Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Polymers 71 (2008) 324–329
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Short communication

# Atomic force microscopy imaging of novel self-assembling pectin–liposome nanocomplexes

Pornsak Sriamornsak [a,b,*], Nartaya Thirawong [a,b], Jurairat Nunthanid [a,b], Satit Puttipipatkhachorn [c], Jringjai Thongborisute [d], Hirofumi Takeuchi [d]

[a] Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand
[b] Pharmaceutical Biopolymer Group (PBiG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand
[c] Department of Manufacturing Pharmacy, Faculty of Pharmacy, Mahidol University, Bangkok 10400, Thailand
[d] Laboratory of Pharmaceutical Engineering, Gifu Pharmaceutical University, 5-6-1 Mitahora-Higashi, Gifu 502 8585, Japan

Received 30 March 2007; received in revised form 17 May 2007; accepted 17 May 2007
Available online 24 May 2007

**Abstract**

Self-assembling pectin–liposome nanocomplexes (PLNs) were prepared by a simple mixing of cationic liposomes with pectin solution. Nanostructures of liposomes, pectin, and PLNs were observed by atomic force microscopy (AFM). The AFM images of pectin show a chain-like structure with a small number of branches while those of liposomes show a spherical form. The AFM images also provided a direct evidence for association of cationic liposomes on the pectin chain. This was confirmed by the FTIR analysis.


*Keywords:* Atomic force microscopy (AFM); Liposomes; Nanocomplex; Pectin; Self-assembly

## 1. Introduction

Polyelectrolyte complexes (PECs) are formed by the electrostatic interaction between a polyelectrolyte and oppositely charged polyelectrolyte in aqueous solution. PECs have been long investigated from the standpoint of the polyacid–polybase interaction, stoichiometry, self-assembly (Ohkawa, Takahashi, & Yamamoto, 2000), and have numerous applications such as membranes, coatings, surfactants, and microcapsules (Hales & Pochan, 2006). In addition, the formation of PECs to the formation of building blocks is expected to find important applications in nanoscience. Owing to the practical and economical aspects for the rational use of PECs, as well as the theoretical interest of such systems in soft condensed matter science, nanochemistry, biology, and medicine, a better understanding of the key factors controlling the complexation between oppositely charged macromolecules is therefore important.

Recently, the interactions between charged lipids and oppositely charged biopolymers have been studied (Antonietti & Wenzel, 1998; Raviv et al., 2005). The highly ordered structures formed by polyelectrolyte–lipid complexes are of great interest in material sciences as templates and building blocks for hierarchical supramolecular assembly (Antonietti & Wenzel, 1998). Takeuchi, Yamamoto, Niwa, Hino, and Kawashima (1994) reported the preparation of chitosan-coated liposomes, in which the chitosan (i.e. cationic polysaccharide) is believed to cover the surface of the liposomes (i.e. vesicles composed of one or more phospholipid bilayers) by forming the ion-complex with dicetylphosphate in the liposomal formulation. Diebold et al. (2007) described the complexes between liposomes and chitosan nanoparticles which formed by combining those components. However, the structures formed when

* Corresponding author. Address: Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand. Tel.: +66 34 253912x2317; fax: +66 34 255801.
*E-mail address:* pornsak@email.pharm.su.ac.th (P. Sriamornsak).

0144-8617/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2007.05.027

charged liposomes are complexed with oppositely charged electrolytes have not been morphologically examined and yet to be understood.

Microscopic analysis at the single molecule level can be achieved by scanning probe methods such as atomic force microscopy (AFM). It has evident advantages for the detection and characterization of heterogeneous populations with regards to the amount of material required for a study as well as to the level of information that can be acquired. AFM is capable of resolving individual molecules so that differences in size (length, diameter, etc.) and conformation (stiffness, aggregation, and association, mode of adsorption to a substrate, etc.) between neighboring polymers can be visualized directly, thus making possible the characterization of the heterogeneity of a molecular population at the level of single polymers. AFM has become a standard tool for the characterization of heterogeneity at the nanoscale, finding applications in every aspect of surface and interface science (Hansma et al., 1997). In the field of single biomolecular characterization, AFM imaging has been used to identify the existence of extended branches in pectin (Morris et al., 2001; Round, Rigby, MacDougall, Ring, & Morris, 2001).

Pectin is a naturally occurring water-soluble polysaccharide which is found in the cell wall of most plants. Though it is a heterogeneous polysaccharide, pectin contains linear chains of (1–4)-linked α-D-galacturonic acid residues (Rolin, 1993). The galacturonic acids have carboxyl groups, some of which are naturally presented as methyl esters and others which are reacted with ammonia to produce carboxamide groups. The degree of esterification (DE) and degree of amidation (DA), which are both expressed as a percentage of carboxyl groups (esterified or amidated), are an important means to classify pectin. Due to its biocompatibility, biodegradability, and non toxicity, pectin represents an attractive biopolymer for a variety of pharmaceutical and biomedical applications. Pectin has shown promise in engineering drug carriers for oral drug delivery (Sriamornsak, 1998; Sriamornsak, Sungthongjeen, & Puttipipatkhachorn, 2007; Sriamornsak, Thirawong, & Puttipipatkhachorn, 2005). Chemically, the structure of pectin is full of carboxyl groups. This may allow the interaction between pectin and the oppositely charged membranes or liposomes. The objective of this study was then to examine the morphological arrangement of pectin–liposome nanocomplexes (PLNs) using different types of pectin by AFM images.

## 2. Materials and methods

### 2.1. Materials

Different types of pectin were donated by Herbstreith & Fox KG (Germany). High methoxy pectin, type CU201 (referred as CU201) contains 70% DE and MW of 200 kDa. Low methoxy pectin, type CU701 (referred as CU701) has 38% DE and MW of 80 kDa while low methoxy amidated pectin, type CU020 (referred as CU020) has 29% DE, 20% DA and MW of 150 kDa. Distearoylphosphatidylcholine (DSPC) and stearylamine (SA) were purchased from Oil & Fats (Japan) and Tokyo Kasei (Japan), respectively. Cholesterol (Chol) was supplied from Sigma Chemical Co. (USA). All other chemicals were of analytical grade and were used as received without further purification.

### 2.2. Preparation of cationic liposomes and self-assembling pectin–liposomes nanocomplexes (PLNs)

Cationic multilamellar liposomes were prepared by the hydration method of lipid film as described previously by Takeuchi, Matsui, Yamamoto, and Kawashima (2003) with some modifications. In brief, the mixture of DSPC, SA, and Chol in molar ratio of 8:0.2:1 was dissolved in a small amount of chloroform. The solution was placed in a rotary evaporator at 40 °C until a thin film was obtained then allowed to stand over night in vacuum chamber to ensure completely solvent removal. The thin film was hydrated with phosphate buffer (pH 6.8, 66.7 mM) by vortexing to obtain multilamellar liposomes. In order to reduce particle size, the multilamellar liposomes were then passed through an extruder (LipoFast-Pneumatic, Avestin, Canada) using 0.2 μm polycarbonate membrane filters (Nucleopore®, Whatman, USA) to generate submicron-sized liposomes. In order to prepare PLNs, an equal volume (i.e. 2 mL) of the liposomal suspension and pectin solution (at the concentration of 1.0% w/v in 66.7 mM phosphate buffer solution, pH 6.8) was mixed by vortexing to obtain 0.5% w/v of pectin as a final concentration.

### 2.3. Atomic force microscopy (AFM)

For imaging, samples were diluted with filtered deionized water to 2–4 μg/mL. An aliquot (2 μL) of the diluted sample solutions was immediately spread on freshly cleaved mica surfaces. The sample was then allowed to dry at ambient temperature (25 °C) for 20 min before imaging at a scan speed of 2 Hz. Tapping mode was carried out using a multimode NanoScope IIIa AFM (Digital Instruments, USA) equipped with Phosphorus (n) doped Si (Veeco, model RTESP) cantilever with a quoted spring constant of 20–80 N/m. Several images of different zones were examined since AFM images are generally limited to small scanned areas. Height mode was used for image analysis. The correction of the images by commercial image processing software (Adobe Photoshop, Version 6.0.1, Adobe Systems Inc., USA) with glowing edges or finding edges mode enabled a reduction of the noise, as shown on the right-hand side of the original AFM images.

### 2.4. FTIR spectroscopic analysis

The FTIR spectra of the liposomes, SA and their physical mixture as well as co-precipitated mixture were

recorded (Nicolet 4700, USA) to determine the interaction between component of liposomes (e.g. SA) and pectin by either KBr disc method or casting on ZnSe crystal. Physical mixture (1:1 in weight ratio between component of liposomes and pectin) was prepared by mixing with vortex mixer for 2 min. In the preparation of the complex between pectin and materials used for liposomes, the co-precipitation method was performed. Briefly, the pectin aqueous solution (0.1% w/v) was dropped into ethanolic solution of DSPC or SA or Chol (0.1% w/v). The wet precipitated particles were then placed on ZnSe crystal and kept at room temperature until dry before determination at wave number of 4000–400 $cm^{-1}$.

## 3. Results and discussion

In this work, a novel self-assembling nanocomplexes between pectin and cationic liposomes, i.e. PLNs, has been prepared using a similar manner with the ion-complex formation of carbopol with positively charged ions on the surface of the liposomes (Takeuchi et al., 2003). Cationic liposomes containing SA were prepared since the SA led to the complexation between liposome particles and pectin molecules when mixing the liposome samples with the pectin dispersion.

The high resolution of the AFM has potential for characterizing the heterogeneous structures of pectin, including linear, branching, blocks or polymers, as well as the structures of its association. Due to the soft nature of all samples, AFM with a tapping mode was used to characterize pectin, liposomes, and PLNs morphology and structure. In this mode, an oscillating probe tip taps, at high frequency (ca. 305 kHz), the samples while scanning it. Advantages of this mode are that low forces and minimal damage enable it to image soft samples in air. Moreover, lateral forces are practically eliminated so that the deformation of the sample is minimal and lateral resolution is on the order of 1–5 nm (Fishman, Cooke, & Coffin, 2004). The image processing software was used to enhance the quality of the images using glowing edges or finding edges in grayscale mode. Fig. 1 shows AFM images of different types of pectin, i.e. CU201, CU701, and CU020. Chain-like structures with a small number of branches were imaged using AFM. Branched structures were distinguished from over-lapping molecules by measuring the heights of the chains. In general, the heights of the chain rose by twice when two chains crossed over one another. At genuine branch points the height remained unchanged (Yang, Feng, An, & Li, 2006).

When the cationic liposomes were examined by AFM, spherical structures of the small unilamellar vesicles were observed (Fig. 2). The size of cationic liposomes was measured from the AFM images. The geometric mean diameter and height were $68 \pm 12$ nm and $37 \pm 9$ nm, respectively. It should be borne in mind that even though no rigorous fixation is required for the preparation of AFM samples, the results may be influenced by lipid spreading or flattening of liposomes, which are soft and deformable, to some extent.

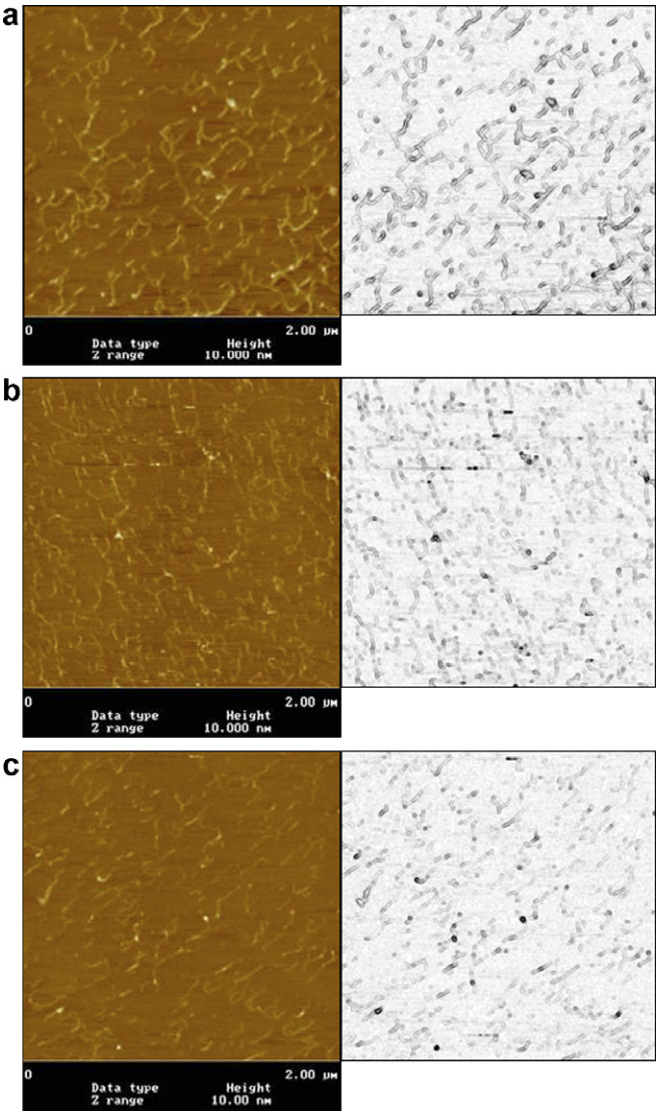


Fig. 1. Topographical (left) and equivalent processed (right) images from atomic force microscopy (AFM) of pectin dispersion: (a) CU201; (b) CU701; and (c) CU020.

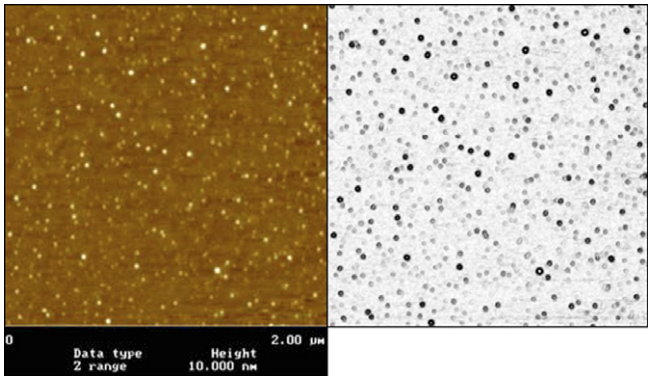


Fig. 2. Topographical (left) and equivalent processed (right) images from atomic force microscopy (AFM) of cationic liposomes.

Hence, the height of liposomes measured from AFM images was less than their diameter due to the flattening

of liposomes while spreading onto the mica surface. In fact, their structure was spherical, as reported when determined by other methods. The dehydration as a result of drying process during sample preparation may also affect the decrease in size. Wangerek et al. (2001) reported the smaller size of lipoplexes, cationic lipids forming liposomes when mixed with DNA form a self-assembling complex, when determined from AFM images, compared to those determined by dynamic light scattering.

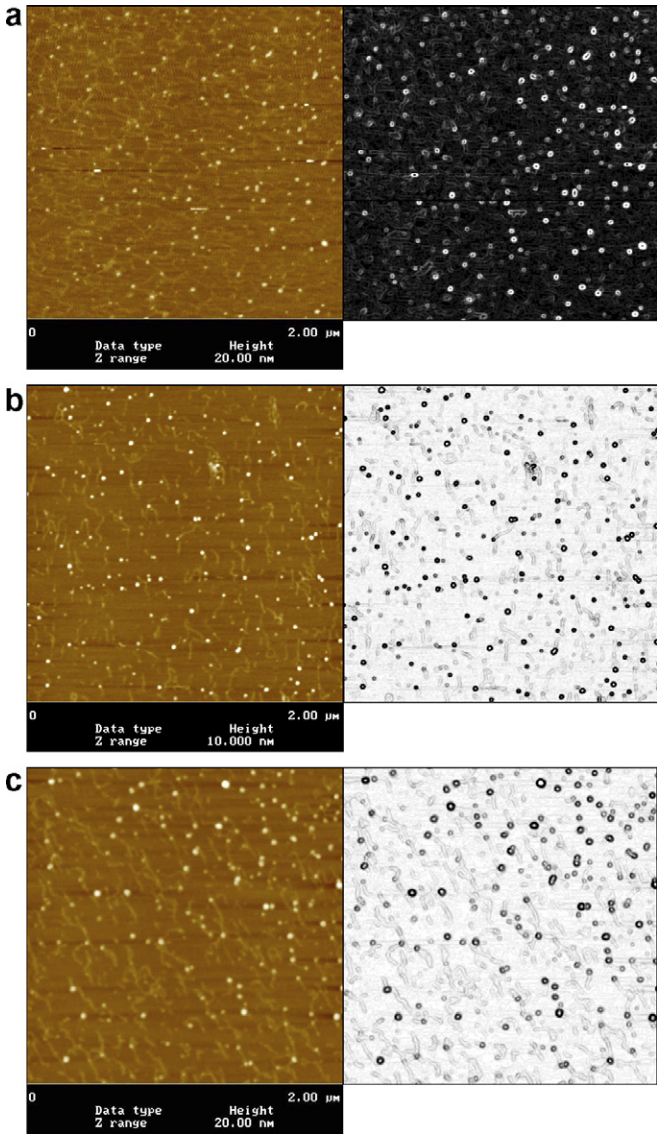


Fig. 3. Topographical (left) and equivalent processed (right) images from atomic force microscopy (AFM) of pectin–liposome nanocomplexes (PLNs) using different pectins: (a) CU201; (b) CU701; and (c) CU020.

Due to the complexity of self-assembly, it is reasonable to expect a hierarchical self-assembled variety of structures, including clusters of aggregated particles, polymer-coated particles, tubular and lamellar, or multilamellar structures. The attachment or association of cationic liposomes on the pectin chain forming self-assembling supramolecular complexes (i.e. PLN) was visualized (Fig. 3). These AFM images provided a direct morphological evidence of PLNs. When PLN structures formed between different pectins and liposomes were compared, similarities could be observed. Fig. 4 demonstrates the proposed model of the interaction between pectin and cationic liposomes to form PLNs, derived from the AFM imaging results. This is different from those of lipoplexes which the AFM images exhibited a large variety of different structures such as loops of DNA extending from a central core of liposomes, globular structures, and so on (Wangerek et al., 2001).

As a result of the carboxylic groups of pectin interacted with the amine groups of SA, the complexes between pectin and cationic liposomes occurred which was confirmed by the FTIR analysis (Fig. 5). In Fig. 5, the FTIR spectra of pectin and SA alone and in physical mixture as well as in coprecipitates at the same weight ratio were detected. In the FTIR spectrum of pure pectin (i.e. CU701), the characteristic absorption bands were observed at 1748 $cm^{-1}$ assigned to C=O stretching of COOR group and 1615 $cm^{-1}$ which assigned to C=O stretching of COOH group (Séné, McCann, Wilson, & Grinter, 1994). In the FTIR spectrum of SA, the characteristic absorption bands at 3332 $cm^{-1}$ assigned to $NH_2$ stretching, 2918 $cm^{-1}$, and 2850 $cm^{-1}$ assigned to CH stretching and 1647 $cm^{-1}$ assigned to $NH_2$ bending were detected (Giovagnoli et al., 2003). The combination of absorption bands between pectin and SA were observed in the physical mixture. Some significant differences could be seen in the FTIR spectrum of the coprecipitates. The broader peak at 3368 $cm^{-1}$ assigned to $NH_3^+$ stretching of primary amine was observed and it was also overlapped with OH stretching of pectin (Silverstein & Webster, 1998). The $NH_2$ bending of SA structure at 1647 $cm^{-1}$ could not be detected in the coprecipitates spectrum. Additionally, the absorption band

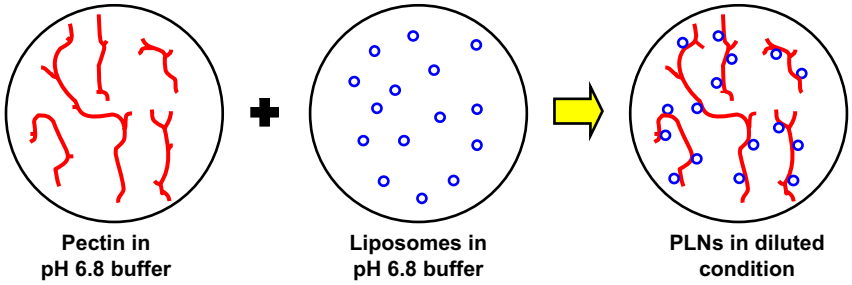


Fig. 4. Schematic representation of the formation of pectin–liposome nanocomplexes (PLNs).

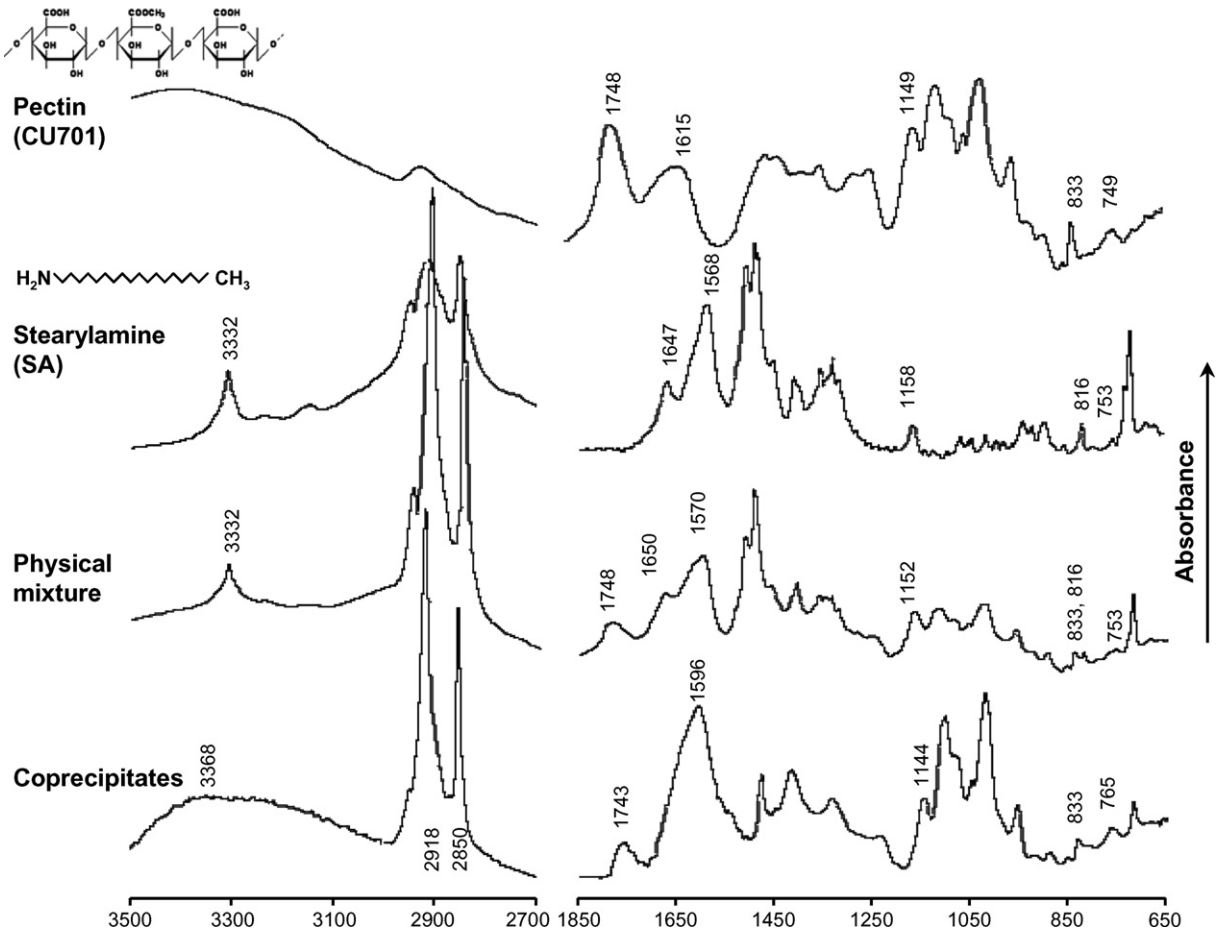


Fig. 5. FTIR spectra of pectin, stearylamine, their physical mixture and coprecipitates.

at 1596 $cm^{-1}$ corresponded to $COO^-$ was observed. These results indicate that COOH groups of pectin might be ionized and dissociated to $COO^-$ groups, which could form complexes with the protonated amine groups ($NH_3^+$) through electrostatic interaction. The other components of liposomes (i.e. DSPC and Chol) did not show any change in FTIR spectra of their coprecipitates, compared to physical mixture spectra (data not shown). The interaction of pectin and the whole particles of cationic liposomes could not be distinguished from the FTIR spectra as the peaks of pectin, Chol, and DSPC were overlapped (data not shown). The IR spectra of coprecipitates of pectin and SA, however, provided an evidence of the electrostatic interactions between the positively charged liposomes containing SA and the negatively charged pectin molecules, which strongly supported the formation of PLNs.

## 4. Conclusion

PLNs could be prepared by a simple mixing of cationic liposomes with pectin solution. Nanostructures of liposomes, pectin, and PLNs could be visualized by AFM. The AFM images provided a direct evidence for association of cationic liposomes on the pectin chain. FTIR results also confirmed the electrostatic interactions between the positively charged SA in liposomes and the negatively charged pectin chain. We are continuing our experiments with these systems, in particular to investigate their mucoadhesive properties to the gastrointestinal tract and the pharmacological effect of drug-loaded PLNs.

## Acknowledgments

The authors thank the Thailand Research Fund and Commission of Higher Education, Thailand for the research funding (Grant No. RMU4880042). Financial support from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0102/2548) to NT and PS is gratefully acknowledged. We also thank Herbstreith & Fox KG (Germany) who kindly donated the pectin samples.

## References

Antonietti, M., & Wenzel, A. (1998). Structure control of polyelectrolyte–lipid complexes by variation of charge density and addition of cholesterol. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 135*, 141–147.

Diebold, Y., Jarr, M., Saez, V., Carvalho, E. L. S., Orea, M., Calonge, M., et al. (2007). Ocular drug delivery by liposome–chitosan nanoparticle complexes (LCS–NP). *Biomaterials, 28*, 1553–1564.

Fishman, M. L., Cooke, P. H., & Coffin, D. R. (2004). Nanostructure of native pectin sugar acid gels visualized by atomic force microscopy. *Biomacromolecules, 5*, 334–341.

Giovagnoli, S., Blasi, P., Vescovi, C., Fardella, G., Chiappini, I., Perioli, L., et al. (2003). Unilamellar vesicles as potential capreomycin sulfate carriers: Preparation and physicochemical characterization. *AAPS Pharm Sci Tech., 4*, article 69. Available from: http://www.aapspharmscitech.org.

Hales, K., & Pochan, D. J. (2006). The many facets of polyelectrolytes and oppositely charged macroions complex formation. *Current Opinion in Colloid & Interface Science, 11*, 268–272.

Hansma, H. G., Kim, K. J., Laney, D. E., Garcia, R. A., Argaman, M., Allen, M. J., et al. (1997). Properties of biomolecules measured from atomic force microscopy images: A review. *Journal of Structural Biology, 119*, 99–108.

Morris, V. J., Mackie, A. R., Wilde, P. J., Kirby, A. R., Mills, E. C. N., & Gunning, A. P. (2001). Atomic force microscopy as a tool for interpreting the rheology of food biopolymers at the molecular level. *Food Science and Technology, 34*, 3–10.

Ohkawa, K., Takahashi, Y., & Yamamoto, H. (2000). Self-assembling capsule and fiber formations of polyion complexes of chitosan and poly(α,L-glutamic acid). *Macromolecular Rapid Communications, 21*, 223–225.

Raviv, U., Needleman, D. J., Li, Y., Miller, H. P., Wilson, L., & Safinya, C. R. (2005). Cationic liposome–microtubule complexes: Pathways to the formation of two-state lipid–protein nanotubes with open or closed ends. *Proceedings of the National Academy of Sciences, 102*, 11167–11172.

Rolin, C. (1993). Pectin. In R. L. Whistler & J. N. Bemiller (Eds.), *Industrial gums: Polysaccharides and their derivatives* (pp. 257–293). New York: Academic Press.

Round, A. N., Rigby, N. M., MacDougall, A. J., Ring, S. G., & Morris, V. J. (2001). Investigating the nature of branching in pectin by atomic force microscopy and carbohydrate analysis. *Carbohydrate Research, 331*, 337–342.

Séné, C. F. B., McCann, M. C., Wilson, R. H., & Grinter, R. (1994). Fourier-transform Raman and Fourier-transform infrared spectroscopy: An investigation of five higher plant cell walls and their components. *Plant Physiology, 106*, 1623–1631.

Silverstein, R. M., & Webster, F. X. (1998). *Spectroscopic identification of organic compounds*. New York: John Wiley & Sons, Inc., p. 102.

Sriamornsak, P. (1998). Investigation of pectin as a carrier for oral delivery of proteins using calcium pectinate gel beads. *International Journal of Pharmaceutics, 169*, 213–220.

Sriamornsak, P., Sungthongjeen, S., & Puttipipatkhachorn, S. (2007). Use of pectin as a carrier for intragastric floating drug delivery: Carbonate salt contained beads. *Carbohydrate Polymers, 67*, 436–445.

Sriamornsak, P., Thirawong, N., & Puttipipatkhachorn, S. (2005). Emulsion gel beads of calcium pectinate capable of floating on the gastric fluid: Effect of some additives, hardening agent or coating on release behavior of metronidazole. *European Journal of Pharmaceutical Sciences, 24*, 363–373.

Takeuchi, H., Matsui, Y., Yamamoto, H., & Kawashima, Y. (2003). Mucoadhesive properties of carbopol or chitosan-coated liposomes and their effectiveness in the oral administration of calcitonin to rats. *Journal of Controlled Release, 86*, 235–242.

Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., & Kawashima, Y. (1994). Mucoadhesion of polymer-coated liposomes to rat intestine in vitro. *Chemical & Pharmaceutical Bulletin, 42*, 1954–1956.

Wangerek, L. A., Dahl, H. H. M., Senden, T. J., Carlin, J. B., Jans, D. A., Dunstan, D. E., et al. (2001). Atomic force microscopy imaging of DNA–cationic liposome complexes optimized for gene transfection into neuronal cells. *The Journal of Gene Medicine, 3*, 72–81.

Yang, H. S., Feng, G. P., An, H. J., & Li, Y. F. (2006). Microstructure changes of sodium carbonate-soluble pectin of peach by AFM during controlled atmosphere storage. *Food Chemistry, 94*, 179–192.