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Synthesis and Characterization of Polymer Nanocarriers for the Targeted Delivery of Therapeutic Enzymes

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Abstract

Protein drugs, such as recombinant enzymes useful for detoxification and replacement therapies, have extraordinary specificity and potency. However, inherently inadequate delivery to target sites and rapid inactivation limit their medical utility. Using chaperone polymeric particles designed within an injectable size range (sub-micron) may help solve these shortcomings. Such nanocarriers would (i) prevent premature inactivation of encapsulated therapeutic protein cargoes, (ii) provide a carrier that can be surface decorated by targeting ligands, and (iii) optimize sub-cellular localization of the drug. This chapter describes the techniques successfully employed for the preparation of polymer nanocarriers (PNC) loaded with the antioxidant enzyme, catalase, and targeted to endothelial cells. Methods of PNC synthesis, loading with catalase, characterization, coupling of a targeting moiety, and in vitro testing of the enzymatic and targeting activities are provided here. Advantages and disadvantages of specific designs are discussed. Due to the modular nature of the targeting methodology employed, it is believed that these protocols will provide a solid foundation for the formulation of a wide variety of enzymatic drug targeting strategies.

Keywords

Polymer; biomaterials; nanocarriers; drug delivery; enzyme therapy; catalase; antioxidants; oxidative stress; vascular endothelium

1. Introduction

Due to high potency and specificity of their activities, protein drugs are rapidly gaining clinical use. Recent advances in the biotechnology of recombinant human proteins can provide large amounts of homogenous, GMP-standard protein therapeutics. Among current clinically used protein therapies fibrinolytic plasminogen activators (1, 2), interferons and cytokines (3–5) growth factors and hormones (6–9), proteases and their inhibitors such as serpins (10–12), and diverse enzymes (e.g., lysosomal hydrolases for replacement therapies) (13–15) are making substantial progress. Further examples include an extensive and rapidly growing list of antibodies, antibody fragments, and their conjugates with toxins and other biologically active agents (16–18). Interestingly enough, although slightly longer clinical testing and approval phases seem to be the trend (19), the number of FDA approvals of proteins for therapeutic applications increased dramatically in the last decade (20). This reflects an unprecedented translation of protein drugs from the realm of small biotech start-ups into specialized resourceful departments of big pharmaceutical companies.

However, protein drugs are still in need of further improvement, particularly in the area of their delivery. They are typically expensive (e.g., cost of a recombinant plasminogen

activator for fibrinolytic therapy is ~\$1,000). Therefore, both economic and biological reasons (e.g., immune response) restrict the practicality of multiple injections for chronic interventions. Even at a single injection, the higher the dose of protein therapeutics, the higher the cost and probability of side effects. Unfortunately, high doses are required when pharmacokinetics is unfavorable due to factors such as rapid elimination via renal clearance and hepatic uptake, which turns out to be the case for most protein therapeutics.

In addition, due to the specific nature of their effects (e.g., enzymatic conversion of selected substrates or binding to selected ligands) many, if not all, protein drugs require localization in specific areas of the body – an organ, tissue, cell, or even sub-cellular compartment. However, only a few protein drugs have a natural affinity to their desired site of therapeutic action and the major fraction of injected dose represents a potentially harmful waste. Even when these drugs reach the site of action, factors such as inactivation by inhibitors and proteolytic degradation greatly limit activity and duration of therapeutic effect.

Therefore, the optimization of protein delivery remains an extremely important, yet challenging and not fully achieved, goal. Through the addition of a carrier or vehicle, therapeutic protein delivery can be optimized in several key ways. First, loading into and, in some cases, even coupling to the surface of the carriers can protect proteins from inactivation by proteases, inhibitors, and other aggressive components of blood and/or other compartments in the body (21). Second, by creating a barrier between a drug on one side and blood and non-target tissues, cells, or organs on another side, a carrier can minimize potentially harmful side effects and avoid eliciting an immune response, both of which are commonly inherent of protein therapeutics. Third, loading into long-circulating carriers decelerate blood clearance of drugs that otherwise are rapidly excreted and also protect the cargo against premature inactivation en route to the target (22). Fourth, by varying size, mechanical properties, and surface decoration, targeted delivery of carrier cargoes to therapeutic sites can be achieved by either passive mechanisms (e.g., utilizing mechanical or electrostatic retention in capillaries or effect of enhanced permeability and retention in areas of inflammation and tumors) (23, 24) or active mechanisms (e.g., utilizing affinity moieties on the surface of carriers that bind to specific determinants in targets) (18, 25, 26). This feature can even extend to achieving more adequate sub-cellular addressing of cargoes, utilizing specific endocytic pathways and intracellular sorting and traffic machinery (27), depicted in Fig. 9.1 for nanocarriers coated with targeting antibodies. Finally, slowly degrading carriers produce a prolonged and more stable profile of drug activity in the target site (28).

This chapter describes the methodology and protocols to formulate polymer nanocarriers (PNC) targeted to endothelial cell adhesion molecules (CAM) designed for enzyme encapsulation and thus protection from lysosomal degradation. While the immobilization of *active* enzymes within PNC has been elusive, the freeze–thaw double emulsion solvent evaporation technique described in this chapter has demonstrated ample loading of catalytically active enzyme (catalase) and protection of this cargo from proteolytic degradation (28). To conjugate targeting CAM antibodies to the PNC formed in this method, a biotin–streptavidin conjugation scheme is used. This protocol attaches antibody–streptavidin conjugates to PNC-containing biotinylated polymer through a single-step addition process, avoiding undesirable aggregation of PNC. These techniques taken together are believed to provide a solid basis for the design of any targeted PNC enzyme delivery strategy.

2. Materials

2.1. Equipment

1. Rotovap (Safety Vap 205; Buchi, Switzerland).
2. Freeze dryer (RCT 60; Jouan, Winchester, VA).
3. Homogenizer (Kinemetica Polytron 3100 with a 7 mm PDTA3007/2 generator; Brinkmann Instruments, Westbury, NY).
4. Dynamic light scattering 90 Plus Particle Sizer (Brookhaven Instruments Corp., Holtsville, NY).
5. UV-VIS spectrophotometer (Cary 50 UV-Vis; Varian, Palo Alto, CA).
6. Microplate reader (Model 2550-UV; Bio-Rad Labs, Hercules, CA).
7. Gamma counter (1470 Wallac Wizard; PerkinElmer, Wellesley, MA).
8. Fluorescence microscope.
9. Desalting Quick Spin Protein Columns (Roche Applied Science, Indianapolis, IN).
10. Laboratory low-speed centrifuge.
11. General cell culture equipment: CO₂ incubators, sterile hood, etc.

2.2. Reagents

All reagents obtained from Sigma-Aldrich (St. Louis, MI), unless otherwise stated.

1. Diamine polyethylene glycol (PEG) (Nektar, Huntsville, AL).
2. 5,000 MW mono-methoxyPEG (mPEG) (Polysciences, Warrington, PA).
3. Lactide (3,6-dimethyl-1,4-dioxane-2,5-dione).
4. Stannous octoate.
5. Poly(lactic co glycolic acid) free acid ended (PLGA – 5050 DL 4A) (Alkermes, Cambridge, MA).
6. *N,N'*-Dicyclohexylcarbodiimide (DCC).
7. Dichloromethane (DCM).
8. Polyvinyl alcohol (PVA) or Pluronic® F68.
9. Catalase from bovine liver (Calbiochem, San Diego, CA).
10. Hydrogen peroxide (H₂O₂).
11. Sodium hydroxide (NaOH).
12. Hydrochloric acid (HCl).
13. Lactate oxidase.
14. 10-Acetyl-3, 7-dihydroxyphenoxazine(Amplex red; Molecular Probes, Eugene, OR).
15. Horseradish peroxidase (HRP) (Calbiochem).
16. Barium chloride (BaCl₂).
17. Potassium iodide (KI).

18. Iodine (I₂).
19. Succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) (Pierce, Rockford, IL).
20. *N*-Succinimidyl-*S*-acetylthioacetate (SATA) (Pierce).
21. Streptavidin from *Streptomyces avidinii* (Calbiochem).
22. Biotin *N*-hydroxysuccinimide ester (Biotin-NHS) (Pierce).
23. Murine monoclonal anti-PECAM antibody (clone 62 was generously provided by Dr. Nakada; Centocor, Malvern, PA).
24. Non-immune mouse IgG (Jackson ImmunoResearch Labs, West Grove, PA).
25. *N, N*-Dimethylformamide (DMF).
26. Dimethyl sulfoxide (DMSO).
27. Hydroxylamine-HCl.
28. Pronase (Protease type XIV – bacterial from *Streptomyces griseus*).

2.3. Buffers, Media, and Cells

1. Phosphate-buffered saline (PBS): 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.2.
2. Human umbilical vein endothelial cells (HUVEC; Lonza, Basel, Switzerland).
3. REN human mesothelioma cells and REN-hPECAM cells transfected with human PECAM (kindly provided by Dr. Steve Albelda, University of Pennsylvania).
4. HUVEC culture medium: M199 medium (GIBCO, Grand Island, NY), 10% FCS (GIBCO) supplemented with 100 µg/mL heparin (Sigma, MO), 2 mM L-glutamine (GIBCO), 15 µg/mL endothelial cell growth supplement (Upstate, Lake Placid, NY), 100 U/mL penicillin, and 100 µg/mL streptomycin.
5. REN cell culture medium: RPMI 1640 medium (GIBCO), 10% fetal bovine serum (Sigma), 2 mM L-glutamine (GIBCO), 100 U/mL penicillin, and 100 µg/mL streptomycin. For REN-hPECAM cells the medium was supplemented with 0.5 mg/mL G418-sulfate.
6. Lysis buffer: 1% Triton X-100, 1.0 N NaOH.

3. Methods

3.1. Preparation of Biotinylated PEG-PLA/PLGA

1. To remove small (MW < 1 kDa) compounds, dissolve 5 g PLGA (38 kDa) into 100 mL dichloromethane and add solution drop wise to a 1 L bath of cold ether, precipitating the PLGA polymer. Perform the same step with the 20 g D, L-lactide in place of PLGA.
2. Filter out precipitates and dry under nitrogen in a rotovap followed by a freeze dryer.
3. Add the PEG diamine and PLGA in a 1:6 molar ratio (e.g., 2 g PLGA to ~100 mg of 3.4 kDa PEG) into a common vessel such as a 250 mL Erlenmeyer flask containing a magnetic stir bar.
4. Remove excess water from this powder mixture by freeze drying overnight.

5. Remove powder from freeze dryer and cap with a rubber septum. Purge vessel with nitrogen 3–5 times.
6. Using a needleline transfer technique, add 100 mL of anhydrous DCM to polymer powder and fully dissolve by mixing on a stir plate.
7. Prepare 5 mL of a 50 mM solution of DCC in anhydrous DCM. DCC is a highly reactive primary amine reagent. Use exclusively in a hood, or preferably using a glove bag or glove box. Since DCC is readily hydrolyzed, prepare solution immediately before use.
8. Next, add 1.16 mL of the DCC solution to the polymer solution to create a final 1:1.1 PLGA:DCC molar ratio.
9. React under constant stirring overnight.
10. Filter solution through a filter (pore size: 3 μ m) followed by concentrating polymer solution on a rotovap to ~50 mL.
11. Add 500 mL of cold ether to precipitate polymer and dry sample on a rotovap.
12. Add 50 mL of DCM and repeat ether precipitation.
13. To remove unreacted PEG, dissolve polymer in acetone (50 mL). Acetone may need to be heated to fully dissolve polymer.
14. Add, drop wise, acetone solution to cold water.
15. Collect precipitate and freeze dry. The consistency of the polymer may prevent filtration. If this occurs, precipitate can be centrifuged instead (15,000 $\times$ g for 1 h at 4°C).
16. Dissolve 1 g of PEG-PLGA into a 50 mL 1:1 solution DCM:DMSO.
17. Prepare a 50 mM solution of biotin-NHS in DMSO.
18. Add biotin-NHS to the PEG-PLGA solution for a final 1.5:1 biotin-NHS:PEG-PLGA molar ratio.
19. Purify polymer by evaporating off DCM and then adding solution to cold DI water.
20. Collect precipitate, dissolve again into acetone and re-precipitate into cold DI water.
21. Freeze dry polymer and store under desiccation until ready for use.
22. For synthesis of PEG-PLA, add 5 g of the purified D, L-lactide from step 1, with 1.25 g of mPEG to a round bottom flask with a Teflon stir bar. PEG and lactide are mixed in stoichiometric amounts to achieve the desired polymer molecular weights. For example, the above amounts result in a mPEG₅-PLA₂₀ (5 kDa PEG block and 20 kDa PLA block) diblock copolymer. To synthesize mPEG₅-PLA₃₀, simply mix 5 g of lactide with 0.8333 g of mPEG.
23. Seal the flask with a rubber septum and purge with dry nitrogen.
24. Heat the sample to 140°C for 2 h, sealed under dry nitrogen to bring the reagents to a melt and drive off residual water. A three neck, round bottom flask can be used instead, with a Drierite drying column if one is concerned about excessive moisture. Trace water severely hinders the polymerization.
25. Reduce the temperature to 130°C and add stannous octoate at 1% of the total reagent mass (for this example that amounts to about 46.6 μ L, taking into account the density of stannous octoate). Let polymerization proceed for approximately 6 h.

26. Reduce temperature to 25°C and dissolve the polymer in approximately 30 mL of DCM.
27. Precipitate this DCM–polymer mixture into cold diethyl ether, adding drop wise. Repeat twice, collecting filtrate.
28. Freeze dry polymer and store under desiccation until ready for use.

3.2. Polymer Nanocarrier (PNC) Formulation and Catalase Encapsulation

1. Prepare a mPEG-PLA solution containing ~20 mol% biotin-PEG-PLA. To do this, dissolve 5 mg biotinylated mPEG-PLA/PLGA (prepared in **Section 3.1**) and 20 mg mPEG₅-PLA_{20–30} diblock copolymer in 1 mL DCM for a total 25 mg/mL polymer solution, per prep. It should be noted that a mPEG₅-PLA₂₀ polymer yields spherical particles, while mPEG₅-PLA₃₀ results in filamentous particles, as illustrated in Fig. 9.2 (29).
2. Prepare 100 µL of a protein solution (1 mg/mL) solution of catalase in 20 mM PBS. Maximum protein concentration used is limited by the solubility of protein in solution. The key parameter is the volume of the aqueous phase, and should be maintained, for this particular formulation, at ~10% of the primary emulsion (e.g., 100 µL catalase/PBS added to 1 mL of polymer solution).
3. Prepare surfactant solution of PVA or F68 pluronic (2% wt) in 20 mM PBS. Higher % hydrolyzed PVA may not go entirely into solution. If this occurs, filter solution with a 0.2 µm filter.
4. Combine 100 µL catalase solution (aqueous phase) and 1 mL of the polymer–DCM solution (oil phase) in a 12 × 75 mm glass (borosilicate) test tube for the primary emulsion, and place on ice. Use glass test tubes to prevent contamination (e.g., DCM dissolves polystyrene plastic test tubes) and to minimize sample loss due to vessel wall adherence.
5. Add 5 mL of the surfactant solution to a 16 × 100 mm borosilicate test tube for the second emulsion, and place on ice.
6. Add 10 mL of the surfactant solution to a small vessel (a 20-mL scintillation vial will suffice) and place on a stir plate set at 1,400 rpm.
7. Prepare a dry-ice and acetone bath in a small vessel deep enough to submerge the entire liquid portion of the test tube containing the primary emulsion and allow it to equilibrate (–80°C) (*see* Note 1).
8. Set up the 7 mm generator on the homogenizer and wash first with water and then acetone, drying thoroughly. Preset the homogenizer at the desired mixing rate prior to emulsification. For this protocol, set speed to 15,000 rpm.
9. Homogenize the primary emulsion, at 15,000 rpm. Once the homogenizer has reached 15,000 rpm, add the dry ice bath, ensuring that the entire liquid portion of the emulsion is chilled. Homogenize the solution for 1 min. To minimize protein denaturing, minimize the time between starting the homogenizer and submersing into the dry ice bath. Note, however, that adding the dry ice bath before the homogenizer has come up to speed will prematurely freeze the aqueous phase before it can blend with the organic polymer-containing phase.

¹*Formulation.* Do not allow second emulsion test tube containing surfactant to sit on ice longer than 15–30 min prior to the actual second emulsion. When the surfactant temperature of the second emulsion is at 4°C, the addition of the primary emulsion (–80°C) brings the entire solution below the gel point for the surfactant solution resulting in poor mixing.

10. After the primary emulsion is complete, pipette the solution into the 5 mL surfactant containing test tube. Homogenize for an additional 1 min at 15,000 rpm, without the dry-ice acetone bath. The speed of this step will determine the final size of the nanocarriers. Important: minimize the transfer time to enhance final loading, carrier yield, and minimize final PNC size.
11. Add the second emulsion into the 10 mL surfactant solution. Allow to stir at ambient conditions for at least 3 h to allow for DCM evaporation and PNC hardening.
12. Centrifuge the 15 mL sample at $1,000 \times g$ for 10 min to remove large aggregate/microparticle fraction (*see* Note 2).
13. Centrifuge the nanoparticle-containing supernatant at $20,000 \times g$ for 30 min. Minimize excessive centrifugation to avoid irreversible PNC aggregation.
14. Remove the supernatant and resuspend the PNC pellet in 500 μ L PBS.
15. Dilute sample again to 15 mL and repeat steps 13 and 14, in order to remove any residual surfactant.

3.3. PNC Characterization (*see* Note 3)

3.3.1. Loading Determination—Protein loading can be determined either by mass (tracing of radioisotope-labeled protein) or catalytic activity.

Loading determination by tracing catalase mass

1. Radiolabel catalase with Na¹²⁵I (PerkinElmer, Boston, MA) via the Iodogen method (Pierce Biotech., Rockford, IL). Unbound ¹²⁵I is removed from catalase by

²*Serial centrifugation size fractionation.* Serial centrifugation to separate different size populations (28) is best used when synthesized particles possess two distinct population sizes, as is the case in this freeze–thaw solvent evaporation PNC formulation. Centrifugation time and speed can be determined by calculating the time needed to settle larger particles using Stokes' law:

$$V = 2ga^2 \frac{\rho_1 - \rho_2}{9\eta}$$

where V is the settling velocity, g the relative centrifugal force, a the particle radius, ρ_1 and ρ_2 are the particle density and the buffer density, respectively, and η is the buffer viscosity. After centrifugation, the supernatant can be collected and centrifuged at a greater speed to collect the nanoparticle fraction. This procedure is an efficient and rapid method of both size selection and removal of residual surfactant. An example of a centrifugation scheme is represented in Fig. 9.4. To remove any residual surfactant that may be bound to the PNC surface, resuspension of nanocarriers in buffer and an additional centrifugation purification step is recommended. As with any centrifugation fractionation technique, resuspension of PNC can be difficult. Ideally centrifugation rates will be selected such that PNC will be pelleted but not aggregated. However, if aggregation does occur, several laboratory techniques can be used to aid in resuspension. For instance, allow the sample to solvate for 5 min to 1 h with mild vortexing. Allowing the PNC to rehydrate slowly can aid in the hydration of the PNC's PEG shell, enhancing particle–particle repulsion. If, after this period, resuspension is still difficult, application of mild pulse sonication (5 W, 1 s intervals) can be used. However, such energy addition may result in disruption of PNC, releasing cargo and/or denaturing loaded protein.

³*PNC size control.* The final size of PNC is dictated by a balance of both kinetic and thermodynamic forces. Mechanical disruption of the two-phase liquid system results in droplet formation. As more energy is added (either through faster shear rates, longer times, larger impeller diameters) the droplets are disrupted and form smaller droplets. However, as the interface between the two phases increases, the destabilizing energy also increases. Unless there is a surfactant that can function to stabilize these two phases, droplets will start to coalesce and form larger particles. Yet this coalescence is rate limiting and determined by factors including aqueous phase viscosity, polymer phase viscosity, surfactant concentration, and temperature. Figure 9.5 demonstrates this balance between energy and size.

To summarize these effects, smaller PNC can be obtained by higher energy rates and increased surfactant content (either the amphiphilic PNC polymer or added solution surfactant). However, as PNC size decreases, so does the internal volume to surface area ratio. With smaller internal volumes, the likelihood of successfully encapsulating protein drops off significantly. Furthermore, longer duration and higher shear rate homogenizations can denature enzymes, dramatically decreasing recovered activity. As such, ultimate carrier size is selected by a compromise between cellular/vascular compatibility and protein loading efficiency.

gel permeation chromatography (GPC, Biospin 6 columns, Bio-Rad Labs, Hercules, CA).

2. Form PNC, as described in **Section 3.2**, substituting catalase in the primary emulsion with a mixture of ^{125}I -catalase and non-iodinated catalase. Make sure a sufficient amount of ^{125}I -catalase is added, since reliable measurements are obtained only when the radioactivity of the resultant samples is above 100 cpm, which usually means that $\sim 50,000$ cpm/prep should be added.
3. Measure total solution ^{125}I -catalase content in the sample before centrifugation. Centrifuge the samples as outlined above (**Section 3.2**) to separate super and submicron fractions of catalase-loaded PNC. Resuspend PNC pellet to determine radioactivity of the ^{125}I -catalase/PNC with a Wizard 1470 gamma counter (PerkinElmer, Boston, MA).

Loading determination by enzyme catalytic activity: Similarly, to determine loading via enzymatic activity, a catalase activity assay (30) is used, both for the total sample before and resuspended pellet after centrifugation.

1. Add 900 μL of 5 mM H_2O_2 in PBS and 100 μL of enzyme-loaded PNC to a quartz cuvette and mix, avoiding vigorous shaking which results in foaming and errors in measurement. In general, aim for a total volume of 1 mL, but amount of PNC can vary from 10 to 100 μL . If PNC concentration is too high, diffraction and other light scattering phenomena can interfere with the spectrophotometer reading. This can increase background noise and make measurements of samples with low activity difficult.
2. Measure loss of H_2O_2 absorbance (242 nm) over time with a spectrophotometer to determine degradation by catalase associated with carriers. Calculate slopes ($\Delta A/\text{min}$), which correlate with loaded enzymatic activity, of decreasing absorbance. Calculate the activity using following equation:

$$\text{Catalase activity, U/mg} = 23.0(\Delta A/\text{min})/\text{mg protein}$$

3.3.2. PNC Enzyme Protection Assay—PNC protection of encapsulated catalase can be shown via incubation with a proteolytic enzyme, such as pronase (28). Protection can be shown by either preservation of catalase activity, via the above described activity assay in **Section 3.3.1**, or by mass of loaded protein degraded.

1. If determining protection by mass, prepare PNC loaded with ^{125}I -catalase as described above in **Sections 3.2** and **3.3.1**.
2. Incubate final PNC prep with a 0.2% (w/v) pronase solution at 37 °C in a shaker bath set at 60 rpm for 1 h, or longer if desired. Dependent on how many time points one wishes to make, 0.5–1 mL total samples are typically enough.
3. If determining protection by mass, remove samples from shaker bath and centrifuge at $16,000 \times g$ for 20 min for a 1 mL sample. Collect and place in the gamma counter the supernatant containing products of ^{125}I -catalase proteolytic degradation and pellet containing intact/protected protein, encapsulated within PNC.
4. If determining protection by activity, simply measure enzymatic activity before and after incubation with protease, via the activity assay in **Section 3.3.1**.

3.3.3. Morphology Characterization—Adequate characterization of sizing and geometry can be accomplished with dynamic light scattering (DLS) and transmission electron microscopy (TEM).

Dynamic light scattering: From DLS, approximate values of diameter can be obtained. *Relative* concentration, proportional to the number of PNC within a specific volume, can be determined from the average scattering intensity measurements. Please refer to the method by Shuvaev et al. for practical details in experimental theory and data interpretation (30).

1. Add 20 μL of reconstituted PNC to a glass NMR tube and dilute with 200 μL of filtered DI water or buffer. Amount of PNC solution added should be adjusted to keep scattering within the optimum range of the instrument, typically for nanoparticles between 200 and 800 kcps.
2. Place in dynamic light scattering apparatus containing an NMR to Cuvette adapter insert (DLS, 90 Plus Particle Sizer; Brookhaven Instruments, Holtsville, NY). Record for 2 min at a 90° angle.

Transmission electron microscopy: TEM provides a means of validating DLS sizing data and determining actual carrier morphology, (e.g., spherical, branched, or wormlike). TEM is not an effective means of determining concentration since drying phenomena can lead to heterogeneous PNC distribution.

1. Apply 5 μL of sample to a TEM mesh grid (Formvar Film 200 mesh; Electron Microscopy Sciences, Hatfield, PA). Cover only the dull (not polished) grid side, completely with sample.
2. Remove excess by touching edge of grid with filter paper, before drying at ambient conditions. Removal of excess sample promotes a monolayer of PNC on the grid surface, improving image quality. Also be sure to try multiple concentrations/dilutions of PNC samples. If samples are too concentrated, aggregation is common upon grid staining and further limited quality imaging results. Be careful not to touch top surface of grid and introduce any particulate contaminants.
3. Prepare 2% (w/v) uranyl acetate (UA) solution in DI water. Cover with aluminum foil and avoid exposure to light. UA solution stored at 4°C typically yields decent sample staining if used within a week of preparation. Longer storage times often lead to excessive UA precipitation.
4. Filter UA through a $0.1\ \mu\text{m}$ syringe filter, placing a drop on parafilm.
5. Touch the top of the sample – containing grid to UA drop. Turn grid sample-side up and cover to block out light. Do not allow any UA to stain the backside of the grid, as this will obscure imaging. Let sample stain for 5 min, before drop wise washing with filtered DI water.
6. Remove excess water as described in **step 2** (above) and allow grid to dry at ambient conditions for 1 h before imaging with TEM.

It is imperative that fluid UA exposure to light be minimized, as this will lead to contrast agent precipitation and imaging artifacts. When imaging, be sure to use the minimum necessary accelerating voltage to avoid PNC sample melting.

3.3.4. Yield Determination—A convenient method of PNC yield determination is a colorimetric PEG assay based on the PEG-barium iodide complex (31). Alternatively, one could perform a lactic acid enzymatic assay (*see* Note 4)

1. Rapidly hydrolyze a 50 μ L aliquot of PNC sample by adding 200 μ L of 5 N NaOH and reacting overnight at 80 $^{\circ}$ C, before neutralizing with 5 N HCl.
2. Prepare solution A, consisting of 2.4 g barium chloride, 8.0 mL 6 N HCl and 32 mL deionized (DI) water.
3. Prepare solution B, consisting of 800 mg of potassium iodide, 500 mg of iodine, and 40 mL of DI water. This solution is very concentrated and not all of the iodine may go into solution. Preparing a more diluted version is not advised since iodine is volatile and may not store well.
4. Prepare a 0.2 mg/mL calibration solution of PEG in DI water (preferably of the same MW used in the PNC, but this is not essential and can be accounted for in the analysis).
5. In a 96-well plate, prepare a calibration series of 50-0 μ L of the stock PEG solution. Ideally, calibration should be in 10 μ L increments for 50-10 μ L, and 2 μ L increments for 10-0 μ L. This setup is good for the detection of 10-0.1 μ g PEG.
6. Add PEG sample from **Step 1** to wells in appropriate volumes such that approximately 10-0.1 μ g of PEG is present.
7. Dilute sample and calibration volumes to 170 μ L total volume with DI water.
8. Add 40 μ L of solution A to each well and mix thoroughly.
9. Make a 1/5 dilution of solution B and add 40 μ L to each well.
10. Allow to develop for 10 min and measure absorbance at 535 nm on the microplate reader. A 550 nm filter on the reader is adequate.

3.4. Preparation of Antibody– Streptavidin Conjugates

Sulfhydryls are introduced onto the molecule of antibody or control IgG through primary amines using SATA. In parallel, heterobifunctional cross-linker, SMCC, is used to introduce stable maleimide reactive groups into streptavidin molecules. Conjugation takes place as a specific reaction between the SH group of the antibody and the maleimide group of streptavidin. Introducing one sulfhydryl per antibody molecule prevents multivalent cross-linking and aggregation whereas several reactive groups may be introduced into streptavidin to increase the yield of the conjugation. In addition, such a conjugation of antibody molecule to streptavidin limits the availability of biotin-binding sites on streptavidin and thus prevents possible cross-linking of the PNC, in contrast to unconjugated streptavidin that may function to cross-link PNC together. Further, streptavidin can be replaced by less immunogenic neutral avidin derivative, neutravidin.

⁴*Particle yield and mass determination.* Double emulsion PNC synthesis typically results in a bimodal distribution, with a significant fraction of undesired large (>1 μ m) particles. During process optimization, reduction in this loss fraction will be a significant technological hurdle. However, proper surfactant concentration may provide one avenue of achieving higher PNC yields. Increasing PVA surfactant concentration from 0.1 to 4 wt% increased the PNC fractional yield, but also simultaneously increased the carrier diameter as well. This result is most likely due to two contributing factors. With greater PNC mass, the overall surface area between phases is increasing, resulting in a saturation of PVA's surfactant capacity at larger sizes. Also, the increased viscosity of the aqueous phase reduced the rate of droplet breakup under the mixing conditions, further adding to the larger carrier size.

Polymer concentration analysis provides the only reasonable method to determine PNC yield. Yet, the PEG assay approach possesses several potential complications. Polymeric surfactant used in PNC synthesis can participate in the barium iodide complex, resulting in overestimating the actual PNC concentration. This is of particular concern with Pluronic[®], a PEG-containing surfactant. As such, careful particle purification in the form of extra centrifugation steps and/or dialysis may be necessary to determine the actual PNC concentration. PEG assays of the supernatants and dialysates provide an indicator of amount of surfactant released through each step. Once no measurable PEG is found, PNC can be assumed to be clear of surfactant. If dialyzing, keep in mind that Pluronic[®], although less than 9 kDa, is a linear polymer and hence has a hydrodynamic radius on the order of a 500 kDa protein.

Erratic results with the PEG assay may also be obtained in the presence of high protein content (above 3 mg/mL) (31). As an alternative, an enzymatic assay may be utilized for the detection of lactic acid, the monomeric building block of PLA (*see* Note 5).

1. Dissolve IgG (antibody or non-immune IgG) and streptavidin in 0.1 M PBS to a concentration of 5.0 and 6.0 mg/mL, respectively (33.3 and 100 μ M, respectively). Make sure that there is no contamination with amine-containing components such as Tris buffer. Keep proteins on ice.
2. Prepare fresh 40 mM SATA in DMSO (9.25 mg/mL).
3. Add freshly prepared SATA to 200 μ L of IgG in a 1:5 IgG:SATA molar ratio and incubate for 2 h at room temperature.
4. Separate modified protein from unreacted components using desalting Quick Spin Protein Columns (Roche Applied Science, Indianapolis, IN).
5. Add 20 μ L of 0.5 M hydroxylamine-HCl (NH_2OH) to SATA-modified IgG and incubate for 2 h at room temperature before again passing IgG sample through desalting column.
6. Prepare fresh 20 mg/mL (45.8 mM) SMCC in DMF. Add SMCC solution to 100 μ M streptavidin to achieve a final streptavidin:SMCC molar ratio of 1:20 (4.4 μ L SMCC/100 μ L streptavidin). The reaction takes 1 h at room temperature. This step may be done during previous step 5. Separate SMCC-modified streptavidin using desalting column.
7. Conjugation is performed by mixing modified proteins at IgG:streptavidin molar ratio 2:1 and incubation for 1 h on ice.
8. The confirmation of conjugation products may be performed by SDS polyacrylamide gel electrophoresis (4–15% gradient gel; Bio-Rad) and following Coomassie staining, as shown in Fig. 9.3.
9. The conjugates may be frozen in appropriate aliquots (i.e., 50 μ L/tube) and store at -80°C before use.

3.5. Conjugation of PNC and Targeting Antibodies

1. Prepare antibody–streptavidin conjugates as described above in **Section 3.4**.
2. Mix 10 μ L of 25 mg/mL PNC with 70 μ g (total protein mass) of antibody–streptavidin conjugate solution (7 μ L of a 50 μ M conjugate stock solution by streptavidin basis). This results in a 1:10,000 Ratio of PNC:antibodies for 300 nm PNC.
3. Dilute solution to 100 μ L and vortex for 1 min.
4. Incubate under gentle agitation for 1 h at ambient temperature.
5. Dilute sample with 1 mL of PBS and centrifuge ($16,000 \times g$ for 10 min).
6. Resuspend pellet and measure size and binding function based on previously reported methods (32).

3.6. Characterization of Targeted PNC In Vitro – PNC Binding to Cells

3.6.1. Fluorescence Detection of PNC Binding to Cells—To visualize binding and to show its specificity we used epifluorescence imaging.

1. Plate REN and REN-hPECAM cells on 24-well dishes with inserted 12-mm glass coverslips and cultivate to confluence.
2. Add anti-PECAM/PNC to confluent cells and incubate for 1 h in regular cell medium at 37°C .

3. Wash out unbound nanocarriers 5 times with fresh RPMI medium, fix cells, and stain with secondary FITC-labeled antibody against mouse IgG.
4. Visualize the bound nanocarriers in REN-hPECAM cells vs. REN cells as a negative control using fluorescence microscope.

3.6.2. Detection of PNC Binding to Cells by Radioisotope Tracing—

Quantification of the binding to human endothelial cells was performed by radioactive tracing of nanocarriers.

1. Plate HUVEC on 24-well dishes and cultivate until culture is confluent.
2. Add anti-PECAM/PNC or control IgG/PNC labeled with ^{125}I -IgG-SA tracer to cells at serial dilutions, starting with ~25,000 cpm per well and incubate for 1 h in regular cell medium at 37°C.
3. Wash out unbound nanocarriers 5 times with fresh RPMI medium and lyse cells with 1% Triton X-100, 1.0 N NaOH.
4. Transfer cell lysate in glass tubes and measure radioactivity of bound material using Wallac 1470 Wizard™ gamma counter. Bound material is expressed as number of bound particles per cell.

5. Conclusions

Several factors must be weighed when formulating the “optimal” PNC. Properties such as yield, enzyme loading, and PNC sizing can be tuned through process manipulation. Yet these process manipulations (e.g., energy input, surfactant concentration) can have complex and competing effects in terms of sizing and enzyme activity. Along with the notion that the definition of optimal is highly dependent upon the therapy being pursued, it is not possible to know a priori what the ideal compromise in PNC properties is. Coupled with the capacity to vary biodistribution through the non-aggregating coupling of antibodies to the carrier surface, in vivo studies must always be performed as a feedback mechanism to tune the synthesis of carrier for each specific therapeutic goal. Only through this continual interaction between the engineering and the biology can PNC delivery strategies result in clinically relevant solutions.

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Fig. 9.1.

Concept of cell adhesion molecule-targeted PNC delivery to endothelial cells (EC). Antibody (Ab)-coated PNC targeted to cell adhesion molecules (CAM) are internalized via CAM-mediated endocytosis, before trafficking to endo/lysosomes (33). PNC-encapsulated catalase is protected from proteolytic digestion during residence within these recycling compartments. Both internalized and surface-bound PNC can detoxify the highly diffusive reactive oxygen species, hydrogen peroxide (produced under pathological conditions by either adherent white blood cells (WBC) or by endothelial cells (e.g., in mitochondria). Masking of endothelial CAM by the targeted PNC may also attenuate pro-inflammatory vascular adhesion of white blood cells.

Fig. 9.2.

Transmission electron microscopy (TEM) of mPEG-PLA PNC. Re-suspended PNC pellet was diluted 1:4 in DI water before application to TEM grid and subsequent staining with 2% (w/v) uranyl acetate. Scale bar is 500 nm. *Left:* sphere morphologies are produced when larger fractions of amphiphilic diblock copolymer consist of hydrophilic block mPEG. In the *left* figure, a 22:78 MW ratio of mPEG:PLA was used to form PNC. *Right:* Worm/branched morphology was achieved with a higher MW fraction of hydrophobic polymer. In this case, only 7% of the diblock polymer MW consisted of mPEG. In general, increasing the hydrophilic fraction of diblock copolymers can lead to a transition from worm micelle and branched morphologies to more spherical particles, if polymers with near monodisperse MW are utilized (34, 35). Due to the more disperse MWs of polymers synthesized with a ring-opening polymerization (degree of MW polydispersity increases further with higher MW), a slightly more heterogeneous PNC morphology can be observed at higher polymer MW with the system described in this chapter.

Fig. 9.3.

Electrophoretic analysis of anti-PECAM/streptavidin conjugate. Anti-PECAM monoclonal antibody (Ab 62) was conjugated to streptavidin (SA) using heterobifunctional cross-linkers SATA and SMCC (*see* **Section 3.4**). Figure shows Coomassie blue staining of gel electrophoresis in reducing conditions (4–15% PAGE gel, Bio-Rad). A *star* indicates formed conjugated species. Due to denaturing and reducing conditions of the electrophoresis only heavy chains of antibody and only monomers of streptavidin that are directly cross-linked appear as conjugated species. Other heavy and light chains of antibody and non-cross-linked streptavidin monomers migrate as intact bands.

Fig. 9.4.

Centrifugation and size fractionation. The scheme represents approximate centrifugation rates (g) necessary to sediment different size populations in a 10 min centrifugation of PLA-based particles. Higher rate, g , or longer spin time, can bring down smaller particles in the desired sub-500 nm range.

Fig. 9.5.

PNC size decreases with increasing energy input. Mean diameter (measured by DLS) is a function of both energy input (which scales with homogenization rate² t) and polymer surfactant capacity (● 5% and ○ 11% PEG content). When surfactant capacity of system is saturated, further increases in energy input do not decrease PNC size. However, greater PEG content (greater intrinsic surfactant capacity of PNC polymer) results in formulation of even smaller PNC carriers.