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Ionic salt induced morphology and drug release control of insulin incorporated biodegradable PLGA microspheres

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ABSTRACT

Biodegradable polymeric microspheres have been used for microencapsulation of number of drugs for controlled delivery applications. Water-in-oil-in-water (w/o/w) double emulsion has been employed for preparation of drug incorporated poly(lactic-*co*-glycolic acid) (PLGA) microspheres. In the present study, existing double emulsion method was modified by introducing ionic salt in continuous phase of emulsion process. Insulin-incorporated microspheres were prepared from wide range of PLGA concentrations under an identical preparation condition, and the influence of varied concentration of salt on microsphere characteristics was studied. The results demonstrated, the degree of solidification of PLGA was controlled using ionic salt and the prepared formulations showed improved morphology, enhanced encapsulation efficiency and a positive modulation over the drug release characteristic compared to control. The modified method should be useful for elimination of highly porous and collapsed microspheres in the formulations prepared from low range PLGA concentration and should pave the way to improve several microsphere formulations for controlled drug delivery applications. Copyright © 2016 VBRI Press.

Keywords: Biodegradable polymers; microsphere; double emulsion; ionic salt; insulin.

Introduction

Advances in recombinant DNA technology and molecular cloning have introduced a large number of protein based therapeutics [**1**]. Frequent dosing of these drugs is often considered as clinically undesirable because of patient discomfort and poor compliance [**2**]. Therefore, it is much needed to have clinically acceptable delivery systems in order to administer these drugs [**3**]. A successful delivery system should show a suitable controlled release (CR) characteristic and the release of the encapsulated or entrapped drug can further be tuned on the basis of therapeutic duration **[4, 5]**. In this context, biodegradable microspheres are recognized to be highly beneficial **[4-6]**. Biodegradable microspheres are of special interest because of its spherical nature provides high surface area and uniform drug release characteristic. Biodegradable polymers were extensively investigated for the development of microspheres encapsulating the drugs for CR application [**7**]. These polymers have high degree of biocompatibility and can be safely removed from the body [**4-7**]. Biodegradable polyesters such as poly (lactic-*co*-glycolic acid) (PLGA) has been widely used in preparation of CR systems for variety of biopharmaceuticals [**7**]. PLGA exhibits several favorable properties, those are remarkably important for preparation of appropriate formulation of a drug for achieving the CR characteristic **[7, 8]**. Controlled biodegradation, excellent biocompatibility, good mechanical property, ease of processing and high safety are the key advantages [**8**].

Several methods have already been used for preparation of drug incorporated microspheres [**5**]. Water-in-oil-in-water (w/o/w) or double emulsion method has been widely employed for the preparation of CR formulations for hydrophilic drugs (*e.g.* protein based therapuetics) **[5,9]**. Double emulsion is usually preferred in order to reduce the high drug loss and enhance the drug encapsulation efficiency of microsphere formulations **[9]**.The successful formation of efficient microspheres is based on the idea of the control over the solidification of dispersed phase (drug containing organic matrix: PLGA with organic solvent dichloromethane) in continuous phases (surfactant containing aqueous phases: poly(vinyl alcohol) with water) **[10-12]**. In order to obtain the microspheres of high drug loading or encapsulation efficiency, it is highly desirable to achieve moderately fast solidification of dispersed phase during the second emulsion process of standard double emulsion protocol. Moderately fast solidification of dispersed phase prevents the diffusion of entrapped drug into the continuous phase and the aqueous influx to disperse phase [**10, 13**]. The solidification of the dispersed

phase primarily depends on the viscosity of PLGA, which in turn depends on concentration of PLGA [**13**]. Below a certain threshold concentration of PLGA, the viscosity may not be good enough to fasten the solidification of dispersed phase. Therefore, the formed microspheres maintain a semi solid nature for quite a longer duration and the aqueous influx as well as diffusional escape of the drugs from PLGA matrix becomes significant [**10**]. The microspheres prepared from the PLGA concentration below this threshold value results in high drug loss as well as formation of water filled channels or pockets in dispersed phase and leads to the formation of highly porous as well as collapsed microspheres [**10**]. The phenomenon is quite common in double emulsion and greatly affects the release features of several formulations prepared from low range PLGA concentrations [**11**].

In this study, our major objective was to partially modify the double emulsion method to widen the preparation of drug incorporated microspheres from a broad range of PLGA concentrations. To realize the purpose, PLGA microspheres were prepared from various PLGA concentrations by introducing ionic salt sodium chloride (NaCl) in continuous phase of the emulsion process. Human recombinant insulin incorporated microspheres were prepared by the existing double emulsion method as well as the modified scheme as proposed. The microspheres of different size were prepared from four different concentrations (wt%) of PLGA such as 50, 25 and 10 and 5. The hypothesis behind the experimental design was that NaCl could generate osmotic pressure in continuous phase and an optimal pressure could balance the pressure inside dispersed phase and continuous phase to facilitate microsphere formation. To test the hypothesis, the impact of different salt concentration (0 M, 0.5 M and 1.0 M) on the microsphere characteristics (morphology, size, drug encapsulation efficiency and *in vitro* drug release) was compared.

Experimental

Materials

The Poly(lactic-*co*-glycolic acid) (PLGA) (LG molar ratio: 50:50, Mw: 20 kDa), dichloromethane, poly(vinyl alcohol) (86-90 mol% hydrolysis), recombinant human insulin, sodium chloride (NaCl), and hydrochloric acid (HCl) were purchased from Wako Pure Chemicals Ltd., Japan. Micro-BCA protein assay kit was purchased from Pierce Biotechnology, USA. Phosphate buffer saline (PBS) (10 ×, pH 7.4) was purchased from Nacali Tesque Inc, Japan. All these materials were used as received without further purification. Deionized water from Millipore water system in the laboratory was used for preparing all the solutions for this study.

Methods

Preparation of microspheres

Human recombinant insulin incorporated PLGA microspheres were prepared by double emulsion method [**14**]. In this study, different concentrations of PLGA such as 50, 25, 10 and 5 wt% was chosen for preparation of insulin incorporated microspheres. Dichloromethane was used as the solvent for preparation of PLGA solution. Insulin stock solution was prepared by dissolving 20 mg of human recombinant insulin in 1 mL of 0.01N HCl as an internal aqueous phase (w_1). Briefly 50 µl of w_1 was dispersed in 1 mL of PLGA solution (o) (prepared by dissolving the necessary wt% of PLGA in dichloromethane). Primary (w_1/o) emulsion was prepared by vigorous mixing for 1 min on a vortex device (Vortex genie, Fischer, Pittsburgh, PA) at setting 10. The resultant w_1/o emulsion was further emulsified in 2 ml of 3% (w/v) PVA at the same vortex speed for another 2 min. The resultant double emulsion (w_1/o/w_2) was added in dropwise to 200 mL of 0.5% (w/v) of PVA and stirred magnetically at 300 rpm for overnight at room temperature (RT) inside a laboratory fume hood. The microspheres were collected after centrifugation and washed for three times with deionized water by centrifugation at 1500 rpm for 15 min. The collected microspheres were frozen at -80 ºC and freeze-dried with a freeze dryer (S P Industries Inc., Japan) below 5 kPa for 48 h. The prepared microspheres were used as control against the formulations prepared using salt in continuous phases of emulsion process.

The insulin incorporated microspheres were further prepared by modifying the above preparation scheme by maintaining a definite concentration of NaCl (0.5 M and 1.0 M) in both the aqueous continuous phases of the double emulsion process. The insulin incorporated microsphere processing was followed from each PLGA concentration at each concentration of NaCl.

Microsphere morphology

The morphology of insulin incorporated microspheres was investigated with a scanning electron microscope (SEM, JSM-5610, JEOL Ltd., Tokyo, Japan). Freeze-dried microspheres were evenly dispersed over a carbon adhesive mounted over an aluminum stub. The prepared samples were sputtered with a thin layer of platinum for 500 s prior to SEM observation.

Size analysis

The freeze-dried microspheres (20 mg) were suspended in 1 mL of deionized water. The aggregation of the microspheres was prevented by placing the samples under ultrasonic waves using an ultrasonicator (Branson Ultrasonics, USA) for 5 min prior to analysis. The samples were analyzed for their size by using a laser diffraction particle analyzer (Shimadzu SALD 7000, Japan). The experiments were performed in triplicate for each sample to determine the average size (µm) of the microsphere formulations. Data are presented as means ± standard deviation (n = 3)

Insulin encapsulation efficiency

The confirmation of insulin loading as well as insulin quantification was carried out by Micro-BCA protein assay using a commercially available kit **[14, 15]**. Firstly, 10 mg of dried insulin incorporated microspheres were dissolved in 1 mL of dichloromethane at RT. Insulin was extracted into 0.01 M HCl under vigorous shaking for 2 min in a high speed vortex device at setting 10. The suspension was allowed to settle for 5 min at RT. The supernatant aqueous

phase containing insulin was extracted by separating from remaining organic phase. The supernatant solution (150 µL) was used for the insulin quantification. Briefly, 150 µL of albumin (BSA) standard in duplicate and individual samples in triplicate were added to individual wells of a 96-well plate. The assay working agent (150 µL) was added to each of the wells containing standards as well as samples. The plate was covered with a sealing tape and the mixing of the solutions in the wells was ensured by shaking the plate in a micro plate shaker for 30 s. The microplate was incubated at 37 ºC in an incubator for 2 h. Following the incubation period, the plate was cooled to RT and the absorbance was measured at 562 nm with a microplate reader. The blank absorbance was subtracted and the insulin concentration (µg/mL) of the unknown samples was measured by comparing with standard curve (R^2=0.99) obtained from BSA standard. Total quantity of the incorporated insulin was calculated. The encapsulation efficiency [**16**] of the formulations was calculated using following equation.

Insulin encapsulation efficiency (%) = [total amount of residual insulin in the yield (g)]/ [initial amount of insulin added during microencapsulation (g)] × 100.

The experiments were performed in triplicate for each formulation and the encapsulation efficiencies (%) of the formulations are presented as means ± standard deviation (n = 3).

In vitro insulin release

In vitro insulin release was studied in PBS (pH = 7.4) at 37 ºC [**14**]. Briefly, 30 mg of microspheres from each formulation were weighed in a 2 mL tube. Then, 1.2 mL of sterile PBS was added to each of the tubes containing microspheres. The tubes were tightly capped and incubated in a shaking water bath incubator (Taitec Corporation., Japan) at 37 ºC with a shaking speed of 50 rpm. After pre-determined time points of 1, 2, 4, 8, 12, 16, 20, 24 and 28 days, 1 mL of release medium was collected and replaced with equivalent volume of fresh PBS. Release experiments were performed for 4 weeks. The insulin amount in the released medium was quantified by Micro-BCA. The cumulative release (%) was calculated from insulin concentration obtained from Micro-BCA and was plotted against time (days) to draw the release curves. The experiments were performed in triplicate for each formulation and the data points in the curve are presented as means ± standard deviation (n = 3).

Results and discussion

The microsphere formulations obtained under different preparation conditions were observed under SEM, and the representative SEM photomicrographs of the microspheres are shown in **Fig. 1**. Microspheres were prepared under the two different conditions from various PLGA concentrations 1 using salt free continuous phases (control) and 2 salt (NaCl: 0.5 M and 1.0 M) based continuous phases. Control microspheres obtained from four different PLGA concentrations are represented in **Fig. 1** (A-D). The microspheres prepared from 50 wt% and 25 wt% of PLGA showed smooth and spherical morphology with visible surface pores. The pore formation in microspheres could be due to the slow polymer precipitation. Slow precipitation of PLGA leads to delayed solidification of the microspheres which might cause the diffusion of large amount of the drugs to continuous phase and subsequent formation of surface pores [**17**]. Visibility of surface pores and the formation of collapsed microspheres in the formulations were gradually increased with decrease in PLGA concentration and the formation of porous and collapsed microspheres were clearly evident in the formulations from 5 wt% and 10 wt% of PLGA. The formulations from 25 wt% of PLGA showed a larger number of surface pores compared to the formulations prepared from 50 wt% PLGA. However, the formulations prepared on further reduction of PLGA concentration (10 wt% and 5 wt%) showed a large number of collapsed microspheres. In order to facilitate the formation of the smooth and spherical microspheres from all the PLGA concentrations under an identical preparation condition, the microsphere preparation was carried out by introducing NaCl in aqueous

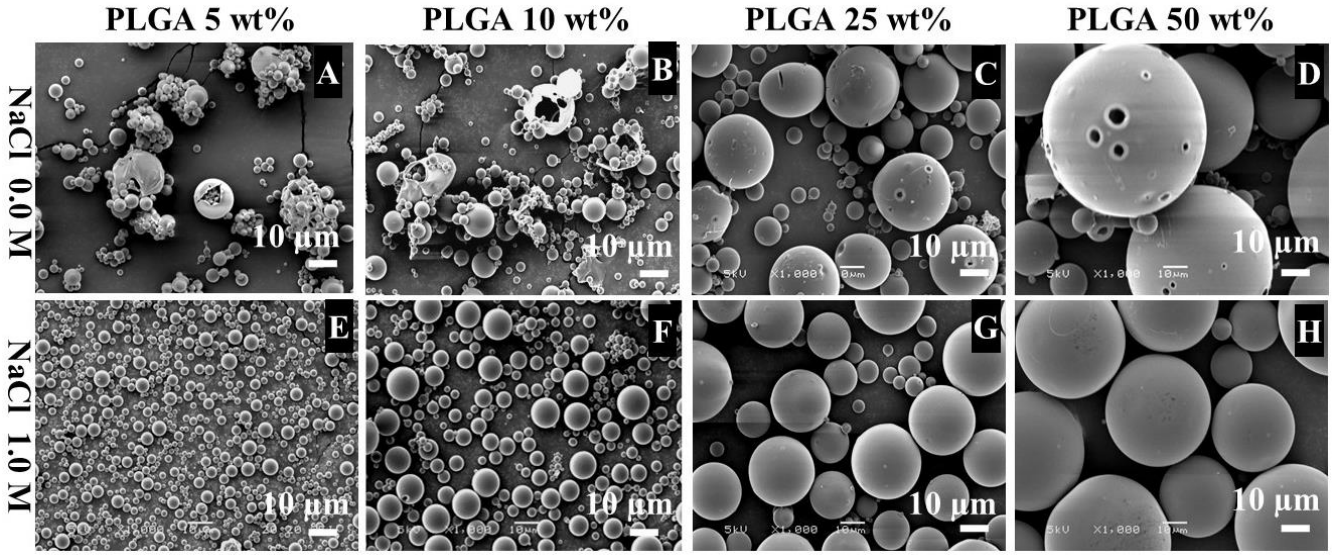

Fig. 1. SEM photomicrographs of insulin incorporated PLGA microspheres from 50 wt% (A, E), 25 wt% (B, F), 10 wt% (C, G) and 5 wt% (D, H) of PLGA at NaCl free (A-D) and those at 1.0 M NaCl (E-H) concentration.

continuous phase of emulsion process. Two different concentrations of salt such as 0.5 M and 1.0 M were used for preparation of insulin incorporated microspheres from all the four different PLGA concentrations. The representative SEM photomicrographs of the microspheres prepared using 1.0 M of the salt are represented in **Fig. 1** (E-H). It was observed that the surface pores in the microsphere formulations from each of PLGA concentration were reduced by increasing the salt concentrations from 0 M to 0.5 M (data not shown) and was completely eliminated using high concentration (1.0 M) of salt. Using a high concentration of ionic salt in continuous phase, the formation of smooth and spherical microspheres was favored from the lowest PLGA concentration (5 wt% PLGA) and the formation of collapsed microspheres in the formulations was entirely eliminated. The results demonstrated that the use of common salt showed to enhance the stability of the microspheres prepared from low range PLGA concentrations and should be useful to control the size of microspheres from a broad range of PLGA concentration. The effect could be due to the fast polymer precipitation on the surface of the dispersed phase which might have caused the prevention of drug loss into the continuous phase. The reason for fast polymer precipitation should be due to the tailored osmotic pressure generated at continuous phase by addition of NaCl [**11, 17**].

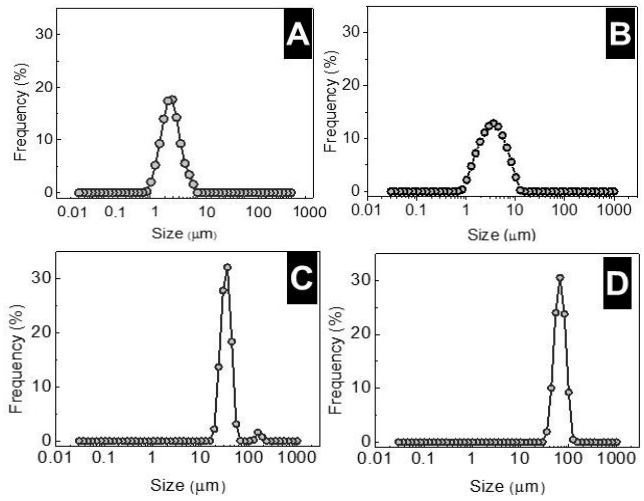

Fig. 2. Microsphere size distribution of the formulations prepared by addition of 1.0 M NaCl in continuous phase of emulsion process: 5 wt% (A), 10 wt% (B), 25 wt% (C) and 50 wt% (D) of PLGA.

The laser particle size analysis showed a narrow size distribution of the microspheres prepared from all the PLGA concentrations using salt in aqueous continuous phase of emulsion process. **Fig. 2** shows the laser particle size analysis of the microspheres prepared using 1.0 M salt in continuous phase. Using salt based approach, the size and size distribution of the microspheres was well controlled by controlling the concentration of PLGA at a broad range. All the microsphere formulations obtained via salt based condition also showed a narrow size distribution and indicated the homogeneity of the microspheres in formulations which should be good for controlled release application [**18**].

In our experiments, the size of the microsphere was controlled in the range from (3.0 ± 0.7) μm to (45.2 ± 1.8) μm by changing the PLGA concentration from 5 wt% to 50 wt% respectively (**Table 1**). A decrease in the average size of the microspheres was observed with increase in NaCl concentration. The results indicated that the microspheres became more and more compact with increase in salt concentration and could be due to increase in built up osmotic pressure at continuous phase **[11, 19, 20]**.

Table 1. Average size and drug encapsulation efficiencies of the microspheres.

PLGA concentration (wt %)	Microsphere size and insulin encapsulation efficiencies at different continuous phase salt concentrations					
	Average microsphere size (μm)			Insulin encapsulation efficiency (%)		
	0 M NaCl	0.5 M NaCl	1.0 M NaCl	0 M NaCl	0.5 M NaCl	1.0 M NaCl
5	5.9±0.6	5.0±0.7	3.00±0.7	21.1±2.6	43.1±1.9	54.3±3.2
10	8.6±0.3	6.0±0.3	5.6±0.3	33.8±1.5	52.2±2.1	61.5±1.2
25	29.1±0.8	25.7±0.6	24.6±0.6	60.8±4.0	72.6±2.9	76.7±2.3
50	49.1±1.3	46.0±1.5	45.2±1.8	89.3±1.8	97.9±0.7	98.6±1.1

The prepared microspheres were further evaluated in terms of their insulin encapsulation efficiency and control over the release of insulin at physiological condition. It was observed that the encapsulation efficiencies of the formulations prepared from the low range PLGA concentrations (5 wt% and 10 wt%) were significantly enhanced with increase in salt concentration (**Table 1**). However, the use of salt did not significantly affect the encapsulation efficiency of the formulation prepared from 50 wt% of PLGA and a moderate increase was observed for the formulation prepared from 25 wt% PLGA. A big jump in encapsulation efficiencies of all the formulations was noticed with variation of salt concentration from 0 M to 0.5 M and the changes did not follow the similar pattern with further variation from 0.5 M to 1.0 M. The reason could be the initial variation in salt concentration from 0 M to 0.5 M, which might be sufficient to stabilize most of the microspheres in the formulations. The increase in encapsulation efficiencies of salt based preparations could be due to the generation of optimal osmotic pressure gradient between the continuous and dispersed phase **[11, 19,20]**.

The release of insulin from all the formulations was studied for 4 weeks. The release curves were prepared for each PLGA concentration at salt free as well as salt based preparation condition and were compared (**Fig. 3**). For the microsphere formulations from each PLGA concentration, the insulin release was prolonged with increase in continuous phase salt concentration and the phenomenon was more significant from the release curves obtained from the formulations prepared from low range PLGA concentrations (5 wt% and 10 wt%). The initial burst from all the formulations was decreased with increase in salt concentration and was significantly reduced for the formulations obtained from 5 wt%, 10 wt% and 25 wt% of PLGA concentration. Almost all the insulin was released within a week period from the control preparations of 5 wt% and 10 wt% of PLGA. However, the release for similar formulations prepared using 1.0 M salt was prolonged for 12 and 14 days period respectively (5 and 8 days for control; 7 and 11 days for 0.5 M NaCl)). This shows an increased trend in sustained release duration with increase in salt concentration.

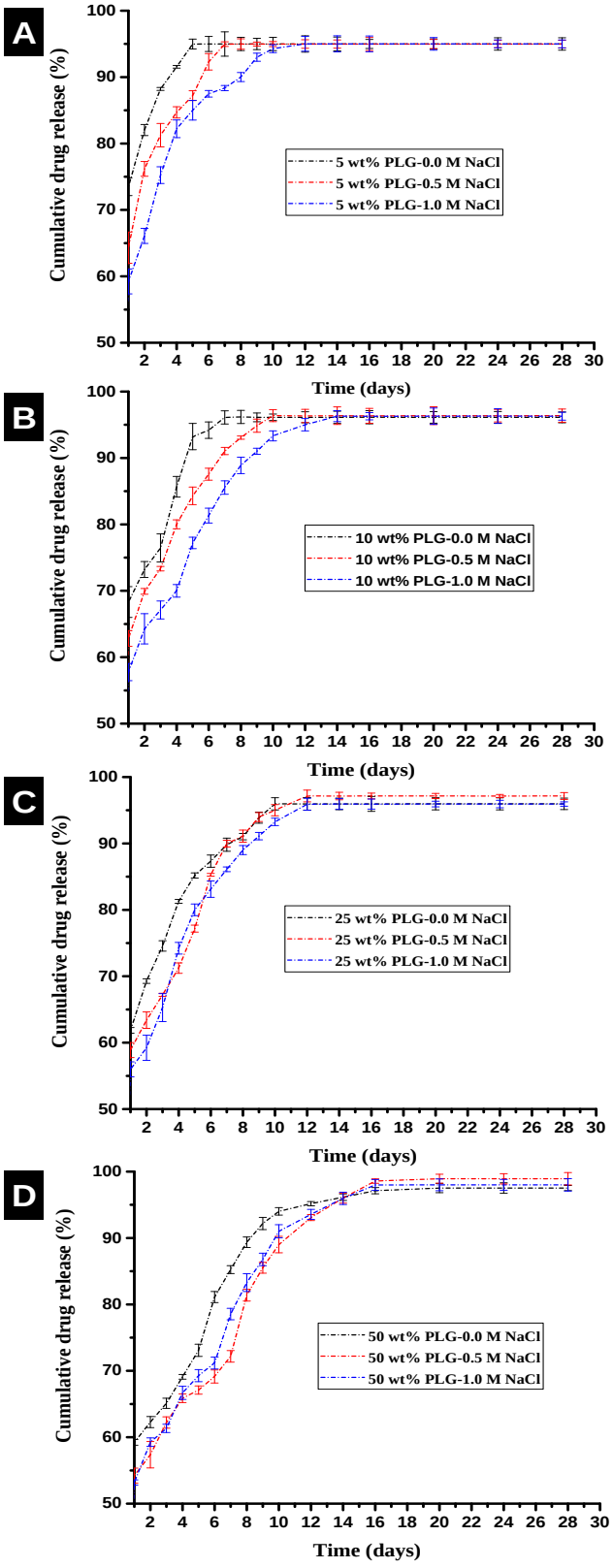

Fig. 3. Insulin release profiles from PLGA microsphere formulations under control and salt based preparation conditions: 5 wt% (A), 10 wt% (B), 25 wt% (C) and 50 wt% (D) of PLGA.

The phenomenon was more significant in the case of the formulations prepared from low range PLGA concentrations. However, no significant differences (other than reduction of initial burst) in release curves were observed among the control and experimental formulations prepared from 50 wt% of PLGA and a moderate increase in sustained release duration was observed among the different release curves obtained from 25 wt% PLGA.

In summary, the use of NaCl was useful to control the osmotic pressure gradient between the continuous and dispersed phases during the emulsion process, which was further useful to control the solidification of PLGA. An optimal pressure gradient generated by an optimal value of NaCl concentration should determine the best formulation condition of microspheres prepared from a unique range concentration of PLGA.

Conclusion

Insulin incorporated PLGA microspheres were prepared from wide range of PLGA concentrations by use of common ionic salt in continuous phase of emulsion process. The smooth and spherical morphology of the microspheres were restored by elimination the highly porous and collapsed microspheres from the formulations. Using modified preparation scheme, the microsphere sizes were well controlled for the variation of PLGA concentration. The formulations prepared using the modified scheme showed significant enhancement in insulin encapsulation efficiencies. All the formulations prepared using modified preparation scheme showed a better control over the insulin release than salt-free preparations. The technology should be useful for industrial applications to establish a unique preparation scheme to produce the controlled release formulations from a range of PLGA concentrations.

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Author's contributions

Conceived the plan: G. Chen, N. Kawazoe and HN; Performed the expeirments: HN; Data analysis: HN; Wrote the paper: HN. Author have no competing financial interests.

References

1. Walsh, G.; *Nat. Biotechnol*. **2000**, *18*, 831. **DOI:** 10.1038/78720
2. Jin, J.; Sklar, G.E.; Oh, V.M.S.; Li, S.C.; *Ther. Clin. Risk Manage*. **2008**, *4*, 269. **DOI**: 10.2147/TCRM.S1458
3. Lu, Y.; Chen, S.; *Adv. Drug Delivery Rev*. 2004, 56, 1621. **DOI**: 10.1016/j.addr.2004.05.002
4. Ye, M.; Kim, S.; Park, K.; *J. Controlled Release* **2010**, *146*, 241. **DOI**:10.1016/j.jconrel.2010.05.011
5. Freiberg, S.; Zhu, X.; *Int. J. Pharm*. 2004, 282, 1. **DOI**: 10.1016/j.ijpharm.2004.04.013
6. Crotts, G.; Park, T.G.; *J. Controlled Release* 1995, 35, 91. **DOI**:10.1016/0168-3659(95)00010-6
7. Cohen, S.; Yoshioka, T.; Lucarelli, M.; Hwang, L.H.; Langer, R.; *Pharm. Res*. **1991**, *8*, 713. **DOI**: 10.1023/A:1015841715384
8. Mundargi, R.C.; Babu, V.R.; Rangaswamy, V.; Patel, P.; Aminabhavi, T.M.; *J. Controlled Release* **2008**, *125*, 193. **DOI**:10.1016/j.jconrel.2007.09.013
9. Yang, Y.Y.; Chung, T.S.; Bai, X.L.; Chan, W. K.; *Chem. Eng. Sci*. **2000**, *55*, 2223. **DOI**: 10.1016/S0009-2509(99)00503-5
10. Yeo, Y.; Park, K.; *Arch. Pharmacal Res*. 2004, 27, 1. **DOI**: 10.1007/BF02980037

11. Mezzenga, R.; Folmer, B.M.; Hughes, E.; *Langmuir* **2004**, *20*, 3574. **DOI**: 10.1021/la036396k
12. Jiang, G.; Thanoo, B.; DeLuca, P.P.; *Pharm. Dev. Technol*. **2002**, *7*, 391. **DOI**: 10.1081/PDT-120015040
13. Freitas, S.; Merkle, H.P.; Gander, B.; *J. Controlled Release* **2005**, *102*, 313. **DOI**:10.1016/j.jconrel.2004.10.015
14. Andreas, K.; Zehbe, R.; Kazubek, M.; Grzeschik, K.; Sternberg, N.; Bäumler, H.; Schubert, H.; Sittinger, M.; Ringe, J.; *Acta Biomater.* **2011**, *7*, 1485. **DOI**:10.1016/j.actbio.2010.12.014
15. Ubaidulla, U.; Khar, R.K.; Ahmad, F.J.; Sultana, Y.; Panda, A.K.; *J. Pharm. Sci*. **2007**, *96*, 3010. **DOI**: 10.1002/jps.20969
16. Hrynyk, M.; Martins-Green, M.; Barron, A.E.; Neufeld, R.J.; *Int. J. Pharm.* **2010**, *398*, 146. **DOI**: 10.1016/j.ijpharm.2010.07.052
17. Yeo, Y.; Park, K.; *Arch. Pharmacal Res*, **2004**, *27*, 1. **DOI**: 10.1007/BF02980037
18. Berkland, C.; Kim, K.K.; Pack, D. W.; *J. Controlled Release* **2001**, *73*, 59. **DOI**: 10.1016/S0168-3659(01)00289-9
19. Pistel, K.; Kissel, T.; *J. Microencapsulation* **2000**, *17*, 467. **DOI**: 10.1080/026520400405723
20. Zhang, J.; Zhu, K.; *J. Microencapsulation* **2004**, *21*, 775. **DOI**: 10.1080/02652040400008465