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Preparation of PLGA nanoparticles containing estrogen by emulsification–diffusion method☆

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Abstract

Nano-sized poly (D,L lactide-co-glycolide) (PLGA) particles, widely used as a biodegradable polymeric carrier, containing estrogen were prepared employing emulsification–diffusion method. Estrogen was chosen as a model drug. The preparation method consists of emulsifying a solution of polymer and drug in the aqueous phase containing stabilizer, previously saturated, followed by adding excess water. Influence of process variables on the mean particle size of nanoparticles has been studied. It was clarified that the type and concentrations of stabilizer, homogenizer speed, polymer concentration determined the size of PLGA nanoparticles. Especially when didodecyl dimethyl ammonium bromide (DMAB) was used as a stabilizer, estrogen containing nanoparticles of smaller than 100 nm was obtained.

Keywords: Poly (D,L lactide-co-glycolide) (PLGA); Nanoparticle; Estrogen; Emulsification–diffusion method; Didodecyl dimethyl ammonium bromide (DMAB)

1. Introduction

Thirty to 50% of patients undergoing percutaneous coronary balloon angioplasty (PTCA) procedures develop reoclusion within 3–6 months. This process of arterial reobstruction is called restenosis. Although a number of factors are involved in the pathogenesis of vascular restructuring, the final feature of restenosis is the proliferation and migration of smooth muscle cells from the media to the intima [1]. In order to inhibit vascular smooth muscle cell proliferation, drugs must be delivered at a high concentration for a prolonged period of time [2,3].

However, local drug delivery rather than systemic administration might be a more effective way to obtain higher tissue drug levels and at the same time decrease the potential adverse systemic

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drug associated side effects [4]. Several studies showed that direct infusion of drug solutions into the targeted artery site using various infusion catheters do not provide adequate retention of therapeutic levels in the arterial wall over time. Medications injected in a solution state diffuse out of the arterial wall too quickly to have a therapeutic effect. Furthermore, the approach of direct infusion of drug solution into artery is not easily applicable for those therapeutic agents which are poorly water soluble [5,6].

One of the most promising dosage forms as potential formulations for site-specific drug delivery system including drug targeting has been nanoparticles. Especially interest has been focused on the use of particle formation prepared from polyesters such as poly (D,L lactide-co-glycolide) (PLGA), poly (D,L lactide) (PLA), poly glycolide (PGA). This is largely due to their biocompatability and to their resorbability through natural pathways [7]. This is also related to the fact that polymers issued from glycolic acid and lactic acid polymers are approved by FDA. These polymers do not require surgical removal after completion of drug release.

Three techniques have been usually used for the preparation of nanoparticles based on preformed biodegradable polymers: (a) solvent-evaporation procedure (Gurny et al.); (b) salting-out procedure (Bindschaedler et al.); (c) nanoprecipitation procedure (Fessi et al.) [8–10]. A process of emulsification followed by solvent evaporation is the most widely used technique for preparing nanoparticles containing drugs. This conventional technique usually consists of three major steps: emulsification step of a water-immisible organic solution with an aqueous phase containing stabilizers: removal step of the solvent by extraction: and isolation step of nanoparticles by filtration or centrifugation [11,12]. Nevertheless, several difficulties have been showed using these techniques when working with toxic solvents (solvent-evaporation) and salts that are incompatible with bioactive compounds (salting-out). And these techniques were not useful to reduce the particle size and were impossible to produce nanoparticles with diameter less than 100 nm.

Recently, Leroux et al. [13] developed a new method called emulsification–diffusion with poly(lactic acid) (PLA), using a partially water-soluble solvent, which is the well accepted benzyl alcohol. In this method, o/w emulsion is formed in the presence of stabilizing colloids, and the addition of sufficient quantity of water induce the diffusion of the solvent and the precipitation of polymer as nanoparticles (250–600 nm). Poly vinyl alcohol (PVA) or gelatin was used as a stabilizer. To overcome the drawback of using toxic solvent, benzyl alcohol, D. Quintanar-Guerrero et al. investigated the emulsification–diffusion method using propylene carbonate (PC) as partially water-soluble solvent [14]. However, as formerly, the use of this technique usually results in large particles (i.e. 100–450 nm).

The aim of the work reported here was to determine the formulation conditions suitable for production of drug-loaded PLGA nanoparticles having a size potentially suitable for arterial uptake. Although exact range of particle size is a matter for conjecture, the optimum particle size range for arterial uptake is below 100 nm diameter. In this paper, the preparation and optimization of an emulsification–diffusion technique for PLGA nanoparticles, using propylene carbonate as a partially water-soluble solvent is investigated. The effect of processing conditions on the mean particle size of nanoparticles was determined to design optimum process conditions to prepare drug-loaded PLGA nanoparticles of less than 100 nm in size. In this study, 17β-estradiol benzonate (estrogen) was chosen as a model antiproliferative agent.

2. Materials and methods

2.1. Materials

Poly(D,L lactide-co-glycolide) (PLGA) with a weight-average molecular weight of 75 000–120 000, whose copolymer ratio of D,L-lactide to glycolide is 75:25 was purchased from Sigma (St Louis, MO). 17β-estradiol benzoate (estrogen) with a molecular weight of 376.5 (Genexol, Sam Yang Genex, Seoul, Korea) was used as a model

drug in this study. Junsei Chemical Co. (Japan) supplied propylene carbonate (PC) as a solvent. Didodecyl dimethyl ammonium bromide (DMAB) was obtained from Aldrich (Aldrich Chemical Co., Milwaukee, WI). Poly(vinyl alcohol) (PVA, molecular wt. 30 000–70 000) was purchased from Sigma Chemical Co. Distilled water was of Milli-Q quality (Millipore, USA-Bedford, MD). All organic solvents were either HPLC grade or American Chemical Society analytical grade reagents.

2.2. Preparation of PLGA nanoparticles

The PLGA nanoparticles were prepared using the emulsification–diffusion method. Fig. 1 shows the mechanism for the formation of PLGA nanoparticles by this technique. After the mutual saturation of the two phases, the partially water-soluble solvent containing PLGA and water containing stabilizer, both liquids are in the state of thermodynamic equilibrium (a) Stirring causes the dispersion of the solvent solution as globules in equilibrium with the continuous phase: the stabilizing agent is then adsorbed on the large interfacial area created; (b) The addition of water to the system destabilizes the equilibrium; (c) It causes the solvent to diffuse to the external phase. During this transport of solute, new globules of nanometer size are produced which gradually become poorer in solvent; (d). As a result, the polymer of the globules aggregates because of the presence of a new, continuous non-solvent phase.

In detail, 200 mg of PLGA was dissolved in 10 ml of PC. The organic phase was added into 20 ml of an aqueous phase containing stabilizer. After mutual saturation of organic and continuous phase, the mixture was emulsified for 7 min with a high speed homogenizer (Omni international waterbury, CT, USA). In order to allow for diffusion of PC into water, water (80 ml) was subsequently added to o/w emulsion solution under the moderate magnetic stirring, leading to the nanoprecipitation of PLGA particles. PC was removed by dialysis, thereafter nanoparticles were lyophilized to produce a homogeneous free-flowing powder. To formulate nanoparticles loaded with estrogen, the drug (3 mg) was added in the initial step of nanoparticle formation, followed by the same sequence as above. The basic recipe for the preparation of PLGA nanoparticles is given in Table 1.

2.3. Particle size analysis

The mean particle diameter of the nanoparticles was assessed by light scattering method (Zeta plus, Brookhaven Inst. Co., USA). Particle size

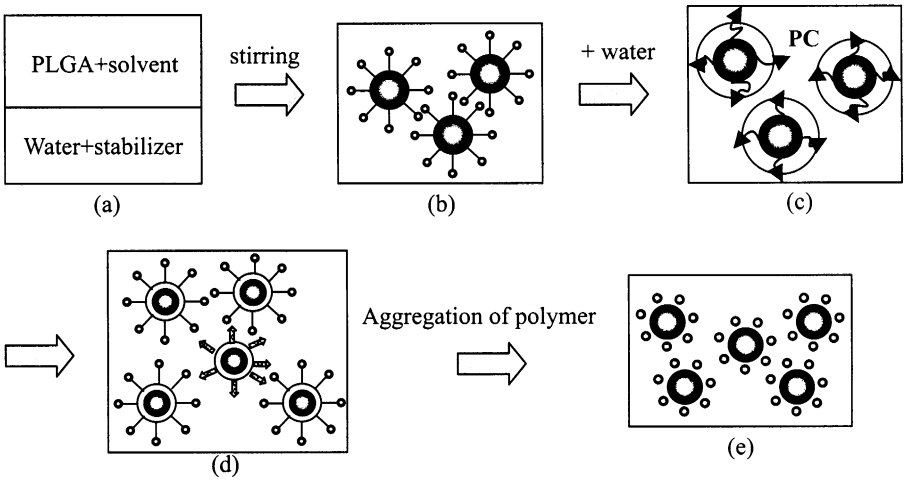

Fig. 1. Schematic description of the proposed formation mechanism of PLGA nanoparticles by emulsification–diffusion method.

Table 1
The basic recipe for the preparation of PLGA nanoparticles

	Ingredients	Amount
Organic phase	PLGA	Variables[a]
	Estrogen	3 mg
	PC	10 ml
Aqueous phase	D.D.I. water	20 ml
	Stabilizer (DMAB, PVA)	Variables[b]
Emulsifying (for 7 min)	Homogenizer speed	Variables[c]

[a] PLGA concentrations were varied 1, 2, 3, 4 (w/v%) with respect to the amount of solvent.
[b] DMAB concentrations were 1, 2, 3, 4 (w/v%) and PVA concentrations were 2.5, 5.0, 7.5, 10.0 (w/v%) with respect to the amount of D.D.I. water.
[c] Homogenized speed was 4800, 8000, 11 200, 13 600 (rpm).

was expressed as number-weighted mean diameter in nanometers and was obtained from the measurements of at least three bathes of nanoparticles unless stated other wise.

3. Results and discussion

3.1. Estrogen content of nanoparticles

The estrogen content of nanoparticles was analyzed using UV spectrophotometer (Shimazu) at 297 nm. Estrogen-loaded nanoparticles were centrifuged at 14 000 rpm for 40 min. The residual estrogen in the supernatant was analyzed using UV spectrophotometer. Nanoparticles formulated by the emulsification–diffusion method have an encapsulation efficiency of 67% based on standard estrogen concentration curve.

3.2. Effect of stabilizer type and concentration

The effect of stabilizer type and concentration on the mean particle size of nanoparticles was investigated. In emulsification–diffusion method, the stabilization of droplets and 'protonanoparticles' after diffusion process is important to avoid coalescence and the formation of agglomerates. When the interface is formed, drive to lower the energy of the system and to hinder the coalescence of particles is the adsorption of materials such as stabilizer at interface. Various stabilizers selected based on pharmaceutical purpose such as P.E.G. (Mw 8000), tween 80, gelatin, dextran (Mw 6000), pluronic L-63, PVA were tested with the emulsification–diffusion method. But in all cases except PVA, no PLGA nanoparticle formation was formed [15,16].

Vinod Labhasetwar et al. [1] demonstrated that surface modification of nanoparticles with DMAB optimally enhances arterial drug (U-86) levels compared to other modifications and the unmodified nanoparticles. The mechanism of enhanced arterial drug (U-86) levels with DMAB surface modification is explained due to the change of anionic surface charge of unmodified nanoparticles into cationic surface charge. The cationic nature of the surface modified nanoparticles probably has increased ionic interactions with the negatively charged glycosaminglycan enriched arterial wall, thus facilitating their arterial uptake and retention. In addition, since DMAB is a mild surface active agent, it could transiently change the permeability of the arterial vasculature and facilitate nanoparticle internalization into the arterial wall. So based on this research, we introduced DMAB as stabilizer into emulsification–diffusion method. There was no coalescence and stable nanoparticle dispersion could be obtained.

Fig. 2 shows the influence of stabilizer concentration when DMAB and PVA were used as stabilizer, respectively. The mean particle size was found to decrease sharply in the 1–2 (w/v%) range of DMAB and 2–4 (w/v%) range of PVA. Above each concentration, only a small quantity of stabilizer is adsorbed at the interface, the excess remains in the continuous phase, and does not play any significant role in the emulsification [17]. The mean particle size of nanoparticles prepared using DMAB, as stabilizer is smaller than that of PVA. The product consists of spherical and discrete particles in the nanometer size range. Increasing concentration of stabilizer resulted in a decreased mean particle size of nanoparticles. This is in agreement with results obtained by H. Rafati et al. using PVA as stabilizer by w/o/w emulsion–evaporation procedure [18].

3.3. CMC and pyrene solubilizing ability of DMAB and PVA

HLB value, CMC, solubilizing ability, and stabilizer type affect surfactant ability. HLB value is hydrophile-lipophile balance and stabilizer used in emulsion system has HLB value ranged from 18 to 20. CMC is critical micelle concentration. Above CMC, stabilizer forms micelle. In nonionic stabilizer system, particle is stabilized by steric hindrance, but in ionic stabilizer system, particle is stabilized by not only steric hindrance but also electrostatic repulsion. To explain the difference of DMAB and PVA, CMC and pyrene solubilizing ability was measured. Figs. 3 and 4 show the UV absorbance of pyrene and the surface tension of stabilizer solution as a function of stabilizer concentration. Pyrene is an aromatic hydrocarbon exhibiting a very low solubility in pure water ($[Py]_{s,water} = 7 \times 10^{-7}$ M) and is largely used as a probe for the study of micelles and other hydrophobic aggregate in water. The increase in the UV absorbance of pyrene with stabilizer concentration indicated the enhanced solubility of pyrene due to the formation of polymer aggregates like micelles in the aqueous solution. Also, a gradual decrease and leveling off of surface tension as a function of stabilizer concentration indicated that stabilizer formed aggregates. As shown in Figs. 3 and 4, at the same concentration, DMAB has

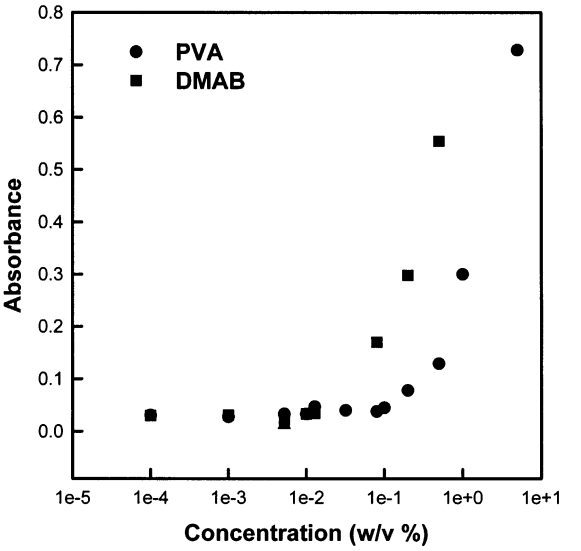

Fig. 3. UV absorbance of pyrene at 372 nm (DMAB), 360 nm (PVA) as a function of concentration (wt% based on total).

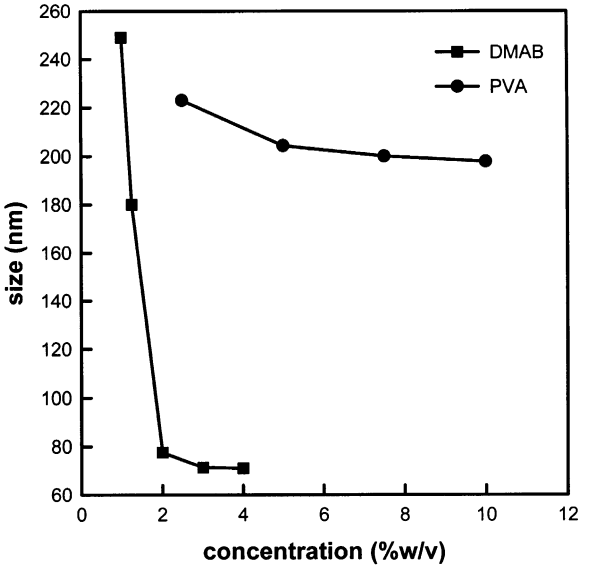

Fig. 2. The influence of the percentage stabilizing agent in the external phase on the mean particle size of PLGA nanoparticles.

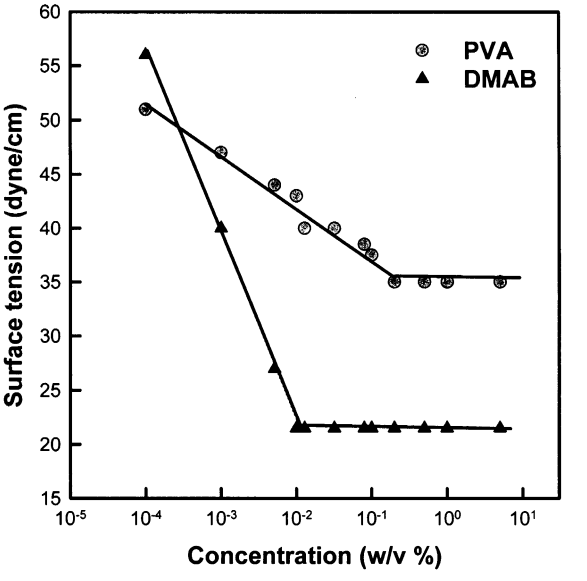

Fig. 4. Surface tension of DMAB and PVA solution as a function of concentration (wt% based on total).

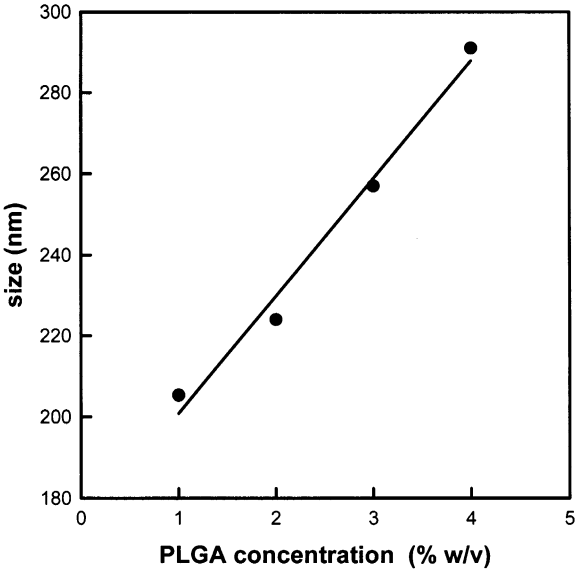

Fig. 5. Effect of PLGA concentration on the mean particle size of PLGA nanoparticles (at the concentration of 2.5 (w/v%) of PVA).

much higher pyrene solubility and much lower CMC than PVA. This fact means that DMAB forms aggregate at the very low concentration and solubilizes organic polymer solution more than PVA. These results can explain the difference of DMAB and PVA.

3.4. Effect of polymer concentration

For these evaluations, PVA as stabilizer was used at a constant concentration of 2.5 (w/v%). PLGA concentrations were varied from 1.0 to 4.0 (w/v%). Fig. 5 shows the effect of polymer concentration on the particle size of nanoparticles. Polymer concentration in the internal phase was a crucial factor in increasing the size of nanoparticles, as its concentration was increased. This is in agreement with the findings of E.J.A.M Schlicher et al. [19]. PC diffuses from the solution into the water carrying some PLGA molecules with it. So as the PLGA concentration increase, the amount of PLGA based on PC increase. Therefore particle size increase at a fixed other conditions. Also as polymer concentration increases, viscosity of organic solution increases. High viscous resistance to the shear forces hinder the nanoparticle formation.

3.5. Effect of Homogenizer Speed

The influence of homogenizer speed on the mean particle size of nanoparticles was also studied. Allemann et al. established that the final size of the nanoparticles in the process of salting-out depends on the globule size throughout the emulsification process. PLGA nanoparticles was prepared using PVA as stabilizer at a constant concentration of 5.0 (w/v%). And homogenization time was fixed at 7 min. The results are shown in Fig. 6. As expected, a decrease of nanoparticle mean size correlated with an increase of homogenizer speed. But above 12 000 rpm, there was no reduction of particle size.

3.6. Effect of temperature of adding D.D.I. water

The important factor influencing on the particle size is diffusion. Stokes–Einstein proposed diffusion coefficient as follows:

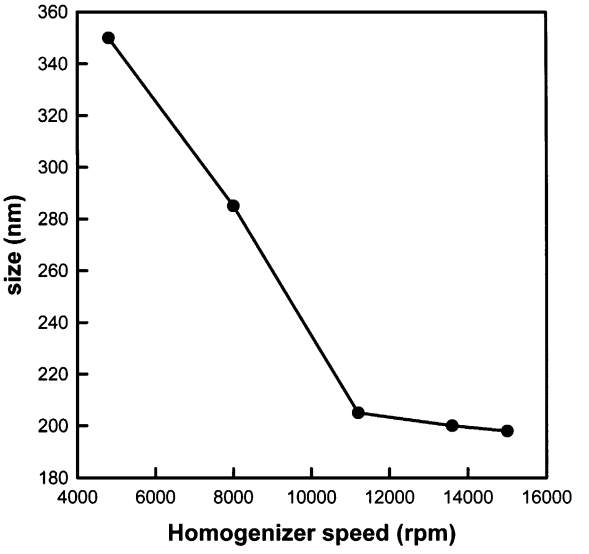

Fig. 6. The influence of homogenizer speed on the mean particle size of nanoparticles (at the concentration of 5.0 (w/v%) of PVA).

Table 2
Effect of temperature of adding D.D.I. water on the mean particle size of nanoparticles

Stabilizer[a]	Temperature of adding water[b](°C)	Mean particle size (nm)	Polydispersity
DMAB	25	78	0.023 ± 0.012
	47	68	0.032 ± 0.015
	60	65	0.056 ± 0.019
PVA	25	204	0.064 ± 0.028
	47	173	0.078 ± 0.041
	60	170	0.063 ± 0.034

[a] The concentration of DMAB was 2.0 (w/v%) and the concentration of PVA was 5.0 (w/v%).
[b] Adding rate of D.D.I. water was 16 ml s^{-1}.

$$D_{AB} = \frac{kT}{6\pi r \eta_B}, \quad (1)$$

where D_{AB} ($cm^2\ s^{-1}$) is mutual diffusion coefficient, k ($= 1.3805 \times 10^{-23}$ J K^{-1}) is Boltzman coefficient, T (K) is Kelvin temperature, r is hydrodynamic volume radius, η_B (cP) is viscosity of continuous phase.

Wilke–Chang also proposed diffusion coefficient:

$$\frac{D_{AB}}{T} = \frac{7.4 \times 10^{-8} (\phi_B M_B)^{0.5}}{\eta_B V_A^{0.6}}, \quad (2)$$

where M_B (g mol^{-1}) is molecular weight of solvent, V_A ($cm^3\ mol^{-1}$) is molar volume of the solute A at its normal boiling temperature and ϕ_B is the association factor of solute B.

Diffusion coefficient is proportional to Kelvin temperature of system and the effect of viscosity of continuous phase is reverse. The particle size was decreased as temperature of adding D.D.I. water increased. The results are given in Table 2 as the effect of temperature of adding water. Because high temperature promoted diffusion, particle size decreased. This result means that rapid solvent exchange precipitate polymer quickly.

3.7. Effect of adding rate of D.D.I. water and speed of magnetic stirrer

The influence of adding rate of D.D.I. water and speed of magnetic stirrer also has been studied. Table 3 shows the effect of adding rate of D.D.I. water. No difference was found in both using DMAB and PVA. Fig. 7 shows the effect of speed of magnetic stirrer. Magnetic stirring rate can be changed by size of spin bar, amount of solution, and so on. So we fixed other variables and used arbitrary unit. The decrease of nanoparticle mean size correlated with increase of magnetic stirring rate. The promoting of mixing by mechanical shear stress of magnetic stirring decreased particle size.

4. Conclusions

The present work has shown that drug containing nanoparticle formation by the emulsification–diffusion method. It demonstrates the potential process to control the size of PLGA nanoparticles. The nanoparticle formation process was to be related to the reduction of globule size due to the rapid diffusion of solvent. The significant step for success of this method is the first stage of the process. The stability and the size of droplets formation during the stage are important factor. Preparative variables such as the type and concentrations of stabilizer, homogenizer speed, polymer concentrations, could be the crucial factors for the formation of PLGA nanoparticles. Especially, the use of DMAB as stabilizer was effective to reduce the particle size below 100 nm. Also diffusion coefficient is proportional to Kelvin temperature of system and the effect of viscosity of continuous phase is reverse. The particle size was decreased as temperature of adding D.D.I. water increased.

Table 3
Effect of adding rate of D.D.I. water on the mean particle size of nanoparticles

Stabilizer[a]	Adding rate of water[b] (ml s^{-1})	Mean particle size (nm)	Polydispersity
DMAB	16	78	0.091 ± 0.017
	0.03	76	0.087 ± 0.031
PVA	16	204	0.103 ± 0.017
	0.03	220	0.081 ± 0.030

[a] The concentration of DMAB was 2.0 (w/v%) and the concentration of PVA was 5.0 (w/v%).
[b] Temperature of adding D.D.I. water was 25°C.

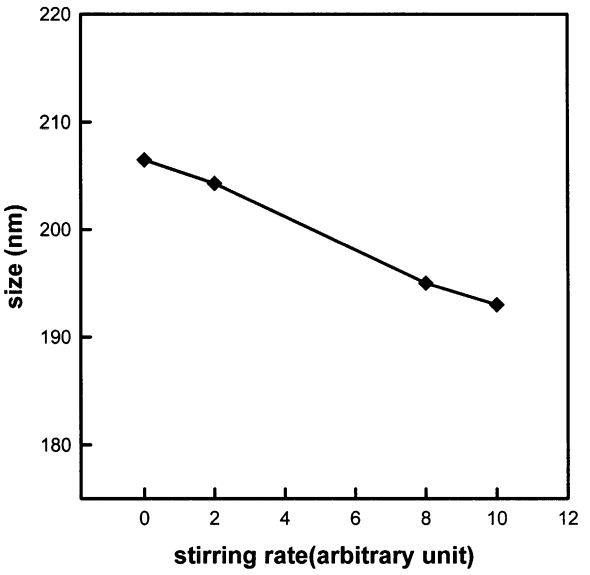

Fig. 7. Effect of stirring rate on the mean particle size of nanoparticles (arbitrary unit was used).

Acknowledgements

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