.69 ± 0.27, 99.50 ± 1.98, and 101.63 ± 1.19%, respectively. The recovery results exhibited good accuracy of the used analytical method as the mean value at each concentration fell within the range of 98–102%. The precision of the assay procedure expressed as % RSD's ($n = 3$) at 30.0, 10.0, 2.5, and 1.0 μg/mL were 0.65, 0.61, 0.76, and 1.76% for within-run, and 1.74, 1.29, 0.48, and 1.97% for between-run, respectively. The % RSD at each concentration over the specified range was within an acceptable criterion of 2.0% in all cases.

Results and discussion

Physicochemical characterization of phospholipid vesicle–bound lysozyme

Particle size and zeta potential

Particle sizes of phospholipid vesicle–bound lysozyme were in the range of 173–532 nm, as shown in Table 1. It was obviously shown that incorporation of more cholesterol into the formulation generally yielded larger size. Cholesterol was known to enhance the rigidity of bilayer membrane above the phase transition temperature of the constituent phospholipid, resulting in an increased elastic modulus, which inhibited curving of the bilayers.[31–33] In addition, it could be anticipated that the vesicle-bound lysozyme would impose strong influence on the net surface charge. Since lysozyme is a highly basic protein with isoelectric point (pI) = 11.16,[34] it would exhibit positive charge at pH 7.4 used and certainly exert electrostatic Coulombic interaction with the negatively charged bilayer membrane as previously suggested.[33]

Binding efficiency

Basically, the interaction of protein and lipid vesicles can be mediated by many kinds of forces such as electrostatic and/or hydrophobic forces or by covalent bond. Besides, the physicochemical properties of protein molecules themselves such as size, shape, charge, the presence of hydrophobic patches on the surface, as well as the conformation of the protein can affect the binding characteristics.[35] It can clearly be seen from Table 1 that lysozyme could readily be bound to negatively charged vesicles. Since lysozyme is a highly basic protein that exhibits positive charge at pH less than its pI as mentioned earlier, electrostatic attraction with negatively charged vesicles would eventually be the major component in this case and followed by high binding efficiency.[33,36] Cholesterol content appeared to have no effect on the binding efficiency of all phospholipid formulations studied.

Secondary structure of phospholipid vesicle–bound lysozyme

Lysozyme has been widely used as the model protein in many studies and its secondary structure has also been evaluated.[37–39] It has previously been reported that the secondary structure of lysozyme in solution neither significantly altered in various buffers with different pH's, nor in various salt concentrations.[33] The content of α-helix from circular dichroism spectra of lysozyme both at 0.05 and 0.1 mg/mL solutions were around 28–31%, 22–27%, 23–28%, and 22–27% in water, acetate, phosphate, and glycine buffers, respectively. A slight decrease, though not significant, in α-helix concurrently with an increase in β-sheet could be designated in buffer solutions compared to that in water, which became leveled off with the increasing salt concentrations. It was shown in this study that secondary structure of lysozyme bound to negatively charged phospholipid vesicles with various lipid compositions did not significantly differ from that in phosphate buffer pH 7.4 as illustrated by FTIR spectra in Figure 1. The α-helical content calculated from peak position at 1658 cm^{-1} of lysozyme in buffer solution and that bound to negatively charged vesicles were 29.2 ± 2.6 and 27.9 ± 3.6%, respectively.

Permeation across Caco-2 cell monolayers

It was evidenced that TEER values of cell monolayers before and after experiments were not significantly different, which indicated the unchanged integrity of cell monolayer. The permeation profiles across Caco-2 cell monolayers from the apical to the basolateral side after 3 h of incubation at 37°C of lysozyme solution and lysozyme bound to phospholipid vesicles were shown in Figure 2.

Table 1. Physicochemical properties and Caco-2 cells permeability of phospholipid vesicle–bound lysozyme.

	Mean ± SD*					
Formulation	Particle size (nm)	Zeta potential (mV)	% Binding efficiency	% Binding capacity	% Cumulative amount permeated after 3 h	$P_{app} \times 10^6$ (cm/sec)
Lysozyme solution	–	–	–	–	0.3 ± 0.1	0.1 ± 0.0
Bilayers-bound lysozyme**						
10:0:1	172.9 ± 1.0	–34.6 ± 1.6	35.5 ± 2.1	10.0 ± 0.6	6.0 ± 3.6	2.8 ± 3.0
8:2:1	333.4 ± 3.9	–32.9 ± 1.5	30.4 ± 0.9	9.9 ± 0.3	1.0 ± 0.2	0.3 ± 0.1
7:3:1	532.5 ± 2.5	–30.5 ± 1.3	32.2 ± 0.8	10.4 ± 0.3	1.2 ± 0.3	0.2 ± 0.1

*Average from three determinations.
**Phosphatidylcholine: cholesterol: dicetyl phosphate (molar ratio).

of the original trapped radicals. All ESR measurements were carried out using a Varian E9 ESR spectrometer and a flat cell assembly (Varian Inc., Palo Alto, CA). Reactants were mixed in a test tube in a final volume of 0.5 ml at 37°C. The reaction mixture was then transferred to a flat cell for measurement. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate and 1,1-diphenyl-2-picrylhydrazyl as reference standards. The software EPRDAP, version 2.0, was used for data acquisition and analysis.

Fluorometric analysis of superoxide and peroxide formation was performed using DHE and DCF-DA as fluorescent probes. Cells were incubated with the probes (10 μM) at 37°C for 30 min, after which they were washed, resuspended in phosphate-buffered saline, and immediately analyzed for fluorescence intensity using a multiwell plate reader (FLUOstar OPTIMA; BMG Labtech Inc.) at the excitation/emission wavelengths of 485/610 nm for DHE measurements and at 485/530 nm for DCF measurements.

Plasmids and Stable Transfection. FLIP, SOD, and glutathione peroxidase (GPx) plasmids were generously provided by Dr. Christian Stehlik (Northwestern University, Chicago, IL). Authenticity of the plasmid constructs was verified by DNA sequencing. Stable transfectants were generated by culturing H460 cells in a 6-well plate until they reached 80% confluence. One microgram of cytomegalovirus-neo vector and 15 μl of LF reagent with 2 μg of FLIP, SOD, GPx, or control pcDNA3 plasmid were used to transfect the cells in the absence of serum. Ten hours later, the medium was replaced with culture medium containing 5% fetal bovine serum, and approximately 36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin and the cell suspensions were plated onto 75-ml culture flasks. The cells were cultured for 4 weeks with G418 selection (400 μg/ml). Resistant transfectants were isolated using cloning cylinders (Bellco Glass, Vineland, NJ) and transferred for expansion and analysis by Western blotting. Stable transfectants were grown in G418-free RPMI medium for at least two passages before each experiment.

Western Blotting. Cell extracts were performed by incubating the cells in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM sodium chloride, 10% glycerol, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture for 30 min on ice. After insoluble debris was pelleted by centrifugation at 14,000g for 15 min at 4°C, the supernatants were collected and analyzed for protein content using bicinchoninic acid assay. Proteins (20 μg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and transferred onto 0.45-μm nitrocellulose membranes. The transferred membranes were blocked for 1 h in 5% nonfat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween-20) and incubated with appropriate primary antibodies at 4°C overnight. Membranes were washed three times with TBST for 10 min and incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immune complexes were detected by chemiluminescence (SuperSignal West Pico; Pierce Biotechnology, Rockford, IL) and quantified by imaging densitometry using UN-SCAN-IT automated digitizing software (Silk Scientific Inc., Orem, UT). Mean densitometry data from independent experiments were normalized to the control.

Immunoprecipitation. Cells were washed after treatments with ice-cold phosphate-buffered saline and incubated in lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 10% glycerol, 0.2% NP40, 100 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture) for 20 min at 4°C. After centrifugation at 14,000g for 15 min at 4°C, the supernatants were collected and the protein content was determined by bicinchoninic acid protein assay. Cleared lysates were normalized, and 60 μg of proteins were incubated with 8 μl of anti-Myc agarose bead (Santa Cruz Biotechnology Inc.) diluted with 12 μl of protein A-agarose for 4 h at 4°C. The immune complexes were washed three times with 500 μl of lysis buffer, resuspended in 2× Laemmli sample buffer, and boiled at 95°C for 5 min. The immune complexes were separated by 10% SDS-PAGE and analyzed by Western blotting as described above.

Statistical Analysis. Data were expressed as means ± S.D. of three or more independent experiments. Statistical analysis was performed using an unpaired two-tailed Student's *t* test at a significance level of $p < 0.05$.

Results

Lipofectamine Induces Apoptosis of Human Lung Epithelial H460 Cells. Cationic liposomes have been shown to induce apoptosis as the primary mode of cell death in various cell types (Aramaki et al., 1999, 2000). To test whether the cationic liposome LF could induce apoptosis in human lung epithelial cells and to determine the underlying mechanism, we first characterized the apoptotic response to LF treatment in H460 cells using Hoechst 33342 and gel electrophoresis DNA ladder assays. Hoechst assay shows that treatment of the cells with LF (0–50 μg/ml) caused a dose- and time-dependent increase in apoptosis over control level (Fig. 1, A and B). Higher doses of LF (>50 μg/ml) decreased apoptotic cell death possibly due to necrosis as

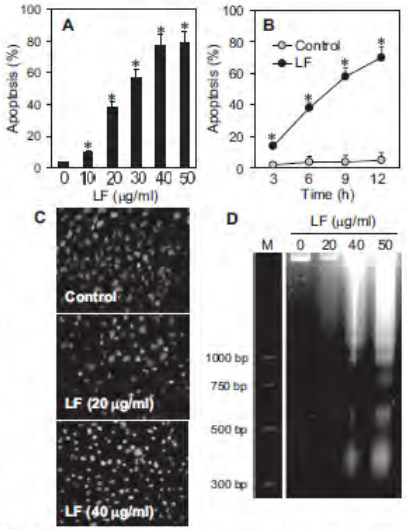

Fig. 1. Lipofectamine induces apoptosis of human lung epithelial H460 cells. A, subconfluent (80%) monolayers of H460 cells were treated with varying concentrations of LF (0–50 μg/ml), and apoptosis was determined by Hoechst 33342 assay after 6 h. B, cell monolayers were treated with LF (20 μg/ml) for various times, and apoptosis was similarly determined. C, representative fluorescence micrographs of cells treated with LF (0, 20, and 40 μg/ml) for 6 h and stained with the Hoechst dye. Apoptotic cells exhibit condensed nuclei with bright nuclear fluorescence (original magnification, 400×). D, cells were treated with varying doses of LF (0–50 μg/ml) and analyzed for DNA fragmentation by gel electrophoresis DNA ladder assay after 6 h. Data are mean ± S.D. ($n = 4$). *, $p < 0.05$ versus nontreated control.

absence of LF, no ESR signal was observed. However, in the presence of added LF, a clear signal consisting of a 1:2:2:1 quartet was detected. Based on line shape and hyperfine splitting of the spectrum, the signal was assigned to the DMPO-$OH^{\cdot}$ adduct, which is indicative of $OH^{\cdot}$ generation. The formation of DMPO-$OH^{\cdot}$ adduct was detected as early as 5 min and peaked at approximately 40 min after the treatment, where it gradually declined to the baseline level (data not shown).

Addition of the $OH^{\cdot}$ scavenger sodium formate strongly inhibited the ESR signal, indicating the specificity of $OH^{\cdot}$ detection. Addition of MnTBAP or CAT also inhibited the signal intensity, indicating that $O_2^{\cdot-}$ and H_2O_2 were generated in LF-treated cells, and that these oxidative species were precursors for $OH^{\cdot}$ generation. The formation of $O_2^{\cdot-}$ and H_2O_2 in the treated cells was confirmed by spectrofluorometry using fluorescent probes DHE and DCF-DA, respectively (Fig. 5B). To determine the cellular source of ROS generation induced by LF, cells were treated with LF in the presence or absence of DPI, a specific inhibitor of NADPH oxidase (Irani et al., 1997; Moungjaroen et al., 2006), or rotenone, a mitochondrial electron transport chain interrupter (Irani et al., 1997; Chen et al., 2003), and their effect on ROS generation was examined by DHE fluorometry. The results show that DPI strongly inhibited LF-induced DHE fluorescence, whereas rotenone showed minimal effect (Fig. 5C). These results suggest that the plasma membrane NADPH oxidase is a key source of superoxide generation induced by LF in the treated cells.

Overexpression of Antioxidant Enzymes Inhibits Lipofectamine-Induced Apoptosis and FLIP Down-Regulation. To confirm the role of ROS in FLIP down-regulation and to test the effect of ROS on LF-induced apoptosis, cells were stably transfected with the antioxidant enzyme SOD, GPx, or control plasmid, and their effects on FLIP expression, ROS generation, apoptosis, and caspase activation by LF were examined. Transfection of the cells with SOD and GPx resulted in a corresponding increase in the antioxidant enzyme expression levels compared with vector-transfected control (Fig. 6A). Overexpression of SOD potently inhibited LF-induced FLIP down-regulation (Fig. 6B) and $O_2^{\cdot-}$ generation (Fig. 6C) compared with vector-transfected control. Such overexpression also inhibited apoptosis (Fig. 7A) and caspase-8 and -9 activation (Fig. 7, B and C) induced by LF. Consistent with our earlier catalase studies, overexpression of GPx showed less inhibitory effects on LF-induced FLIP down-regulation, $O_2^{\cdot-}$ generation, apoptosis, and caspase activation. Together, these results suggest that although H_2O_2 is involved in LF-induced apoptosis, $O_2^{\cdot-}$ is the major regulator of cell death and FLIP down-regulation induced by LF.

Down-Regulation of FLIP Is Mediated through ROS-Dependent Ubiquitination. FLIP has been shown to be down-regulated by ubiquitination and proteasomal degradation in various apoptosis conditions (Kim et al., 2002; Perez and White, 2003; Chanvorachote et al., 2005). To determine whether this regulatory pathway is involved in the down-regulation of FLIP by LF and whether ROS plays a role in this process, we performed immunoprecipitation studies analyzing FLIP ubiquitination in cells treated with LF in the presence or absence of antioxidants. Cells were transiently transfected with ubiquitin and myc-tagged FLIP plasmids, and the resulting immune complexes were analyzed by SDS-PAGE immunoblotting using anti-ubiquitin antibody. Figure 8A shows that in the absence of LF, minimal ubiquitinated FLIP was produced. Upon LF treatment, the level of ubiquitinated FLIP was greatly increased. Pretreatment of the cells with MnTBAP strongly inhibited the ubiquitination of FLIP, whereas CAT was less effective. These results indicate the role of $O_2^{\cdot-}$ as the major mediator of FLIP ubiquitination, consistent with its role in LF-induced FLIP down-regulation (Fig. 4B). We also performed apoptosis studies in cells treated with LF in the presence and absence of MnTBAP and CAT (Fig. 8B). The results show that at the same treatment doses, MnTBAP strongly inhibited the apoptotic effect of LF, whereas catalase showed less inhibitory effect. These results are in good agreement with our earlier antioxidant enzyme overexpression studies and support the role of ROS, particularly $O_2^{\cdot-}$, in apoptosis and FLIP down-regulation by LF.

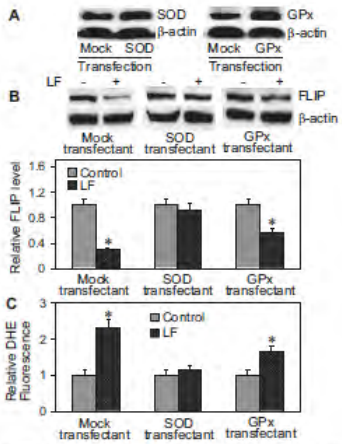

Fig. 6. Effects of antioxidant enzyme overexpression on Lipofectamine-induced FLIP down-regulation and ROS generation. A, H460 cells were stably transfected with SOD, GPx, or control plasmid as described under *Materials and Methods*. Cell extracts were prepared and analyzed for antioxidant enzyme expression by Western blot using antibodies specific for SOD and GPx. β-Actin was used as a loading control. B, transfected cells were treated with LF (20 μg/ml) and analyzed for FLIP expression after 6 h by Western blotting. C, transfected cells were similarly treated with LF and analyzed for ROS generation after 0.5 h. Plots are mean ± S.D. ($n = 4$). *, $p < 0.05$ versus nontreated control.

Discussion

Cationic liposomes have been successfully used in gene transfection and are considered as promising tools for gene delivery. However, limited understanding of their cytotoxicity and the underlying mechanism is the major drawback in the successful application of these agents. LF is one of the most widely used cationic liposomal agents due to its high transfection efficiency compared with several other transfecting agents such as Lipofectin, DEAE-dextran, and DOTAP

8. CONCLUSIONS

- Summarize only the crucial results and their interpretation which appear to be the novel findings.
- It is not the same as abstract.

Conclusion

Proteins as macromolecules could exhibit themselves in many levels of structure which could be influenced by micro-environmental factors such as ionic strength and pH. It was demonstrated in this study that lysozyme conformation still remained intact after being bound to phospholipid vesicles. The permeation across Caco-2 cell monolayers of phospholipid vesicle–bound lysozyme was demonstrated to be significantly enhanced with a size-dependent manner.

Conclusions

This novel, one-step method for the preparation of liposomes by freeze-drying at room temperature involved utilizing CBN as a volatile, solid, inert carrier, which was eventually eliminated from the mixture of phospholipid microparticles by sublimation. The optimal processing conditions used in the sublimation process of CBN were at a temperature of 25–30°C and a pressure of 1.5–2.0 mBar for 8 hours. Numerous formulation parameters influencing the properties of the reconstituted liposomes, such as reconstitution time, particle size, zeta potential, and vesicle type, as well as lamella structure, were studied and evaluated. This proposed method could simply be applied and could be promising in liposome preparations.

9. REFERENCES

Two different styles are widely used:

- **Numbering in one consecutive series by order of mention in the text.**
- **Mentioning in the text by chronological order of the year published.**

Most internationally used citation style:

- **Sarisuta N, Kumpugdee M, Muller BW, Puttipipatkhachorn S. 1999. Physico-chemical characterization of interactions between erythromycin and various film polymers. Int J Pharm 186: 109-118.**

Introduction

Rapid advancements in biotechnology have posed new challenges for pharmaceutical research scientists to develop delivery systems for peptide (>3 amino acids) and protein drugs. To date pharmacologically active macromolecules have been limited to be administered orally since they have several limitations owing to poor intestinal membrane permeability, size limitation, intestinal metabolism, hepatic metabolism, and in some cases solubility limitations.[1] Since most macromolecules being proved as therapeutic agents are hydrophilic in nature, no passive diffusion via transcellular pathway can take place. Unfortunately, they are also restricted to paracellular transport because of their large size. Molecular mass of drugs will not affect the bioavailability when it ranges from 500–700 Da. Increasing however beyond this range such as protein or peptide, bioavailability would decrease abruptly. Another obstacle of oral absorption of protein or peptide drugs apart from the hepatic first pass metabolism is proteolytic degradation, which would occur not only in the intestinal lumen but also at the brush border membrane, in the mucosal cell.[2,3]

Two main categories to modify the epithelial permeability are enhancing transcellular or paracellular transports by coadministration of absorption enhancers such as cell membrane permeabilization (e.g. bile salts, surfactants) or tight junction openers (e.g. zolunar occludin toxins, sodium caprate, chitosan derivatives).[4] These approaches have some limitations since once tight junctions are opened or membranes are permeabilized, transport is enhanced not only of the drugs but also of undesirable substances. The next strategy to improve the permeability is using formulation approaches composing the particulate

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Introduction

Liposomes, which are microscopic phospholipid vesicles with a bilayered membrane structure, have received a lot of attention during the past 30 years as pharmaceutical carriers of great potential. To date, many new developments in the area of liposomal drugs, from clinically approved products to newly experimental applications, with gene delivery and cancer therapy are still the principal areas of interest (Torchilin, 2005). Some liposomal products have been approved for clinical use in several countries, such as doxorubicin (Doxil®, Sequus Pharmaceutical Inc., USA), daunorubicin (Daunoxome®, Nexstar Pharmaceutical Inc., USA), and amphotericin B (Ambisome®, Nexstar Pharmaceutical Inc., USA) (Torchilin, 2005; Sharma and Sharma, 1997). Doxil, a long-circulating liposomal formulation, was the first liposomal product approved for clinical use in the United States. Anthracycline glycosides, both doxorubicin and daunorubicin, are highly effective antineoplastic drugs, but they can cause severe cardiac toxicity in humans. Long-circulating liposomal formulations of anthracyclines have been shown to improve the therapeutic index of the drugs against a variety of solid tumors by not only reducing cardiac toxicity, but also increasing drug accumulation in tumors (Sharma and Sharma, 1997). Amphotericin B, a drug of choice for the treatment of seriously systemic fungal infections, is, however, associated with high rates of serious side effects, including nephrotoxicity, thrombophlebitis, hypokalemia, and anemia. These side effects limit the dose levels to be achieved and are the major reasons for failure or discontinuation of therapy. Liposomes of amphotericin B (Ambisome), available as lyophilized reconstitute powder for injection, have been shown to possess a superior therapeutic index over the deoxycholate-based formulation (Fungizone®, Bristol-Myers Squibb, USA), mainly due to a decrease in

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10. APPENDIX

- Location for numerous tables and illustrations, computer data, questionaire results, complicated statistics, mathematical proofs, or detailed descriptions of special equipment.

Manuscript Writing

- Research must be composed of body, voice, and mind.
- According to our current graduate programs, it is imperative for all students to have their research articles published in journals (national for Master and international for Doctoral) in order to fulfill the program requirement.

Manuscript Writing

- **Twelve Steps** to developing an effective first draft of your manuscript.
- You should now have detailed notes you can use to write your draft paper.
- If you don’t have one already, it may help to prepare an outline for each section which includes a number of major headings, sub-headings and paragraphs covering different points.

Manuscript Writing

- Some people recommend that you begin with the **Introduction** and continue in order through each section of the paper to help ensure flow.
- Others suggest that you begin with the easiest sections, which are usually the **Methods** and **Results**, followed by the **Discussion**, **Conclusion**, **Introduction**, **References** and **Title**, leaving the **Abstract** until the end.
- The main thing is to begin writing and begin filling up the blank screen or piece of paper.

Twelve Steps to Developing an Effective First Draft of your Manuscript

1. Consolidate all the information
2. Target a journal
3. Start writing
4. Write quickly
5. Write in your own voice
6. Write without editing
7. Keep to the plan of your outline

Twelve Steps to Developing an Effective First Draft of your Manuscript

8. Write the paper in parts
9. Put the first draft aside
10. Revise it
11. Revise for clarity and brevity
12. Be consistent

Twelve Steps to Developing an Effective First Draft of your Manuscript

1. **Consolidate all the information.**

   Ensure you have everything you need to write efficiently, i.e., all data, references, drafts of tables and figures, etc.

Twelve Steps to Developing an Effective First Draft of your Manuscript

2. Target a journal.

Determine the journal to which you plan to submit your manuscript and write your manuscript according to the focus of the targeted journal.

The focus may be clearly stated within the journal or may be determined by examining several recent issues of the targeted journal.

Twelve Steps to Developing an Effective First Draft of your Manuscript

3. Start writing.

When writing the first draft, the goal is to put something down on paper, so it does not matter if sentences are incomplete and the grammar incorrect, provided that the main points and ideas have been captured.

Write when your energy is high, not when you are tired.

Try to find a time and place where you can think and write without distractions.

Twelve Steps to Developing an Effective First Draft of your Manuscript

4. Write quickly.

Don’t worry about words, spelling or punctuation at all at this stage, just ideas.

Keep going.

Leave gaps if necessary.

Try to write quickly, to keep the flow going.

Use abbreviations and leave space for words that do not come to mind immediately.

Twelve Steps to Developing an Effective First Draft of your Manuscript

5. Write in your own voice.

Expressing yourself in your own way will help you to say what you mean more precisely.

It will be easier for your reader if they can "hear" your voice.

Twelve Steps to Developing an Effective First Draft of your Manuscript

6. **Write without editing.**

Don't try to get it right the first time.

Resist the temptation to edit as you go.

Otherwise, you will tend to get stuck and waste time.

If you try to write and edit at the same time, you will do neither well.

Twelve Steps to Developing an Effective First Draft of your Manuscript

7. Keep to the plan of your outline.

Use the headings from your outline to focus what you want to say.

If you find yourself wandering from the point, stop and move on to the next topic in the outline.

Twelve Steps to Developing an Effective First Draft of your Manuscript

8. Write the paper in parts.

Don’t attempt to write the whole manuscript at once, instead, treat each section as a mini essay.

Look at your notes, think about the goal of that particular section and what you want to accomplish and say.

Twelve Steps to Developing an Effective First Draft of your Manuscript

9. Put the first draft aside.

Put aside your first draft for at least one day.

The idea of waiting a day or more is to allow you to “be” another person.

It is difficult to proofread and edit your own work; a day or more between creation and critique helps.

Twelve Steps to Developing an Effective First Draft of your Manuscript

10. Revise it.

Revise it and be prepared to do this several times until you feel it is not possible to improve it further.

The objective is to look at your work not as its author, but as a respectful but stem critic.

Does each sentence make sense?

In your longer sentences can you keep track of the subject at hand?

Do your longer paragraphs follow a single idea, or can they be broken into smaller paragraphs?

These are some of the questions you should ask yourself.

Twelve Steps to Developing an Effective First Draft of your Manuscript

11. Revise for clarity and brevity

Revise sentences and paragraphs with special attention to clearness.

For maximum readability, most sentences should be about 15-20 words.

For a scientific article, paragraphs of about 150 words in length are considered optimal.

Avoid using unnecessary words.

Twelve Steps to Developing an Effective First Draft of your Manuscript

12. Be consistent.

Often a manuscript has more than one author and therefore the writing may be shared.

However, the style needs to be consistent throughout.

The first author must go through the entire manuscript and make any necessary editorial changes before submitting the manuscript to the journal.

Fourteen Steps to Writing an Effective Discussion Section

The purpose of the Discussion is to

- state your interpretations and opinions,
- explain the implications of your findings, and
- make suggestions for future research.

Its main function is to

- answer the questions posed in the Introduction,
- explain how the results support the answers and,
- how the answers fit in with existing knowledge on the topic.

Fourteen Steps to Writing an Effective Discussion Section

Discussion is considered the heart of the paper and usually requires several writing attempt.

The organization of the Discussion is important.

Before beginning you should try to develop an outline to organize your thoughts in a logical form.

You can use a cluster map, an issue tree, numbering, or some other organizational structure.

The steps listed below are intended to help you organize your thoughts.

Fourteen Steps to Writing an Effective Discussion Section

To make your message clear, the discussion should be kept as short as possible while clearly and fully stating, supporting, explaining, and defending your answers and discussing other important and directly relevant issues.

Care must be taken to provide a commentary and not a reiteration of the results.

Side issues should not be included, as these tend to obscure the message.

No paper is perfect; the key is to help the reader determine what can be positively learned and what is more speculative.

Fourteen Steps to Writing an Effective Discussion Section

1. Organize the Discussion from the specific to the general: your findings to the literature, to theory, to practice.
2. Use the same key terms, the same verb tense (present tense), and the same point of view that you used when posing the questions in the Introduction.
3. Begin by restating the hypothesis you were testing and answering the questions posed in the Introduction.

Fourteen Steps to Writing an Effective Discussion Section

4. Support the answers with the results.

   Address all the results relating to the questions, regardless of whether or not the findings were statistically significant.

5. Describe the patterns, principles, and relationships shown by each major finding/result and put them in perspective.

   The sequencing of providing this information is important; first state the answer, then the relevant results before citing the work of others.

   If necessary, point the reader to a figure or table to enhance the “story”.

Fourteen Steps to Writing an Effective Discussion Section

6. Clearly stating why they are acceptable and how they are consistent or fit in with previously published knowledge on the topic.
7. Defend your answers, if necessary, by explaining both why your answer is satisfactory and why others are not.

   Only by giving both sides to the argument can you make your explanation convincing.
8. Discuss and evaluate conflicting explanations of the results.

   This is the sign of a good discussion.

Fourteen Steps to Writing an Effective Discussion Section

9. Discuss any unexpected findings.

   When discussing an unexpected finding, begin the paragraph with the finding and then describe it.

10. Identify potential limitations and weaknesses and comment on the relative importance of these to your interpretation of the results and how they may affect the validity of the findings.

    When identifying limitations and weaknesses, avoid using a apologetic tone.

Fourteen Steps to Writing an Effective Discussion Section

11. Summarize concisely the principal implications of the findings regardless of statistical significance.
12. Provide recommendations (no more than two) for further research.

    Do not offer suggestions, which could have been easily addressed within the study, as this shows there has been inadequate examination or interpretation of the data.

Fourteen Steps to Writing an Effective Discussion Section

13. Explain how the results and conclusions of this study are important and how they influence our knowledge or understanding of the problem being examined.
14. Discuss everything, but be concise, brief, and specific in your writing of the Discussion.

Journal Impact Factors

- Journal Impact Factor is from Journal Citation Report (JCR), a product of Thomson ISI (Institute for Scientific Information).
- JCR provides quantitative tools for ranking, evaluating, categorizing, and comparing journals.
- The impact factor is one of these; it is a measure of the frequency with which the "average article" in a journal has been cited in a particular year or period.

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10. หลักเกณฑ์การประเมินผลข้อเสนอการวิจัย ของส่วนราชการและรัฐวิสาหกิจที่เสนอของบประมาณ ประจำปี 2547 ตามมติคณะรัฐมนตรี สำหรับผู้ทรงคุณวุฒิ กองวิเคราะห์โครงการและประเมินผล สำนักงานคณะกรรมการวิจัยแห่งชาติ ตุลาคม 2545

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11. ทิศทางการวิจัยและแผนวิจัย ปีงบประมาณ 2544-2547 กับนโยบายและแนวทางการวิจัยของชาติ ฉบับที่ 6 (พ.ศ. 2545-2549) เพื่อใช้ในการประเมินผลข้อเสนอการวิจัยของส่วนราชการและรัฐวิสาหกิจที่เสนอของบประมาณ ประจำปี 2547 ตามมติคณะรัฐมนตรี กองวิเคราะห์โครงการและประเมินผล สำนักงานคณะกรรมการวิจัยแห่งชาติ กรกฎาคม 2545