

## Selection of the solvent system for the preparation of poly(D,L-lactic-co-glycolic acid) microspheres containing tumor necrosis factor-alpha (TNF- $\alpha$ )

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### Abstract

Dichloromethane (DCM) and acetonitrile (ACN) are the most commonly used solvents for polylactic acid (PLA) or poly(lactic-co-glycolic acid) (PLGA). In order to select a suitable solvent system for the preparation of PLGA microspheres containing tumor necrosis factor-alpha (TNF- $\alpha$ ), the stability of TNF- $\alpha$  when mixed with DCM and ACN under various phase conditions was investigated. When the TNF- $\alpha$  solution was emulsified into DCM to form a W/O emulsion prior to solvent evaporation using the W/O/W technique, a significant loss in activity of TNF- $\alpha$  was found. When the TNF- $\alpha$  was dispersed as a dry powder in the DCM phase, the protein was inactivated due to immediate hydration under the conditions of the solid/O/W system. Since TNF- $\alpha$  also was inactivated in a buffered saline containing ACN, the stability of the protein in microspheres prepared from an anhydrous solvent system was studied using ACN as the polymer solvent. Multiphase microspheres prepared by an anhydrous multiple emulsion process had a significantly higher loading efficiency of intact TNF- $\alpha$  than conventional matrix-type microspheres prepared by an anhydrous method using TNF- $\alpha$  powder and ACN. © 1998 Elsevier Science B.V.

**Keywords:** Acetonitrile; Anhydrous solvent evaporation system; Dichloromethane; Matrix-type microspheres; Multiphase microspheres; Poly(D,L-lactic-co-glycolic acid); Stability of TNF- $\alpha$

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### 1. Introduction

Many drugs, including insulin, the interferons, the interleukins and tumor necrosis factor (TNF), are now synthesized in large quantities by gene

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recombination techniques. In many cases, the desired therapeutic efficacy of these drugs requires multiple administration since these macromolecules have a short biological half-life when administered intravenously. Various kinds of biocompatible devices for sustained and controlled release of drugs have been investigated. Silicone was extensively studied as an implant material for sustained release of contraceptive steroids (Chien, 1983). However, implanted devices of non-degradable materials must be removed surgically after therapy has been completed or after the supply of drug from the device has been depleted. Biodegradable and biocompatible polymeric materials can be used in the design of implantable sustained release devices which do not require surgical removal. Such erodible composites may take the form of pellets (Stewart II and Schellekens, 1986; Yamakawa et al., 1990), rods (Sanders et al., 1986) and microspheres (Kreuter and Liehl, 1981; Gautier et al., 1992) for the delivery of biologically active peptides, proteins and other molecules.

Poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are currently the most widely used biodegradable polymers in implantable sustained release devices. For poorly water-soluble drugs, an O/W solvent evaporation method has been successfully employed to prepare microspheres of high drug content. For water soluble drugs, drug loading will decrease due to the partitioning of the drug into the external aqueous phase (Bodmeier and McGinity, 1987a,b). Several researchers have made modifications to the conventional solvent evaporation method and have successfully prepared PLA or PLGA microspheres which contain water-soluble compounds such as quinidine sulfate (Bodmeier and McGinity, 1987a,b), luteinizing hormone-releasing hormone (LHRH), and agonists (Csernus et al., 1990; Ogawa et al., 1988). Other biodegradable microspheres containing steroids (Beck et al., 1985; Redmon et al., 1989), anticancer agents (Juni et al., 1985), antibiotics (Sampath et al., 1992) and other peptides (M-Garcia et al., 1988; Wakiyama et al., 1992) have also been reported.

The water-in-dichloromethane (DCM)-in-water (W/O/W) emulsion technique has been the pre-

ferred method for the encapsulation of water-soluble drugs with the solvent evaporation technique (Heya et al., 1991; Yamakawa et al., 1992) and has resulted in higher loading efficiency of such drugs than the O/W process. The oil-in-oil (O/O) emulsion system was reported by Jalil and Nixon (1989, 1990) to prepare PLA microspheres with a higher drug loading efficiency than those obtained with the W/O/W emulsion system. These workers used mineral oil as the continuous external phase of the solvent evaporation medium and ACN as the dispersed oil phase. The PLA microspheres contained phenobarbitone as the model drug.

A knowledge of drug stability during the preparation of biodegradable microspheres, particularly for proteins and peptides, is important since drug stability may be compromised both by the pH of the medium for preparation of a dosage form and the physical conditions of the process. The objectives of this study were to investigate the influence of solvent systems on the stability of a protein drug and to develop a successful method to prepare the microspheres and to optimize drug loading in the microspheres. TNF- $\alpha$  was selected as the model protein, since it is highly water soluble and chemically unstable. The solvent effects on the stability of TNF- $\alpha$  in solution and in a dried powder form, when dispersed in either DCM or ACN, were investigated in order to select an optimized method to prepare PLGA microspheres with minimal loss of activity of the TNF- $\alpha$ . PLGA microspheres containing TNF- $\alpha$  powder dispersed in the polymer matrix (matrix-type microspheres) were prepared by the O/W, W/O/W and O/O emulsion solvent evaporation methods to investigate the effect of the solvents on the stability of the protein. The multiphase microspheres including TNF- $\alpha$  were also prepared by the multiple emulsion solvent evaporation system reported by Iwata and McGinity (1992, 1993).

## 2. Materials and methods

### 2.1. Chemicals

TNF- $\alpha$  ( $M_r$  17 000 by SDS–polyacrylamide gel electrophoresis, IP = 5.9) was synthesized at

Dainippon Pharmaceutical Co., Osaka. PLGAs of  $M_r$  7000 and 57 000 were purchased from Wako Pure Chemical Industries, Osaka, and Birmingham Polymers, AL, respectively. Sorbitan sesquioleate (SO-15EX) was purchased from Nikko Chemicals, Tokyo. Sorbitan monooleate (Span 80), polyoxyethylene (20) sorbitan monooleate (Tween 80), and polyoxyethylene (10) octylphenyl ether (Triton X-100) were purchased from Wako Pure Chemical Industries, Osaka. Ethylcellulose (10 mPa·s) and Eudragit RS-100 were obtained from Shin-etsu Chemical, Tokyo and Higuchi, Tokyo, respectively. Mineral oil (light) was purchased from Nakarai Tesque, Kyoto.

## 2.2. Preparation of TNF- $\alpha$ powder

Fine TNF- $\alpha$  powder was prepared by lyophilization of a W/O emulsion where the aqueous phase was phosphate-buffered saline (0.02 M, pH 7.0) containing TNF- $\alpha$  at  $8 \times 10^6$  to  $9 \times 10^6$  JRU/ml (3.2–3.6 mg/ml) and gelatin at 100 mg/ml. Aqueous phase (50 ml) was emulsified into 100 ml cyclohexane containing 1 g SO-15EX as an emulsifying agent using a probe type ultrasonic homogenizer (US-300, Nihonseiki, Tokyo). This emulsion was placed in a round-bottom flask and rapidly frozen by immersion in a bath maintained at  $-40^\circ\text{C}$  for 1 h. The frozen emulsion was subsequently lyophilized for 24 h at  $-5^\circ\text{C}$  and then dried at  $20^\circ\text{C}$  for 3 h. The resulting dried mass was a powder with a mean size of approximately  $5\ \mu\text{m}$  consisting of TNF- $\alpha$ , gelatin, SO-15EX, and the buffering salts.

TNF- $\alpha$  powder with a particle size of approximately  $150\ \mu\text{m}$  was prepared by the lyophilization of 50 ml phosphate-buffered saline (0.02 M, pH 7.0) containing TNF- $\alpha$  at  $8 \times 10^6$  to  $9 \times 10^6$  JRU/ml (3.2–3.6 mg/ml) and gelatin at 200 mg/ml. The obtained cakes of lyophilized TNF- $\alpha$  were comminuted by passage through a  $250\text{-}\mu\text{m}$  stainless steel screen.

## 2.3. Preparation of a sample solution for determination of TNF- $\alpha$ recovered from O/W and W/O emulsions and PLGA microspheres

The DCM-in-PBS (O/W) emulsion consisted of 4 ml DCM dispersed in 18 ml PBS which

also included 1% w/v gelatin and 0.1% w/v Triton X-100. This emulsion was prepared using an ultrasonic homogenizer, then 2 ml TNF- $\alpha$  original solution ( $6.85 \times 10^5$  JRU/ml) was added and mixed to yield a final emulsion of 24 ml. After incubation for 1 h at  $37^\circ\text{C}$ , the TNF- $\alpha$  in the aqueous phase was separated by centrifugation at 16 000 rpm for 30 min to prepare the sample solution.

When either DCM, cyclohexane or soybean oil was present as the continuous external phase of the W/O emulsion, 1 ml of the original solution of TNF- $\alpha$  containing 1% w/v gelatin was dispersed by the ultrasonic homogenizer for 15 s at room temperature into 5 ml of either pure DCM, cyclohexane or soybean oil. The DCM phase also contained either PLGA (16.7% w/w), ethylcellulose (8.3% w/w) or Eudragit RS-100 (16.7% w/w). After incubation at  $37^\circ\text{C}$  for 1 h, the dispersed aqueous phase of each W/O emulsion was separated by ultrasonic treatment into 10 ml PBS containing 1% w/v gelatin and 0.1% w/v Triton X-100. Centrifugal separation at 16 000 rpm for 1 h was conducted to prepare the sample solution.

To determine the level of TNF- $\alpha$  in the PLGA microspheres, the aqueous phase containing TNF- $\alpha$  was separated from the microspheres by washing the PLGA with acetone three times followed by centrifugation at 4000 rpm for 60 min. This sample was dried under reduced pressure for 2 h. The dried precipitate was then dissolved in PBS containing 1% w/v of gelatin (pH 7.0) to prepare the sample solution.

The concentration of the polymers in the organic solvents is expressed as a weight-in-weight percentage.

## 2.4. Determination of TNF- $\alpha$ in the sample solution

Intact TNF- $\alpha$  was immunologically determined by an enzyme immunoassay (EIA) using a kit supplied by the Research Laboratories of Dainippon Pharmaceutical Co. The activity of TNF- $\alpha$  was expressed in terms of the Japan Reference Unit (JRU). The total amount of dissolved

protein consisting of intact and/or inactivated TNF- $\alpha$  was determined by UV spectroscopy (UV-160, Shimadzu, Kyoto) at 278 nm. The UV spectral pattern of TNF- $\alpha$  was not affected by the TNF- $\alpha$  inactivation caused by exposure to the DCM or ACN, since the primary structure of TNF- $\alpha$  was not altered under such inactivating conditions. The specific activity of TNF- $\alpha$  was expressed as the EIA activity to the total weight of TNF- $\alpha$  in the sample.

*2.5. Evaluation of TNF- $\alpha$  stability in a solution when dispersed in a DCM phase containing PLGA or sorbitan sesqui-oleate at various concentrations*

The gelatin containing TNF- $\alpha$  solution, 0.5 ml ( $6.85 \times 10^5$  JRU/ml, 274  $\mu$ g/ml) was emulsified into 5 ml of the PLGA–DCM solution by ultrasonic homogenization for 15 s at room temperature to prepare a W/O emulsion. After incubation at 37°C for 1 h, the emulsion was homogenized in 20 ml PBS containing 1% w/v gelatin and 0.1% w/v Triton X-100 to separate the W/O emulsion and to form an O/W emulsion. TNF- $\alpha$  was extracted from the outer aqueous phase separated by centrifugation at 16 000 rpm for 30 min and the EIA activity of the protein was determined.

The concentration of PLGA and SO-15EX in the organic solvents is expressed as a weight-in-weight percentage.

*2.6. Preparation of matrix-type microspheres*

*2.6.1. TNF- $\alpha$  powder-in-DCM-in-water solvent evaporation method*

PLGA matrix-type microspheres were prepared by the DCM-in-water (O/W) emulsion method and contained 320 mg TNF- $\alpha$  powder ( $6.15 \times 10^4$  JRU/mg) dispersed in 6.0 g PLGA–DCM solution (33% w/w) (O-phase). The organic solution was then poured through a narrow nozzle (1.2 mm i.d.) into a 2% w/v polyvinyl alcohol solution (W-phase) and agitated at 500 rpm for 24 h at room temperature using a 47 mm three-blade propeller. The hardened microspheres were filtered on a nylon screen (50  $\mu$ m) and washed with distilled water, and then air-dried for 1 h at

room temperature. The microspheres were further dried under reduced pressure ( $< 0.05$  Torr) for 24 h. The free-flowing PLGA microspheres were evaluated microscopically.

*2.6.2. TNF- $\alpha$  powder-in-ACN-in-oil solvent evaporation method*

PLGA microspheres were prepared by an ACN-in-mineral oil (O/O) emulsion system. The ACN solution phase containing 2 g of the polymer at 33–40% w/w and 320 mg TNF- $\alpha$  powder ( $6.15 \times 10^4$  JRU/mg) was poured through a narrow nozzle (1.2 mm i.d.) into 70 g light mineral oil containing Span 80 (1% w/w), using the same apparatus as used for the O/W solvent evaporation process. The O/O emulsion was agitated at 400–500 rpm using a three-blade propeller for 48 h at room temperature. The hardened microspheres were filtered on a nylon screen (50  $\mu$ m) and washed three times with *n*-hexane, 2% w/v Tween 80 solution and distilled water followed by drying through an air stream. The microspheres were then dried under reduced pressure (0.05 Torr) for 24 h. The free-flowing microspheres contained homogeneously dispersed powder particles of TNF- $\alpha$  in the polymer wall.

*2.7. Preparation of multiphase microspheres using a modified solvent evaporation method*

Soybean oil in which aluminum mono-stearate was dispersed at 2% w/w was heated to 140°C to dissolve the dispersed solid in the oil and then cooled at room temperature. A total of 1 g dried gelatin powder (5  $\mu$ m) containing TNF- $\alpha$  ( $6.15 \times 10^4$  JRU/mg) was dispersed by an ultrasonic homogenizer into 3.25 g oil phase containing Span 80 (4% w/w) to obtain a fine oily suspension of the solid-in-oil (S/O) type. The oily suspension (1 g) was poured into 7 g PLGA–ACN solution (28.6% w/w) and dispersed gently to form a solid-in-oil-in-oil (S/O/O) type emulsion. The S/O/O emulsion was poured through a narrow nozzle into 70 g mineral oil containing 0.5% w/w of Span 80, and then agitated. The resulting S/O/O/O multiple emulsion was agitated at 400–500 rpm using a three-blade propeller for 24 h to evaporate the ACN. The hardened microspheres were

filtered on a 50  $\mu\text{m}$  nylon screen and washed three times with *n*-hexane, followed by 2% w/v Tween 80 solution, and then distilled water. The hardened multiphase microspheres were dried under reduced pressure for 24 h.

### 3. Results and discussion

#### 3.1. Stability of TNF- $\alpha$ in the aqueous phase of O/W and W/O emulsions where the O-phase was DCM, cyclohexane or soybean oil

O/W solvent evaporation systems for the preparation of PLGA microspheres require a water-immiscible organic solvent that freely dissolves the PLGA. DCM is the most commonly used solvent for PLGA and is used as the organic phase of the O/W emulsion system to dissolve both the polymer and the drug for the microencapsulation of hydrophobic drugs (Beck et al., 1985). This solvent is also used as the organic phase in the W/O/W emulsion system when water-soluble drugs such as peptides (Csernus et al., 1990) are dissolved in the internal aqueous phase. In the case of TNF- $\alpha$ , which has antitumoral activity in the form of a specific tertiary structure, maintenance of the structural integrity of the protein during the preparation of the W/O/W emulsion is critical. The stability of TNF- $\alpha$  in the internal aqueous phase of the W/O/W multiple emulsion was investigated prior to preparing the PLGA microspheres.

The results in Table 1 describe the stability of TNF- $\alpha$  expressed as the specific activity of TNF- $\alpha$  dissolved in the aqueous phase of either the O/W or the W/O emulsion. The EIA activity and protein concentrations of TNF- $\alpha$  in the original solution were  $6.85 \times 10^5$  JRU/ml and 274  $\mu\text{g/ml}$ , respectively, and the specific activity of the TNF- $\alpha$  prior to emulsification was  $2.50 \times 10^3$  JRU/ $\mu\text{g}$ , as shown in Table 1. The TNF- $\alpha$  activity was unchanged after simple mixing into the O/W emulsion. The original solution was diluted 10 times and was  $6.85 \times 10^4$  JRU/ml after this mixing step. The specific activities of TNF- $\alpha$  in the O/W emulsions where the oil phase consisted of DCM, or PLGA-containing DCM, were similar in value,

$2.44 \times 10^3$  to  $2.47 \times 10^3$  JRU/ $\mu\text{g}$ , as that obtained with the original solution of TNF- $\alpha$ . These results indicated that neither adsorption nor inactivation of TNF- $\alpha$  occurred on the surface of the fine droplets of the DCM phase of this O/W emulsion.

When PLGA, ethylcellulose and the acrylic polymer were dissolved in the DCM phase, the W/O emulsion was stabilized by each polymer due to a reduction in the interfacial tension and increased viscosity of the organic phase. In this W/O emulsion, the original solution of TNF- $\alpha$  was dispersed in the DCM phase. The specific activity of the protein was, however, significantly reduced in the emulsion containing either PLGA or ethylcellulose and an activity of less than 4% of intact TNF- $\alpha$  was obtained. A significantly higher specific activity ( $1.13 \times 10^3$  JRU/ $\mu\text{g}$ , 45.2%) of TNF- $\alpha$  was observed with Eudragit RS-100 under the same phase conditions of the W/O emulsion. When no polymer was present in the DCM phase of the W/O emulsion, the aqueous phase readily separated. The residual activity percentage of the protein from the emulsion containing DCM was 67.6%. Cyclohexane exerted a minimal effect on the TNF- $\alpha$  showing 96.8% residual activity, despite the fact that the aqueous phase was emulsified by SO-15EX in the oil phase even after 1-h incubation at 37°C. Soybean oil, which contained the finely dispersed aqueous phase, also had a slight effect on activity of TNF- $\alpha$  under the same incubation condition.

From these results, it was concluded that the TNF- $\alpha$  interaction at the droplet interface in the W/O emulsion caused a structural rearrangement of TNF- $\alpha$ , and significant inactivation of TNF- $\alpha$  when the dispersed aqueous phase was finely dispersed in the DCM phase. Eudragit RS-100 was found to stabilize the TNF- $\alpha$ .

Fig. 1A shows the residual activity of TNF- $\alpha$  in the aqueous phase of the W/O emulsions, as a function of the concentration of PLGA ( $M_n$  57 000) in the organic phase, DCM. The EIA activity of TNF- $\alpha$  decreased with polymer concentrations greater than 0.05 mg/g in the organic phase, and was extremely low at concentrations greater than 5 mg/g. In contrast to this activity change, the total protein levels in the aqueous phase decreased to 70% of the starting level, even

Table 1  
Effect of phase condition in various emulsions on stability of TNF- $\alpha$  in the aqueous (W) phase

Type of emulsion	Dispersion phase	External phase	Polymer or surfactant in organic phase	Activity of TNF- $\alpha$ in W phase determined by EIA (JRU/ml)	Concentration of TNF- $\alpha$ in W-phase determined by UV ( $\mu$ g/ml)	Specific activity <sup>f</sup> of TNF- $\alpha$ in W-phase (JRU/ $\mu$ g)	Activity ratio to intact TNF- $\alpha$ in the original solution (%)
Original solution <sup>a</sup>	—	Gelatin–PBS	—	$6.85 \times 10^5$	274	$2.50 \times 10^3$	—
O/W	DCM	Gelatin–PBS <sup>b</sup>	—	$6.83 \times 10^4$	27.6	$2.47 \times 10^3$	98.8
O/W	DCM	Gelatin–PBS <sup>b</sup>	PLGA <sup>c</sup>	$6.35 \times 10^4$	26.0	$2.44 \times 10^3$	97.6
W/O	PBS	DCM	—	$3.81 \times 10^5$	226	$1.69 \times 10^3$	67.6
W/O	PBS	DCM	PLGA <sup>c</sup>	$0.20 \times 10^5$	205	$0.10 \times 10^3$	4.0
W/O	PBS	DCM	Ethylcellulose <sup>d</sup>	$0.12 \times 10^5$	196	$0.06 \times 10^3$	2.4
W/O	PBS	DCM	Eudragit RS-100 <sup>e</sup>	$2.48 \times 10^5$	219	$1.13 \times 10^3$	45.2
W/O	PBS	Cyclohexane	SO-15EX <sup>e</sup>	$5.39 \times 10^5$	223	$2.42 \times 10^3$	96.8
W/O	PBS	Soybean oil	—	$6.44 \times 10^5$	279	$2.31 \times 10^3$	92.4

<sup>a</sup> The original solution of TNF- $\alpha$  was diluted 10 times in the aqueous phase of the O/W emulsion. In the case of the W/O emulsion, the original solution was directly dispersed in the O-phase.

<sup>b</sup> Triton X-100 (0.1% w/v) was included as an emulsifier.

<sup>c</sup> Concentration of polymer was 16.7% w/w.

<sup>d</sup> Concentration of polymer was 8.3% w/w.

<sup>e</sup> Concentration of surfactant was 2% w/w.

<sup>f</sup> Specific activity of TNF- $\alpha$  (JRU/ $\mu$ g):

Activity of TNF- $\alpha$  determined by EIA (JRU/ml)

Concentration of TNF- $\alpha$  determined by UV spectroscopy ( $\mu$ g/ml)

with PLGA concentration of 500 mg/g in the organic phase. A stable W/O emulsion was formed when PLGA was present in the DCM at concentrations greater than 0.1 mg/g. The aqueous phase gradually separated in the emulsions that contained PLGA at concentrations below 0.1 mg/g in the DCM phase.

The lower molecular weight ( $M_r$  7000) PLGA exhibited a similar effect, inactivating TNF- $\alpha$  in the W/O emulsion, and displayed a critical PLGA concentration for inactivation between 0.04 and 0.2 mg/g (Fig. 1B). The TNF- $\alpha$  recovery, calculated as the protein level in the aqueous phase, decreased at high concentrations of PLGA for both molecular weights ( $M_r$  57 000 and 7000) in the DCM phase (Fig. 1A and B), due to the denatured TNF- $\alpha$  protein being partly excluded

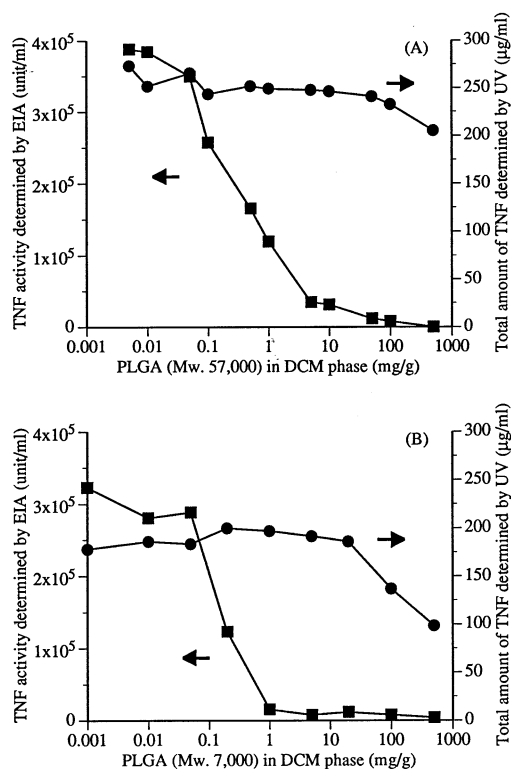


Fig. 1. Changes in TNF- $\alpha$  activity determined by EIA and total protein amount of TNF- $\alpha$  determined by UV spectrophotometry vs. the concentration of PLGA (MW 57 000 (A) and MW 7000 (B)) dissolved in the dichloromethane phase where the TNF- $\alpha$  solution was the dispersed phase of a W/O emulsion.

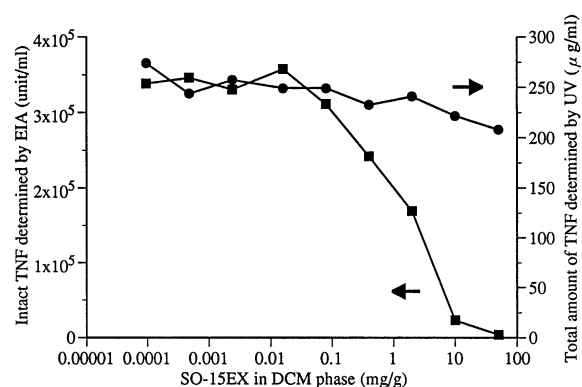


Fig. 2. Changes in TNF- $\alpha$  activity determined by EIA and total amount of TNF- $\alpha$  protein determined by UV spectrophotometry vs. the concentration of sorbitan sesqui-oleate (SO-15EX) dissolved in the dichloromethane where the TNF- $\alpha$  solution was the dispersed phase of a W/O emulsion.

from the aqueous phase by precipitation and/or by adsorption at the interface of the W/O emulsion.

Fig. 2 shows the effect of the hydrophobic surfactant (SO-15EX, HLB = 4.2) in the DCM phase of the W/O emulsion, on the EIA activity and the recovery of TNF- $\alpha$  when no PLGA was present. The EIA activity of TNF- $\alpha$  in the aqueous phase, where the theoretical concentration was  $6.85 \times 10^5$  JRU/ml, decreased gradually after incubation at 37°C for 1 h, as the SO-15EX concentration in the DCM phase increased to levels above 0.05 mg/g. Simultaneously, the aqueous phase was emulsified into small droplets and stabilized by the addition of the SO-15EX above the same concentration level. These results support the findings in Table 1 that demonstrated that the PLGA was not the only excipient that degraded the protein. The denaturation and the EIA activity loss of TNF- $\alpha$  in the inner aqueous phase were also induced by the physical stress on the protein during the preparation of the W/O emulsion.

A TNF- $\alpha$  powder (200 mg) with mean particle size of either 5 or 150  $\mu$ m was dispersed in 2 ml DCM and PLGA–DCM solution and incubated at room temperature for 24 h. The EIA activity was determined after three washings of the TNF- $\alpha$  powder with 10 ml acetone followed by drying

under reduced pressure for 24 h. The activity of the dispersed protein for each particle size was determined. Each specific activity level in the DCM or PLGA–DCM was  $2.47 \times 10^3$  to  $2.56 \times 10^3$  JRU/ $\mu$ g, which was unchanged from the initial level of  $2.50 \times 10^3$  unit/ $\mu$ g (Table 2 (A)). These anhydrous dispersions were expected to circumvent the inactivation caused by the emulsification of the TNF- $\alpha$  solution into the DCM phase during microsphere preparation using a TNF- $\alpha$  powder/O/W solvent evaporation method. However, when the 5- $\mu$ m powder was dispersed in the PLGA–DCM solution (16.7% w/w) and incubated with 10 ml gelatin–PBS at room temperature for 1 h, the specific TNF- $\alpha$  activity was reduced significantly from  $2.50 \times 10^3$  to  $0.16 \times 10^3$  JRU/ $\mu$ g (Table 2 (B)). It was microscopically observed that during the incubation period, the TNF- $\alpha$  powder (5  $\mu$ m), which was dispersed in the DCM phase, swelled when in contact with the water from the gelatin–PBS phase to form a W/O/W emulsion, prior to separation of the TNF- $\alpha$  powder from the DCM phase (organic phase). The TNF- $\alpha$  was extracted from this W/O/W emulsion by ultrasonic homogenization with Triton X-100 (0.1% w/v) into an O/W emulsion and the aqueous phase was separated by centrifugation. The large particle size TNF- $\alpha$  powder (150  $\mu$ m), when dispersed in both the PLGA–DCM solution and the polymer-free DCM, was more stable, and these dispersions resulted in significantly higher residual activity levels even when extracted by the gelatin–PBS solution. This was due to the separation of the TNF- $\alpha$  powder particles from the DCM phase prior to the formation of the finely dispersed W/O emulsion which caused the inactivation of TNF- $\alpha$ . It was concluded from these studies that the TNF- $\alpha$  solution/O/W and TNF- $\alpha$  powder/O/W solvent evaporation systems were unsuitable for the microencapsulation of TNF- $\alpha$  in the intact form.

### 3.2. Stability of TNF- $\alpha$ in aqueous solutions containing ACN

PLGA microspheres were prepared by dissolving the polymer in ACN, which constitutes the dispersion phase of an O/O emulsion solvent

evaporation system, where mineral oil is the external organic medium. The influence of ACN on the stability of TNF- $\alpha$  in aqueous solution or in solid (powder) form was investigated. Fig. 3 shows the changes in the EIA activity of TNF- $\alpha$  in a gelatin–PBS medium, as a function of the amount of ACN in the solution. TNF- $\alpha$  solution ( $6.24 \times 10^6$  JRU/ml) of 0.05 ml was added to 4.95 ml of the medium containing ACN from 0 to 15% w/w. The EIA activity was determined after incubation at 37°C for 15 min, 4 and 20 h. The activity decreased significantly in the mixed solution containing more than 12% w/w ACN after 15 min. TNF- $\alpha$  in solution form was inactivated even at the lowest ACN concentrations following longer exposure times (4 and 20 h). These data indicated that to stabilize the protein, ACN exposure to TNF- $\alpha$  must be circumvented during the preparation of PLGA microspheres through the solvent evaporation process. It was concluded from these studies that the PLGA microspheres could not be prepared by a O/O solvent evaporation system if TNF- $\alpha$  in solution form is directly mixed with PLGA–ACN solution, since TNF- $\alpha$  is significantly inactivated, even with short exposure and in the presence of a low percentage of ACN.

Contrary to the stability results obtained for the protein solution when mixed with ACN (Fig. 3), TNF- $\alpha$  in the solid form (dried powder) maintained its activity when dispersed in PLGA–ACN solution prepared as a O/O emulsion system. The results in Table 3 describes the time profiles of the

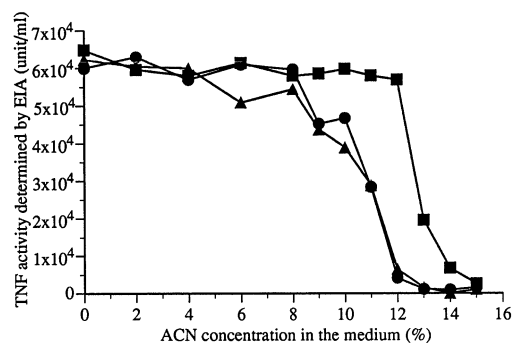


Fig. 3. Changes in activity of TNF- $\alpha$  in a gelatin–PBS containing various amounts of acetonitrile after incubation for 15 min (■), 4 h (●) and 20 h (▲).



Table 2  
Activity of TNF- $\alpha$  recovered by separation of a powder containing TNF- $\alpha$  (A) and by extraction with 10 ml gelatin–PBS (B) from a suspension of the protein powder dispersed in the dichloromethane

Method of recovery	Mean particle diameter ( $\mu\text{m}$ )	Dispersion medium	Activity of TNF- $\alpha$ determined by EIA (JRU/mg)	Weight of TNF- $\alpha$ determined by UV ( $\mu\text{g}/\text{mg}$ )	Specific activity <sup>c</sup> of TNF- $\alpha$ (JRU/ $\mu\text{g}$ )	Percentage of residual activity of TNF- $\alpha$
—	5	—	$6.15 \times 10^4$	24.6	$2.50 \times 10^3$	100.0
—	150	—	$3.48 \times 10^4$	13.9	$2.50 \times 10^3$	100.0
(A)	5	DCM	$6.20 \times 10^4$	24.2	$2.56 \times 10^3$	102.4
(A)	5	PLGA–DCM <sup>a</sup>	$6.10 \times 10^4$	23.9	$2.55 \times 10^3$	102.0
(A)	150	DCM	$3.41 \times 10^4$	13.4	$2.54 \times 10^3$	101.6
(A)	150	PLGA–DCM <sup>a</sup>	$3.43 \times 10^4$	13.9	$2.47 \times 10^3$	98.8
(B)	5	DCM	$5.51 \times 10^4$	26.0	$2.12 \times 10^3$	84.8
(B)	5	PLGA–DCM <sup>a</sup>	$0.32 \times 10^4$	19.5	$0.16 \times 10^3$	6.4
(B)	150	DCM	$3.41 \times 10^4$	13.7	$2.49 \times 10^3$	99.6
(B)	150	PLGA–DCM <sup>a</sup>	$3.02 \times 10^4$	13.5	$2.24 \times 10^3$	89.6

<sup>a</sup> Concentration of polymer was 16.7% (w/w).

<sup>b</sup> Specific activity of TNF- $\alpha$  in the gelatin (unit/ $\mu\text{g}$ ):

Activity of TNF- $\alpha$  in the gelatin powder determined by EIA (JRU/mg)  
Weight of TNF- $\alpha$  in the gelatin powder determined by UV spectroscopy ( $\mu\text{g}/\text{mg}$ )

Table 3

Activity of TNF- $\alpha$  in a dried powder (5  $\mu$ m) dispersed in acetonitrile containing 10% w/w PLGA after various incubation times

Incubation time	TNF- $\alpha$ activity determined by EIA (JRU/mg)	Activity % to the initial
Initial	$6.15 \times 10^4$	100.0
15 min	$6.03 \times 10^4$	98.1
4 h	$6.11 \times 10^4$	99.4
24 h	$6.18 \times 10^4$	100.6
72 h	$5.99 \times 10^4$	97.4

EIA activity of the TNF- $\alpha$  dispersed as a powder. The TNF- $\alpha$ -gelatin powder of 200 mg was dispersed in 10 ml of 10% PLGA-ACN solution and incubated for up to 72 h at room temperature. The TNF- $\alpha$  powder was separated from the ACN medium by centrifugation (3000 rpm, 30 min) and washed three times with ACN. The precipitate was dried under reduced pressure (< 0.05 Torr) for 24 h, then dissolved in 10 ml gelatin-PBS. TNF- $\alpha$  in the resulting solution was determined by EIA after appropriate dilution. The activity of TNF- $\alpha$  in the powder (5  $\mu$ m) was not affected by PLGA-ACN solution in which the TNF- $\alpha$ -gelatin powder was dispersed in this anhydrous system.

It was concluded from these experiments that the anhydrous suspension of TNF- $\alpha$  in a PLGA-ACN solution, which was the dispersed phase in the O/O (ACN-in-mineral oil) solvent evaporation system, stabilized the protein during the preparation of the PLGA microspheres.

### 3.3. Activity of TNF- $\alpha$ in PLGA microspheres prepared by solvent evaporation method using dichloromethane as the polymer solvent

In the microspheres prepared by the TNF- $\alpha$  powder/O/W emulsion solvent evaporation method, the EIA activity of TNF- $\alpha$  could not be detected within the size range 150–250 and 250–500  $\mu$ m where the theoretical content of TNF- $\alpha$  in each size microspheres was  $8.48 \times 10^3$  JRU/mg (Table 4). This inactivation of TNF- $\alpha$  resulted from the hydration of the TNF- $\alpha$  powder dis-

persed in unhardened PLGA-DCM droplets of the microspheres. The TNF- $\alpha$  powder swelled in the presence of water as seen with the droplets of unhardened microspheres. The powder/O/W emulsion eventually changed into a W/O/W emulsion, resulting in significant activity loss of TNF- $\alpha$  as demonstrated in Tables 1 and 2. It was concluded that solvent evaporation in the aqueous medium using DCM as the dispersion phase was not applicable to microsphere preparation containing intact TNF- $\alpha$ .

### 3.4. Activity of TNF- $\alpha$ in PLGA microspheres prepared by solvent evaporation under anhydrous condition

#### 3.4.1. Matrix-type microspheres

The EIA activity of TNF- $\alpha$  in matrix-type microspheres prepared by the O/O emulsion process is listed in Table 4. The TNF- $\alpha$  loading efficiencies ranged from 53.5 to 71.6%. From the TNF- $\alpha$  stability data in Table 3, it was expected that TNF- $\alpha$  when dispersed in the ACN medium would demonstrate high stability. The loading efficiency of TNF- $\alpha$  in the PLGA microspheres, therefore, was calculated from the determined EIA activity of TNF- $\alpha$ . The smaller size microspheres contained smaller amounts of TNF- $\alpha$ . This was due to leakage of the TNF- $\alpha$  powder into the external medium during the evaporation of solvent from the unhardened microspheres, which had an increased specific surface area.

#### 3.4.2. Multiphase microspheres

Multiphase microspheres were prepared through an anhydrous multiple emulsion solvent evaporation process partly modified from the method previously reported by Iwata and McGinity (1992, 1993). The protein incorporated into the microspheres prepared by this W/O/O/O emulsion method showed poor stability due to the diffusion of ACN into the aqueous phase, thereby inactivating TNF- $\alpha$  in the internal aqueous phase.

The EIA activities of TNF- $\alpha$  determined from the multiphase microspheres prepared by the modified (S/O/O/O) method are also listed in Table 4. The loading efficiencies of TNF- $\alpha$  in each size range were significantly higher than those of

Table 4

Comparison of TNF- $\alpha$  loading efficiency between matrix-type and multiphase microspheres prepared by various types of solvent evaporation methods

Type of micro-spheres	Batch no.	Particle size range ( $\mu\text{m}$ )	Loaded amount of TNF- $\alpha$ in microspheres (JRU/mg)	Determined activity of TNF- $\alpha$ in microspheres (JRU/mg)	TNF loading efficiency (%)
Matrix-type (TNF powder/O <sup>a</sup> /W)		100–250	$8.48 \times 10^3$	ND	0
Matrix-type (anhydrous O <sup>b</sup> /O)	1	250–500	$8.48 \times 10^3$	ND	0
		100–250	$8.48 \times 10^3$	$4.53 \times 10^3$	53.4
	2	250–500	$8.48 \times 10^3$	$5.98 \times 10^3$	70.7
		100–250	$8.48 \times 10^3$	$5.82 \times 10^3$	68.8
Multiphase (anhydrous O <sup>b</sup> /O)	1	250–500	$8.48 \times 10^3$	$6.06 \times 10^3$	71.6
		100–250	$4.82 \times 10^3$	$3.77 \times 10^3$	78.2
		250–500	$4.82 \times 10^3$	$4.81 \times 10^3$	99.8
	2	100–250	$4.82 \times 10^3$	$4.11 \times 10^3$	85.3
		250–500	$4.82 \times 10^3$	$4.79 \times 10^3$	99.4

<sup>a</sup> Dichloromethane.

<sup>b</sup> Acetonitrile.

the matrix-type microspheres. The concentration of the PLGA–ACN solution in unhardened multiphase microspheres, however, was at a lower level, thus providing a higher tendency for leakage of the TNF- $\alpha$  from the multiphase microspheres than from the matrix-type microspheres. The TNF- $\alpha$  loading efficiencies of approximately 80% or higher were obtained with the multiphase microspheres in both diameter ranges of 100–250 and 250–500  $\mu\text{m}$ , while less than 75% was incorporated in the 250–500  $\mu\text{m}$  size matrix-type microspheres. This higher TNF- $\alpha$  loading efficiency was attributed to the barrier effect of the soybean oil phase which separated the TNF- $\alpha$  powder from the ACN phase.

#### 4. Conclusions

The stability of TNF- $\alpha$  in the presence of DCM and ACN revealed that TNF- $\alpha$ , when present in an aqueous solution, cannot be processed to maintain protein activity with either the W/O/W system or the O/O system. In the W/O/W system, the DCM phase containing PLGA had a denatur-

ing effect on TNF- $\alpha$  due to an interfacial interaction between the internal aqueous phase and the organic phase. In the hydrous O/O system, ACN in the dispersion phase, when mixed with a TNF- $\alpha$  solution, caused a significant inactivation of the protein during the period needed for the ACN to evaporate and the microsphere to harden. TNF- $\alpha$  was microencapsulated intact by the anhydrous O/O solvent evaporation system employing a suspension of TNF- $\alpha$  powder dispersed in PLGA–ACN solution. The loading efficiency of TNF- $\alpha$  into these matrix-type microspheres, determined by measurement of EIA activity, was about 50–70% of theoretical levels. The PLGA multiphase microspheres containing TNF- $\alpha$  were prepared by means of an anhydrous multiple emulsion system which was prepared by a partially modified procedure of a process previously reported by McGinity and Iwata (1994). This microencapsulation system, for which ACN and mineral oil were also employed as the polymer solvent and evaporation medium, respectively, enabled the incorporation of TNF- $\alpha$  into the microspheres at approximately 80% or higher compared to the theoretical loading.

The anhydrous solvent evaporation system (S/O/O/O) utilized to produce multiphase PLGA microspheres was the most suitable encapsulation process for TNF- $\alpha$ . Future studies will focus on reducing the particle size of the microspheres and on a more detailed investigation of the TNF- $\alpha$  encapsulation properties of multiphase microspheres prepared through the hydrous and anhydrous systems.

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