

Research Paper

Comparison of Diafiltration and Tangential Flow Filtration for Purification of Nanoparticle Suspensions

Gautam Dalwadi,¹ Heather A. E. Benson,¹ and Yan Chen^{1,2}

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Purpose. The study reports evaluation of different purification processes for removing surplus surfactant and formulating stable nanoparticle dispersions.

Methods. Nanoparticle formulations prepared from poly(D,L-lactide-co-glycolide) and polyvinyl alcohol (PVA) were purified by a diafiltration centrifugal device (DCD), using 300K and 100K molecular weight cut-off (MWCO) membranes and a tangential flow filtration (TFF) system with a 300K MWCO membrane. The effects of process parameters including MWCO, transmembrane pressure (TMP), and mode of TFF on nanoparticle purification were evaluated, and two purification techniques were compared to the commonly used ultracentrifugation technique.

Results. Both DCD and TFF systems (concentration mode at TMP of 10 psi) with 300K MWCO membrane removed maximal percent PVA from nanoparticle dispersions (89.0 and 90.7%, respectively). T_{90} , the time taken to remove 90% of PVA in 200-ml sample, however, was considerably different (9.6 and 2.8 h, respectively). Purified nanoparticle dispersions were stable and free of aggregation at ambient conditions over 3 days. This is in contrast to the ultracentrifugation technique, which, although it can yield a highly purified sample, suffers from drawbacks of a level of irreversible nanoparticle aggregation and loss of fine particles in the supernatant during centrifugation.

Conclusions. The TFF, in concentration mode at TMP of 10 psi, is a relatively quick, efficient, and cost-effective technique for purification and concentration of a large nanoparticle batch (≥ 200 ml). The DCD technique can be an alternative purification method for nanoparticle dispersions of small volumes.

KEY WORDS: diafiltration; diafiltration centrifugal device (DCD); nanoparticles; purification; tangential flow filtration (TFF).

INTRODUCTION

The field of nanotechnology, which deals with ultrasmall materials, is highly progressive at present. Its applications in drug delivery, target-specific therapy, molecular imaging, biomarker, biosensor, diagnosis, and many other biomedical fields are rapidly growing. Novel drug delivery systems based on biopolymers provide new opportunities in pharmaceutical formulation for all therapeutic classes of medicine. Improvements in product self-life, patient compliance, therapeutic efficacy, and safety have been demonstrated. Biodegradable polymer materials are used to synthesize novel drug delivery system in the form of nanoparticles, hydrogels, dendrimers, micelles, quantum dots, etc. (1–3).

Recently, nanoparticles have received increasing interest as a delivery system for drugs, contrast agents, proteins, peptides, DNA, vaccines, and other biologically active agents. They are often designed for the purpose of transporting the diagnostic or therapeutic agent to particular

targets in the body following oral or parenteral administration. In these applications, there is an absolute requirement for nanoparticles to be free of toxic impurities.

Nanoparticles can be prepared from either natural or synthetic polymeric materials. There are numerous methods for preparation of drug-loaded nanoparticles. Some involve polymerization of monomers, and others form nanoparticles by manipulation of polymers via processes such as emulsification–solvent evaporation, solvent diffusion, multiple emulsion, salting out, phase inversion, ionic gelation, and nanoprecipitation (4). Depending on the method of preparation, there is a potential that certain impurities, some of which may be toxic, could be present in the final product. These impurities include organic solvents such as dichloromethane, surfactants, emulsifiers or stabilizer, monomer residuals, polymerization initiators, salts, and large polymer aggregates (5). The presence of these impurities will not only cause potential biological intolerance, but may also alter the physicochemical and release characteristics of nanoparticle systems. Effective purification of nanoparticles is therefore a necessary step for controlling the quality and characteristics of nanoparticle products.

A range of approaches have been used for purification of nanoparticles. Filtration through mesh or filters is often employed for removal of large aggregates (6,7). Centrifuga-

¹ Western Australian Biomedical Research Institute, School of Pharmacy, Curtin University of Technology, GPO Box U1987, Perth, Australia 6845.

² To whom correspondence should be addressed. (e-mail: y.chen@curtin.edu.au)

tion or ultracentrifugation techniques are commonly used for removal of organic solvents, free drug, or free stabilizer such as polyvinyl alcohol (PVA) and electrolytes (6,8–10). Dialysis techniques (11), gel filtration (12), ultrafiltration (13), and, more recently, diafiltration (14,15) and cross-flow microfiltration (5,16) have been investigated for the purification of nanoparticles. Centrifugation or ultracentrifugation, in combination with washing nanoparticles with an appropriate medium such as deionized water, is the most common approach to remove large quantities of process impurities (17,18). However, the impact of the centrifugation force can cause caking and difficulties in redispersing nanoparticles (19,20). A significant loss of nanoparticles to the supernatant can also occur when insufficient centrifugation force is applied, resulting in a low yield of nanoparticles. Purification by dialysis is a time-consuming process with a high risk of microbial contamination of the product and inadequate removal of relatively large molecule impurities such as PVA (19). In addition, the dialysis technique can potentially result in premature release of nanoparticle payload during the lengthy purification period. Gel filtration is a faster process but is limited because only a relatively small volume of sample can be processed at a time. In addition, irreversible adsorption of actives onto the column stationary phase and poor resolution between large impurities and small nanoparticles can restrict the use of this technique for purification of drug-loaded particulate formulations. Ultrafiltration, although more efficient than dialysis and gel filtration, can cause nanoparticles to stick together or adhere to the membrane surface, thus leading to a considerable decrease in filtrate flux. Concentration polarization, fouling, and cake formation are primary concerns in ultrafiltration but can be overcome by cross-flow microfiltration (14). Recently, the use of cross-flow microfiltration as a purification technique for nanoparticles has been investigated. Although research into this process is limited, the technique has potential as an efficient purification technique with minimal detrimental effects on nanoparticle size and drug-loading capacity (5,15).

In the present study, we evaluate and compare the feasibility of using a diafiltration centrifugal device (DCD) and a tangential flow filtration (TFF) system for purification of poly(lactide-co-glycolide) (PLGA) nanoparticles containing PVA as an emulsifier/stabilizer. In TFF (also referred to as cross-flow filtration), the nanoparticle dispersion feed stream passes parallel to the membrane face with one portion passing through the membrane (filtrate or permeate), whereas the remainder (retentate or concentrate) is recirculated back to the feed reservoir (21). The characteristics and stability of nanoparticles before and after purification were compared. The purification performance of the DCD and TFF system after repeated use for multiple batches of nanoparticles was also evaluated. In addition, both processes were compared to the commonly used ultracentrifugation method.

MATERIALS AND METHODS

Materials

Chemicals used for preparation of nanoparticles were as follows: PLGA, Purasorb[®] PDLG 85/15, MW: 15,600 Da (Purac Biochem, Gorinchem, Netherlands), PVA (80%

hydrolyzed, MW 9000–10,000 Da), dichloromethane, and dialysis tubing with flat width 40 mm, diameter 25 mm (all from Sigma Chemical Co., St. Louis, MO, USA). The chemicals and reagents used for PVA analysis were boric acid (BDH, Victoria, Australia), iodine (Abbott, Botany, NSW, Australia), and potassium iodide (Selby Scientific, Victoria, Australia). All other chemicals were of analytical grade and were purchased from Sigma Chemical Co. The following devices were used for nanoparticle purification: Macrosep[™] centrifugal devices [Omega[™] 300K and 100K molecular weight cut-off (MWCO) membrane] purchased from PALL Gelmen Science, Minimate[™] Capsule (Pall Corporation, East Hills, NY, USA) with Omega[™] 300K MWCO membrane, Masterflex[®] peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL, USA), Swagelok[®] Pressure gauge (0–80 psi; Fluid Mechanics Ltd, Queensland, Australia), and Allegra[®] centrifuge (Beckman Coulter, Fullerton, CA, USA). Ultrapure water (<0.06 μ S) prepared from a Milli-Q purification system was used in all experiments.

Experimental Setup

The TFF system was set up with the peristaltic pump Minimate[™] capsule and two pressure gauges placed before and after the Minimate[™] capsule as shown in Fig. 1. The Omega[™] membrane has a mean pore size of 30 nm and an effective filtration area of 50 cm². This membrane was chosen for its low protein binding properties as the TFF system is intended for purification of protein and peptide-loaded nanoparticles.

Preparation of PLGA Nanoparticles with PVA as a Stabilizer

Poly(lactide-co-glycolide) nanoparticles were prepared by an emulsification solvent diffusion method (22,23). Briefly, 4% w/v of PLGA was prepared in a solvent mixture consisting of ethanol and acetone (4/6 v/v). An amount of 12.5 ml of this polymer solution was then added into 50 ml of aqueous PVA solution (4% w/v) in a 250 ml glass beaker using a peristaltic pump at a flow rate of 1.7 ml/min. The mixture was stirred continuously at 400 rpm by a propeller mixer during the addition process. The dispersion formed was stirred for a further 30 min to solidify nanoparticles before being transferred into dialysis tubing and purified by dialysis against 1 l freshwater. The water was replaced every 2 h, and the dialysis process was repeated four times in an attempt to remove the residual PVA and organic solvents. After purification, the nanoparticle dispersion was passed through double layers of 53 μ m mesh to remove any aggregates and then freeze-dried under vacuum to obtain powdered nanoparticles.

Selection of an Omega[™] Membrane for the DCD and TFF System

Macrosep[™] centrifugal devices (DCD system used in this study) with Omega[™] 300K and 100K MWCO membranes were compared for their efficiency to purify PVA from nanoparticles. Eight milliliters of PLGA nanoparticle

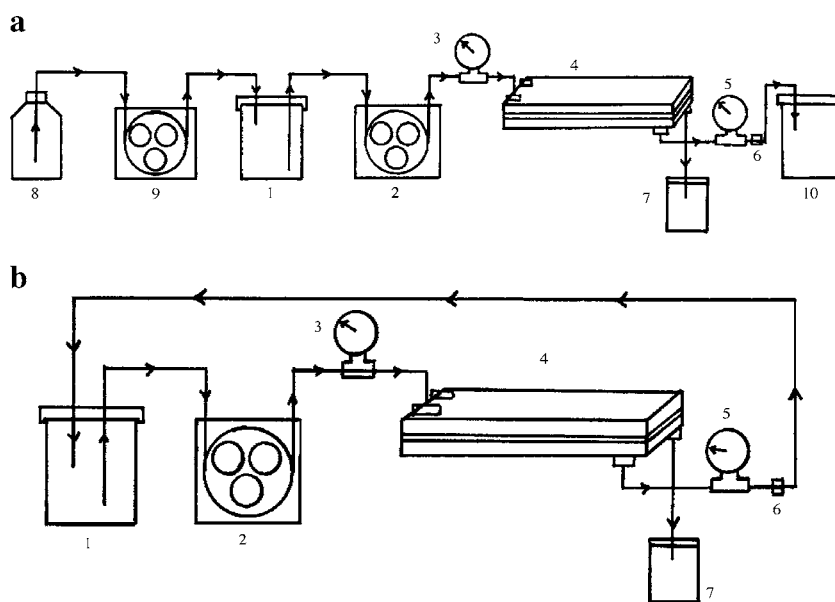


Fig. 1. Schematic representation of TFF purification system setup: (a) diafiltration mode; (b) concentration mode. Components are as follows: (1) sample reservoir; (2) peristaltic pump-1; (3) pressure gauge-1; (4) Minimate™ 300K; (5) pressure gauge-2; (6) screw clamp valve; (7) filtrate collector; (8) dilution reservoir; (9) peristaltic pump-2; (10) retentate collector.

dispersion (0.5 mg/ml) was loaded into the concentrate/retentate chamber of the DCD. The device was then centrifuged at 4500 rpm for 15 min. The filtrate collected was analyzed for PVA content and turbidity, whereas the concentrate was diluted with water to the initial volume to repeat the cycle. At the end of the experiment, the concentrate was diluted to its original volume with water and the PVA content was determined. The membrane that produced the highest purification efficiency was selected for further studies with the DCD and TFF systems.

Adsorption of PVA Onto Omega™ Membrane in DCD System

Polyvinyl alcohol adsorption onto the membrane was studied with repeat purification of 8 ml of PVA solution (0.1 and 0.5 mg/ml) using a fresh Macrosep™ centrifugal device with Omega™ membrane MWCO 300K. The PVA solution was loaded into the concentrate chamber, and the device was centrifuged at 4500 rpm for 15 min. The filtrate collected was analyzed for PVA content, whereas the concentrate was diluted with water to the initial volume to repeat the cycle (seven times). For each PVA concentration, the study was performed in triplicate in a continuous fashion without washing of the device between purification steps. At the end of either the sixth cycle (0.1 mg/ml) or eighth cycle (0.5 mg/ml), when the cumulative filtrate volume reached 25 ml, the concentrate was diluted with water to 8 ml and the PVA concentration was determined.

Purification of Nanoparticles by the DCD System

Two approaches were investigated. In the first approach (continuous diafiltration), water was used to dilute the

concentrate prior to centrifugation. Briefly, 8 ml of nanoparticle dispersion (0.5 mg/ml) was purified using a fresh Macrosep™ centrifugal device with Omega™ membrane MWCO 300K by centrifugation at 4500 rpm for 15 min. The filtrate collected was analyzed for PVA content and turbidity, whereas the concentrate was diluted with water to the initial volume to repeat the cycle (total of seven cycles). At the end of the experiment, the PVA content of the concentrate was determined following dilution to its original volume with water. The DCD system was flushed with deionized water between different purification samples.

In the second approach, the nanoparticle suspension (0.5 mg/ml) was used to dilute the concentrate to the initial volume (rather than water in the previous approach), and the cycle was repeated using the same protocol as described previously. A total of eight cycles were performed. At the end of the eighth cycle, the retentate or concentrate was diluted with water to the initial volume to continue the cycle. This was repeated for the ninth and tenth cycles. At the end of experiment (tenth cycle), the concentrate was diluted with water to its original volume prior to the determination of PVA content. All filtrates collected during the purification process were also analyzed for PVA content.

Purification of Nanoparticles by the TFF System

The purification of nanoparticles was performed with the setup described previously (Fig. 1). Two modes of purification process were investigated: (1) diafiltration and (2) concentration. These methods are similar to continuous diafiltration and discontinuous diafiltration by volume as previously reported in the literature (24). Both were performed at room temperature. The diafiltration process involved a 200 ml feed volume of PLGA nanoparticle dispersion (0.5 mg/ml)

and the transmembrane pressure (TMP) of 2.75 psi. In the diafiltration process, the purified retentate or concentrate was directed to a separate retentate collector (Fig. 1a), and the feed reservoir was diluted by water at the same rate as filtrate or permeate was being generated, hence maintaining a constant volume. The nanoparticle dispersion in the feed reservoir was dispersed by magnetic stirring during the purification process. Samples of filtrate were taken at designated times and analyzed for PVA content and turbidity. At the end of the experiment, both filtrate and retentate were sampled and their PVA content was determined. The purified sample was also characterized for particle size distribution and zeta potential.

The concentration process was performed with a 200 ml feed volume of PLGA nanoparticle dispersion (0.5 mg/ml) and the TMP maintained at either 2.75 or 10 psi. The retentate was directed back to the feed bottle. As more filtrate was generated, the concentration of retentate increased. The filtrate was sampled at designated times to monitor the amount of PVA removed from the nanoparticle dispersion. Once the retentate volume approached the TFF system holdup volume (capsule plus tubings, approximately 15 ml), purification was stopped. Nanoparticles were recovered by flushing the system with 30 ml water (approximately twice the volume of holdup capacity), and the collected retentate was diluted back to 200 ml for characterization of nanoparticle dispersion stability. During the purification process, the filtrate was analyzed regularly for PVA content and turbidity. The purified sample was also assessed for PVA content, particle size distribution, and zeta potential. Purified nanoparticles were lyophilized to yield nanoparticle powder before being used for morphological study.

Between purification of batches, the TFF system was cleaned by 1-h continuous circulation of 0.1 M NaOH followed by flushing with a large volume of water (≥ 1 l). The system was stored in 5% glycerine containing 0.1% sodium azide.

Purification of Nanoparticles by Ultracentrifugation

A nanoparticle dispersion of 0.5 mg/ml was centrifuged at 14,000 rpm for 20 min. The supernatant was removed and replaced with an equal volume of fresh deionized water for redispersing of nanoparticles. The dispersion obtained was then subjected to centrifugation at 14,000 rpm for a further 20 min. This cycle was repeated twice.

Determination of PVA Content

The PVA content in samples was determined by a colorimetric method modified in our laboratory, which is based on the formation of a greenish colored complex between two adjacent hydroxyl groups of PVA and iodine molecule in the presence of boric acid (25). Briefly, the PLGA nanoparticle dispersion (0.5 mg/ml) was prepared by dispersing a known amount of freeze-dried nanoparticles in water. One milliliter of 0.5 mg/ml of PLGA particles was centrifuged at 14,000 rpm for 20 min. Fifty microliters of the supernatant was removed and diluted with 5 ml water. The diluted solution was mixed with 3 ml boric acid (3.8% w/v) and 0.6 ml 0.1 M iodine solution (prepared from iodine and potassium iodide) then made up to 10 ml with water. The UV

absorbance of the final solution was measured by a UV-VIS spectrophotometer at 690 nm. A standard plot of PVA was prepared under identical conditions with concentration ranging from 0 to 35 $\mu\text{g/ml}$. As a control, PLGA water extract was prepared under the same conditions and analyzed. This PVA assay was validated for its linearity, accuracy, specificity, and reproducibility.

To determine the amount of PVA in the filtrate, a known volume of filtrate was firstly diluted with water, then mixed with boric acid, iodine solution, and adjusted to volume with water as described above before being analyzed at the wavelength 690 nm. The amount of PVA in the filtrate and retentate was expressed as a percentage with respect to the total free PVA detected in nanoparticle dispersion before purification [mean \pm standard deviation (SD), $n = 3$ in all cases].

Determination of Turbidity

Turbidity measurement was carried out by comparing the UV absorbance of filtrate and water at 400-nm wavelength using a UV-visible spectrophotometer (Shimadzu 1201) to determine if any nanoparticles passed through the membrane.

Characterization of Nanoparticles

Particle Size and Zeta Potential

Nanoparticle size and zeta potential were determined using a Zetasizer 3000HS (Malvern Instruments, Worcestershire, UK). The particle size measurement was performed by photon correlation spectroscopy (PCS) at 25°C with a detection angle of 90°, and the raw data were subsequently correlated to Z average mean size using a cumulative analysis by the Zetasizer 3000HS software package. The zeta potential of particles was determined by laser Doppler anemometry. All analyses were performed on samples appropriately diluted with water. For each purification, the mean \pm SD of three samples was obtained. All purified nanoparticle dispersions were diluted with water to concentrations similar to that of unpurified samples and then characterized.

Field Emission Scanning Electron Microscopy

Purified and unpurified nanoparticle powder samples were placed on metallic studs with double-sided carbon tapes and coated with platinum by a sputter coater (JFC-1300, Auto Fine Coater, Jeol, Tokyo, Japan) for 40 s in a vacuum at a current intensity of 40 mA. The effect of purification by TFF on morphology of particles was observed using field emission scanning electron microscopy (FESEM; JEOL JSM 6700F electron microscope).

RESULTS AND DISCUSSION

Assay Validation of PVA Analysis by the Colorimetric Method

The PVA assay used in the present study was adopted from the colorimetric method developed by Finely (25). This

Table I. PVA Removal by Omega™ Membrane with 300K MWCO

Concentration of PVA standard solution (mg/ml)	PVA removed in filtrate (%)	PVA retained in retentate (%)	PVA adsorbed onto membrane (%; mg)	Total PVA removed ^a (%)
0.1	61.2	0.5	38.3; 0.31	99.5
0.1	70.6	0.7	28.7; 0.23	99.3
0.1	74.4	2.0	23.6; 0.19	98
Mean ± SD, n = 3	68.7 ± 6.7	1.1 ± 0.8	30.2 ± 7.4	98.9 ± 0.8
0.5	87.2	2.1	10.7; 0.43	97.9
0.5	88.9	4.4	6.7; 0.27	95.6
0.5	87.0	3.8	9.2; 0.37	96.2
Mean ± SD, n = 3	87.7 ± 1.0	3.4 ± 1.2	8.9 ± 2.0	96.5 ± 1.2

^aTotal percent PVA removed is obtained from percent PVA remained in the retentate subtracted from initial 100% PVA level.

assay was validated for linearity, accuracy, specificity, and reproducibility over the concentration range of 0–35 µg/ml. The assay was found to be linear over this concentration range with a correlation coefficient (r^2) greater than 0.996. The assay variation was small when the measurements were performed on different days with PVA concentration below 25 µg/ml (relative standard deviation, RSD < 2.0%). When PVA concentration was above 25 µg/ml, PVA was prone to form insoluble precipitates in the presence of iodine. In the present study, all samples were diluted with water to a concentration below 25 µg/ml to minimize assay variation. To minimize interference in PVA assay by the matrix, the aqueous extract of a known amount of PLGA was also analyzed as a control.

The accuracy of the PVA assay was determined by measuring the recovery of a known amount of PVA added to the purified PLGA nanoparticles whose PVA level had been previously determined. A recovery of 100.3% with RSD of 4.6 ($n = 3$) was obtained, indicating that the PVA assay was accurate.

Formulation of Nanoparticles with PVA as a Stabilizer and PVA Removal by Dialysis

Poly(lactide-co-glycolide) nanoparticles used in the present study were formulated by the emulsification solvent diffusion technique in the presence of PVA (4%), an emulsifier and stabilizer employed in the formulation. The quantity of PVA in the formulation is high, being four times that of PLGA. It has been suggested that the presence of a stable thick layer of PVA on the surface of nanoparticles plays an important role in stabilizing nanoparticles during cross-flow filtration and freeze-drying processes (16). However, the excess amount of PVA in the nanoparticle formulation can have effects not only on the physical properties of particles such as size, zeta potential, surface hydrophobicity, drug loading, and release, but also on cellular uptake of particles (9). Despite frequent reports of using centrifugation and ultracentrifugation with water washing techniques for removing PVA, our experience showed that the centrifugation technique suffers from a high risk of causing nanoparticle caking and difficulty in redispersion (19). Furthermore, it is very difficult to recover all nanoparticles of fine size even with ultracentrifugation. Other authors also raised the same concern on the impact of the centrifugation forces on the redispersibility and morphology of polylactide (PLA)-based

nanoparticles (20). To overcome this problem, we initially utilized a dialysis technique to remove PVA. However, only 17.6% PVA was removed after 8 h dialysis with the external phase changed every 2 h (19). In the present study, such nanoparticles were then subjected to further purification by diafiltration or TFF system.

Nanoparticles were analyzed for free PVA content prior to purification by the colorimetric method described previously. Free PVA content in the nanoparticle sample was found to be 80.4 ± 3.1% w/w (Table I).

Purification of PLGA Nanoparticles—Selection of Omega™ Membrane

The Minimate™ capsule with Omega™ membrane (polyethersulfone-based) is available in a range of MWCO. The capsule has an effective filtration area of 50 cm² with low protein adsorption characteristics. The manufacturer claims that it can filter a range of volumes and is capable of performing sequential concentration and diafiltration steps using the same device and allows easy scale-up (26). To select an efficient Omega™ membrane for separation of nanoparticles from PVA molecules of 10 kDa, we compared the centrifugal devices (i.e., DCD) containing Omega™ membranes with 100K and 300K MWCO. In this comparison study, centrifugal devices of both membranes were loaded with an equal quantity of nanoparticle dispersion (8 ml of 0.5 mg/ml nanoparticles) and were subjected to continuous diafiltration that involved adding water to the retentate to maintain the same volume after each centrifugation (24). Although the molecular weight of PVA (10 kDa) is only 1/10 or 1/30 of the MWCO of the Omega™ membranes, there was a considerable difference in the purification efficiency between the two MWCO membranes as shown in Fig. 2. In 20 ml of filtrate volume, about 78% PVA was removed in the filtrate with the Omega™ membrane 300K but only 13% PVA with the membrane 100K. At the end of purification, the concentrate contained 8.6% of PVA in the device with 300K membrane, indicating that about 12.7% PVA was adsorbed onto the device membrane. Such membrane adsorption of PVA has been reported previously by Limayem *et al.* (5). Based on the data obtained from this continuous diafiltration study, the Omega™ membrane with 300K MWCO was selected for all subsequent purification of nanoparticles by the DCD and TFF systems.

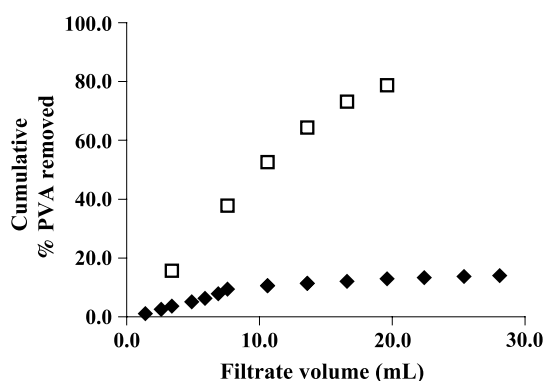


Fig. 2. A comparison of Omega™ membranes with MWCO 100K (♦) and 300K (□) for purification of PLGA nanoparticle (8 ml × 0.5 mg/ml) from PVA using Macrosep™ centrifugal devices (DCD continuous diafiltration approach).

It was not anticipated that the percentage of PVA removed by the 100K membrane would be so low as the MWCO of the membrane is ten times the size of the PVA molecules. We speculate that this may be attributed to fouling of the membrane by self-association of PVA molecules because of inter- and intrachain hydrogen bonding formed between the polar hydroxyl groups in the PVA molecules (27). In addition, change in shape of the PVA molecules (28) during the concentration process may contribute to the low filtration percentage.

Purification by the DCD System

Adsorption of PVA to the DCD

Adsorption of PVA to the Omega™ membrane with 300K MWCO was studied by continuous exposure of PVA standard solutions to the DCD with the selected membrane. The continuous diafiltration or constant volume diafiltration approach was adopted involving washing out PVA in the retentate by adding water to the retentate at the same rate as the filtrate was being generated (24). There was a much lower level of percent PVA being filtrated, indicating significant adsorption of PVA by the membrane (Table I; Fig. 3), when 0.1 mg/ml PVA was subjected to purification by DCD using a fresh membrane. However, close examination of the amount of PVA being adsorbed onto the membrane revealed that the absolute amount of adsorbed PVA was similar for both 0.1 and 0.5 mg/ml PVA solution (Table I). The difference in percent PVA removed or adsorbed was a result of different PVA concentration used. As expected, initial exposure led to a high percentage of PVA adsorption onto the membrane, and continuous dilution only slowly washed off adsorbed PVA. After the sixth diafiltration volume, 61.2% PVA had been recovered in the filtrate, with 38.3% adsorbed onto the membrane. Subsequent exposure of the same concentration of PVA solution showed lower PVA adsorption. The amount of PVA in the filtrate of the subsequent experiment was marginally higher than the initial experiment. However, the triplicate filtration of 0.5 mg/ml PVA solution with the same membrane showed consistent total PVA removal profile of $96.5 \pm 1.2\%$, indicating the membrane saturation by the PVA adsorption (Table I). The

PVA membrane adsorption, calculated based on the PVA content detected in both filtrate and retentate, was $8.9 \pm 2.0\%$ with 0.5 mg/ml PVA solution after eight diafiltration volumes. This is equivalent to an average of 0.36 mg PVA adsorbed per device. The slow release of PVA from the membrane after continuous dilution of retentate with water also indicates that this portion of PVA was absorbed strongly to the membrane. We conclude that once the membrane is saturated with PVA, the removal profile of PVA is likely to be consistent.

Purification of Nanoparticles by the DCD

The same DCD system previously used for PVA solution was then employed for purification of three replicate batches of nanoparticles (0.5 mg/ml) using the continuous diafiltration approach. With about 25 mL filtrate, $79.7 \pm 3.2\%$ was removed, suggesting that removal of PVA in filtrate was consistent (Fig. 4). The turbidity measurement of the filtrate showed no difference to that of a control (water), confirming that no nanoparticles passed through the membrane. Analysis of PVA level in the retentate showed that $11.0 \pm 2.5\%$ remained in the sample, indicating that membrane adsorption of PVA was $9.1 \pm 5.6\%$. As this is similar to that of the 0.5 mg/ml PVA solution described previously, it further supports the conclusion that repeated use of the DCD does not affect the purification performance of the DCD. However, it also showed that the residual amount of PVA in the retentate is more difficult to remove as shown by the curve in Fig. 4.

In a separate experiment, the purification of nanoparticles was performed by continuous loading of nanoparticles in the retentate. In this case, the PVA removal profile showed a linear relationship with filtrate volume, suggesting that PVA removal is concentration dependent (Fig. 5). Neither PVA nor nanoparticles caused blockage of the 300K membrane. Following saturation of the membrane with PVA, the subsequent removal of PVA by the filtrate was constant (Fig. 5). Importantly, the presence of a high concentration of nanoparticles did not affect performance of the membrane.

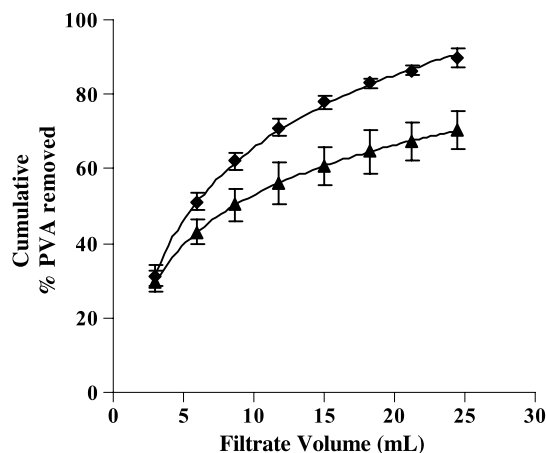


Fig. 3. PVA removal in filtrate by and adsorption onto the DCD Omega™ membrane with 300K MWCO with continuous diafiltration approach. ▲: 0.1 mg/ml PVA solution ($n = 3$); ◆: 0.5 mg/ml PVA solution. The symbols represent mean \pm SD ($n = 3$).

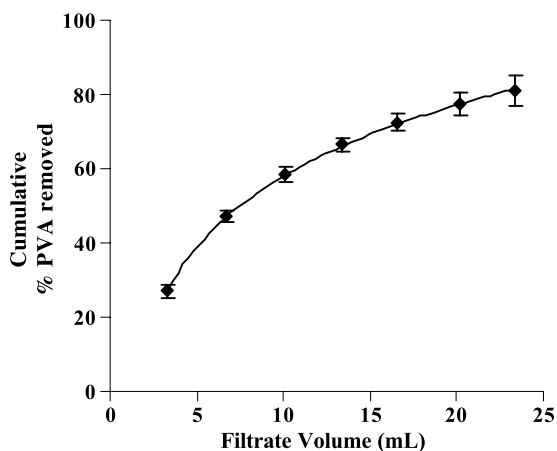


Fig. 4. Purification of nanoparticles (8 ml of 0.5 mg/ml NPs) by DCD using the Omega™ membrane with 300K MWCO with continuous diafiltration approach. The symbols represent mean \pm SD ($n = 3$) of PVA removed in filtrate.

Purification of Nanoparticles by TFF System—Diafiltration Versus Concentration Process

To compare the effect of purification mode of TFF, we studied both diafiltration and concentration processes with 200 ml of 0.5 mg/ml NP dispersion, purified for 170 min (2.8 h) at TMP of 2.75 psi, with the feed diluted with water at the same rate as the filtrate was being generated (Fig. 1a). The diafiltration process removed $68.3 \pm 1.7\%$ PVA in total with about 32% PVA remained with nanoparticles after 2.8 h (Fig. 6, Table III), indicating that the process was slow but reproducible. Employing a concentration mode with the same TMP (2.75 psi) only marginally improved the rate of PVA removal in the filtrate (about 5% increases) but failed to decrease the amount of PVA remaining with nanoparticles, despite a significant reduction in membrane absorption of PVA (Table III). However, the PVA removal profile of the TFF in concentration mode showed a remarkable linear

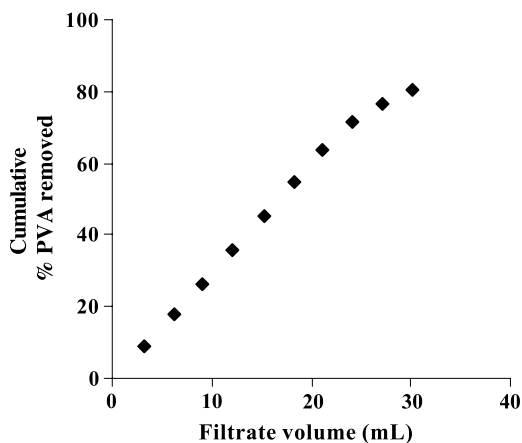


Fig. 5. Purification of nanoparticles (8 ml of 0.5 mg/ml) by DCD using the Omega™ membrane with 300K MWCO. Retentate was diluted with 0.5 mg/ml nanoparticles of the same batch after each cycle up to eighth cycle. In the last two cycles, retentate was diluted with water before centrifugation. Values represent percent PVA removed in filtrate.

relationship against time and filtrate volume (Fig. 6) with correlation coefficient $r^2 \geq 0.9999$. This is distinctively different from that of diafiltration mode in TFF and DCD. The latter two showed a good fit to a logarithmic equation. Based on the calculation of slope of the linear regression line of percent PVA removed against time, we estimated that the average rate of PVA removal by filtrate is 13.0%/h for diafiltration mode and 19.8%/h for concentration mode (Table II). However, in the case of diafiltration mode, rate of removal is decreasing as a function of time as shown in Fig. 6; to compare the two modes more accurately, we then used the mathematical equations derived from the best-fit curves to calculate T_{90} , the time taken for removal of 90% PVA. Based on T_{90} , the concentration mode is almost nine times more efficient than the diafiltration mode (Table III). Although the concentration mode is considered as a more efficient process than the diafiltration mode at 2.75 psi, T_{90} of 5.0 h is undesirable, as it is still too long for purification. To further increase purification efficiency, we employed TMP of 10 psi in further experiments.

Our observations of the PVA removal profiles are in contrast to a previous report by Limayem *et al.* (5) who observed a substantial reduction in both filtrate flow rate and PVA removal rate for processes such as diafiltration and concentration during the purification period. We believe that the discrepancy could be due to differences in the process conditions employed and the nature of the membrane. The affinity of the membrane to PVA could be different as Limayem *et al.* used a cellulose-based membrane in contrast to the polyethersulfone membrane in our study. Furthermore, in comparison to that of Limayem *et al.*, a relatively low concentration of nanoparticle dispersion (0.5 mg/ml), which contained about 80% w/w PVA, was used in our study. Nevertheless, this comparison of two purification modes does suggest that the concentration process could be a good alternative to diafiltration as it reduces the purification process time because of its progressive reduction in process volume. Therefore, it is possible to obtain the purified retentate in a small concentrated volume.

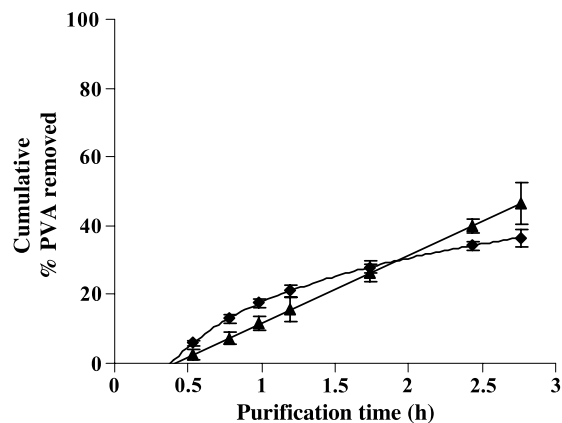


Fig. 6. Comparison of purification of nanoparticle dispersions by concentration and diafiltration process using a Minimate™ TFF capsule with 300K MWCO membrane with TMP of 2.75 psi. Two hundred milliliters of 0.5 mg/ml nanoparticle samples was purified each time. \blacktriangle : Purification by concentrated mode; \blacklozenge : purification by diafiltration mode. The symbols represent mean \pm SD ($n = 3$) of PVA removed in filtrate.

Table II. Effect of Purification Process Parameters on PVA Removal by the TFF System

Mode of purification	TMP (psi)	Free PVA level in nanoparticle sample before purification (% w/w \pm SD)	Average rate of PVA removal in filtrate ^a (%/h \pm SD)
Diafiltration	2.75	80.4 \pm 3.1	13.0 \pm 1.5
Concentration	2.75	80.4 \pm 3.1	19.8 \pm 3.7
Concentration	10.0	80.4 \pm 3.1	33.7 \pm 1.8

Purification was performed with 200 ml nanoparticle dispersions of 0.5 mg/ml, $n = 3$.

^a Average PVA removal rate was calculated based on the slope of the linear regression line of percent PVA removed against time.

Effect of TMP on Purification of Nanoparticles by TFF System—Concentration Mode

In this study, we used the TFF system in concentration mode with the TMP of 10 psi, which is about 30% capacity of the current TFF capsule, to purify three 200-ml batches of nanoparticle dispersions (0.5 mg/ml). All samples showed similar linear PVA removal profiles in the filtrate (Fig. 7) using the same TFF capsule. Compared to the concentration mode at 2.75 psi, current purification conditions not only removed more PVA in the filtrate, but also further reduced the level of PVA adsorption onto the membrane (Table III). Consequently, the PVA content in the nanoparticle dispersion was reduced from initial value of 80.4 \pm 3.1% w/w to 7.5 \pm 2.7% w/w in 170 min with T_{90} of 2.8 h. This demonstrates that purification of nanoparticles by TFF in concentration mode at 10 psi is considerably faster than that in the diafiltration or concentration mode performed at 2.75 psi.

The PVA removal data obtained from this study were directly proportional to the purification time or filtrate volume. No decrease in PVA removal by the filtrate, in the concentration mode, was observed in this study as demonstrated by the linear relationship obtained from the plot of cumulative percent PVA removed against time ($r^2 = 0.9999$; Fig. 7). This suggests that there was no obvious membrane fouling or particle caking, phenomena often seen in other

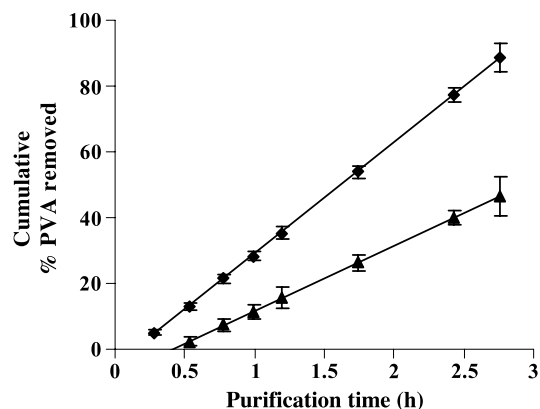


Fig. 7. Comparison of purification of nanoparticle dispersions at different TMP by concentration process using a Minimate™ TFF capsule with 300K MWCO membrane. Two hundred milliliters of 0.5 mg/ml nanoparticle samples was purified each time. ♦: Purification at 10 psi TMP; ▲: purification at TMP of 2.75 psi. The symbols represent mean \pm SD ($n = 3$) of PVA removed in filtrate.

cross-flow filtration systems (29). Our sample size with respect to the membrane area (2.0 mg/cm²) is much lower than those reported in the literature, such as 23 mg/cm² (5) and 71.3–3571.4 mg/cm² (29). In addition, a flux of 13.5 l/m² h was used in our study. This flux is below the critical flux value that can cause formation of particle layers on the membrane surface (caking), leading to a reduction in performance of the filtration process (30). We believe that both low sample loading and flux may have contributed to the characteristics of the PVA filtration profile observed in our study.

On repeated use of the TFF system, the capsule showed a consistent purification performance, indicated by the linearity of the purification profile and small standard deviation values (Fig. 7, Table III). Measuring both PVA levels in the filtrate as well as retentate (i.e., nanoparticle dispersion after purification), their sum produced values close to 94% with the concentration mode at TMP of 10 psi. However, the deviation in mass balance is much greater in TFF system operated at 2.75 psi. We attribute such deviation to the membrane adsorption of PVA

Table III. Comparison of Nanoparticle Purification by Different Processes

Purification process/mode	TMP (psi)	T_{90} (h)	PVA remained with nanoparticles after purification (% w/w)	PVA distribution (%)			
				Removed in filtrate	Remained with nanoparticles	Adsorbed onto the membrane	Total PVA removed ^a (%)
TFF diafiltration	2.75	49.4	25.5 \pm 1.4	39.4 \pm 2.6	31.7 \pm 1.7	28.9 \pm 3.6	68.3 \pm 1.7
TFF concentration	2.75	5.0	30.4 \pm 1.7	44.6 \pm 6.7	37.8 \pm 2.1	17.5 \pm 2.5	62.1 \pm 2.1
TFF concentration	10.0	2.8	7.5 \pm 2.7	84.6 \pm 8.9	9.3 \pm 3.4	6.1 \pm 7.2	90.7 \pm 3.4
DCD Macrosep 300K	N/A	9.6 ^b	8.8 \pm 2.0	79.9 \pm 3.2	11.0 \pm 2.5	9.1 \pm 5.6	89.0 \pm 2.5
Ultracentrifugation	N/A	3.3 ^c	1.0 \pm 0.01	98.7 \pm 0.01	1.3 \pm 0.01	N/A	98.7 \pm 0.01

All purifications were performed with triplicate batches ($n = 3$) of 200 ml nanoparticle dispersion (0.5 mg/ml) with free PVA level of 80.4 \pm 3.1% w/w. Values are mean \pm SD. T_{90} , the time taken to remove 90% of initial PVA in nanoparticle dispersion, is calculated using the mathematical equation obtained from the line or curve of best fit to the data in Figs. 4, 6, and 7. N/A: not applicable.

^a Total PVA removed is obtained from percent PVA retained on nanoparticles subtracted from 100% PVA level.

^b This value was calculated based on centrifugation capacity of eight DCD devices (8 ml per device) per run.

^c This value was estimated based on centrifugation capacity of 100 ml per run.

Table IV. Physical Stability of Nanoparticle Dispersions at Ambient Conditions After Purification by Different Processes

Purification process/mode	TMP (psi)	Size (nm)		Zeta (mV)	
		Before	After	Before	After
TFF diafiltration	2.75	300.2 ± 2.1	Day (0) 305.8 ± 4.4 Day (3) 301.6 ± 1.7	-20.83 ± 1.42	Day (0) -19.7 ± 1.4 Day (3) -19.6 ± 1.1
TFF concentration	2.75	286.5 ± 2.3	Day (0) 284.13 ± 0.60 Day (3) 291.7 ± 3.17	-22.7 ± 3.21	Day (0) -21.5 ± 5.2 Day (3) -19.9 ± 3.4
TFF concentration	10.0	276.0 ± 5.3	Day (0) 275.3 ± 6.5 Day (3) 271.5 ± 4.57	-31.73 ± 4.4	Day (0) -21.8 ± 2.6 Day (3) -22.3 ± 2.2
DCD Macrosep 300K	N/A	293.0 ± 3.2	Day (0) 295 ± 9.37 Day (3) 290 ± 5.4	-29.40 ± 2.7	Day (0) -13.6 ± 1.3 Day (3) -12.5 ± 2.6
Ultracentrifugation	N/A	292.3 ± 6.6	Day (0) 307.2 ± 8.4 Day (3) 312.5 ± 21.4	-22.43 ± 2.6	Day (0) -35.3 ± 1.7 Day (3) -18.2 ± 1.1

N/A: not applicable. Values are mean ± SD, $n = 3$.

(Table III) as postulated previously in the literature (5). The turbidity of filtrate for all samples was found to be similar to the control (water) with RSD < 2.0%, indicating that nanoparticles did not permeate through the membrane. This demonstrates that TFF can efficiently separate free PVA from nanoparticles.

Comparison of Different Purification Process and Their Effects on Stability and Characteristics of Nanoparticles

Table III summarizes purification of an equal quantity of nanoparticle dispersion by different techniques. Based on T_{90} values, the TFF system with concentration mode at 10 psi showed the highest purification efficiency. DCD, on the other hand, produced purified nanoparticles with a similar level of PVA but was more time consuming because of its limitation in handling a large volume of sample. In our view, DCD is a viable alternative for purification of a small batch size, but it does require more manpower for handling samples. In comparison, the TFF system can be totally automated and is highly cost effective for processing a large batch of nanoparticles in both the laboratory and industry.

Ultracentrifugation, although used commonly to remove a high level of PVA, can negatively impact on both the final yield (data not reported here) and stability of the nanoparticle dispersion. In our case, at centrifugation speed of 14,000 rpm for 20 min, a considerable amount of nanoparticles remained in the supernatant, which appeared turbid. In addition, small amounts of nanoparticles aggregated after ultracentrifugation and failed to redisperse after vigorous mixing. These aggregates were not used for particle size measurement as they settled rapidly to the bottom of the tube. For the three samples purified by ultracentrifugation, the PVA level remaining with the purified nanoparticles was measured, and the level in supernatant was estimated by subtraction (Table III). Because of the significant loss of nanoparticles to the supernatant, we believe that the data on ultracentrifugation could likely have overestimated PVA removal.

Physical characteristics of nanoparticles before and after purification are presented in Table IV. Samples purified by ultracentrifugation appeared to be larger in particle size (14 nm bigger) and are less stable in both particle size and

zeta potential. This could be due to the excessive removal of PVA or the impact of the process itself. In contrast, only a marginal change in particle size (1–5 nm) occurred after purification using either the DCD or TFF technique. Such change, however, is within the day-to-day size measurement

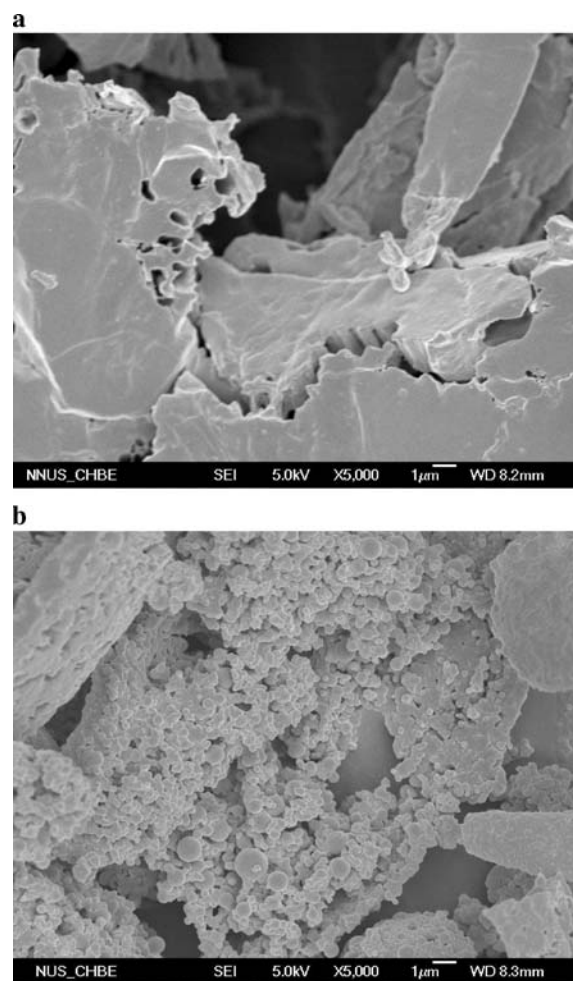


Fig. 8. FESEM micrographs of PLGA nanoparticles before (a) and after (b) TFF purification.

error and cannot be credited to the purification process. The purification process removes only free PVA in the dispersion, whereas PVA molecules closely associated with nanoparticles are not expected to be removed by this process. The analysis of PVA in retentate showed that a percentage of free PVA remained in the dispersion at the end of the purification process (Table III). Those remaining PVA molecules are expected to contribute to the stability of the nanoparticle dispersion (16). The stability study of all batches showed no significant difference (evaluated by 95% confidence intervals) in the particle size distribution of purified and unpurified nanoparticle dispersions during the 3 day storage at room temperature (Table IV). Substantial removal of free PVA did not have a significant impact on the stability of the dispersion over a 3 day period. This is in agreement with Quintanar-Guerrero's hypothesis that a stable thick coating layer of PVA provides resistance against destabilization (16).

Individual nanoparticles, mostly spherical in shape, were seen with purified nanoparticles (by TFF with concentration mode at 10 psi) as shown by FESEM images. Because of the presence of excessive PVA, nanoparticles were not visible by FESEM with the unpurified sample (Fig. 8).

Examination of the zeta potential of the nanoparticle dispersions before and after purification (Table IV) suggests that surface negativity of the nanoparticles seems to be changed as a result of purification. However, analysis of 95% confidence intervals of the data suggested that the significant change in zeta potential only occurred to the samples purified by DCD and ultracentrifugation methods. As PVA molecules have been shown to exhibit a negative zeta potential in water (31), it is not surprising that their removal is associated with the reduction of the negativity of the zeta potential in the dispersion. This trend, however, was not seen with the samples purified by ultracentrifugation. Overall, the change in zeta potential does not quantitatively correlate with reduction of PVA in the nanoparticle dispersion.

Overall, purification of nanoparticles by TFF did not cause any apparent physical destabilization of the nanoparticles. Physical properties such as particle size and the dispersibility of nanoparticles were not affected by the purification process. Negativity of zeta potential was reduced, but no clear quantitative trend was established. Compared to the DCD system, TFF, when operated in concentration mode at TMP of 10 psi, is capable of purification of large sample sizes with automated and continuous operation. It offers faster and more efficient purification approaching 91% removal of free PVA with minimum manpower.

CONCLUSIONS

In the present study, we investigated the use of DCD and TFF for purification of nanoparticles to remove excess PVA and compared the techniques to the commonly used ultracentrifugation technique. The results of this study show that a TFF capsule with a high MWCO membrane can efficiently remove approximately 91% PVA molecules in 2.8 h by the concentration mode under TMP of 10 psi. The purification process was more efficient than dialysis and DCD (for large sample batches) and had less impact on the yield, size, and stability of nanoparticles than ultracentrifugation. The percentage of PVA removed by filtrate showed a linear relation-

ship with filtrate volume and time. Neither membrane fouling nor particle caking was observed during the purification process. Increasing TMP from 2.75 to 10 psi improved the PVA purification rate without affecting performance. The TFF system showed a high reproducibility in the purification of multiple batches of nanoparticles. Nanoparticle stability and particle size were not affected by the purification process, although zeta potential was reduced as a result of removal of PVA. We conclude that TFF, when operated in concentration mode at a high TMP, is a useful purification method for efficiently removing large molecules while producing stable nanoparticle dispersions in a laboratory scale. It also has potential for scale-up to meet industry requirements as the membrane surface can be increased easily. The DCD technique, on the other hand, can be a good alternative purification method for nanoparticle dispersions of small volumes because of its limited volume-handling capacity.

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