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Novel curcumin-loaded chitosan-polyelectrolyte complexed nanoparticles and their characteristics

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Abstract−Curcumin was incorporated into oil/water (o/w) emulsion by dissolving it in soybean oil to cover the surface of the oil droplets with a pH-sensitive polyelectrolyte complex (PEC) composed of chitosan and fucoidan molecules. This curcumin-loading efficiency of the suspension system was investigated. The size and zeta potential distributions of novel curcumin-loaded chitosan nanoparticles self-assembled with fucoidan (CFNPLC) were assessed at various pH and fucoidan-to-chitosan mass ratio (FCMR). The release behavior of curcumin from CFNPLC was confirmed quantitatively by the superimposition of relevant release behavior. The release of curcumin from CFNPLC prepared with a chitosan solution of pH 6 for one to two days was slower than that from CFNPLC prepared with a chitosan solution of pH 3.7 for five to 10 hrs. This was attributed to the higher affinity of a chitosan molecule to curcumin molecules at a higher pH. The centrifugation of release medium accelerated the release of curcumin droplet from the surface of CFNPLC into the release medium much faster than a conventional curcumin release. Not to mention a high stability of curcumin blended with soybean oil, encapsulated in CFNPLC, the advantage of the CFNPLC is addressed in such a way that curcumin releasing period of a conventional curcumin delivery using CFNPLC, is supposed to be extended for much longer time than one to two days of the curcumin release period in this study. Thus, CFNPLC can bring about an enhanced effect of curcumin bioavailability resulting from the high curcumin stability and its extended release because it was dissolved in soybean oil and CFNPLC sustained slow curcumin-release for more than days in the oral drug (curcumin) delivery system. Therefore, CFNPLC can be treated mainly as a food-functional additive or healthy dietary product for tumor patients.

Keywords: O/W Emulsion, CFNPLC, Polyelectrolyte Complex, Curcumin, Chitosan, Fucoidan

INTRODUCTION

Curcumin is a hydrophobic molecule isolated from the rhizomes of *Curcuma longa* (*turmeric*) with proven bioactivity as an anti-inflammatory, antimutagenic, anti-oxidant, apoptosis-inducing agent, and anti-cancer agent [1]. Curcumin has been associated with a range of biological activity, including the modulatory activity of P-glycoprotein, by inhibiting both its function and expression, despite the instability of curcumin [2]. On the other hand, its delivery is quite challenging because of its low aqueous solubility and rapid metabolic degradation rate inside the human body [3]. In particular, at acidic and neutral pH, curcumin has been reported to have an aqueous solubility as low as 11 ng/mL in an aqueous buffer (pH=5.0) [4]. Moreover, curcumin has a very low bioavailability after oral administration [5] because of the poor absorption, rapid metabolism, especially by glucuronidation conjugation, and rapid elimination [6]. Degradation reactions dramatically alter the curcumin structure and properties affecting its pharmacokinetic and pharmacodynamic behavior [7]. Hence, pure curcumin is unstable to chemical degradation in aqueous alkaline solutions above pH 7. Therefore, innovative nanoformulations have been introduced and developed to overcome these problems. The food matrices used for the oral delivery system of curcumin include solid lipid nanoparticles (NPs) [8], nanoemulsions [9-11], liposomal dispersions [12], and casein dispersions [4]. After incubation at 37 °C for one month, curcumin-loaded emulsion showed little change, and more than 85% of the curcumin was retained by emulsions stored and under acidic conditions. In contrast, their yellow color faded when stored under alkaline conditions, with 62, 60, and 53% retained by emulsions stored at pH 7.0, 7.4, and 8.0, respectively [13]. Among those oral curcumin delivery systems, curcumin incorporated in oil/water (o/w) emulsions improved the water dispersibility and chemical stability against degradation [13]. In addition, curcumin was reported to be more stable in organic solvents than in water and showed the maximum stability in ethanol than in other organic solvents, such as DMSO, isopropanol, 1,4-dioxane, and ethylene glycol [14].

Yue et al. [15] reported that the surface charge affects the cellular uptake and intracellular trafficking of chitosan-based NPs, such that positively charged NPs improve the internalization rate because the positively charged NPs prefer to contact with a negatively charged cell surface. Lee et al. reported the characteristics of chitosan-fucoidan polyelectrolyte complex NPs [16-19], using polyelectrolyte complexation between chitosan and fucoidan in chitosan/fucoidan mixed solutions at various pH and fucoidan to chitosan mass ratios (FCMRs). Samrot et al. [20] and Jahromi et al. [21] examined curcumin-release using chitosan/anionic polymer NPs. Samrot et al.

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[20] and Jahromi et al. [21] prepared curcumin-loaded chitosan NPs with anionic polymers, such as carboxymethyl cellulose and tripolyphosphate, respectively.

Administering the optimized chitosan/anionic polymer NPs is important to promote greater intestinal uptake for oral delivery systems to swell and disintegrate after intestinal uptake. In this study, curcumin was incorporated into o/w emulsions by dissolving it in soybean oil to cover the surface of the oil droplets with a pH-sensitive polyelectrolyte complex composed of chitosan and fucoidan molecules and convert the o/w emulsions to a suspension with a dispersed phase of novel curcumin-loaded chitosan NPs self-assembled with fucoidan(CFNPLC). The preparation procedure of the novel CFNPLC was optimized and the prepared CFNPLC was characterized by Fourier transform infrared spectroscopy, scanning electron microscopy, transmission electron microscopy, size and zeta potential distribution, and the curcumin-release behavior *in vitro* under a range of conditions. This suspension system was investigated to obtain the curcumin-loading efficiency and the size and zeta potential distributions of CFNPLC at various pH and FCMRs.

MATERIALS AND METHODS

1. Materials

All the chemicals used in the present work were of analytical grade unless otherwise specified. Curcumin (mixture of demethoxycurcumin and bisdemethoxycurcumin, 98+%) was purchased from Acros Organics in the USA. Chitosan (deacetylation degree of 75-85%, viscosity (0.5% in 5% acetic acid) of 5-20 cps, case # 0321-6250) were purchased from Showa Chemicals Japan. Acetic acid was purchased from Merck in Germany. Fucoidan from *Fucus vesiculosus* (F5631-1G) was purchased from Sigma-Aldrich. Tween 80 was purchased from Reagents DUKSAN. Ethanol, NaOH and HCl were purchased from OCI Company Ltd. in Korea. Soybean oil (soybean 100%) was purchased from Ottogi Soybean Oil in Korea. Ascorbic acid and butylated hydroxytoluene were purchased from Sigma-Aldrich.

2. Preparation of Curcumin-loaded Chitosan NPs Self-assembled with Fucoidan (CFNPLC) in the o/w Emulsion of Curcumin-loaded Soybean Oil

By dissolving 100 mg of chitosan in 0.2%w/v acetic acid, a 0.1%w/v chitosan solution was prepared to make a final volume of 100 mL. To each of five 40 mL vials containing 10 mL of a 0.1%w/v chitosan solution (in 0.2%w/v acetic acid [pH 3.7]), 0.5, 0.75, 1.0, 1.5 and 2.0 g of 5% Tween 80 was added, followed by sufficient stirring for solubilization. Upon dissolving 100 mg of curcumin in 100 mL of soybean oil, 1 mL of a 0.1%w/v curcumin solution was added to each of the resulting Tween 80-added solutions, which were then homogenized at 12,000 rpm for 3 min to prepare five o/w emulsions termed A-1, A-2, A-3, A-4, and A-5, respectively. In addition, a 0.1%w/v fucoidan solution was prepared by dissolving 100 mg of fucoidan in deionized water to make a final volume of 100 mL. Upon homogenization, 2 mL of a 0.1%w/v fucoidan solution with a FCMR of 1 : 0.2 was added to each o/w emulsion, which was then stirred for 30 min to prepare CFNPLC in the o/w emulsions. Therefore, the o/w emulsions of curcumin-loaded soybean oil were prepared to measure the size of the oil droplets as a discrete phase and to compare their size with the size of CFNPLC in the o/w emulsions of A-1, A-2, A-3, A-4, and A-5, to determine the optimal input of 5% Tween 80.

To study the effect of the pH of the chitosan solution, the pH of 10 mL of a 0.1%w/v chitosan solution (in 0.2%w/v acetic acid) was adjusted to 2.0, 3.0, 5.0, or 6.0 with either 1 N NaOH or HCl. Therefore, chitosan solutions were prepared at pH 2.0, 3.0, 5.0, and 6.0, as well as an unadjusted pH of 3.7. After determining the optimal input of 5% Tween 80, the process of preparing CFNPLC was performed repeatedly with the optimal input of 5% Tween 80, in the same way except that the pH of the 0.1%w/v chitosan solution and the input volume of 0.1%w/v fucoidan solution were different. As process variables, the pH of the 0.1%w/v chitosan solution was set to 2, 3, 3.69, 5, and 6 and the input volume of the 0.1%w/v fucoidan solution was set to 1, 2, 6 and 10 mL, to give FCMRs of 1 : 0.1, 1 : 0.2, 1 : 0.6 and 1 : 1 for the CFNPLC prepared in the o/w emulsion.

3. Preparation of Simple o/w Emulsion of Curcumin-loaded Soybean Oil

Ten mL of demineralized water and 0.5 g of 5% Tween 80 in a 40 mL vial were solubilized by sufficient stirring to prepare a micellar solution. After dissolving 100 mg of curcumin in 100 mL of soybean oil, 1 mL of a 0.1%w/v curcumin solution (i.e., oil mixture) was added to the micellar solution, which was then homogenized at 12,000 rpm for 3 min to prepare the o/w emulsion of B-1.

4. Recovery Process of CFNPLC

The CFNPLC samples were retrieved by centrifuging the previous o/w emulsions for 15 min to separate them from an aqueous medium and oil droplets. Subsequently, the retrieved CFNPLC were frozen at −75 °C for freeze-drying with a lyophilizer (Il Shin Bio Base, FD8512). To facilitate and improve their separation efficiency, the o/w emulsion system was sonicated before centrifugation in an ultrasonic water bath (JAC-4020, KODO) for 5 min to disintegrate the aggregated oil droplets because of the difficulty in separating CFNPLC from the oil droplets in the o/w emulsions in the presence of any oil droplets of a similar size or larger than that of CFNPLC in the o/w emulsion.

5. Estimation of the Curcumin-loading Efficiency

According to the aforementioned CFNPLC preparation procedure, CFNPLC were formed in triplicate using a chitosan solution at pH of 2.0, 3.0, 3.7 (i.e., unadjusted), 5.0, and 6.0 and 0.1%w/v fucoidan solutions at input volumes of 1, 2, 6, and 10 mL. After stirring the corresponding o/w emulsion for 30 min and adding each input volume of 0.1%w/v fucoidan solution, the resulting mixture was sonicated to recover the CFNPLC and centrifuged at 13,000xg for 15 min to separate CFNPLC. The resulting supernatant was separated and sonicated for 5 min again for accurate UV-Vis spectrophotometric analysis of the curcumin loading efficiency in CFNPLC. Subsequently, 1 mL of the supernatant was dissolved in 9 mL of ethanol to measure the absorbance at 425 nm using a UV-Vis spectrophotometer (UV-VIS 160, Shimadzu) to estimate the concentration of the curcumin remaining in the solution after CFNPLC complexation. One mL of the supernatant resulting from the preparation of soybean oil-only (without curcumin)-loaded chitosan NPs self-assembled with fucoidan (CFNPLS) was dissolved in 9 mL of ethanol and used as a blank for the UV-Vis spec-

trophotometry. The procedure of CFNPLS preparation was the same as that of the corresponding CFNPLC preparation except for no addition of curcumin. The standard curves were calibrated with the absorbance measurements of 0.001 to 0.01 mg curcumin/mL ethanol at 425 nm. The curcumin-loading efficiency was calculated using Eq. (1):

$$\text{Curcumin-loading efficiency (\%)} = \frac{\text{Curcu}^{o} - yV}{\text{Curcu}^{o}} \times 100 \quad (1)$$

where Curcu^{o}, y, and V denote the amount of curcumin input, the curcumin concentration in the supernatant, and the volume of the supernatant, respectively.

6. Size Distribution, Zeta Potential, and SEM Analysis

The size and zeta potential of the CFNPLC in the prepared suspensions were measured using a zeta potential meter (Malvern, Zetasizer, Nano ZS) in triplicate. The morphology of the CFNPLC was examined by SEM (Hitachi High-Technologies, SU-8220). One droplet of the suspension containing redispersed CFNPLC was deposited on a glass slide and dried in air at room temperature. The surface morphology of the CFNPLC was then observed by SEM at an accelerating voltage of 15.0 kV.

7. FTIR Analysis

Pellets of freeze-dried CFNPLC were prepared by mixing KBr powder with the freeze-dried CFNPL-powder at a ratio of 100 : 1 and compressing the resulting mixtures into pellets. The FTIR spectra (Perkin Elmer, Frontier) were obtained from the 4,000 to 400 cm^{-1} region at room temperature. The spectra of pure chitosan, fucoidan, and curcumin were also obtained to analyze the interaction involved in forming their complex.

8. TEM Analysis

The morphology of CFNPLC was observed by TEM (Hitachi High-Technologies, H-7600) at 100 kV. One droplet of the suspension containing redispersed CFNPLC was deposited on a carbon-coated grid and dried in a vacuum oven for 24 h at 25 °C.

9. Curcumin-release Studies

Two types of CFNPLC were prepared: 1) a chitosan solution of pH 3.7 and FCMR of 1 : 1, and 2) a chitosan solution of pH 6.0 and FCMR of 1 : 1. Five milligrams of freeze-dried CFNPLC was dispersed at a stirring rate of 150 rpm in 5 mL of each mixture of 1) phosphate buffer solutions (PBS) at pH 7.4 containing 1%w/v ascorbic acid and 0.0001%w/v butylated hydroxytoluene, (60%) and ethanol (40%), and 2) PBS (60%) of pH 3.0 containing the same, and ethanol (40%), as curcumin-release media incubated in a shaking incubator at 37 °C. Curcumin was soluble in ethanol and showed the highest stability compared to the other organic solvents examined [14]. The release medium of curcumin was prepared using the procedure reported by Hosseini et al. [22], in which a mixture (6 : 4) of PBS (60%) at pH 7.4 and ethanol (40%) was used because of the insolubility of oregano essential oil released from the chitosan NPs in PBS.

In the curcumin-release study, serial samples were taken simultaneously at predetermined times from the same media containing CFNPLC and CFNPLS as the curcumin-release samples and blank samples, respectively, for UV-Vis spectrophotometric analysis. In addition, ethanol was also used as a blank for UV-Vis spectrophotometry.

Similarly, two types of chitosan NPs self-assembled with fucoidan unloaded with any oil droplet of curcumin solution in soybean oil (CFN), were prepared at the same pH of the chitosan solution as well as the same FCMR as in the curcumin-release study of CFNPLC, in the same manner as CFNPLC except for excluding the curcumin solution in soybean oil. Five milligrams of freeze-dried CFN was dispersed in the same media as in the curcumin-release study of CFNPLC. In the CFN release study, serial samples were taken at the same predetermined times as in the curcumin-release study, from the same medium containing CFN as the release samples for UV-Vis spectrophotometric analysis, where ethanol was used as a blank.

The *in vitro* release behavior of curcumin was observed in the release media for 54 h to estimate the amount of curcumin available *in vivo*. The medium containing CFNPLC was centrifuged, as described by Hosseini et al. [22], for 5 min at 9,000 rpm to detach a released curcumin-droplet on the surface of CFNPLC at predetermined times: 1, 2, 3, 4, 5, 6, 10, 22, 30, 46, 50, and 54 h. Serial samples of 1 mL were withdrawn from their supernatant at each time. Fresh medium (1 mL) was added to the curcumin-release medium in the shaking incubator. In addition, the medium containing CFNPLS for the blank samples and CFN were treated in the same manner as the medium containing CFNPLC. Each withdrawn sample was added to 9 mL of 100% ethanol and vortexed for 1 min for UV-Vis spectrophotometry at 425 nm. The drug release studies were performed such that both the cumulative amount of curcumin released from CFNPLC and the cumulative curcumin-release (%) were calculated at each sampling time using Eqs. (2) and (3), respectively. Release studies of CFN were also performed to determine the cumulative amount released, as if curcumin molecules had been released, according to the standard curves of curcumin based on UV-Vis spectrophotometry analysis at 425 nm. The converted cumulative release (%) was calculated at each sampling time in accordance with Eqs. (2) and (3), respectively:

$$\text{Cumulative amount of curcumin released} = v\Sigma_{j=1}^{n-1} C_j + C_n V \quad (2)$$

where

j=iterative sampling time from, 1^{st} to $(n-1)^{th}$ time.

C_j=curcumin concentration of the sample taken at the j^{th} sampling time from the medium containing CFNPLC.

v=sampling volume taken from the medium containing CFNPLC.

V=volume of the medium containing CFNPLC.

$$\text{Cumulative curcumin release (\%)} = \frac{\text{Cumulative amount of released curcumin}}{\text{Curcumin amount loaded in CFNPLC}} \times 100 \quad (3)$$

In Eq. (3), the ratio of the cumulative amount of released curcumin to the curcumin amount loaded in CFNPLC may be fractionated as a product of the ratio of the cumulative amount of released curcumin to the curcumin input amount and the reciprocal of the curcumin-loading efficiency.

RESULTS AND DISCUSSION

1. Determination of the Optimal amount of Emulsifier in the Preparation of CFNPLC

CFNPLC was prepared in o/w emulsions, A-1, A-2, A-3, A-4,

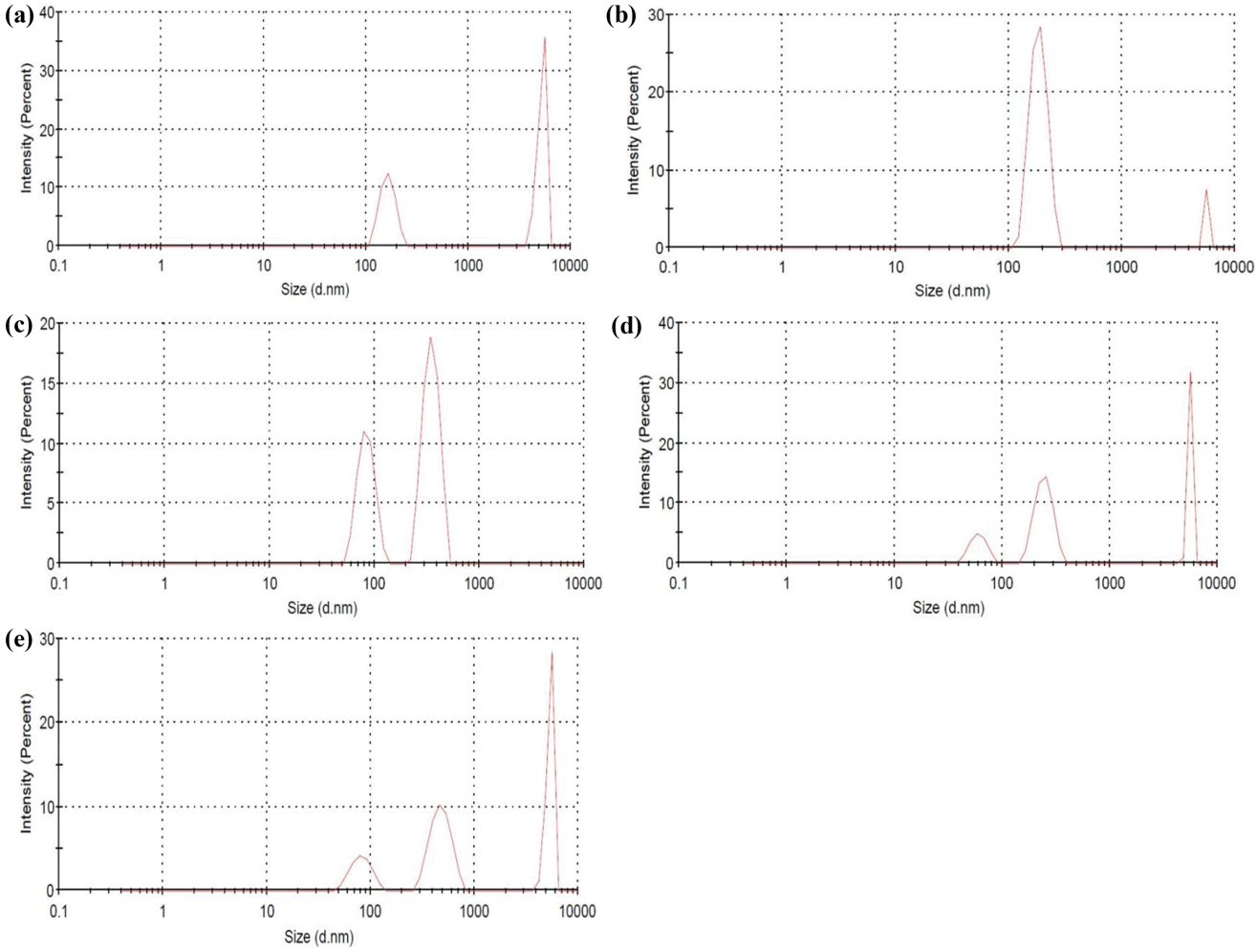

Fig. 1. Size distribution of NPs, including CFNPLC prepared in o/w emulsions of A-1 (a), A-2 (b), A-3 (c), A-4 (d) and A-5 (e) with various amounts of emulsifiers (5% Tween 80): (a) 0.5 g; (b) 0.75 g; (c) 1 g; (d) 1.5 g; (e) 2 g.

and A-5. Fig. 1(a) to (e) show the sizes of the NPs, including CFNPLC in the o/w emulsions of A-1 (5% Tween 80, 0.5 g), A-2 (5% Tween 80, 0.75 g), A-3 (5% Tween 80, 1 g), A-4 (5% Tween 80, 1.5 g) and A-5 (5% Tween 80, 2 g), respectively. The o/w emulsion of B-1 of curcumin-loaded soybean oil as a discrete phase was prepared to measure the size of the oil droplets to determine the optimal input of 5% Tween 80 by comparing their size with the size of CFNPLC in the o/w emulsions of A-1, A-2, A-3, A-4, and A-5. Fig. 2 shows the size distribution of the oil droplets in the o/w emulsion of B-1. Comparing the size distributions of the o/w emulsions of A-1 in Fig. 1(a) and B-1 in Fig. 2 shows that that the peaks of the size distribution in Fig. 2 are composed of those of discrete oil droplets of approximately 150 nm, micelles of 5% Tween 80 of 55 nm and oil droplet-aggregates of more than 1,000 nm up to 5,000 nm. Accordingly, both peaks of 150 nm and 5,000 nm in Fig. 1(a) correspond to CFNPLC or discrete oil droplets and aggregates of oil droplets, respectively. The size of the oil droplets could be controlled in inverse proportion to the amount of emulsifier, as demonstrated by the experimental result in Fig. 1(b), showing that the peak of 150 nm strengthened to become the main peak. In comparison, the peak at 5,000 nm weakened to become a minor

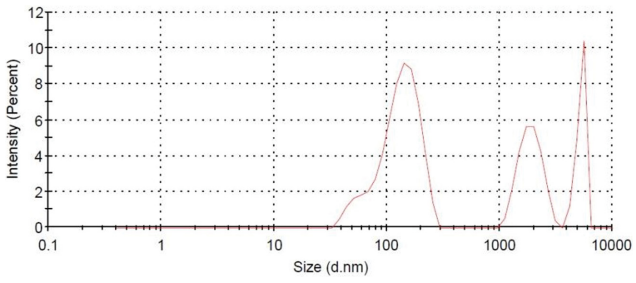

Fig. 2. Size distribution of oil droplets in the o/w emulsion of B-1 with 0.5 g of emulsifier (5% Tween 80).

peak as the added amount of 5% Tween 80 was increased from 0.5 g to 0.75 g. Therefore, the fractions of aggregated and discrete oil droplets decreased and increased, respectively, with increasing amount of 5% Tween 80. As the amount of Tween 80 was increased further from 0.75 g to 1 g, as shown in Fig. 1(c), the peak of the aggregated oil droplets disappeared, while the peak of the discrete oil droplets moved to the left at 80 nm, showing the reduced size of the discrete oil droplets. In addition, the peak of CFNPLC ap-

peared at ca. 350 nm. As the amount of 5% Tween 80 added was increased further from 1 g to 1.5 g, as shown in Fig. 1(d), three peaks arose at ca 65 nm, 250 nm, and 5,500 nm, representing micelles of 5% Tween 80 and discrete oil droplets, CFNPLC, and aggregates, respectively. Thus, the peak of aggregated oil droplets at 5,500 nm reappeared, as shown in Fig. 1(d), even though it disappeared in Fig. 1(c). The hydrophobic parts of the chitosan chains were assumed to be associated with emulsion oil droplets and the formation of polymer bridges connecting the droplets, resulting in bridging flocculation. A similar mechanism was previously reported for chitosan flocculating bacteria [23]. Moreover, the interaction between the hydrophobic part of the surfactant and the emulsion-destabilizing polymer plays a role in bridging flocculation [24].

Therefore, the appearance of a peak of aggregated oil droplets at 5,500 nm (Fig. 1(d)) can be interpreted as the inception stage of bridging flocculation to have the peak of aggregated oil droplets at 5,500 nm appear as the added amount of 5% Tween 80 was increased from 1 g to 1.5 g. Finally, these three peaks were retained as the addition was increased from 1.5 g to 2 g, as shown in Fig. 1(e). The two peaks of discrete oil droplets and CFNPLC in Fig. 1(e) were in a similar position to those in Fig. 1(c). Therefore, based on the separateness of the overlapped peak of discrete oil droplets from that of CFNPLC, and the disappearance of the peak of aggre-

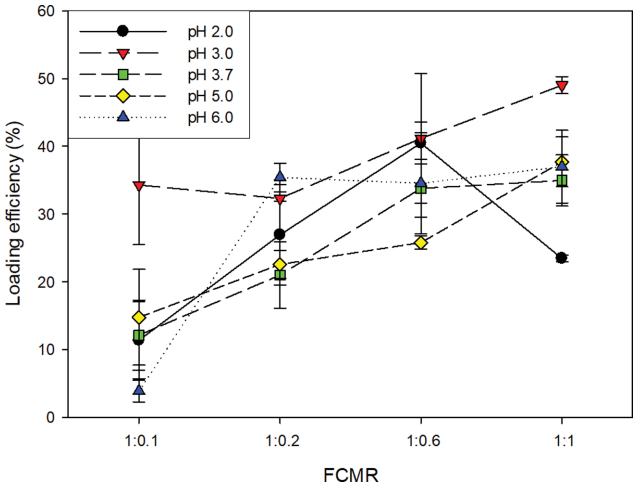

Fig. 3. Curcumin-loading efficiency of CFNPLC formed in triplicate, using the chitosan solution of pH 2.0, 3.0, 3.7 (i.e., unadjusted), 5.0 and 6.0 with FCMRs of 1 : 0.1, 1 : 0.2, 1 : 0.6 and 1 : 1.

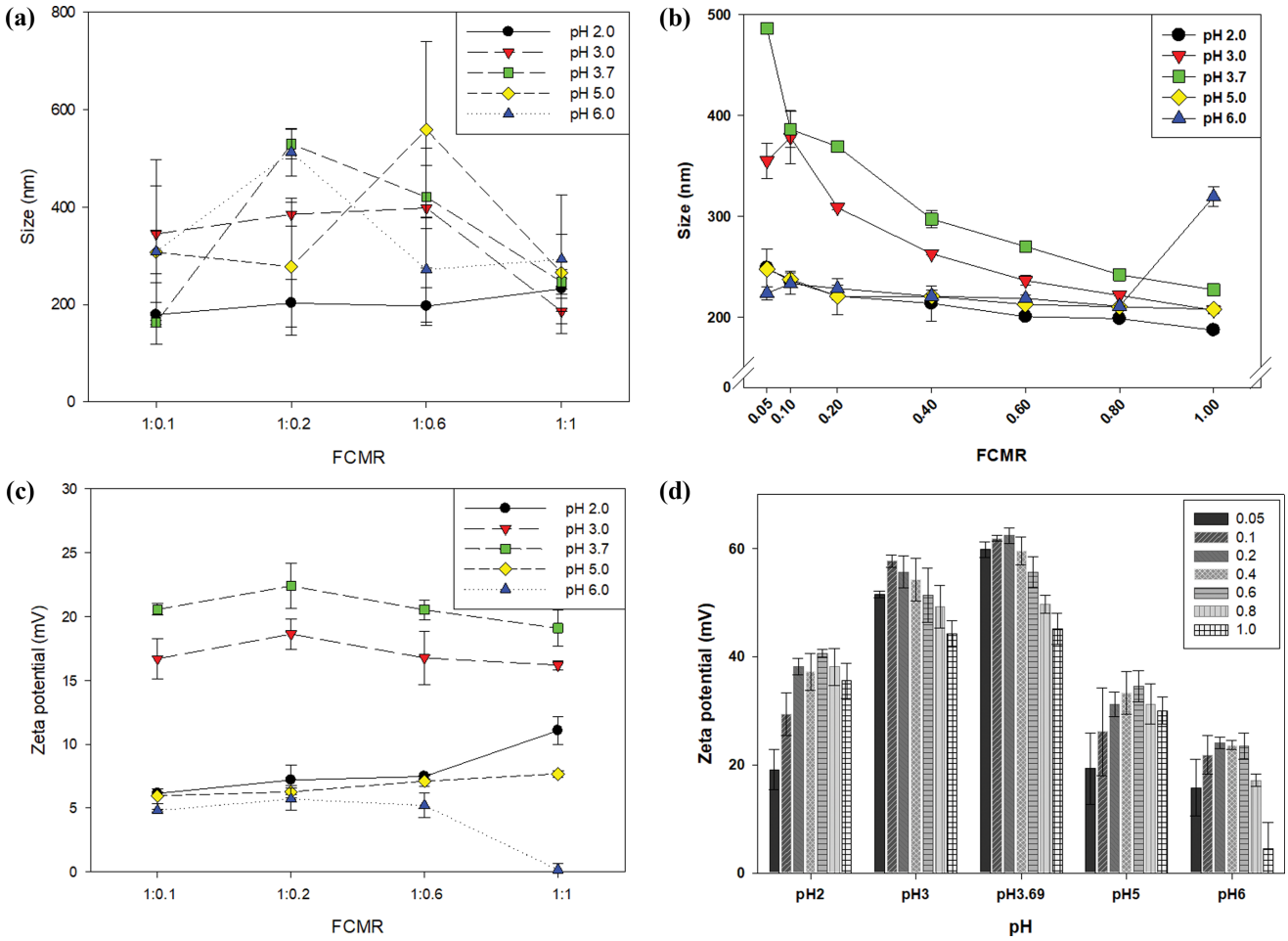

Fig. 4. Dependencies of CFNPLC and CFN, formed in triplicate, characteristics on FCMR nd pH of chitosan solution: 1. Size distribution ((a) CFNPLC in this study; (b) CFN [18,19]); 2. Zeta potential distribution ((c) CFNPLC in this study; (d) CFN [18]).

gated oil droplets in terms of the size distribution, the optimal condition on the added amount of emulsifier was determined to be 1 g of 5% Tween 80.

2. Curcumin-loading Efficiency

CFNPLC was formed in triplicate using the chitosan solution of pH 2.0, 3.0, 3.7 (i.e., unadjusted), 5.0, and 6.0 with 1, 2, 6, and 10 mL input volumes of the 0.1%w/v fucoidan solution. After measuring the curcumin concentration in the supernatant of the o/w emulsion containing the suspended CFNPLC, sonicated before centrifugation, with the standard curve (i.e., Y=133.38X+0.009 (R^2=0.9996)), the curcumin-loading efficiency was estimated using Eq. (1), as shown in Fig. 3. The error of absorbance due to soybean oil was less than 3%, which may be ignored.

The curcumin content in CFNPLC was determined by estimating the curcumin-loading efficiency, as shown in Fig. 3. As FCMR increased, the curcumin-loading efficiency increased for chitosan solutions of pH 3.0, 3.7, and 5.0. In contrast, for chitosan solutions of pH 2.0 and 6.0, as the FCMR increased, the curcumin-loading efficiency increased at low FCMR but decreased or reached a plateau at high FCMR. At FCMR=1 : 1, the curcumin-loading efficiency was the highest at ca. 50% and the lowest at ca. 25% at pH 3.0 and pH 2.0, respectively. Curcumin-loading efficiency of ca. 35% was achieved for CFNPLC with FCMR of 1 : 1 prepared with a chitosan solution of pH 3.7 and 6.0, as shown in Fig. 3. Therefore, considering the initial input amount of curcumin of 1 mg, the amount of curcumin loaded in both CFNPLC was ca. 0.35 mg.

3. Mean Size and Zeta Potential of CFNPLC

The optimal amount of emulsifier added was determined to be 1 g of 5% Tween 80 based on the separateness of the overlapped peak of discrete oil droplets from that of CFNPLC and the disappearance of the peak of aggregated oil droplets in terms of the size distribution. Nevertheless, more than two peaks were occasionally observed in the size distribution of both micelles of 5% Tween 80 and discrete oil droplets, CFNPLC, and aggregated oil droplets, where the peak of CFNPLC between 150-450 nm was selected with their percentage intensity. The sizes of CFNPLC were averaged from triplicate measurements using their intensity as a weight. Fig. 4(a) shows the dependency of the CFNPLC size on the FCMR and pH for all chitosan solution pH and all FCMRs. Table 1 lists the polydispersity index (PDI) for their size distribution. The hydrogel particle size of CFNPLC ranged from 163 nm to 559 nm, as shown in Fig. 4(a), which was similar to the size range of CFN, as shown in Fig. 4(b) [18,19], previously prepared in an earlier study by simple polyelectrolyte complexation with the same range of pH of the chitosan solution and FCMR. On the other hand, because the o/w emulsion process preceded the polyelectrolyte complexation in the present study, compared to previous studies [18,19], the size distribution of CFNPLC prepared with chitosan solutions at pH 3.0, 3.7, 5.0, and 6.0 behaved most differently at FCMR of 1 : 0.6, 1 : 0.1, 1 : 0.6 and 1 : 0.2, respectively, from those in previous studies [18,19].

Table 1. PDI for the size distribution of FCMR at various pHs and FCMRs

pH of chitosan	FCMR			
	1 : 0.1	1 : 0.2	1 : 0.6	1 : 1
2.0	0.0204	0.0586	0.0388	0.0077
3.0	0.0838	0.0041	0.0944	0.0589
3.69	0.0008	0.0034	0.0239	0.0107
5.0	0.0213	0.2548	0.1041	0.0888
6.0	0.3793	0.0089	0.1584	0.2053

Unlike their size distribution, the zeta potential of CFNPLC was dependent on both pH and FCMR. Fig. 4(c) shows the dependency of CFNPLC zeta potential on the FCMR and pH. The zeta potential of CFNPLC increased in the order of pH 3.7, 3.0, 2.0, 5.0, and 6.0 at any given FCMR, which was the same as that reported previously [18], as shown in Fig. 4(d). In addition, the pattern of the zeta potential variation, according to FCMR, was also consistent with that in a previous study [18] except for the magnitude of zeta the potential. The maximum zeta potential at each pH ranged from 5.7 mV to 22.4 mV in this study, compared to 23.5 mV to 62.0 mV in the previous study [18]. This reduced CFNPLC zeta potential in this study was attributed to the entrapment of curcumin blended with soybean oil without an electric charge in CFNPLC, which was not found in the previous study [18].

4. FTIR Spectrum of CFNPLC for Curcumin, Chitosan, and Fucoidan

Fig. 5 shows the FTIR spectra of chitosan (a), fucoidan (b), curcumin (c), and CFNPLC (d). Because chitosan and fucoidan are both polysaccharides, their FTIR spectra (Figs. 5(a) and (b)) show the characteristic polysaccharide bands in the region of 900-1,150 cm^{-1}, which corresponds to the ring stretching, C-O stretching, asymmetric bridge oxygen stretching vibrations of the saccharide structure [25-27]. Both chitosan and fucoidan have characteristic peaks at 2,800-2,950 cm^{-1} and 3,200-3,500 cm^{-1}, which were assigned to the presence of aliphatic and hydroxyl groups, respectively [28, 29]. Despite the above similarity in the structure of chitosan and fucoidan, they both have typical peaks in their characteristic groups. For example, the characteristic peaks at 1,594 cm^{-1} in the spectrum of chitosan (Fig. 5(a)) are attributed to amino groups, and those at 1,650 cm^{-1}, 1,376 cm^{-1}, and 1,324 cm^{-1} are assigned to the amide I, amide II, amide III bands, respectively [25,30-33]. On the other hand, the spectrum of fucoidan (Fig. 5(b)) has a peak at 1,221 cm^{-1}, which was assigned to the S=O stretching vibration of the sulfate group and the additional peak at 823 cm^{-1} was attributed to the O-4 sulfates [34]. The peak at approximately 1,633 cm^{-1} in the spectrum of fucoidan (Fig. 5(b)) may be due to the presence of 2-O-acetyl groups in the structure [29,35,36].

Both FTIR spectra of curcumin (Fig. 5(c)) and CFNPLC (Fig. 5(d)) showed a keto group of curcumin at 1,114 cm^{-1} and 1,085 cm^{-1}, respectively. Fig. 5(d) shows the amide I, amide II, and amide III bands of chitosan at 1,640 cm^{-1}, 1,376 cm^{-1}, and 1,324 cm^{-1}, respectively, in the spectrum of CFNPLC. On the other hand, the amino group peak of chitosan at 1,594 cm^{-1} disappeared. The peak at 842 cm^{-1} for the O-4 sulfates of fucoidan shifted to 871 cm^{-1}. The peaks at 1,633 cm^{-1} corresponding to 2-O-acetyl groups of fucoidan shifted to 1,640 cm^{-1} to overlap with the peak of amide I in the spectrum of CFNPLC. Owing to the polysaccharide nature of both chitosan and fucoidan, the common saccharide peak at

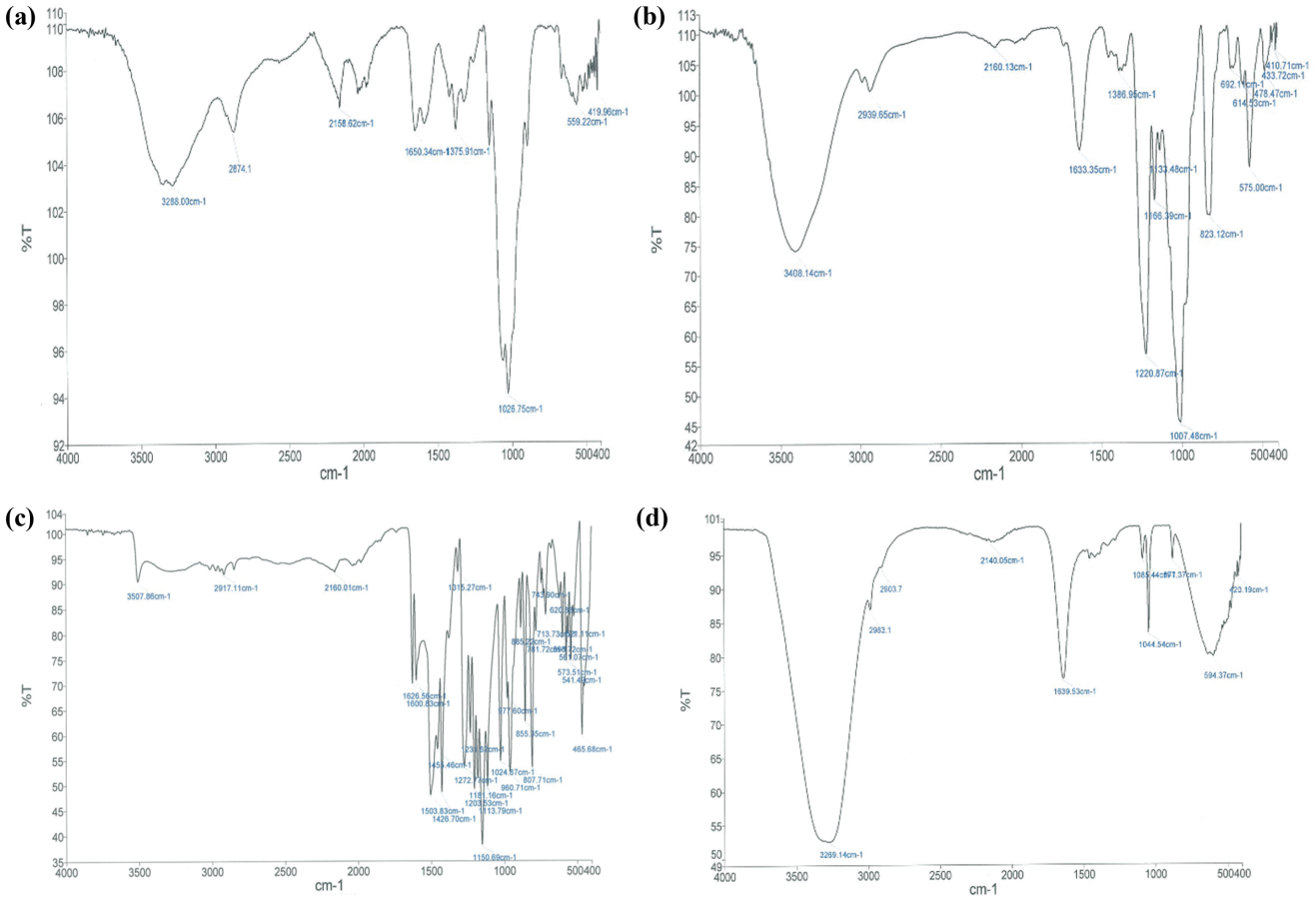

Fig. 5. FTIR spectra: (a) Chitosan; (b) Fucoidan; (c) Curcumin; (d) CFNPLC showing the keto group of curcumin at the wavenumber of 1,085 cm^{-1}.

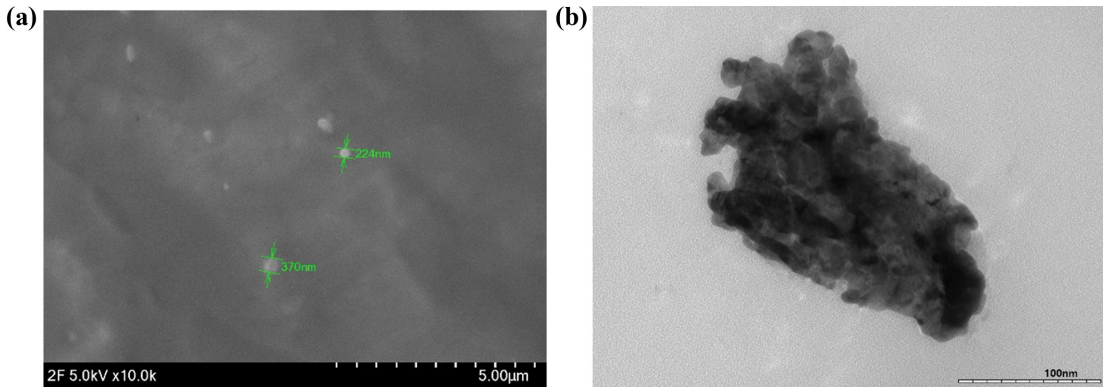

Fig. 6. Analysis on CFNPLC prepared using the chitosan solution of pH 2.0 with FCMR of 1 : 1: (a) SEM image; (b) TEM image which shows dark spots of encapsulated curcumin-loaded micelle in CFNPLC with the size of 200-250 nm indicated by its scale bar of 100 nm.

900-1,150 cm^{-1}, the hydroxyl peak at 3,200-3,500 cm^{-1}, and the aliphatic peak at 2,900-2,983 cm^{-1} were present in the spectrum of CFNPLC.

5. Morphology of CFNPLC

Lyophilization induces inter-NP aggregation during the freezing step because of the significant entanglement of polymer chains as the freezing interface propagates [37]. Instead of lyophilizing the hydrogel-NPs, one drop of their suspension was placed on glass and dried at room temperature to avoid forming a freeze-drying-induced NP-matrix, as described by Lee and Lim [17]. The prepared CFNPLC was observed by a field emission SEM (FESEM, Hitachi High-Technologies, SU-8220) and a TEM (Hitachi High-Technologies, H-7600), as shown in Figs. 6(a) and (b), respectively, where the morphology of CFNPLC consisted of round and oval shapes. In particular, encapsulated curcumin-loaded micelles in CFNPLC appeared as dark spots, as shown in Fig. 6(b), consistent

with the results reported elsewhere [38].

6. Curcumin-release Studies

The behavior of curcumin release from CFNPLC prepared with both a chitosan solution of pH 3.7 and FCMR of 1 : 1 and chitosan solution of pH 6.0 and FCMR of 1 : 1 was observed for 54 h. Although Wang et al. [39] reported the complete degradation of curcumin in PBS of pH 7.2, its rapid degradation was not attributed to chemical decomposition but rather to the precipitation of curcumin [40]. A previous study reported that owing to changes in the molecular structure of curcumin, it tended to crystallize out of aqueous solutions at pH below 7(4), which may cause heterogeneity in the solution. The formed curcumin crystals were relatively large (10-50 μm) and prone to rapid sedimentation [4]. Thus, the curcumin solutions were more heterogeneous at acidic and neutral pH because of different aggregated forms of curcumin [41]. In this release study, curcumin-containing oil droplets released from the surface of CFNPLC were detached into the curcumin-release medium by centrifugation at each sampling time, which may have caused the heterogeneity in the curcumin-release medium incubated in a shaking incubator at 37 °C, composed of the following: 1) PBS (60%) of pH 7.4 and ethanol (40%) or 2) PBS (60%) of pH 3.0 and ethanol (40%). Curcumin, which is soluble in ethanol, showed the maximum stability in ethanol among organic solvents assessed [14].

Fig. 7 shows the curcumin-release behavior of CFNPLC prepared with both chitosan solution of pH 6.0 (△) and FCMR of 1 : 1 and both chitosan solution of pH 3.7 (▲) and FCMR of 1 : 1 in the release medium of the mixture of PBS (60%) of pH 7.4 and ethanol (40%), in which ethanol was used as a blank for UV-Vis spectrophotometry. The smooth behavior of curcumin release in this curcumin-release experiment (Fig. 7) indicates the absence of any spatial heterogeneity in the release medium caused by the centrifugation-forced detachment of curcumin-containing oil droplets released from the surface of CFNPLC at each sampling time. On the other hand, the total cumulative amount of curcumin released in Fig. 7 (△, ▲) was ca. 1.8 mg and 1.5 mg, respectively, which led to an exaggerated payload of curcumin in CFNPLC, whose yield was 5-15 mg considering the curcumin-loading efficiency of ca. 35%, as shown in Fig. 3. This exaggerated payload resulted from an overestimation of the UV-Vis absorption value by as much as the absorbance due to turbidity resulting from ethanol-induced aggregation or possible precipitation in the curcumin-release samples owing to the use of ethanol as a blank in UV-Vis spectrophotometry. In this study, UV-Vis absorption value of a sampled aqueous Tween 80 solution in ethanol at 425 nm turned out negligible below detectable range even when 1 g of 5% Tween 80 was dissolved in 5 mL of demineralized water.

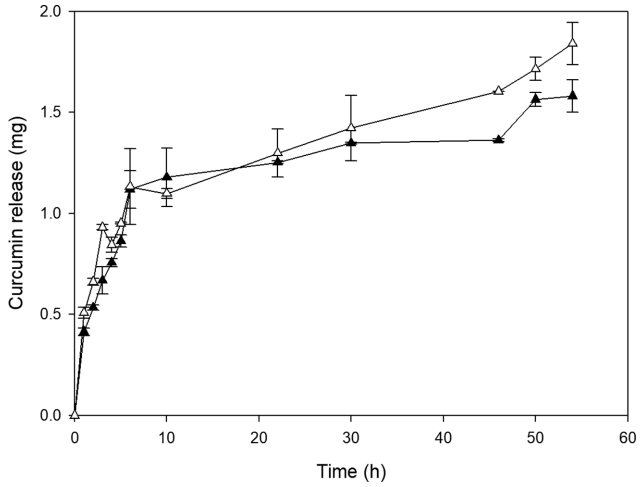

Fig. 7. Curcumin-release behavior, in duplicate curcumin-release study, of CFNPLC prepared with both chitosan solution of pH 6.0 (△); pH 3.7 (▲), and FCMR of 1 : 1, in which ethanol was used as a blank for the analysis of UV-Vis spectrophotometry, in the release medium of the mixture of phosphate buffer solutions (PBS) (60%) of pH 7.4 and ethanol (40%).

For reference, CFN was prepared using a chitosan solution at the same pH, 5 mg of which was placed in the same release medium and underwent the release experiment under the same conditions, as shown in Fig. 7. Although PBS is excellent for physiological science, the phosphate in a PBS-buffered sample may precipitate if the sample is mixed with ethanol. The resulting absorbance was possibly attributed to turbidity resulting from the precipitation from PBS medium in ethanol as well as the ethanol-induced aggregation of each fucoidan molecule released in PBS medium due to centrifugation and the disintegration of CFN. However, the ethanol-induced aggregation of each fucoidan molecule remaining in a distilled water did not show any significant absorbance at 425 nm upon its polyelectrolyte complexation with chitosan molecules without adding any emulsifier like Tween 80 [unpublished data]. Therefore, the resulting absorbance was attributed to turbidity resulting from the precipitation from PBS medium in ethanol, which was affected by fucoidan molecules and Tween 80 released in PBS medium due to centrifugation and the disintegration of CFN.

The release studies of CFN in this study were performed such that the ultimate cumulative amounts into which the absorbance was converted, as if the absorbance had resulted from the released curcumin molecules, according to the standard curves of curcumin at 425 nm, were shown in Figs. 9(a) (○) and (b) (○) to be ca. 1.3 mg.

Fig. 8 represents the curcumin-release behavior of CFNPLC prepared with a chitosan solution at both pH 6.0 (a) and pH 3.7 (b), and the FCMR of 1 : 1 in the release medium of the mixture of PBS (60%) and ethanol (40%). The curcumin-loading efficiency of both CFNPLC was ca. 35% as shown in Fig. 3. The release behavior of A-1 (Fig. 8(a)) and B-1 (Fig. 8(b)) was examined in the release medium of pH 3.0-PBS and ethanol, under which condition of release medium, a polyelectrolyte complex of chitosan and fucoidan was reported not to disintegrate. Therefore, the curcumin release for A-1 (Fig. 8(a)) and B-1 (Fig. 8(b)) was only ca. 10%. In the case of the release medium of the mixture of pH 7.4-PBS and ethanol, B-2 (Fig. 8(b)) showed that the curcumin-release behavior was unsteady for relatively fast 10 h incubation to reach much higher value of curcumin release than B-1 (Fig. 8(b)). This was attributed to the preparation of CFNPLC with a chitosan solution of pH 3.7. Moreover, a polyelectrolyte complex of chitosan and fucoidan disintegrated in the medium of pH 7.4. In contrast, A-2 (Fig. 8(a)) showed a slower release of curcumin than B-2 (Fig.

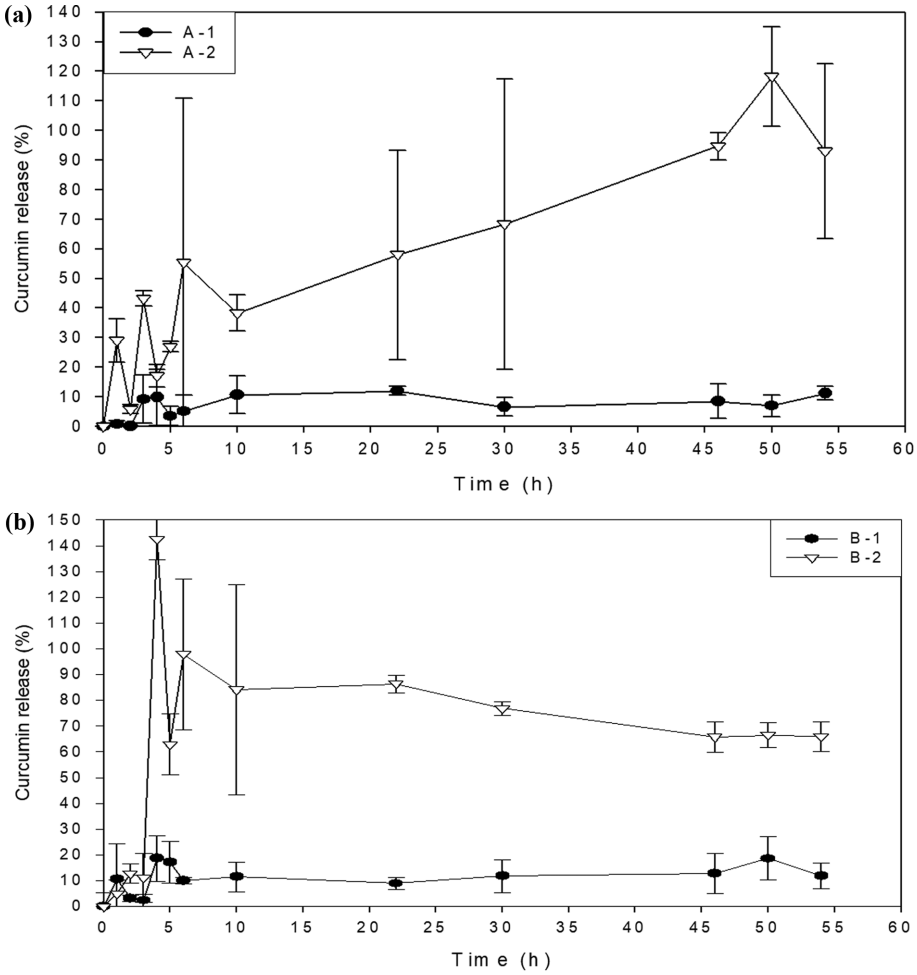

Fig. 8. Curcumin-release behavior of CFNPLC prepared with FCMR of 1 : 1 using following conditions; (A-1) a chitosan solution of pH 6.0 in the release medium of 60% PBS (pH 3.0) + 40% ethanol, (A-2) a chitosan solution of pH 6.0 in the release medium of 60% PBS (pH 7.4) + 40% ethanol, (B-1) a chitosan solution of pH 3.7 in the release medium of 60% PBS (pH 3.0) + 40% ethanol and (B-2) a chitosan solution of pH 3.7 in the release medium of 60% PBS (pH 7.4) + 40% ethanol. The experiment was performed in duplicate.

8(b)), suggesting that CFNPLC prepared with a chitosan solution of pH 6.0 showed a slower curcumin release than that of CFNPLC prepared with a chitosan solution of pH 3.7. This was attributed to the higher affinity of a chitosan molecule to curcumin molecules at higher pH [42]. In Fig. 8, the high standard deviation of cumulative released curcumin was caused not by experimental error but by release system-inherent error in this curcumin release experiment. The peak with high standard deviation indicates that highly concentrated curcumin-including oil droplets detached from the surface of CFNPLC into the release medium, were sucked into either of duplicated samples frequently, particularly at the incipient stage of predetermined sampling time in the release experiment, which was considered in the cumulative curcumin-release profile at the next sampling time. In fact, it was also true and reflected in the profiles of cumulative curcumin release in Fig. 7 from the perspective that the magnitude of standard deviation at each predetermined sampling time in both A-2 and B-2 (Fig. 8) was found to be very similar, as shown in Fig. 9, to those at each predetermined sampling time in curcumin release behavior of CFNPLC prepared with both chitosan solution of pH 6.0 and pH 3.7 (Fig. 7), respectively. The higher standard deviation of the cumulative curcumin release of A-2 (Fig. 8(a)) compared to the other conditions may be due to the higher affinity of chitosan molecules to curcumin at pH 6.0 than at pH 3.7, as well as the asynchronous disintegration of CFNPLS in the medium of the blank for UV-Vis spectrophotometric analysis, to that of CFNPLC used as a curcumin-carrier in the curcumin-release medium, particularly at the latter stages of the curcumin release experiments. The asynchronous disintegration would result in a notable difference in the amount of fucoidan molecules and Tween 80 released between the curcumin-release medium and the medium of the blank. However, it is unlikely that the asynchronous disintegration may be the reason for the higher standard deviation of the cumulative curcumin release of A-2 (Fig. 8(a)) than those of B-2 (Fig. 8(b)) particularly at the latter stages of the curcumin release experiments because blanks used in both curcumin release behaviors having the similar magnitude of standard deviation, of CFNPLC prepared with chitosan solution of pH 6.0 (Fig. 7), and A-2 (Fig. 8(a)) were ethanol and CFNPLS, respectively. Moreover, curcumin molecules released from CFNPLC may have brought more pieces

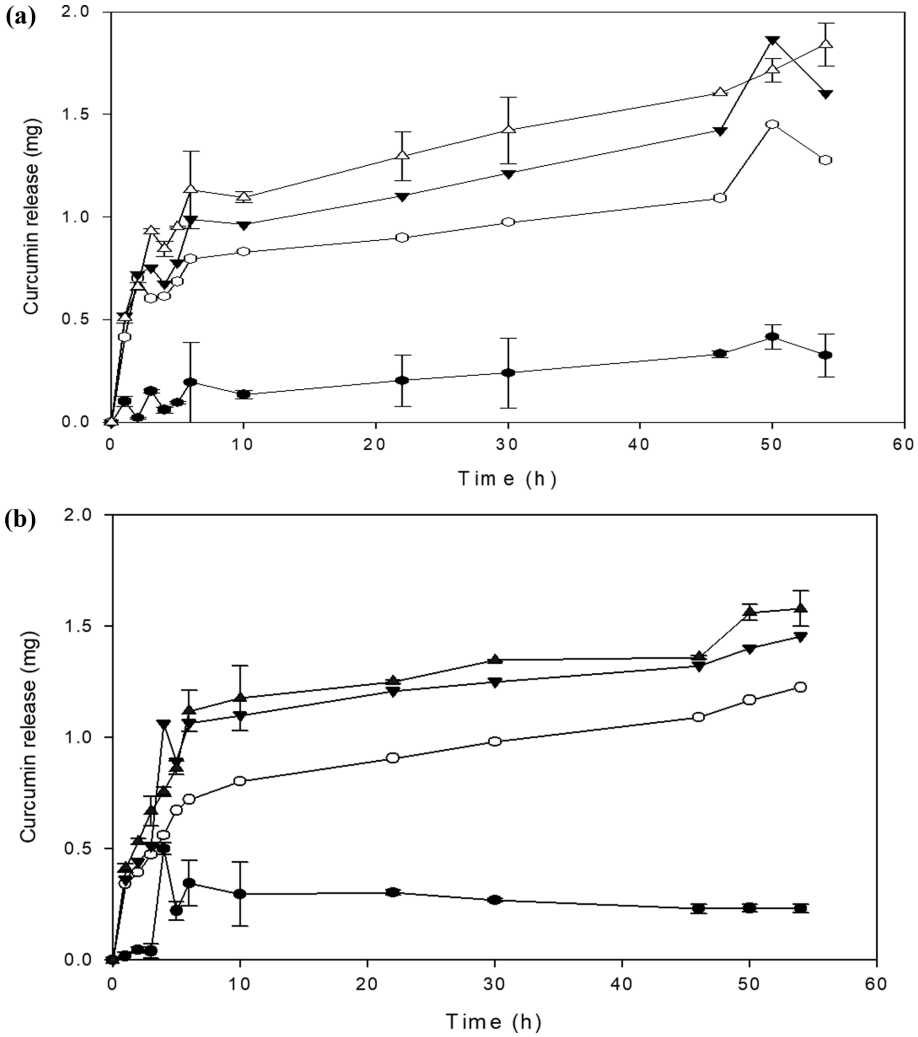

Fig. 9. Comparison between the release behavior of CFNPLC (△ (a) and ▲ (b)), as shown in Fig. 7, and the release behaviors (▼, (a) and (b)) into which the curcumin-release behaviors of CFNPLC (●, (a) and (b)), as shown in Fig. 8, were superimposed on those, for reference, of CFN (○, (a) and (b)), respectively, in which ethanol was used as a blank for UV-Vis spectrophotometric analysis; Both CFNPLC and CFN were prepared with both chitosan solution of pH 6.0 (a); pH 3.7 (b) and FCMR of 1:1, and incubated in the release medium of the mixture of pH 7.4-PBS (60%) and ethanol (40%).

of tiny debris of CFNPLC including chitosan molecules, which generated extra turbidity, having higher affinity to curcumin [42] at pH 6.0 than at pH 3.7.

The total cumulative amount of curcumin-release, as shown in A-2 (Fig. 8(a) (or Fig. 9(a) (●))) and B-2 (Fig. 8(b) (or Fig. 9(b) (●))), was 0.33 mg and 0.23 mg, respectively. The CFNPLC yield of 5-15 mg indicates that the mass of CFNPLC-loaded curcumin was 0.12-0.35 mg, which covers the range of the cumulative amount of curcumin-release, as shown in A-2 (Fig. 8(a)) and B-2 (Fig. 8(b)). [The mass of CFNPLC-loaded curcumin was obtained as the mass of curcumin input (i.e., 1 mg) multiplied by the curcumin-loading efficiency (i.e., ca. 35%) and the ratio of input mass of CFNPLC in the release experiment (i.e., 5 mg) to the yield of CFNPLC (i.e., 5-15 mg).].

Thus, the curcumin-release behavior of CFNPLC using ethanol as a blank, as shown in Figs. 7 (△, ▲), resulted in the exaggerated payload of curcumin in CFNPLC, by as much as the cumulated amount, as shown in Figs. 9(a) (○) and (b) (○), respectively. Therefore, the curcumin-release behavior of CFNPLC, as shown in Figs. 8 A-2 and B-2, was superimposed on those converted from the absorbance of CFN release behavior, as shown in Figs. 9(a) (○) and (b) (○), respectively. As a result of the superimposition, the release behavior can be obtained, as shown in Fig. 9(a) (▼) and (b) (▼). The release behavior of Fig. 9(b) (▼) was almost identical to that in Fig. 7 (▲) (or Fig. 9(b) (▲)), while the release behavior of Fig. 9(a) (▼) was close to that in Fig. 7 (△) (or Fig. 9(a) (△)). Moreover, the difference between the release behavior of Fig. 7 (△) (or Fig. 9(a) (△)) and Fig. 9(a) (▼) may be explained by curcumin molecules being released from CFNPLC, producing tiny debris of CFNPLC including chitosan molecules, which generates extra turbidity, that have a higher affinity to curcumin [42] at pH 6.0 than at pH 3.7. Thus, the curcumin-release behavior of CFNPLC was verified quantitatively with the aid of superimposition, as shown in Fig. 9.

CONCLUSION

Curcumin was incorporated into o/w emulsions by dissolving it with soybean oil to cover the surface of the oil droplets in the o/w emulsions with a pH-sensitive polyelectrolyte complex composed of chitosan and fucoidan molecules and convert the o/w emulsions into a suspension with a dispersed phase of CFNPLC. The curcumin-loading efficiency of this suspension system and the size and zeta potential distributions of CFNPLC at various pH and FCMRs were investigated. At FCMR of 1 : 1, the curcumin-loading efficiency was highest at ca. 50% and lowest at ca. 25% at pH 3.0 and pH 2.0, respectively. Curcumin-loading efficiency of ca. 35% was achieved for CFNPLC with FCMR of 1 : 1 prepared with a chitosan solution of pH 3.7 and 6. The hydrogel particle size of CFNPLC ranged from 163 nm to 559 nm, which was similar to the size range of the chitosan-fucoidan NPs prepared in a previous study [18,19] by simple polyelectrolyte complexation with the same ranges of pH and FCMR. The zeta potential of CFNPLC increased in the pH order of 3.7, 3.0, 2.0, 5.0, and 6.0 at any given FCMR, which was the same as reported elsewhere [18]. In addition, the pattern of their zeta potential variation, in this study, according to FCMR, was also consistent with that in a previous study except for the magnitude of the zeta potential. The reduced CFNPLC zeta potential in this study was attributed to the entrapment of curcumin blended with soybean oil without an electric charge in CFNPLC, which did not occur in the previous study [18].

The CFNPLC preparation procedure was optimized and the prepared CFNPLC was characterized by FTIR spectroscopy, SEM, and TEM. In addition, the release of curcumin from CFNPLC prepared with a chitosan solution of pH 6.0 for one to two days was slower than that from CFNPLC prepared with a chitosan solution of pH 3.7 for five to 10 hrs. This was attributed to the higher affinity of a chitosan molecule to curcumin molecules at a higher pH. Thus, CFNPLC can bring about an enhanced effect of curcumin bioavailability, resulting from high curcumin stability because it was dissolved in soybean oil and sustained slow curcumin-release for days in the oral drug (curcumin) delivery system. Therefore, CFNPLC can be treated mainly as a food-functional additive or healthy dietary product for tumor patients.

The curcumin-release behavior of CFNPLC was explained. CFNPLC prepared with a chitosan solution at both pH 6.0 (A) and pH 3.7 (B) and FCMR of 1 : 1 released slowly and rapidly, respectively. Approximately 100% of the amount of curcumin encapsulated in the curcumin-release medium of the mixture of pH 7.4-PBS (60%) and ethanol (40%) was released. On the other hand, the CFNPLC released only 10% of the amount of curcumin encapsulated in the curcumin-release medium of the mixture at pH 3.0-PBS (60%) and ethanol (40%). In this study, the curcumin-release behavior of CFNPLC was confirmed quantitatively by the superimposition of the relevant release behavior.

Not to mention the high stability of curcumin blended with soybean oil, encapsulated in CFNPLC, the advantage of the CFNPLC is regarded to be a quite slow release of curcumin. In the curcumin release study of the CFNPLC, the medium containing CFNPLC was centrifuged as described by Hosseni et al. [22], to detach a released curcumin-droplet on the surface of CFNPLC at predetermined times. In this manner, the centrifugation of the media accelerated the release of curcumin droplet from the surface of CFNPLC into the release medium much faster than a conventional curcumin release without centrifugation. Therefore, the curcumin releasing period of a conventional curcumin delivery using CFNPLC is supposed to be extended for much longer time than one to two days of the curcumin release period in this study.

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