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# Microencapsulation of dissociable human growth hormone aggregates within poly(D,L-lactic-co-glycolic acid) microparticles for sustained release

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**Abstract**

For the sustained release formulation of recombinant human growth hormone (rhGH), dissociable rhGH aggregates were microencapsulated within poly(D,L-lactic-co-glycolic acid) (PLGA) microparticles. rhGH aggregates were first produced by adding a small volume of aqueous rhGH solution into a partially water miscible organic solvent phase (ethyl acetate, EtAc) containing PLGA. These rhGH aggregates were then microencapsulated within PLGA polymer phase by extracting EtAc into an aqueous phase pre-saturated with EtAc. Release profiles of rhGH from these microparticles were greatly affected by changing the volume of incubation medium. The released rhGH species consisted of mostly monomeric form having a correct conformation. This study reveals that sustained rhGH release could be achieved by microencapsulating reversibly dissociable protein aggregates within biodegradable polymers. 

*Keywords:* Human growth hormone; Poly(D,L-lactic-co-glycolic acid); Biodegradable polymer; Sustained release

## 1. Introduction

Recently, sustained release formulation of various therapeutic proteins has been attempted to reduce the necessity of the multiple injections given to the patients. The most widely used method is to microencapsulate proteins within biodegradable poly(D,L-lactic-co-glycolic acid) (PLGA) polymeric microspheres (Cleland, 1998; Putney and Burke, 1998). Although many different formulation approaches have been examined, protein release profiles from PLGA microspheres seem to be unsatisfactory due to inherent protein stability problems, such as aggregation and non-specific adsorption that occur during the formulation process, as well as the release period (Crotts and Park, 1997a,b). Double emulsion solvent evaporation method based on water-in-oil-in-water ($W_1/O/W_2$) emulsion has been popularly used to encapsulate various proteins within PLGA microspheres (Cleland and Jones, 1996). This method, however, elicits severe protein instability

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problems during the encapsulation process. Particularly, protein molecules dissolved in an inner water phase ($W_1$) irreversibly aggregate to a great extent as they are continuously exposed onto an interface between water ($W_1$) and oil (O) phases (Maa and Hsu, 1997; Maa et al., 1998). It is conceivable that unfolded protein molecules at the interface form undissociable aggregates (Bam et al., 1998). In our previous study, it was shown that recombinant human growth hormone (rhGH) microencapsulated by the double emulsion solvent evaporation method was in a highly aggregated state within PLGA microspheres, resulting in a non-release behavior over an extended period after showing an initial burst (Kim and Park, 1999).

rhGH has been used for treating pediatric hypopituary dwarfism, which is administered for several years daily or three times a week. Sustained release formulation has great potential to enhance patient compliance, as well as to increase rhGH efficacy. Recently, PLGA microsphere formulation encapsulated with rhGH was marketed, which was prepared with a cryogenic spray drying process (Johnson et al., 1996; Lee et al., 1997). The addition of zinc as a rhGH stabilizer was claimed to be a critical formulation factor to form reversible insoluble aggregates of zinc–rhGH complex. It appears that the formation of zinc-induced rhGH dimeric aggregates plays a role in stabilizing the rhGH during the formulation and subsequently exhibiting continuous monomeric rhGH release from microspheres (Cunningham et al., 1991; Tracy, 1998). From this point of view, rhGH aggregation pathway can be divided into two classes: reversibly dissociable and irreversibly undissociable depending on how rhGH is aggregated. The conventional W/O/W method for rhGH microencapsulation leads to the latter type of aggregation within PLGA microspheres, while the zinc ion induced rhGH aggregation results in the former type of aggregation.

In this study, it was hypothesized that if rhGH could be aggregated in a reversibly dissociable form, it is possible to encapsulate the rhGH aggregates within PLGA microspheres to achieve a sustained release for a desired period. The encapsulation of the pre-formed rhGH aggregates is expected to minimize the formation of irreversible rhGH aggregates during the formulation process in contrast to that of native rhGH that tends to aggregate irreversibly during the W/O/W process. A partially water miscible organic solvent, EtAc, was used to induce the formation of reversible rhGH aggregates. PLGA microparticles containing the rhGH aggregates were tested for the controlled release of rhGH as a function of the volume of incubation medium. It was postulated that the volume of the incubation medium affected the rhGH release rate by shifting the dissociation tendency of rhGH monomer from the aggregates. Structural integrity and conformation of released rhGH species were characterized by size exclusion chromatography (SEC) and circular dichroism (CD) spectroscopy, respectively.

## 2. Materials and methods

### 2.1. Materials

rhGH was obtained from Dong-A Pharmaceutical Co. (Seoul, Korea). Two PLGA having a lactic/glycolic molar ratio of 50/50, Resomer RG502 and RG502H were purchased from Boehringer Ingelheim (Ingelheim, Germany). RG502 was an end-capped PLGA with weight average molecular weight of 12 000 and RG502H was an end-uncapped PLGA with that of 8600. Pluronic L-121 was donated by BASF (Parsippany, New Jersey). Polyvinylalcohol (PVA; 88% hydrolyzed, MW 13 000–23 000) was from Aldrich (Milwaukee, MO). Micro-BCA assay kit was obtained from Pierce (Rockford, IL). All other chemicals were of analytical grade.

### 2.2. Methods

#### 2.2.1. Preparation and characterization of solvent induced rhGH aggregates

Various aqueous volumes of rhGH solution (4.41 mg/ml) were added into 5 ml of deionized water saturated with ethyl acetate (EtAc) or dichloromethane (DCM). The adding volume was varied at 0, 0.2, 0.5, 0.7, and 1 ml. After 3 h, the transmittance determined as a measure of rhGH

solubility was detected at 600 nm by using a spectrophotometer (Beckman DU-600). The size of rhGH insoluble particles was analyzed by using a dynamic light scattering technique (Zeta-plus, Brookhaven, New York). In order to study the effect of shear on the character of solvent induced rhGH aggregates, 0.4 ml of aqueous solution containing 2.5 mg rhGH was emulsified in 13 ml of solvent (DCM or EtAc) by using a homogenizer (Fisher Scientific PowerGen 700) for 10, 30, 60, and 120 s at 1000 rpm. The primary emulsion was added to 100 ml of deionized water and the solution was stirred overnight for solvent evaporation. Precipitate was removed by centrifuging for 10 min at 10 000 rpm. Soluble rhGH fraction was determined by micro-BCA assay method.

*2.2.2. Microparticle preparation*

Salt-free freeze-dried rhGH ($\sim$140 mg) dissolved in 0.5 ml of 10 mM phosphate buffer (pH 8) was added to 2.5 ml of DCM or EtAc. The organic phase contained 800 or 1000 mg of PLGA blend and 2% (w/v) Pluronic L-121. The PLGA blend was composed of equivalent weight ratio of RG502H and RG502. The primary emulsion was briefly generated by using a homogenizer (Tekmar Co., Model SDT 1810). The stabilized primary emulsion was immediately re-emulsified within 230 ml of 5 mM citrate buffer (pH 5.3) containing 1% (w/v) PVA, which was saturated with DCM or EtAc, by using a high speed homogenizer (Fisher Scientific PowerGen 700) for 10 min at 2000 rpm. The secondary emulsion was placed under a magnetic stirring condition for 12 h. The hardened microparticles were centrifuged, washed, freeze-dried and stored at $-20$ °C. The size distribution and morphology of the microparticles were observed by using a particle counter system (PAMAS-2120, USA) and a scanning electron microscopy (SEM, Philips 535M), respectively.

*2.2.3. Determination of rhGH loading within PLGA microparticles*

The loading percent of rhGH within PLGA microparticles was determined in triplicate after dissolving 10 mg of microparticles in 3 ml of 0.1 N NaOH solution containing 0.5% (w/v) SDS as previously reported (Kim and Park, 1999). The protein content in the clear solution obtained after 1 day incubation at 37 °C was analyzed by a micro-BCA method as described in our previous report (Kim and Park, 1999).

*2.2.4. rhGH release experiment*

Twenty milligrams of freeze-dried microparticles were placed in three different volumes (1, 5, 10 ml) of PBS (pH 7.4) containing 0.01% (w/v) sodium azide with 0.02% (w/v) Tween 20. At pre-determined time intervals, the supernatant was collected to determine the amount of the released protein in the incubation medium. Fresh buffer solution was replenished at each sampling time to maintain a constant pH in the incubation medium.

*2.2.5. Size-exclusion chromatography and circular dichroism spectroscopy*

The amount of soluble aggregates in released rhGH in the medium was quantitated by SEC. The conformational change of native and released rhGH was determined by far UV CD spectrum taken at 25 °C with Jasco CD (Model Jasco, 720-A) as previously reported (Kim and Park, 1999).

## 3. Results and discussion

It has been generally known that protein molecules dissolved in an aqueous medium are precipitated upon contact with water miscible organic solvents (Rothstein, 1994). Organic solvent induced protein precipitation occurs by unfolding of protein molecules and their subsequent aggregation as a result of lowering dielectric constant in the aqueous medium. The self-aggregated and precipitated protein species are generated mainly by hydrophobic interaction between the exposed hydrophobic moieties, and they are reversibly dissociated upon removing organic solvents from the aqueous medium. The treatment of organic solvent for protein precipitation was routinely used in bio-separation process (Rothstein, 1994; Cao et al., 2000). Based on this fact, it was hypothesized that if reversibly dissociable rhGH aggregates could be generated by exposing an aqueous rhGH

solution to an appropriate water miscible solvent, they could be encapsulated within the PLGA microspheres.

The effect of two organic solvents, EtAc and DCM, on the aggregation behavior of rhGH dissolved in aqueous solution was tested. The former is more water miscible than the latter. EtAc and DCM can be dissolved in water up to 8.7 and 1.6% (v/v) at 20 °C, respectively. Various rhGH amounts dissolved in deionized water were exposed to water phase saturated with EtAc or DCM. Saturated solubilities of water in EtAc and DCM are 3.3 and 0.24% (v/v), respectively. Table 1 shows that water phase saturated with EtAc, induced decrease in transmittance value with increasing the rhGH amount, while DCM did not affect the change in transmittance. This reveals that rhGH was aggregated and precipitated, when exposing to a partially water-soluble organic solvent in accordance with the conventional thought. The size of rhGH aggregate particles is around 2–3 μm in diameter as determined by a dynamic light scattering method. These insoluble particles were fully re-solubilized upon dilution with excess amount of water, suggesting that they were reversibly dissociable rhGH non-covalent aggregates.

Because proteins have a surface-active property due to their micelle-like structures, they tend to unfold and subsequently aggregate upon exposure to different interfaces, such as solid/liquid, gas/liquid, and liquid/liquid. Double emulsion solvent evaporation process, which has been extensively used for protein encapsulation with biodegradable microspheres, requires the formation of primary water-in-oil emulsion droplets. The primary emulsion step in the double emulsion process generates a water/oil interface. Onto this interface, the encapsulated proteins in the water phase presumed to be continuously unfolded and aggregated as long as the discrete two liquid phases having different surface tensions are present. When a minimally water soluble organic solvent, such as DCM, is used for the preparation of the primary emulsion, a DCM/water volume ratio of 416, as calculated from the solubility of water in DCM, is an upper critical value for producing a liquid/liquid interface. Thus, DCM as an oil phase for the generation of primary emulsion droplets inevitably yields an oil/water interface concomitantly having dispersed water phase droplets saturated with DCM. On the other hand, using EtAc as an oil phase for the formation of primary emulsion generates an EtAc/water interface, only when EtAc/water volume ratio is below 29.3. Below the volume ratio of 29.3, inner water phase droplets are partially saturated with EtAc, leading to the protein aggregation mainly through unfolding onto the interface of EtAc/water. Above the volume ratio of 29.3, however, it is expected that hydrated protein molecules in $W_1$ phase instantaneously becomes protein aggregate particles upon exposing to EtAc phase due to the disappearance of the $W_1$ phase. In this case, there might be no discrete formation of a water/oil interface, but tiny hydrated protein aggregates existed in suspension within EtAc medium. It should be mentioned that the above prediction for the formation of water/oil interface was simply based on the solubility of organic solvents in water in the absence of rhGH. Thus, in the presence of an excess amount of pre-hydrated protein in the inner water

Table 1
Characteristics of rhGH aggregates in deionized water saturated with organic solvents

| | Final rhGH concentration (mg/ml) | Effective diameter (μm) | Transmittance (%) |
|---|---|---|---|
| Water saturated with EtAc | 0.00 | ND | 100.0 |
| | 0.17 | ND | 99.6 |
| | 0.40 | 2.27 | 72.4 |
| | 0.54 | 2.79 | 56.5 |
| | 0.74 | 2.29 | 46.8 |
| Water saturated with DCM | 0.74 | ND | 99.9 |

ND: not detected.

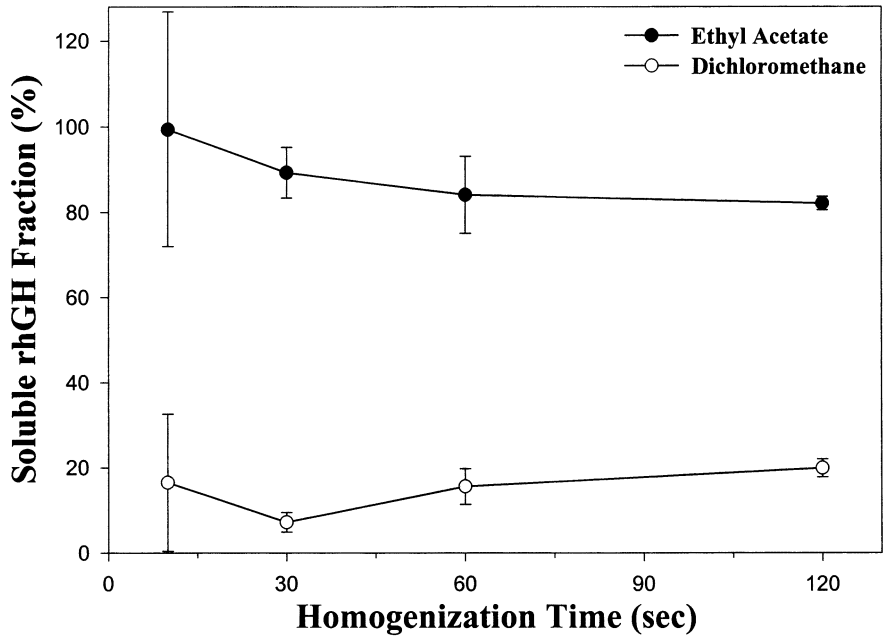


Fig. 1. Effect of two organic solvents on the re-solubilization behavior of rhGH as a function of homogenization time during a simulated process.

phase, the required solvent/water volume ratio for the formation of an interface should be changed to a greater extent, because a large fraction of water molecules are tightly bound to proteins, not being available for the liquid–liquid mixing with organic solvents.

Fig. 1 shows the effect of DCM and EtAc on the re-solubilization of rhGH as a function of homogenization time during a simulated process of double emulsion solvent evaporation process in which PLGA polymer was not incorporated into the organic phase. The solvent/water volume ratio was set at 32.5 (13 ml/0.4 ml) for this experiment. Under this condition of relatively low rhGH concentration, a water/oil interface exists for DCM, but not for EtAc. It can be seen that the DCM as a continuous oil phase results in extensive irreversible rhGH aggregation, while EtAc induces much reduced irreversible rhGH aggregation after the simulated emulsion and solvent evaporation process. It is reasonable to say that an interface formed between DCM and water phases played a critical role in the non-dissociable rhGH aggregation. Initially solubilized rhGH in an inner water phase ($W_1$) was presumably to be denatured and aggregated in an irreversible manner at the interface of $W_1/O$. EtAc shows much higher soluble rhGH fraction with increasing the homogenization time. This is likely attributed to the fact that the absence of $W_1/O$ interface did not contribute to the surface induced rhGH aggregation. The rhGH aggregation in a reversible form took place as a result of EtAc induced protein precipitation mechanism as mentioned above.

In order to see the effect of EtAc on rhGH release behaviors from PLGA microparticles, two different formulations were prepared by using different kinds of organic solvents as listed in Table 2. Formulation A is a typical recipe for protein encapsulated microspheres using DCM. Formulation B is for microparticles prepared by using EtAc. The pre-saturation of the aqueous water phase with the selected solvent plays an important role in determining the morphology of PLGA microparticles, because it controls the solvent removal rate from embryonic microsphere droplets to the aqueous phase. A SEM picture of the formulation B microparticles is illustrated in Fig.

2. The formulation A microspheres were individually spherical as reported elsewhere, but the formulation B microparticles were composed of aggregates of sub-micron size small nanoparticulates. The size distributions of the two different microspheres are shown in Fig. 3. The formulation A microspheres are around 100 μm in diameter, but the formulation B microparticles are much smaller having a multi-modal size distribution. The reduced size of the formulation B microparticles in a highly aggregated state, with the addition of EtAc in the oil phase, as well as in the outer water phase, can be caused by the effect of EtAc on rhGH aggregation in the primary emulsion step and the development of embryonic microspheres in the secondary emulsion process. In the primary emulsion process, the use of EtAc as an organic phase might induce the formation of reversibly dissociable rhGH aggregates within the inner aqueous emulsion droplets, because of a water miscible solvent induced rhGH aggregation mechanism (Rothstein, 1994; Cao et al., 2000). On the other hand, the use of DCM might result in the irreversibly dissociable rhGH aggregate within the inner aqueous droplets by the interface induced rhGH aggregation. The presence of EtAc in the outer aqueous phase at a saturation concentration permitted a slower solidification of embryonic microspheres. This was because the escaping tendency of EtAc from a dispersed phase to an aqueous medium phase was delayed to a greater extent, compared to that of more volatile DCM. Early surface solidification of the formulation A microspheres by a rapid solvent removal and subsequent polymer precipitation process near the droplet surface is responsible for the resultant large microspheres sizes. As reported previously (Kim and Park, 1999), the interior structure of the formulation A microspheres was highly porous as a result of the double emulsion process. The de-

Table 2
Formulation and characteristics of two different microparticles

| Formulation | hGH Weight (mg) | PLGA weight (mg) | Solvent | $W_2$ Phase saturation condition | Actual loading (%) | Encapsulation efficiency (%) |
|---|---|---|---|---|---|---|
| A | 142 | 1000 | DCM | DCM | 12.2 | 98.4 |
| B | 137 | 800 | EtAc | EtAc | 11.2 | 76.7 |

DCM, dichloromethane; EtAc, ethyl acetate.

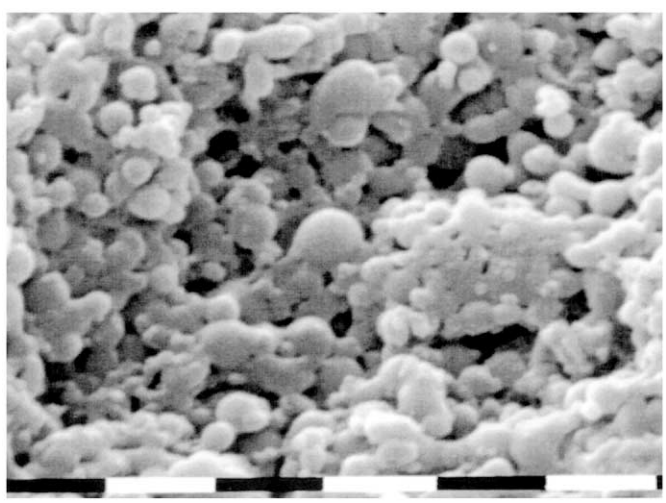

Fig. 2. SEM picture of the formulation B microparticles. Scale bar is 1 μm.

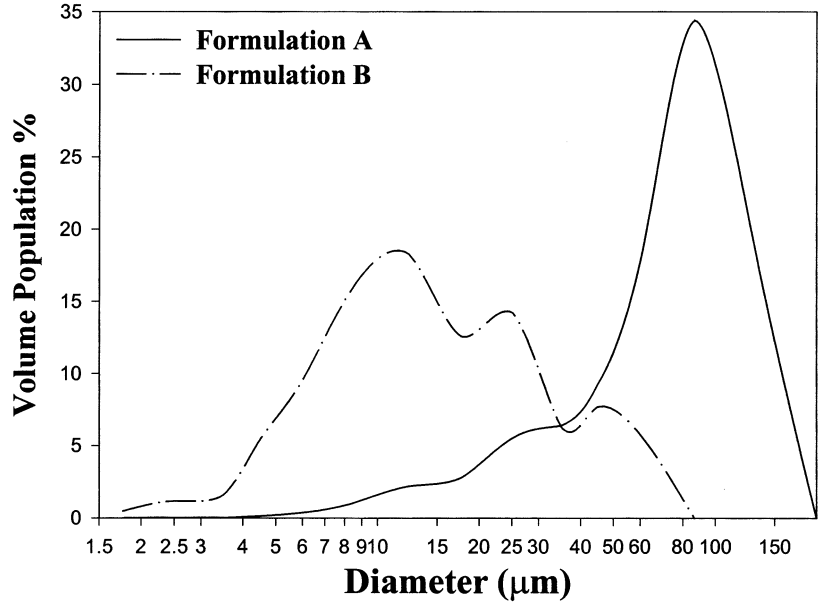


Fig. 3. Size distributions of the two different microparticles.

layed solidification of formulation B microparticles made the embryonic emulsion droplets continuously reduce their size to the size of initially produced rhGH aggregates. This is the most plausible reason for the formulation B microparticles being small. It can be said that for the formulation B microparticles, PLGA polymer coated around the outer surface of rhGH sub-micron precipitate particles. The aggregation of individual sub-micron particles is likely to form aggregates resulting in a broad range of size distribution. Residual amount of EtAc in the microparticles might contribute the self-aggregation of sub-micron particles during the freeze-drying process. However, they are free flowing powder after the freeze-drying. Schematic illustrations of W/O/W and A(aggregate)/O/W methods are shown in Fig. 4. It should be noted that in the formulation of microparticles, the volume ratio of oil phase to water (2.5 ml/0.5 ml = 5) was far less than the formerly mentioned critical volume ratio for the generation of oil/water interface for the two solvents. Nevertheless, finely suspended rhGH aggregates were produced in the primary emulsion step of the formulation B due to the presence of an excess amount of pre-hydrated rhGH molecules in the inner water droplets dramatically reduced the water fraction miscible with EtAc. This resulted in the formation of reversible protein aggregates without generating the O/W interface. Although the A/O/W formulation used in this study is relevant to a solid/oil/water (S/O/W) method (Morita et al., 2000), the present formulation has an advantage over the conventional S/O/W formulation. The A/O/W method is a one-step process for encapsulation of fine protein particles within PLGA microparticles. The fine protein aggregates were formed during spontaneous mixing of a water miscible organic solvent with an aqueous protein solution. This approach does not require an additional process to produce fine protein particles that requires in the S/O/W method.

Fig. 5 shows rhGH release profiles from two different microparticles. It can be seen that formulation B microparticles exhibit a better release profile than formulation A microspheres. As previously reported in our lab (Kim and Park, 1999), rhGH release profiles from the microspheres prepared by the double emulsion solvent evaporation method demonstrate an initial burst and followed by a very slow release due to the protein aggrega-

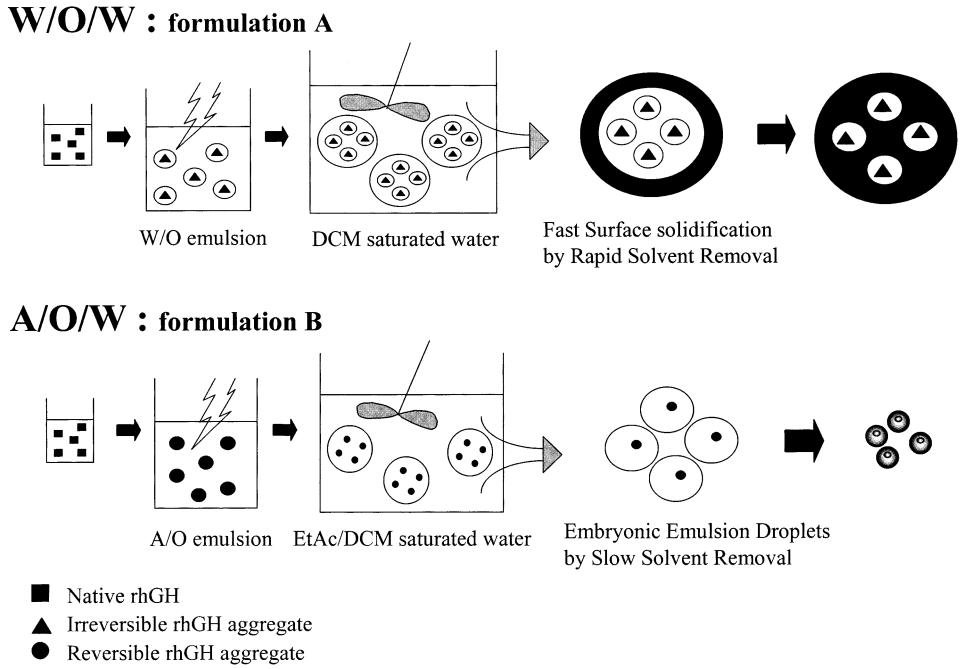


Fig. 4. Schematic illustrations of the preparation methods for the formulation A prepared by W/O/W (water-in-oil-in-water) double emulsion method and the formulation B prepared by A/O/W (aggregate in oil-in-water) method.

tion and adsorption problems occurred during the formulation process. The new formulation based on using EtAc in both oil (O) and outer water phase ($W_2$) suggests that the formation of irreversible protein aggregation within microspheres can be circumvented. Reversibly, dissociable rhGH aggregates encapsulated with a biodegradable PLGA coating layer are likely to be dissolved out slowly. Thus, PLGA polymer plays a role as a temporary diffusion barrier in controlling the rhGH release rate from microparticles. Different sizes of the two microspheres are also likely to contribute to the release profiles, because of their different surface areas.

Fig. 6 shows rhGH release profiles from two formulations as a function of incubation medium volume. Since it was hypothesized that the release rate of rhGH was dependent on a thermodynamic equilibrium between reversibly dissociable rhGH aggregates and rhGH monomer, it was expected that change in the incubation medium volume affected the rhGH dissolution rate from the microparticles. Decreased concentration of

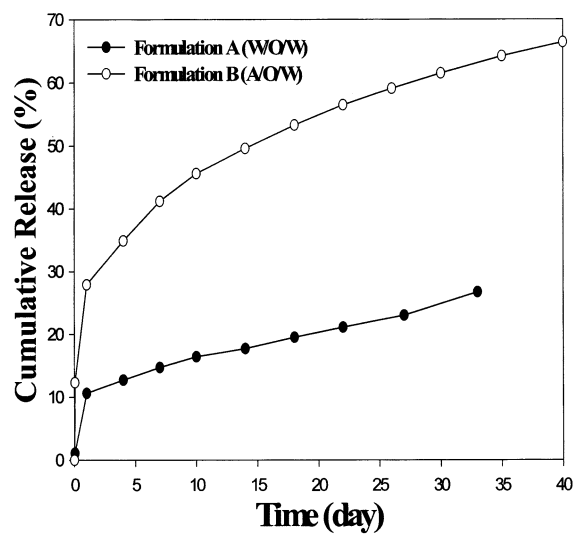


Fig. 5. Cumulative rhGH release profiles from the two different microparticles. Formulation A was prepared by W/O/W method and formulation B was prepared by A/O/W method.

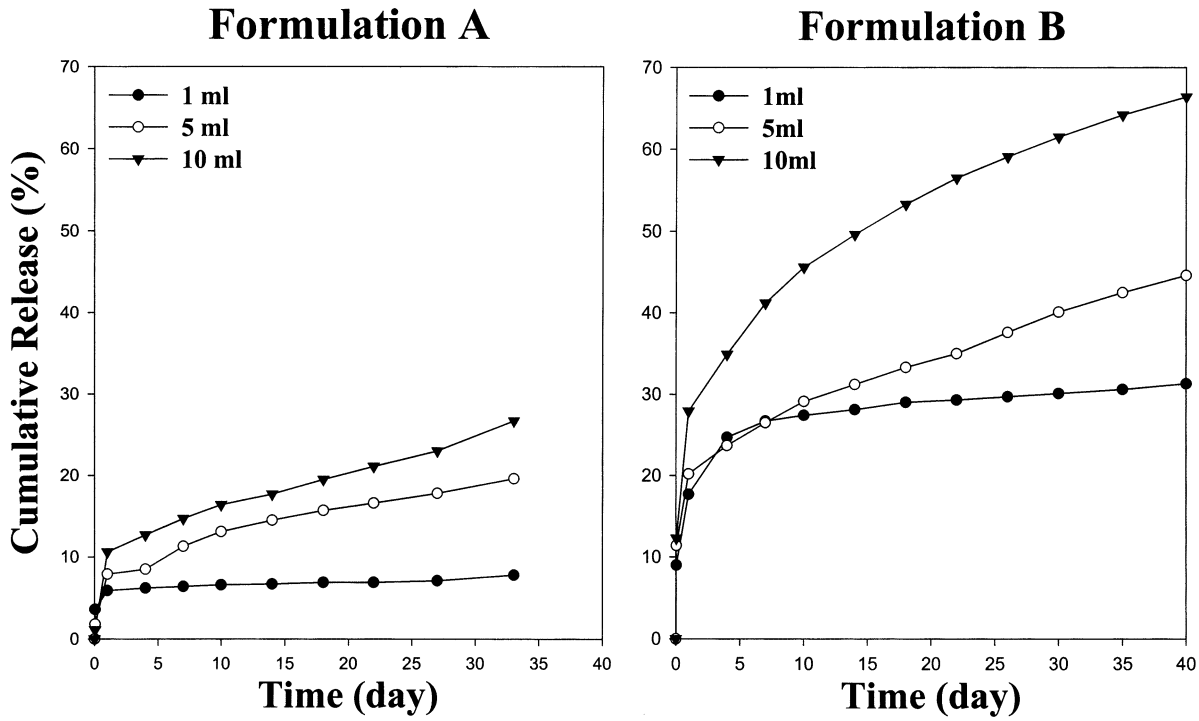


Fig. 6. Cumulative rhGH release profiles from the two different microparticles as a function of incubation medium volume. Formulation A was prepared by W/O/W method and formulation B was prepared by A/O/W method.

monomeric rhGH in the incubation medium shifts the equilibrium towards dissociating more rhGH monomer species from rhGH aggregates. The formulation B microparticles show that more amount of rhGH is released in response to increasing the volume of incubation medium than the formulation A microspheres, suggesting that the rhGH release was caused by slow dissolution of rhGH aggregates in a thermodynamically controlled manner. This suggests that the formulation B microparticles contained more reversible rhGH aggregates than the formulation A microspheres. To confirm whether the released rhGH was a monomeric form, the collected samples after 1-h incubation were subjected to SEC. The result from the SEC showed that all the samples regardless of the incubation medium volume demonstrated a major rhGH monomer peak with over 92% homogeneity. In addition, the release samples had native rhGH conformation as determined by CD spectra.

In conclusion, a new formulation approach for encapsulation of rhGH within PLGA microparticles was examined, which was based on the use of EtAc in organic phase, as well as in the aqueous extracting medium phase. The resultant rhGH microparticles were different from previously reported microspheres, which have been traditionally prepared by double emulsion solvent evaporation method using DCM. Reversibly dissociable rhGH aggregate particles could be coated with PLGA polymer, and they exhibited more sustained rhGH release profiles. Modulated rhGH release amount in response to the concentration of rhGH in the incubation medium implies that rhGH release from microparticles occurred via a rhGH dissolution controlled mechanism.

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