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Preparation and evaluation of poly(L-lactic acid) microspheres containing rhEGF for chronic gastric ulcer healing

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Abstract

Biodegradable microspheres containing recombinant human epidermal growth factor (rhEGF) were prepared using poly(L-lactic acid) by a solvent evaporation method based on multiple w/o/w emulsion. Encapsulation efficiency and initial release were influenced by the amount of polymer, inner water phase volume and osmotic pressure difference between inner water phase and outer water phase. The effect of osmotic pressure difference between inner water phase and outer water phase in w/o/w emulsion on particle size, porosity and in vitro release of rhEGF from microspheres were also studied. Microspheres prepared with the optimized osmotic pressure, polymer amount and inner water volume produced 21% initial release on the first day with 92% encapsulation efficiency. The blood concentration of rhEGF was maintained at constant levels for 9–11 days after a single subcutaneous (s.c.) administration of rhEGF microspheres. The gastric ulcer healing effect of a single s.c. administration of rhEGF microspheres was increased 1.44-fold compared with twice a day s.c. administration of rhEGF saline solution after 11 days. The enhanced curative ratio of rhEGF loaded microspheres may be due to the optimized osmotic pressure, high encapsulation efficiency and sustained release pattern.

Keywords: rhEGF; Microsphere; Poly(L-lactic acid); Encapsulation efficiency; Osmotic pressure difference; Chronic gastric ulcer healing

1. Introduction

rhEGF is a single-chain polypeptide containing 53 amino acid residues (M_w=6045) and three disulfide bridges [1]. rhEGF stimulates the proliferation and differentiation of epithelial tissues such as the intestinal mucosa, corneal epithelial tissue, lung and trachea epithelia [2]. Moreover, rhEGF was able to inhibit gastric acid secretion [3–7] and protect gastroduodenal mucosa against tissue injury induced by ulcerogenic agents [8–11]. We have reported that oral bioadhesive gels containing rhEGF is effective against induced acute and chronic gastric ulcers in rats [12], but the healing efficacy was not complete. When rhEGF was administered orally, systemic effect cannot be expected because of its susceptibility to enzyme breakdown and its poor GI membrane transport ability [13]. So, it is necessary to develop a long-acting systemic rhEGF delivery system for chronic gastric ulcer [14]. Many different approaches have been used to maintain certain plasma levels using sustained-release dosage forms.

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In recent years, poly(DL- or L-lactic acid) (PLA) has been investigated extensively as a polymer of microspheres for injectable sustained release drug delivery [15–18].

High encapsulation efficiency and low initial release is the most important factors in the development of sustained release microspheres containing water-soluble drugs. We have noticed that phosphates remained in rhEGF as raw material, which is used as a lyoprotectants in the process of lyophilization of rhEGF manufacturing. The phosphates existed in inner water phase to create the different osmotic pressure between the inner and outer water phase of double (w/o/w) emulsion, and the resultant water influx occurred from outer to inner phase in order to reach the osmotic pressure balance in the preparation of rhEGF microspheres. Microspheres encapsulation efficiency and initial release were greatly affected in this process and microspheres' morphology and characteristics were also changed.

The aim of this study was to develop and characterize rhEGF-loaded microspheres in order to obtain a single injectable depot system for chronic gastric ulcer healing. First of all, the influence of the polymer amount, osmotic pressure difference between inner phase and outer phase and the amount of inner phase volume on microspheres characteristics (encapsulation efficiency, particle size and porosity) were studied for optimization of the microsphere system. Then with regard to the optimized microspheres, the plasma concentration profile of rhEGF after single depot injection was detected during the 11 days and the chronic gastric ulcer healing effects was also compared by extracting data from multiple subcutaneous (s.c.) injections of rhEGF solutions.

2. Materials and methods

2.1. Materials

rhEGF was supplied by Daewoong Pharm. (Seoul, South Korea). The human EGF Quantikine® kit was purchased by R&D Systems (Minneapolis, MN, USA), Poly L-lactide, M_w=85 000–160 000 and 9-aminoacridine were obtained from Sigma (St. Louis, MO, USA). Polyvinyl alcohol (Shinyo Pure, Tokyo, Japan), methylene chloride (Junsei Pure, Tokyo, Japan) and acetic acid (Wako Pure, Tokyo, Japan) were used. All other chemicals were of reagent grade and used without further purification.

2.2. Preparation of rhEGF/PLA microspheres

rhEGF-incorporating PLA microspheres were prepared by a solvent-evaporation method [19,20]. Corresponding to 3 mg of rhEGF was dissolved in 0.1 ml of water, and 150 mg of polymer (PLA) was dissolved in 3 ml of methylene chloride. These two solutions were mixed and emulsified by a homogenizer under ice cooling temperatures to form a primary w/o emulsion. This primary emulsion was dispersed in 300 ml of water containing 1% polyvinyl alcohol and 0.3 M phosphate by stirring at a rate of 1200 rpm with a mechanical stirrer. The resultant w/o/w multiple emulsion was stirred continuously for 3 h at room temperature (25°C) to remove the methylene chloride. The hardened microspheres were collected by centrifuging and washed with distilled water. They were freeze dried and stored at −20°C in a desiccator. The composition of the polymer, inner phase and outer phase systems for preparing rhEGF microspheres is shown in Table 1.

2.3. Determination of rhEGF

rhEGF content of the microspheres was determined by methylene chloride and acetic acid extraction method [21]. Extracted rhEGF was analyzed by HPLC methods described previously by our laboratory [13]. Briefly, samples were injected into a 50-μl sample loop. Separation was achieved on a 10-μm reversed phase C_{18} column (μ-Bondapak; 3.9×300 mm, 10 μm) and eluted by a gradient

Table 1
Compositions of the polymer inner phase–outer phase systems for preparing rhEGF microspheres

Composition	Standard microsphere	Modified microsphere
Inner water volume (ml)	0.1	0.1
rhEGF (mg)	3	3
PLA dissolved in 3 ml methylene chloride (mg)	150	150
Phosphate concentration in 300 ml water solution containing 1% PVA outer phase (M)	0	0.3

mobile phase: starting from 5 min of 93% Mobile phase A (7% Mobile phase B) followed by 27 min of linear gradient to 58% Mobile phase A (42% Mobile phase B) and then continuing on for 5 min before returning to 93% Mobile phase A. The flow rate was 1.2 ml/min. Mobile phase A consisted of 90% 10 mM K_2HPO_4 (pH 6.5) and 10% acetonitrile. Mobile phase B consisted of 30% 10 mM K_2HPO_4 (pH 6.5) and 70% acetonitrile. Detection was monitored using a UV detector at 214 nm.

2.4. Effect of osmotic pressure on the tracer influxing into microspheres

Different osmotic pressure between the inner phase and outer phase in the encapsulation procedure by a solvent-evaporation method affected, critically, the encapsulation efficiency of rhEGF microspheres. 9-Aminoacridine as a tracer was added to the outer phase. The amount of 9-aminoacridine going into the inner phase of microspheres was extracted by methylene chloride and acetic acid extraction method and detected by HPLC method. Samples were injected into a 50-μl sample loop. Separation was achieved on a 5-μm reversed phase C_{18} column (Shiseido Capsell Pak C_{18}; 4.6×250 mm, 5 μm). The mobile phase consisted of a mixture of distilled water and acetonitrile (17:83) containing 0.1% triethylamine (pH 6.5) and delivered at the flow rate of 1.0 ml/min. Effluents were monitored using a UV detector at 262 nm.

2.5. Shape and particle size distribution of rhEGF/PLA microspheres

The shape, inner porosity, and surface of the microspheres were observed with a scanning electron microscope (Hitachi S 510, Tokyo, Japan). The microspheres were coated with pure gold using an ion coater (Eiko Engineering IB-3, Tokyo, Japan) in a vacuum (0.1 Torr) under high voltage (800–1500 V and 8 Am). Average particle size and its distribution were determined using a Coulter Counter (CASY™, Scharfe System, Germany). Microspheres were suspended in isotonic saline solution. After appropriate dilution with the same solution, the suspension was forced to flow through a capillary. The resistance change by a particle was electronically scaled and counted.

2.6. rhEGF release test

About 2–4 mg of microspheres were put into a vial containing 1 ml of 1/30 M phosphate buffer and 0.01% polysorbate 80. The vial was immersed in a shaker bath maintained at 37.0±0.1°C and was shaken horizontally. Samples were centrifuged and dried by lowering the pressure at the 1, 2, 3, 5, 7, 9, 11 experimental day, respectively. rhEGF remaining in microspheres was extracted by methylene chloride/acetate acid and the amount of rhEGF was determined by HPLC as described above.

2.7. Animal study

Male Sprague–Dawley rats weighing 250 g were used. The modified rhEGF microspheres (corresponding to 220 μg/kg of rhEGF) was subcutaneously administered to the dosal site of rats once on the first day of the experiment. As a control experiment, rhEGF saline solution was single subcutaneously administered at a dose of 50 μg/kg. Blood samples were collected from the tail vein at intervals on designated day. The concentration of rhEGF in plasma was measured by the ELISA method using Quantikine® kit. The non-compartmental pharmacokinetic parameters, area under the drug concentration–time curve (AUC), was calculated from plasma data.

2.8. Chronic gastric ulcer healing test

Chronic gastric ulcer healing test was investigated in rats after inducing chronic gastric ulcers using the acetic acid method. To induce chronic gastric ulcers, approximately 70 μl of 75% acetic acid was injected directly into the serous surface of the stomach. After 24 h following the induction of ulcers, the rats as a test group were injected subcutaneously with microspheres, the rats as control group were injected with rhEGF in saline solutions and the rats with no treatment were left as a normal group. In order to evaluate the ulcer treatment, the rats were killed on the first day, sixth and 11th day after the induction of ulcers. The area of ulcerated mucosa was measured (in m^2) and the curative ratio was calculated from ulcer area using following equation.

Table 2
Effects of polymer amount on rhEGF encapsulation efficiency and initial release of rhEGF from rhEGF microspheres[c] on the first day

L-PLA (mg)	Encapsulation efficiency (%)[a]	Initial release (%)[b]
75	27.7±4.2	75.6±6.3
150	70.9±1.8	75.6±3.7
300	5.9±3.4	13.0±5.1

[a] Encapsulation efficiency = actual loading/theoretical loading × 100 (%).
[b] Initial release ratio = rhEGF remaining in microsphere after 1 day (μg/mg)/actual loading × 100 (%).
[c] The formulation was same as standard microsphere (Table 1) with variation of L-PLA amount.

$$\text{Curative ratio} = \frac{\text{area of ulcer without treatment} - \text{area of ulcer after treatment}}{\text{area of ulcer without treatment}} \times 100$$

3. Results and discussion

3.1. Effect of polymer amount on encapsulation efficiency

By increasing the amount of polymer from 75 to 150 mg, the encapsulation efficiency of rhEGF was increased from 27.7 to 70.9%. However, when the amount of polymer was increased to 300 mg, the encapsulation efficiency was decreased by 5.9% as shown in Table 2. It might be that the viscosity of organic phase in the primary w/o emulsions was so high that the final w/o/w emulsions could not be obtained by mechanical stirring. This result indicates that encapsulation efficiency is increased with increasing of polymer within a certain range.

3.2. Effect of osmotic pressure on encapsulation efficiency and initial release

rhEGF agent contains phosphates, which is used as lyoprotectants in the process of lyophilization of rhEGF manufacturing. In the preparation of microsphere, phosphates affect microsphere formation due to osmotic pressure differences between the inner and outer phase of w/o/w emulsion. This different osmotic pressure affects the microspheres formation and influences the physicochemical properties of rhEGF microspheres as illustrated in Fig. 1. When the osmotic pressure of the inner phase is greater than that of the outer phase result in increasing the influx of water from outer phase, then the microspheres might be porous and have a large particle size (Fig. 1A). But when there is no osmotic pressure difference between inner phase and outer phase, the

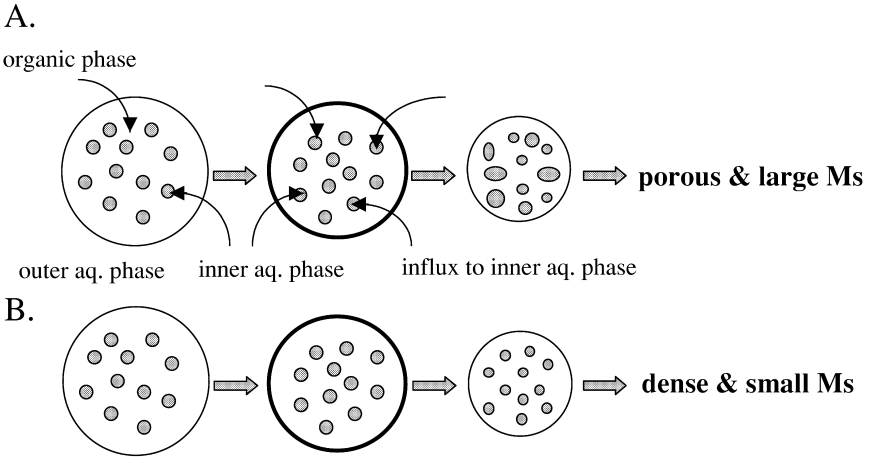

Fig. 1. Schematic illustration of the effects of osmotic pressure on the formation of microspheres. Dark colored lines indicate solidified polymer. (A) Osmotic pressure of inner phase is higher than that of outer phase. (B) There is no osmotic pressure difference between the inner phase and outer phase.

microspheres might be dense and have a small particle size (Fig. 1B).

To elucidate the osmotic pressure effect, 9-aminoacridine, as a influx tracer was dissolved in outer phase. The effect of phosphates concentration in outer phase on the amount of influx tracer from outer phase is shown in Fig. 2. When phosphate concentration increased from 0 to 0.3 M in outer phase, the tracer (9-aminoacridine) influx to microspheres was decreased from 0.78 to 0.23 mg. This result indicated that the amount of phosphate in the outer phase induce the different osmotic pressure between inner phase and outer phase and affect drug loading and initial release.

When phosphate concentration increased from 0.1 to 0.3 M in the outer phase, the encapsulation efficiency of rhEGF was increased from 79.5 to 93.0% and the initial release on the first day was decreased from 57.6 to 21.7% as shown in Fig. 3. In the event of the osmotic pressure difference was not adjusted, rhEGF was lost into the outer water phase and pore formation resulted from influx of water from the outer phase.

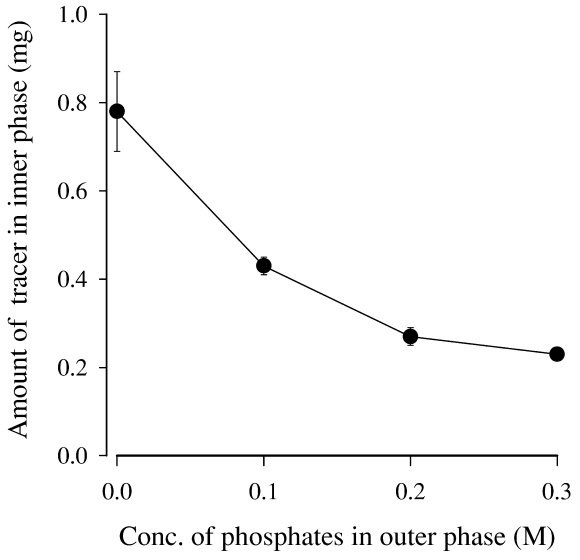

Fig. 2. Effect of phosphate concentration on immigrated amount of influx-tracer (9-aminoacridine) from outer aqua phase. Microspheres were prepared with addition of phosphates into outer aqua phase.

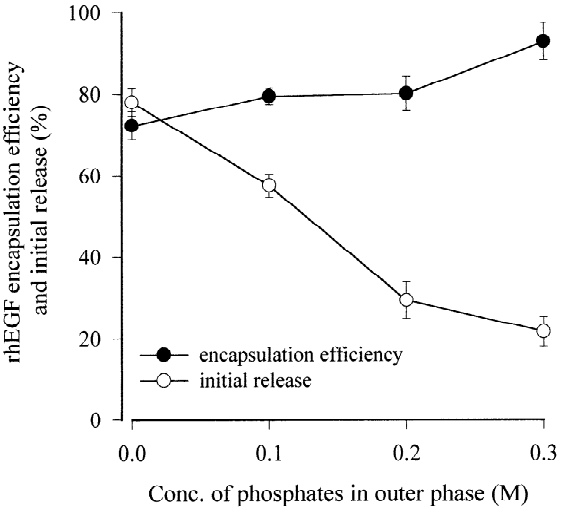

Fig. 3. Effect of phosphates concentration in outer phase on rhEGF encapsulation efficiency and its initial release on the first day.

3.3. Effect of inner phase volume on encapsulation efficiency and initial release

The effect of inner water volume on rhEGF encapsulation efficiency and initial release is shown in Table 3. When the inner water volume was increased from 0.1 to 0.3 ml, the encapsulation efficiency was decreased from 70.9 to 22.6% and initial release was increased from 75.6 to 82.0%, respectively. Increasing the volume of the inner water phase in the primary w/o emulsions can

Table 3
Effects of inner water volume on rhEGF encapsulation efficiency and initial release of rhEGF from rhEGF microspheres[c] on the first day

Inner water volume (ml)	Encapsulation efficiency (%)[a]	Initial release (%)[b]
0.1	70.9±1.8	75.6±3.7
0.2	66.8±5.5	82.9±2.1
0.3	22.6±0.9	82.0±2.8

[a] Encapsulation efficiency = actual loading/theoretical loading ×100 (%).

[b] Initial release ratio = rhEGF remaining in microsphere after 1 day (μg/mg)/actual loading×100 (%).

[c] The formulation was same as standard microsphere (Table 1) with variation of inner water volume.

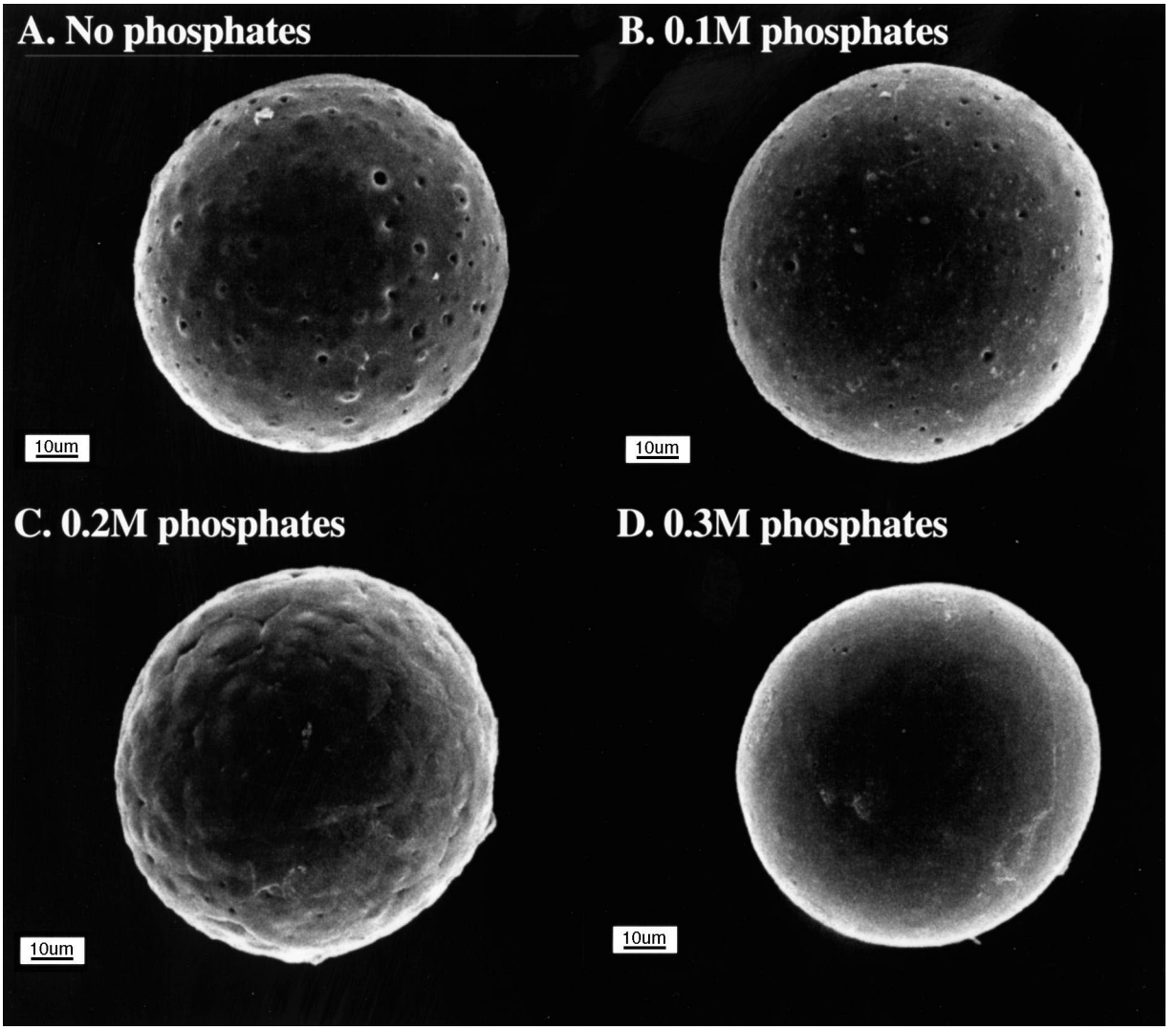

Fig. 4. Scanning electron micrographs of rhEGF microspheres prepared with standard conditions (Table 1) and variation of different phosphates concentration in outer phase.

relatively increase the internal cave volume and surface pore size of microspheres, thus reducing the encapsulation efficiency by drug loss and increase initial release.

3.4. Characterization of rhEGF/PLA microspheres by SEM

Microspheres prepared without balancing osmotic pressure resulted in microspheres having many pores on its surface and a rough surface as shown in Fig. 4. But when balancing osmotic pressure, the surface of microspheres was smooth and compact with few pores. SEM imaging of the internal structure of microspheres was shown in Fig. 5. Microspheres prepared without balancing osmotic pressure resulted in internal structure having very large holes produced from influx of outer water.

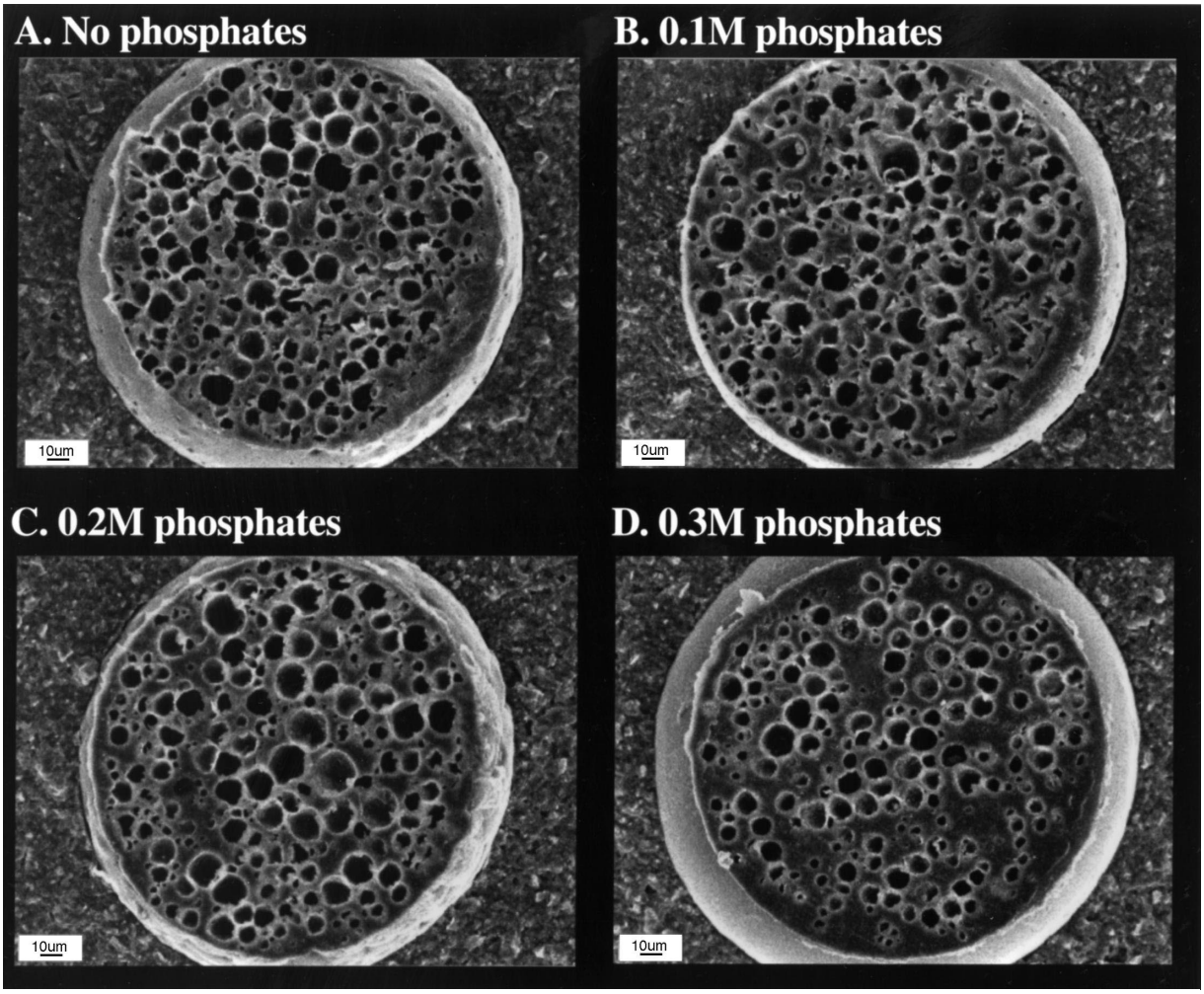

Fig. 5. Scanning electron micrographs of rhEGF microspheres internal structure prepared with standard conditions (Table 1) and variation of different phosphates concentration in outer phase.

3.5. Particle size distribution

Adjusting osmotic pressure with addition of phosphates from 0 to 0.3 M into outer water phase, results in the reduction of mean particle size from 131 to 75 μm. It might be that the inner water volume was decreased by the reduced influxed-water from the outer water phase during the microspheres solidifying process. This can result in reduced microsphere size as shown in Fig. 6.

3.6. Release test of rhEGF/PLA microspheres

The amount of rhEGF released from microspheres was calculated by determining remaining rhEGF content in microspheres after designed time interval due to rhEGF instability in the medium [22]. rhEGF released from the modified microspheres (Table 1) was sustained for 11 days as shown in Fig. 7. The release manner of microspheres might be governed mainly by diffusion because PLA microspheres almost kept their shape and size after 11 days.

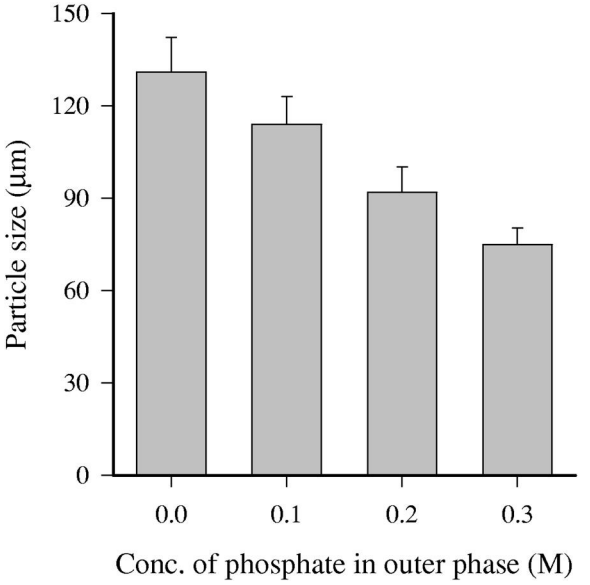

Fig. 6. Particle size distribution of rhEGF microspheres prepared in standard conditions (Table 1) and different phosphates concentration in outer phase.

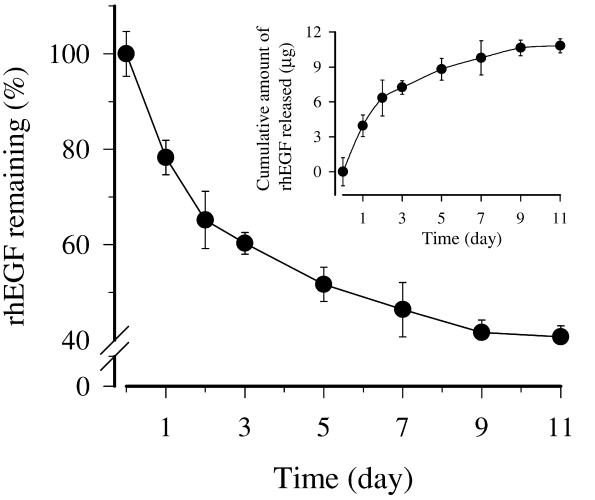

Fig. 7. rhEGF remaining in microspheres (modified microsphere, Table 1) as a function of release time during the 11 days. Plot in small box represents rhEGF cumulative release from 1 mg rhEGF microspheres.

3.7. Pharmacokinetic analysis

The plasma concentration–time profiles of rhEGF after single s.c. injection of rhEGF saline solution and single depot injection of rhEGF microspheres to rats were shown in Fig. 8. The AUC of rhEGF after a single depot s.c. administration of microspheres (220 μg/kg of rhEGF) was 124.49 ng·day/ml and C_{max} was above 10 ng/ml. When rhEGF saline solution was administered s.c. (50 μg/kg) to rat, the t_{max}, C_{max} and $t_{1/2}$ were 17.5±2.2 min, 23.6±0.8 ng/ml and 31.4±2.2 min, respectively. It would appear that the plasma concentration of rhEGF from microspheres sustained and maintained comparative high level during the 11days.

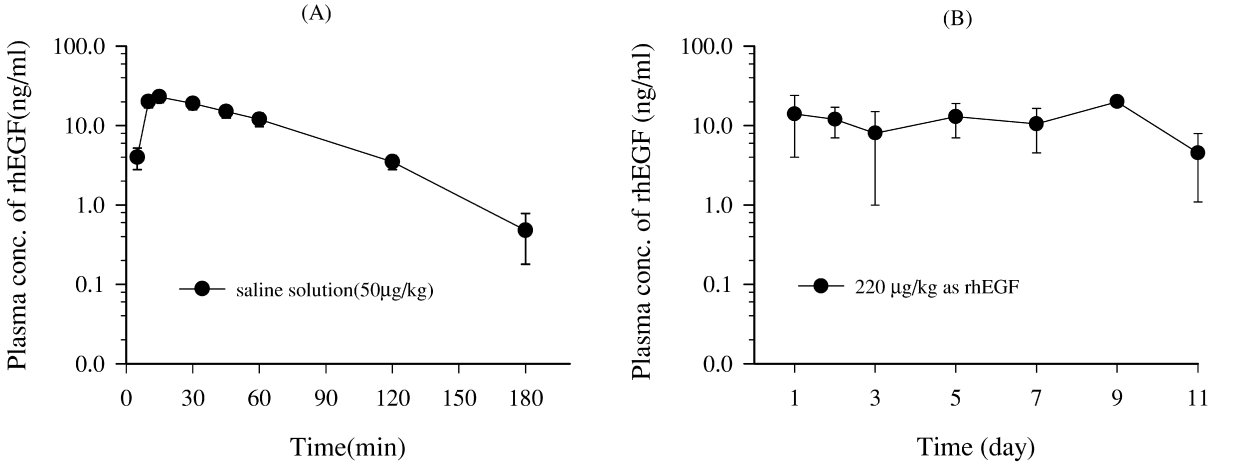

Fig. 8. Plasma concentration of rhEGF after single s.c. administration of rhEGF saline solution (A) and single depot injection of rhEGF microspheres (B).

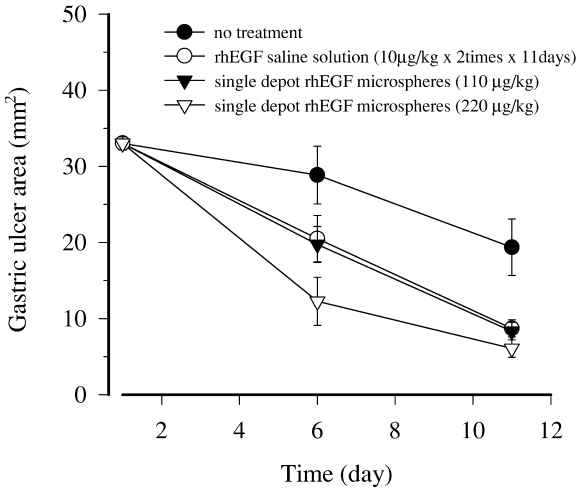

Fig. 9. The area of gastric ulcers induced by the acetic acid reduced at sixth and 11th days after s.c. administration of single depot rhEGF microspheres (110 and 220 μg/kg as rhEGF), rhEGF saline solution (10 μg/kg) twice/day for 11days, compared to no rhEGF treatment group.

3.8. Chronic ulcer healing test

Gastric ulcer healing effect was determined by the measurement of the area of ulcerated mucous after single-depot s.c. administration of rhEGF microspheres or rhEGF saline solution during the 11days. To test chronic gastric ulcer healing, rhEGF saline solution was injected at a dose of 10 μg/kg bi-daily (total 220 μg/kg during 11 days) to chronic gastric ulcer rats as a control group, and two kinds of single depot rhEGF microspheres were injected at a dose of 110 and 220 μg/kg as a test A and test B group. The decline of ulcer area of the test A group was similar to that of the control group (The curative ratio was 40.2±7.1 vs. 37.9±9.2 at 6 day; 74.7±3.4 vs. 73.6±3.4 at 11 day); however, in the test B group, decline of ulcer area was faster than that of the control group about 2-fold (The curative ratio was 62.9±9.6 vs. 37.9±9.2 at 6 day) as shown in Fig. 9 and Table 4. It is indicated that the single depot administration of rhEGF microspheres was effective for chronic gastric ulcer healing than multiple rhEGF saline injections. And this depot system could sustain rhEGF blood plasma levels for 11 days. Fig. 10 shows representative photographs of gastric ulcers induced by the acetic acid method and its healing effect after single-depot-rhEGF microsphere s.c. administration at a dose of 220 μg/kg to rats.

4. Conclusions

Osmotic pressure difference between inner phase and outer phase greatly affected the physicochemical characteristics of the resultant rhEGF/PLA microspheres. rhEGF/PLA microspheres with its high encapsulation efficiency, low initial release, continued plasma concentration and high curative ratio was prepared by adjusting the inner water volume, amount of PLA, and added phosphate amount into the outer phase.

The enhanced curative ratio of rhEGF by using optimized microspheres in this study may have been achieved through a combination of factors including the high encapsulation efficiency, low initial release and 11-day sustained release pattern. This system might be applicable to treat chronic gastric ulcers by

Table 4
Curative ratio of gastric ulcer on the sixth and 11th day after single depot rhEGF microspheres and rhEGF saline solution s.c. administration to rats

Time (day)	No treatment	rhEGF saline solution[a]	Single depot rhEGF microspheres[b]	
		(10×22 μg/kg)	110 μg/kg	220 μg/kg
6	12.6±11.5	37.9±9.2*	40.2±7.1*	62.9±9.6**
11	41.4±11.2	73.6±3.4*	74.7±3.4*	81.6±3.4*

[a] rhEGF saline solution was injected to rats twice a day at a dose of 10 μg/kg for 11 days.
[b] The formulation was same as modified microsphere (Table 1).
Significantly different from the no treatment group by Student's *t*-test: *$P<0.05$; **$P<0.01$.

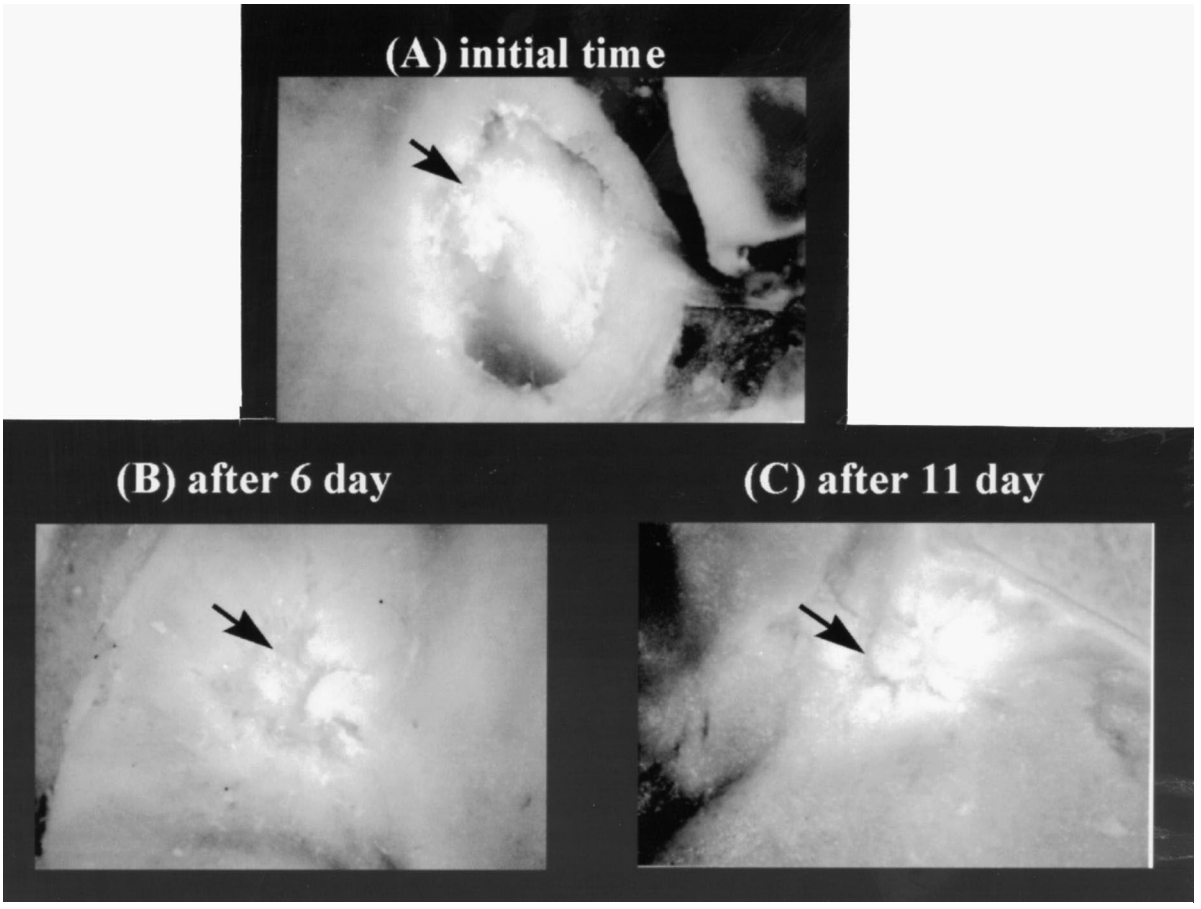

Fig. 10. Representative photographs of gastric ulcers and its healing effect at sixth and 11th days after single-depot-rhEGF microsphere s.c. administration. Arrows indicate ulcer area.

a single s.c. depot injection. This rhEGF/PLA microsphere system can also help to improve other types of therapy for rhEGF treatment such as skin and corneal injury.

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