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Optimized stability retention of a monoclonal antibody in the PLGA nanoparticles

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ABSTRACT

Low efficiency and stability problems have been major issues in the formulation of engineered monoclonal antibodies (mAbs) for a variety of therapeutic uses, which may be severer for applying to encapsulation into nanoparticle (NP). In this study, the formulation and stabilizing conditions to encapsulate a potential mAb (3D8 scFv) into biodegradable poly(lactic-*co*-glycolic acid) (PLGA) NPs were investigated. And the effect of stabilizers on the stability of 3D8 scFv was investigated with the 3D8 scFv that was recovered from the primary emulsion in the double emulsion process. The conformational stability of the recovered 3D8 scFv was evaluated by circular dichroism (CD) and fluorescence spectroscopy. The DNA binding and hydrolyzing activities of the recovered 3D8 scFv were evaluated by enzyme-linked immunosorbent assay (ELISA) and agarose gel electrophoresis, respectively. The results conclude that mannitol was the most suitable stabilizer for retaining stability and activity of 3D8 scFv in the process of the PLGA NP preparation. Finally, obtained results suggest that this systematic process can provide efficient delivery system of 3D8 scFv as well as other potential mAbs or proteins for therapeutic uses, which is likely to be useful for intracellular delivery requiring sustained release.

1. Introduction

Therapeutic use of monoclonal antibody (mAb) is one of emerging areas of biopharmaceutical applications, in which eighteen approved mAbs were put on the market and applied over hundred times to clinical tests (Reichert et al., 2005). Owing to such efforts, more advanced technologies for the formulation and delivering of mAbs are required for related potential applications. For therapeutic target, adequate formulation and delivery systems of mAbs are requisite for preserving the unstable structure and controlled release of mAbs (Daugherty and Mrsny, 2006). Even though the injectable particle systems for protein delivery have been developed with microparticles/nanoparticles based on biodegradable polymers, such as poly(lactic-*co*-glycolic acid) (PLGA) (Cegnar et al., 2004; Alonso, 1996; Gaspar et al., 1998) and poly(lactic acid) (PLA) (Gref et al., 2001), the mAb delivery has not been applied to those yet.

Biodegradable microparticles have attracted much attention as carriers of various protein drugs because of controllable properties, which were mainly fabricated by double emulsion (water-in-oil-in-water, WOW) and evaporation–extraction method. But, this method has caused severe problems in drug stability, which is derived from exposure to a few stresses in the emulsification step (Daugherty and Mrsny, 2006; Cegnar et al., 2004). These stresses in general include WO interfaces, high pressure, temperature gradient, shear force, and free radicals, which cause aggregation, denaturation of mAbs and following inactivation of mAbs (Griebenow and Klibanov, 1996; Sah, 1999a,b). To resolve these stability problems, incorporating an additive into the degradable particle has been widely used (van de Weert et al., 2000a,b).

An anti-DNA mAb that has a DNA binding and hydrolyzing activity, denoted by 3D8 scFv, was reported recently by Kwon and coworkers (Kim et al., 2006). For therapeutic applications, the engineered 3D8 scFv of which the site of action is intracellular region should be delivered through cellular membrane into cytoplasm. Intracellular delivery of mAbs through systemic route requires delivery systems based on biodegradable nanoparticle (NP). However, the NPs may exhibit other problems in addition to the inactivation of mAb due to instability and lower loading efficiency, etc. (Cegnar et al., 2004; Alonso, 1996; Kang and Singh, 2003). Also, physical properties of NPs should be modulated to gain some benefits for the systemic delivery, such as enhanced permeation and retention (EPR) effect, longer half-life, efficient cellular uptake and lower renal clearance (Torchilin, 2006; Prabha et al., 2002; Vasir and Labhasetwar, 2007).

Antibody formulation is in general known to require individual condition and innovative techniques depending on their species, even though knowledge and technologies of the formulation of

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many potential antibodies have been accumulated (Alonso, 1996). In the present study, the optimized formulation conditions and stability retention to prepare the 3D8 scFv-loaded PLGA NPs with high therapeutic effect were investigated. To evaluate the retention of the 3D8 scFv stability, formulated 3D8 scFv is recovered from primary emulsion and characterized by a variety of methods, which enables to select an optimum stabilizer. We hypothesized that physical properties of PLGA NPs and the formulation efficiency can be optimized by modulating various formulation conditions. It is expected that the suggested systematic process containing the preparation of 3D8 scFv-loaded PLGA NP and evaluations of the 3D8 scFv stability and activity (Fig. 1) can suggest a prototype of the mAbs encapsulation into biodegradable NPs with high efficiency.

2. Materials and methods

2.1. Materials

Poly(lactic-*co*-glycolic acid) (PLGA) (RG504H) was purchased from Boehringer Ingelheim. Rabbit IgG, alkaline phosphatase-conjugated goat anti-rabbit IgG antibody, micro-BCA (bicinchoninic acid) reagent kit were purchased from Pierce Biotechnology. Bovine serum albumin (BSA), poly(vinyl alcohol) (PVA, 80% hydrolyzed), *p*-nitrophenyl phosphate (*p*-NPP), D-mannitol, D-(+)-trehalose dihydrate, (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD), trypsin protease, poly(ethylene glycol) (PEG, M_w = 600) were purchased from Sigma. Heparin sodium salt and sodium dodecyl sulphate (SDS) were purchased from Acros and Junsei chemical, respectively. Plasmid Miniprep kit was purchased from Intron Inc. 3D8 scFv mAb was provided from Kwon's group (Kim et al., 2006).

2.2. Preparation of 3D8 scFv-loaded PLGA NPs

3D8 scFv-loaded PLGA NPs were prepared using the solvent evaporation process involved the formation of double emulsion (w/o/w) according to a previous report, as shown in Fig. 1 (Alonso et al., 1993). 3D8 scFv (500 μg) was dissolved in 100 μl of 10 mM phosphate buffered saline (PBS) solution (pH 7.2) and subsequently various additives (10%, w/v) were added to the solution to stabilize 3D8 scFv. The mixture was then emulsified in 1 ml of methylene chloride solution containing PLGA (50 mg/ml) by sonication (70 W, 0 cycle, Ms73 tip, 60 s) using a homogenizer (Bandelin Sonoplus, UW2070) in an ice bath to form a primary w/o emulsion. Then, 2 ml of 3% (w/v) aqueous PVA solution was added to the emulsion and the mixture was re-emulsified by sonication (70 W, 0 cycle, Ms73 tip, 60 s) in ice bath. The resulting secondary w/o/w emulsion was then poured into 100 ml of a 0.3% (w/v) aqueous PVA solution under vigorous agitation to remove organic solvent for 1 h. The solution was filtered through bottle top filter with pore size of 0.45 μm and the filtrate was ultracentrifuged at 21,000 rpm for 20 min at 4 °C to isolate PLGA nanoparticle, followed by washing several times with distilled water and lyophilizing.

2.3. Characterizations of 3D8 scFv-loaded PLGA NPs

The 3D8 scFv content in PLGA NP was determined by hydrolysis method (Hora et al., 1990). Briefly, 5 mg of lyophilized PLGA NP was digested in 4 ml of a 0.1N aqueous NaOH solution containing 5% sodium dodecyl sulfate (SDS) at room temperature for 15 h until a clear solution was obtained. The loading amount of 3D8 scFv was determined by the BCA protein assay, which was also converted to loading efficiency. The size of PLGA NPs was measured using dynamic light scattering (DLS, FPAR-1000, Photal, Japan). Freeze-dried PLGA NPs were dispersed in distilled water by bath-sonication. The surface morphology of the PLGA NPs was observed using scanning electron microscopy (SEM, JEOL/JSM-6380, Japan). Freeze-dried PLGA NPs were dried under vacuum for overnight and coated with gold–palladium under an argon atmosphere before the SEM measurement.

2.4. Recovery of 3D8 scFv from the primary emulsion

Fig. 1 shows the recovery process of 3D8 scFv in the middle of the preparation process of PLGA NPs. In the process of primary emulsification, 3D8 scFv was extracted from the organic phase by adding 4 ml of PBS and PEG solution and then centrifuged at 3000 rpm

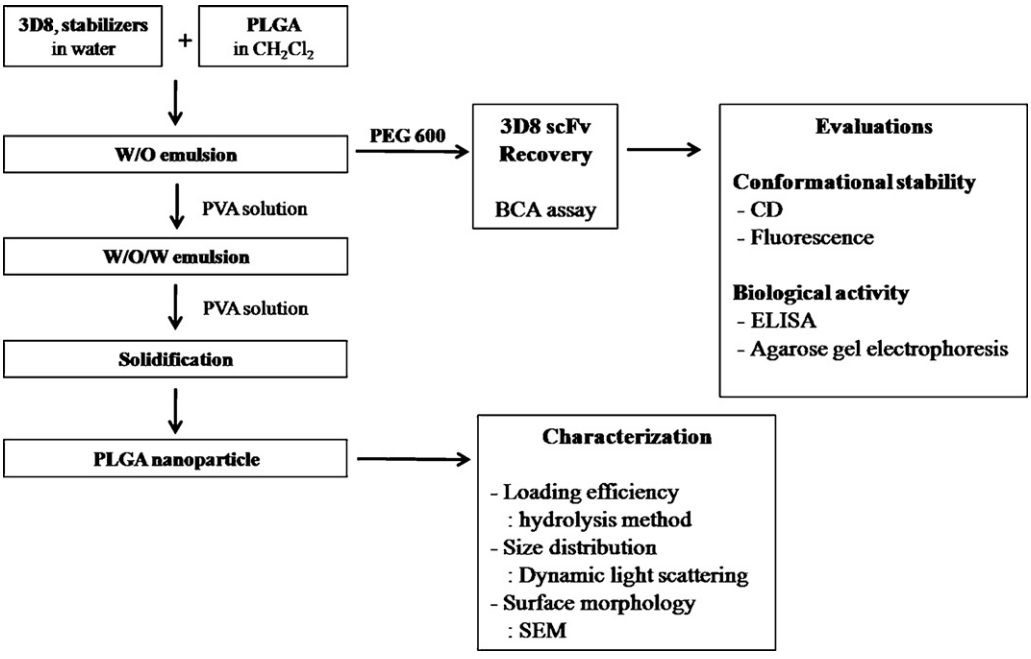

Fig. 1. Processes of 3D8 scFv formulation with PLGA and 3D8 scFv recovery for stability evaluation.

Table 1
The effect of PLGA M_w, polymer concentration and 2nd sonication time on the size of PLGA NPs.

PLGA (M_w)	Polymer concentration (mg/ml)	Sonication time (s)		Size[a] (nm)
		1st	2nd	
50,000	50	10	10	421 ± 21
	50	10	30	265 ± 15
	50	10	60	178 ± 32
	35	10	30	242 ± 20
100,000	50	10	30	350 ± 12

[a] Mean ± S.D. (n = 3).

for 15 min to accelerate phase separation. The recovered aqueous phase was used to evaluate stability and activity of 3D8 scFv.

2.5. Circular dichroism and fluorescence spectroscopy

The secondary structural stability of recovered 3D8 scFv was analyzed in quartz cells with 1 mm path lengths using circular dichroism (CD, JASCO 810 W, Japan). CD spectra were recorded in an average of 10 scans between 190 and 260 nm, with a 1.0 nm bandwidth, a scanning rate of 10 nm/min, a wavelength step of 0.2 nm and a time constant of 2 s. CD band intensities were expressed as molar ellipticities, $[\theta]$ M, in degrees cm^2 $dmol^{-1}$. The extents of secondary structures of 3D8 scFv were determined from the ellipticities using K2d software (Sreerama et al., 2000, 1999; Johnson et al., 1999; Bohm et al., 1992). Fluorescence emission spectra were obtained using a spectrofluorometer (JASCO FP-6500, Japan) to analyze tertiary structures of recovered 3D8 scFv. The samples were excited at 280 nm and the emission spectra were recorded at 300 nm. All spectra measurement were carried out at 25 °C and corrected by subtracting the contribution from the blank solution or emulsion.

2.6. DNA binding assay by enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was carried out using to evaluate DNA binding activity of 3D8 scFv as our previous report (Kim et al., 2006). The 96-well polystyrene microtiter plate was coated with 100 μl of oligodeoxynucleotide with the concentration of 10 μg/ml in a solution (TBS) containing 50 mM Tris–Cl (pH 7.5) and 50 mM NaCl for overnight at 4 °C and washed three times with TBS containing 0.05% Tween-20 (TBST), followed by blocking with TBS containing 3% (w/v) bovine serum albumin (BSA) for 1 h at 37 °C. Then, 3D8 scFv (100 μl of 20 μg/ml) was added to the blocked plate and incubated for 1 h at 37 °C. After washing with TBST, the plate was incubated with rabbit IgG (100 μl of 1 μg/ml) and then with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (100 μl of 1:10,000 dilution). Each incubation step was carried out for 1 h at 25 °C, followed by washing three times with TBST. Finally, *p*-nitrophenyl phosphate (*p*-NPP) solution (1 mg/ml in 0.1 M glycine, 1 mM $ZnCl_2$ and 1 mM $MgCl_2$, pH 10.3) was added to each well and the UV absorbance was recorded at 405 nm with a multi-plate reader.

2.7. DNA hydrolyzing assay by agarose gel electrophoresis

Agarose gel electrophoresis was carried out to investigate the DNA hydrolyzing activity of recovered 3D8 scFv. The pUC19 plasmids were prepared by Plasmid Miniprep kit and used as substrate of 3D8 scFv. More than 95% of the purified pUC19 plasmids existed as a supercoiled form, estimated by 0.7% agarose gel electrophoresis. DNA hydrolyzing reaction was initiated by mixing 3D8 scFv (0.8 μM) with the substrate (2.2 nM) in TBS containing 2 mM $MgCl_2$ or 50 mM EDTA. In all cases, the ionic strength of reaction batch was maintained at 150 mM by adjusting NaCl concentration in the TBS buffer. Enzyme reactions were retained for 1 h at 37 °C and terminated by trypsin protease (20 μg/ml) treatment for 1 h at 37 °C. Samples were loaded on 0.7% agarose gels and stained with ethidium bromide.

3. Results

3.1. The effect of formulation parameters on physical properties of PLGA NPs

The effect of a variety of formulation parameters on physical properties and encapsulation efficiency of PLGA NPs was investigated. In preliminary test, increases in the sonication power showed a tendency to produce smaller and more uniform PLGA NPs without the loss of 3D8 scFv activity (data not shown). Therefore, the sonication power was fixed at the maximum level (70 W) to prepare smaller PLGA NPs. The effect of the PLGA molecular weight on the particle size was investigated. In Table 1, the lower molecular weight (M_w = 50,000) of PLGA provided smaller PLGA NP than the higher one (M_w = 100,000) under the same other conditions. Table 1 also presents the influence of PLGA concentration on the particle size. Under the same other parameters, PLGA concentration did not mainly affect the size of PLGA NPs. On the other hand, the sonication times considerably influenced the size of PLGA NPs. In Table 1, increasing secondary the sonication time resulted in decreases in the size of PLGA NPs at the same primary sonication time. This result means that the secondary sonication time plays an important role to modulate the size of PLGA NPs and enough sonication time is required to prepare smaller PLGA NPs. The influence of the primary sonication time on the size of PLGA NPs can be confirmed in Table 2. Under the same secondary sonication time (60 s), there

Table 2
The effect of sonication times on size, loading amount and loading efficiency of PLGA NPs.

1st sonic time (s)	2nd sonic time (s)	Size (nm)[a]	Loading amount (μg/mg)[a]	Loading efficiency (%)
10	60	189 ± 12	0.45 ± 0.08	4.47
30	60	210 ± 09	0.59 ± 0.04	5.87
60	10	612 ± 25	0.70 ± 0.04	6.98
60	30	450 ± 32	0.68 ± 0.02	6.77
60	60	198 ± 14	0.72 ± 0.07	7.17

[a] Mean ± S.D. (n = 3).

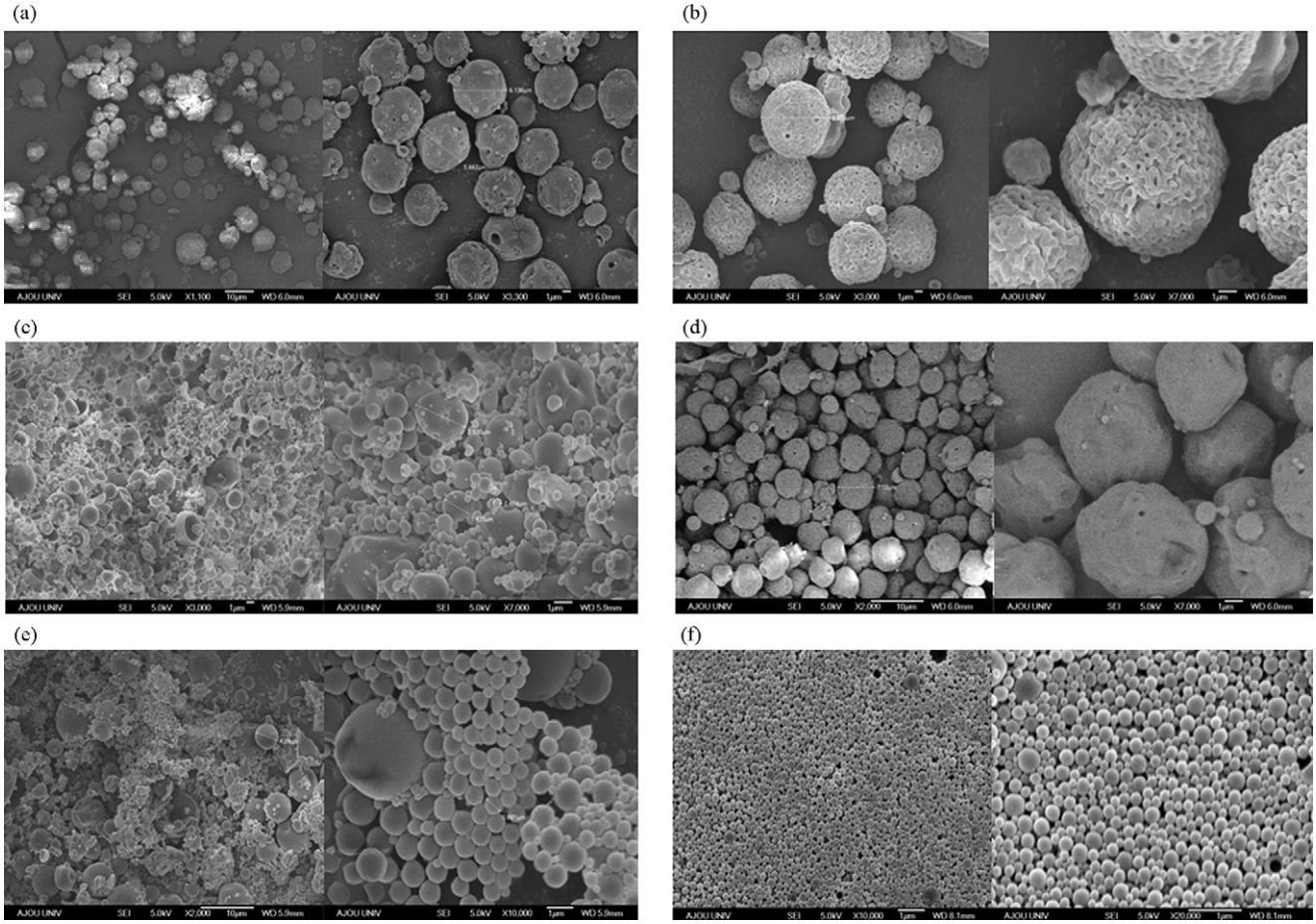

Fig. 2. SEM images of 3D8 scFv loaded PLGA NPs prepared in different experimental conditions. (a) Experimental control: PLGA M_w = 50,000, PLGA/MC ratio (50 mg/ml), sonication tip (Ms73), primary (1st) and secondary (2nd) sonication time (10 and 20 s), (b) PLGA/MC ratio (35 mg/ml), (c) 2nd sonication time (30 s), (d) PLGA M_w (100,000), (e) 1st sonication time (60 s), 2nd sonication time (60 s), (f) optimized preparation conditions: PLGA M_w (50,000), PLGA/MC ratio (50 mg/ml), sonication tip (TT 13), 1st and 2nd sonication time (60 and 60 s), filtration. In (b) and (c), all other experimental conditions are same with those of experimental control (a).

were no any correlation between the primary sonication time and the size of PLGA NPs. Therefore, it is concluded that the secondary sonication time is the predominant parameter to control the size of PLGA NPs and the longer time provide smaller PLGA NPs.

The SEM images exhibit the effect of formulation parameters on the surface morphology and size distribution of PLGA NPs (Fig. 2). As shown in Fig. 2a, the control sample showed quite rough surfaces and heterogeneous size distribution of PLGA NPs. The experimental sample (Fig. 2b) that prepared with the lower PLGA concentration (35 mg/ml) did not show improved results in surface roughness and size distribution as compared with the control sample. However, the sample (Fig. 2c) that prepared by changing only the secondary sonication time to 60 s at conditions of the control sample showed smaller sizes of PLGA NPs, even though it still exhibited heterogeneous size distribution, which was also confirmed the effect in Tables 1 and 2. And Fig. 2d shows images of the PLGA NP prepared with the higher molecular weight of PLGA (M_w = 100,000). The result exhibited PLGA NPs with the smoother surface and more homogeneous size distribution. However, the higher molecular weight of PLGA induced an increase in sizes of PLGA NPs as described above. As shown in Fig. 2e, the smaller PLGA NPs having homogeneous size distribution were produced from increasing both the primary and secondary sonication times to 60 s. Based on these results, the formulation parameters were optimized and the result are shown in Fig. 2f. In this sample, the filtration process was carried out to remove the reduced minor portion of quite larger PLGA NPs that is produced at any formulation conditions.

3.2. The effect of formulation parameters on the loading efficiency of 3D8 scFv

The influence of some formulation parameters on the loading efficiency (LE) of 3D8 scFv was investigated. The primary sonication time affected the loading efficiency of 3D8 scFv. In Table 2, increasing the primary sonication time at the same secondary sonication time (60 s) induced increases in the LE of 3D8 scFv, whereas the secondary sonication time did not affect the LE. Therefore, it can be inferred that the LE of 3D8 scFv would be dominated already at the primary emulsification step. This is probably due to the large size of primary emulsion because too large sized primary emulsion cannot be entrapped into secondary emulsion.

The LEs of 3D8 scFv were obtained from the loading amount (LA) of the 3D8 scFv that was isolated from PLGA NPs through two recovery processes. In Table 3, the hydrolysis method recovers significantly larger amounts of 3D8 scFv than the extraction method. This result indicates that hydrolysis method is more suitable for investigating the LE of 3D8 scFv. The addition of NaCl salt to the secondary emulsion also influenced the LE of 3D8 scFv. The addition of the salt is not so important parameter to optimize the formulation, even though the addition is favorable for the improvement of the LE of 3D8 scFv.

Table 3
The effect of protein recovery method and salt on size, loading amount and loading efficiency of PLGA NPs.

Recovery method	NaCl (0.05 M)	Size (nm)[a]	Loading amount (μg/mg)[a]	Loading efficiency (%)
Hydrolysis method	+	180 ± 21	10.5 ± 0.08	105
	−	180 ± 21	9.6 ± 0.04	96
Extraction method	+	180 ± 21	2.9 ± 0.02	29
	−	180 ± 21	2.5 ± 0.06	25

[a] Mean ± S.D. (n = 3).

Table 4
The recovery amount and rate of 3D8 scFv recovered from the primary emulsion containing PLGA depending on additive.

Additive[a]	with PLGA		without PLGA	
	Recovered amount (μg/ml)[b]	Recovered rate (%)	Recovered amount (μg/ml)[b]	Recovered rate (%)[b]
+Control[c]	60.6 ± 0.02	100.0	60.5 ± 0.02	100.0
−Control[d]	55.6 ± 0.05	93.0	56.2 ± 0.01	92.9
Heparin	56.6 ± 0.01	93.4	57.8 ± 0.01	95.5
HP-β-CD	57.2 ± 0.01	94.4	57.2 ± 0.04	94.5
Trehalose	57.0 ± 0.02	94.1	58.4 ± 0.01	96.5
Mannitol	60.0 ± 0.01	99.0	59.6 ± 0.01	98.5
PEG	55.0 ± 0.04	90.6	56.0 ± 0.05	92.6

[a] Nominal content relative to internal aqueous solution is 10% (w/v).
[b] Mean ± S.D. (n = 3).
[c] +Control is intact 3D8 scFv.
[d] −Control is formulated 3D8 scFv without stabilizer.

3.3. Recovery of 3D8 scFv from the primary emulsion

Table 4 presents the recovery amounts and rates of 3D8 scFv from the primary emulsion in the presence and absence of PLGA. All conditions revealed relatively the high recovery rates of over 90%, regardless of the existence of PLGA and additives in the primary emulsion. The recovery rate in the primary emulsion in which PEG was used as an additive was the lowest, whereas the case of mannitol was the highest. However, no significant changes in the recovery rates of 3D8 scFv were observed by adding additives. This means that the recovery method is effective to evaluate the stability and activity of 3D8 scFv.

3.4. The effect of additives on the conformational stability of 3D8 scFv

Fig. 3 shows Far-UV CD spectra of intact 3D8 scFv and the 3D8 scFv formulated along with mannitol. Two samples shows quite similar shape, even though the intensity was not adjusted to a similar level due to the use of different concentrations. In the spectra, wavelengths at the maximum ellipticity of each sample were obtained and summarized in Table 5 along with other samples in the same manner. Table 5 presents the ratio of secondary structures of 3D8 scFv mAbs at each additive. Ratios of α-helix, β-sheet and random coiled structures in native 3D8 scFv were 7, 49 and 44%, respectively. In case of 3D8 scFv without additives (negative control) revealed almost similar ratios of secondary structures with those of native 3D8 scFv, 8, 48 and 44%, respectively. This result demonstrates that secondary structures of 3D8 scFv were nearly stable under primary emulsification. But, in cases of trehalose and PEG, the ratios of α-helix structure somewhat decreased and those of random coiled structure a little increased, compared with those in other additives. These results indicate that trehalose and PEG might destabilize rather than stabilize secondary structures of 3D8 scFv. Fig. 4 shows fluorescence emission spectra of 3D8 scFv depending on stabilizer species. Each spectrum shows different fluorescence intensity because concentrations of recovered 3D8 scFv were different at all additives. Since the fluorescence intensity is irrelevant to tertiary structure changes of 3D8 scFv, our analysis is focused on variations in emission wavelength maxima. Table 6 summarizes emission wavelength maxima in fluorescence spectra of 3D8 scFv with different additives. The emission wavelength maximum of native 3D8 scFv was 337 nm. This indicates

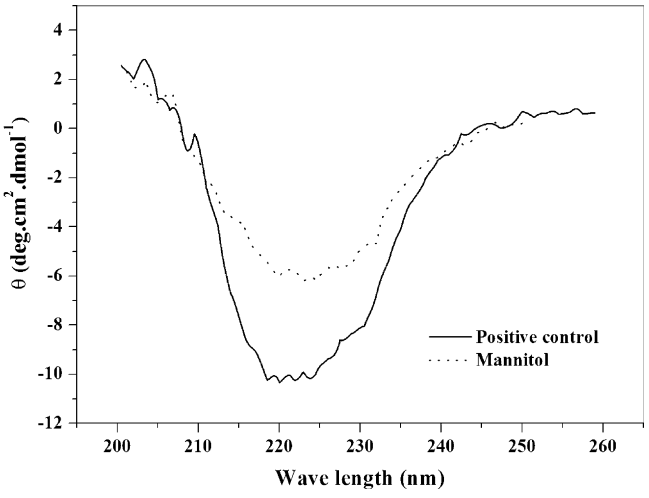

Fig. 3. Far-UV spectra of the 3D8 scFv mAbs recovered from primary emulsion containing PLGA.

Table 5
The ratio of secondary structures of the 3D8 scFv recovered from the primary emulsion containing PLGA depending on additive species.

Additive	α-Helix (%)[a]	β-Sheet (%)[a]	Random coiled (%)[a]
+Control[b]	7 ± 2	49 ± 2	44 ± 2
−Control[c]	8 ± 1	48 ± 1	44 ± 1
HP-β-CD	9 ± 1	47 ± 1	44 ± 1
Heparin	7 ± 1	49 ± 1	44 ± 1
Trehalose	4 ± 2	48 ± 2	48 ± 2
Mannitol	8 ± 2	48 ± 2	44 ± 2
PEG	4 ± 1	48 ± 1	48 ± 1

[a] Mean ± S.D. (n = 3).
[b] +Control is intact 3D8 scFv.
[c] −Control is formulated 3D8 scFv without stabilizer.

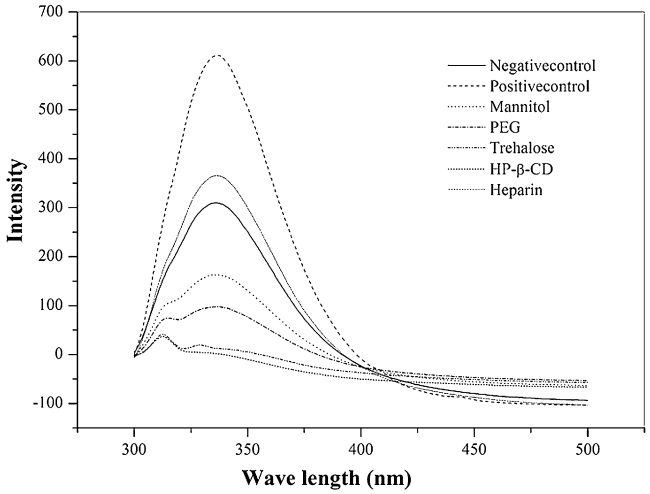

Fig. 4. Fluorescence emission spectra of the 3D8 scFv mAbs recovered from primary emulsion containing PLGA.

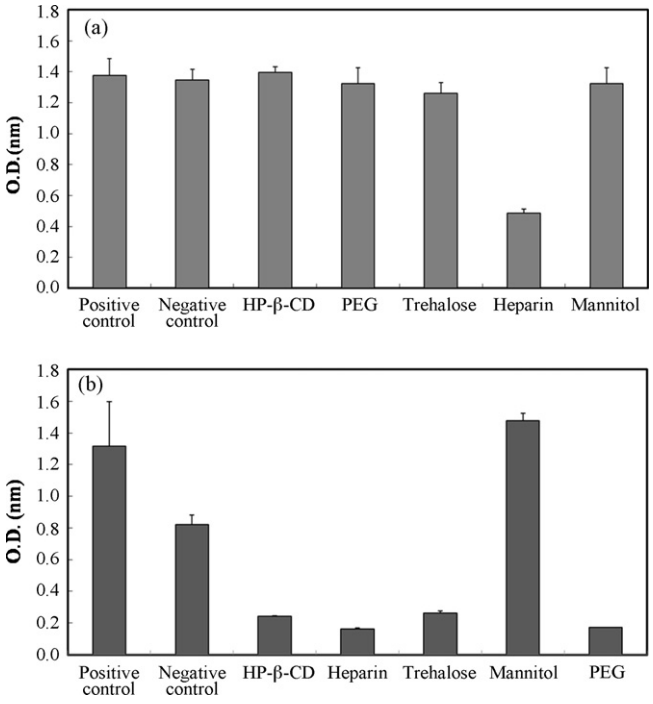

Fig. 5. The DNA binding activity of recovered 3D8 scFv in the absence (A) and presence (B) of PLGA, evaluated by ELISA.

that the tryptophan residue of 3D8 scFv is close to the surface of the protein and is partly exposed to external environments (Sharma and Kalonia, 2003; Eftink, 1994). In cases of heparin, mannitol and PEG, the emission wavelength maxima were around 337 nm. This fact showed that environment of tryptophan residues in 3D8 scFv was not changed, meaning maintaining tertiary structure of 3D8 scFv. However, in cases of other additives, the emission wavelength maxima were red-shifted. In an unfolded protein, the emission wavelength maximum is red-shifted, indicating that tryptophan residues of proteins moved to more polar environment (Kang et al., 2002).

3.5. The effect of additives on the biological activity of 3D8 scFv

Fig. 5 shows DNA binding activity of 3D8 scFv measured by ELISA. In the absence of PLGA in primary emulsion (Fig. 5a), all additives except heparin revealed similar levels in comparison with intact 3D8 scFv, indicating that most additives did not influence on DNA binding activity of 3D8 scFv. Only heparin showed negative effect on the activity under primary emulsion without PLGA. More interestingly, in the presence of PLGA, only mannitol exhibited similar levels of activity against intact 3D8 scFv (positive control). DNA hydrolysis assay provides data exhibiting the extent of DNA hydrolyzing by 3D8 scFv in agarose gel as shown in Fig. 6. DNA is visualized in the gel by the addition of ethidium bromide (symbol 'E' in Fig. 6) that binds to DNA by intercalating between the bases. When 3D8 scFv hydrolyze DNA, the visualized bands should be observed in regions of lower molecular weight, indicating that supercoiled DNA was hydrolyzed to uncoiled state. In control (intact 3D8 scFv), supercoiled band of DNA was not observed, indicating native DNA hydrolyzing activity of 3D8 scFv. Fig. 6 shows that most effective one among various additives was mannitol, which is consistent with results of structural stability and DNA binding activity.

4. Discussion

The control of the size, size distribution and surface morphology is very important for quality, therapeutic effects and biodistribution of PLGA NP. The modulation of the optimal formulation carried out in our study is in order to provide the most suitable combination of good morphology, proper particle size and high drug loading efficiency. Small NPs are preferable because they allow a variety of routes of administration, e.g. intravenous, oral, nasal, ocular, and transdermal routes, etc. (Alonso, 1996). However, decreasing the NP size should be carefully considered because the sonication time for the control of the NP size could have influence on the reduction of protein stability (McClean et al., 2004). To obtain the high loading efficiency is also fundamentally an important parameter for the therapeutic effect using NPs. Sah et al. reported (Sah, 1997) that the extraction methods usually underestimate the actual amount of protein in microspheres because of incomplete extraction of proteins and their loss during the procedures. They also demonstrated that the hydrolysis method was a great advantage in recovering proteins without any loss due to experimental processes.

Even though non-invasive extraction techniques are required to evaluate stability and activity of many potential proteins and Abs, suitable extraction techniques for the recovery of proteins and Abs from the primary emulsion have been little explored (Sah, 1999a,b; Kang and Singh, 2003; Kang et al., 2002). Activity of incorporated proteins may be under- or overestimated when extraction media, such as methylene chloride or dimethyl sulfoxide, dissolve protein

Table 6
Fluorescence results of the 3D8 scFv recovered from primary emulsion containing PLGA depending on additive species.

Additive	+Control[b]	−Control[c]	HP-β-CD	Heparin	Trehalose	Mannitol	PEG
λ_{em}[a] (nm)	337.0	335.6	312.6	335.2	313.0	335.7	336.9

[a] Emission wavelength maximum.
[b] +Control is intact 3D8 scFv.
[c] −Control is formulated 3D8 scFv without stabilizer.

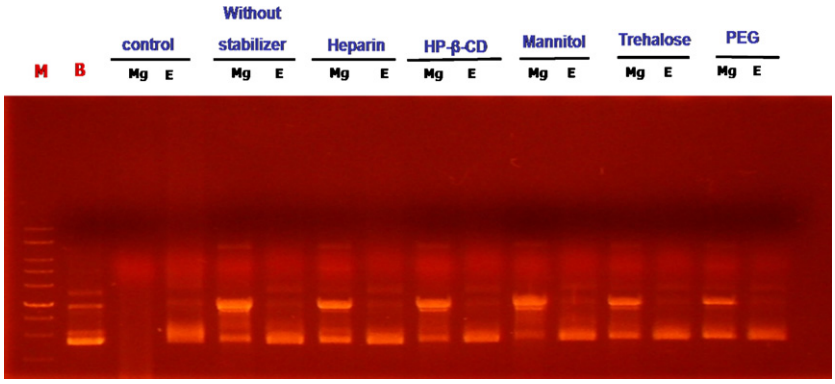

Fig. 6. The DNA hydrolyzing activity of recovered 3D8 scFv from primary emulsion containing PLGA, evaluated by agarose gel electrophoresis. M (Marker), B (Buffer control: DNA, Mg, TBS).

aggregates or cause aggregate formation. Also, irreversible conformational changes of proteins may occurred by the extraction media (Castellanos et al., 2002). The efficiency of protein recovery is generally low (Kang et al., 2002; van de Weert et al., 2000a,b; Johansen et al., 1998; Sharif and O'Hagan, 1995; Sah, 1997) because PLGA tends to precipitate out with encapsulated protein due to the addition of excess buffer (van de Weert et al., 2000a,b). Therefore, in the first step of extraction method for 3D8 scFv recovery, PEG was added to the primary emulsion for the effective breakdown of the emulsion because PEG can be miscible with both water and extraction media (van de Weert et al., 2000a,b). In our study, both cases exhibited high recovery rates. These results indicate that PLGA did not nearly affect recovery rates of 3D8 scFv, not consistent with other previous results (Castellanos et al., 2002; van de Weert et al., 2000a,b). This is probably attributed to the nature of 3D8 scFv. Our fluorescence results that will be in detail described showed that emulsification process induce only denaturation of 3D8 scFv. This result indicates that 3D8 scFv is more susceptible to denaturation than aggregation by various exterior physico-chemical stresses. Therefore, in case of the recovery of 3D8 scFv was comparatively high as compared with other proteins or Abs. Furthermore, several studies have shown that the degree of recovery from protein aqueous solution indicates the extent of protein adsorption at the W/O interface (Castellanos et al., 2002; van de Weert et al., 2000a,b; Johansen et al., 1998). But, significant changes in the recovery rates of 3D8 scFv were not observed depending on stabilizer species including sugars, polyols and hydrophilic polymer. Structural stability of 3D8 scFv recovered from primary emulsion was evaluated by CD and fluorescence analysis. Circular dichroism is an excellent method to analyze the conformation of proteins and peptides (Greenfield et al., 1996). The far-UV spectrum of CD generally reflects secondary structures of protein (Sreerama et al., 1999; Johnson, 1999). Various empirical methods and analyses have been developed for quantitative estimation of the secondary structure content of protein (Bohm et al., 1992; Sreerama et al., 2000; Fink, 1995). Effects of sugars, such as mannitol, sucrose and trehalose, on protein stability in emulsion are controversial (Sah, 1999a,b; Kang and Singh, 2003). Effects of trehalose on human growth hormone and Tetanus Toxoid during emulsification without PLGA were reported (van de Weert et al., 2000a,b; Johansen et al., 1998). According to the reports, stabilizing ability of sugars depended on protein species. Therefore, it is suggested that the evaluation to select optimal additives should be carried out depending on protein species in the CD and fluorescence analysis. In our case, the extent of variations in the secondary structure was very small and under limited ranges of error. Through this experiment, we can conclude that primary emulsification process was not a major cause of the damage of native secondary structure of 3D8 scFv even in the presence of PLGA, indicating almost no denaturation of 3D8 scFv. Although there were nearly no changes in secondary structure of 3D8 scFv, evaluation on structural stability of 3D8 scFv is not reliable (Sharma and Kalonia, 2003; Greenfield, 1996). So, fluorescence analysis was carried out to analyze variations in tertiary structure of 3D8 scFv. Generally, fluorescence analysis is used to probe the tryptophan environments during the equilibrium unfolding process. Monitoring the intensity and emission maximum of tryptophan reveals the relative polarity change of the probe during the unfolding process (Jorgensen et al., 2004). In our fluorescence analysis, we showed that heparin, mannitol and PEG maintained tertiary structure of 3D8 scFv. However, in cases of other additives, the emission wavelength maxima were red-shifted indicating that tryptophan residues of proteins moved to more polar environment (Kang et al., 2002). So, our fluorescence analyses demonstrate that damaged structures of 3D8 scFv under primary emulsification were derived from only denaturation. The biological activity of proteins is closely related with the conformational state. Our group reported that 3D8 scFv has hydrolyzing activities using both double-stranded and single-stranded DNA substrates (Kim et al., 2006). ELISA results depicts that mannitol effectively retained biological activity of 3D8 scFv during primary emulsification and other additives have negative effects on the activity even than the case of no additive. This result is roughly agreement with the results obtained by CD and fluorescence analyses. Some studies have reported that interfacial denaturation highly depends on protein concentration (Johansen et al., 1998; Tambera et al., 2005; Kim et al., 2005) because limited amount of protein irreversibly adsorbed to the interface, in which protein behave as 'self-protectant' at higher concentration (Sah, 1996; van de Weert et al., 2000a,b; Tambera et al., 2005). Most experiments about the stability of protein, high concentration of protein (20–100 mg/ml) were used (Sah, 1996; van de Weert et al., 2000a,b).

The DNA hydrolysis assay suggests that mannitol is the most effective one among various additives, which is consistent with results of structural stability and DNA binding activity. However, trehalose of which the structure is similar with mannitol had no effect or even negative effect on biological activity of 3D8 scFv. These results are consistent with results about structural stability which were obtained by CD and fluorescence analysis. Heparin did not have any effect on biological activity of 3D8 scFv. This result is probably inferred from as follows. Since anionic heparin chain can enclose positively charged surface of 3D8 scFv, 3D8 scFv might be protected from W/O interface by the enclosed heparin chain, resulting in retained structure of 3D8 scFv. But, the strong affinity between heparin and 3D8 scFv might hamper intrinsic ability of 3D8 scFv, resulting in negative effect on biological activ-

ity of 3D8 scFv. Kim and coworkers reported that poly(ethylene glycol)/poly(L-histidine) diblock copolymer (PEG-PH) forms ionic complexes with negatively charged BSA and largely improves the stability (Kim et al., 2005). However, their results may be controversial because they focused on only structural stability of protein via spectroscopic analyses. HP-β-CD is known as a promising stabilizer of protein during primary emulsification (Sah, 1996; Kang et al., 2002). Stabilizing property of HP-β-CD is based on ability to shield hydrophobic amino acid residues of proteins. The hydrophilicity of proteins would increase after the aromatic rings of tryptophan, tyrosine, and phenylalanine inserting into the hydrophobic cavity of CD. Such an increase in the hydrophilicity keeps proteins away from O/W interface, which reduces the aggregation and denaturation of proteins (Sah, 1996). Although PEG is a hydrophilic polymer used as stabilizing agent, our results did not suggest any effects on stability and activity. It was reported that PEG prevented structural perturbation of protein because of preventing protein adsorption on the O/W interface (Castellanos et al., 2003).

In summary, the aim of this study was to investigate the optimized formulation and stability retention of 3D8 scFv for the encapsulation into PLGA NPs. An optimized method was introduced to adjust the processing parameters to give small size NPs, high loading efficiency and to preserve the biological activity of 3D8 scFv. The small particle size (~210 nm), homogeneous size distribution and smooth surface morphology were controlled well by sonication time to produce secondary (w/o/w) emulsion, polymer M_w and concentration. The sonication time to produce secondary emulsion was a main parameter to produce smaller particle size. The higher loading efficiency was resulted from the longer sonication time to produce primary (w/o) emulsion and the addition of NaCl to external aqueous phase. Moreover, hydrolysis method was a great advantage – outstanding differences up to 76% comparing with extraction method – in recovering proteins without any loss due to experimental processes.

And through the systemic evaluations of maintaining the structural stability (CD, fluorescence spectroscopy) and biological activity (ELISA, agarose gel electrophoresis), we revealed that the addition of mannitol to internal aqueous phase as an additive was a very effective way to preserve the stability of 3D8 scFv. Finally, obtained results suggest that this systematic process can provide efficient delivery system of 3D8 scFv as well as other potential mAbs or proteins for therapeutic uses, which is likely to be useful for intracellular delivery requiring sustained release.

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