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Large scale production of vesicles by hollow fiber extrusion: a novel method for generating polymersome encapsulated hemoglobin dispersions

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Abstract

Vesicles, which include both liposomes and polymersomes (polymer vesicles), are being developed as therapeutic drug carriers. In this study, we present a fully scalable low pressure extrusion methodology for preparing vesicles. Vesicles were generated by continuous extrusion through a 200 nm pore diameter hollow fiber (HF) membrane. The first half of this study describes a method for generating empty polymersomes composed of different molecular weight amphiphilic poly (butadiene-*b*-ethylene oxide) (PBD-*b*-PEO) diblock copolymers on a large scale (50 -100 ml) using a HF membrane. Monodisperse empty polymersomes were formed with particle diameters slightly less than 200 nm, which were close to the rated 200 nm pore size of the HF membrane. The second half of this study describes the successful encapsulation of hemoglobin (Hb) inside the aqueous core of polymersomes using the HF extrusion methodology. We demonstrate that polymersome encapsulated hemoglobin (PEH) particles formed by this technique had similar oxygen affinity, cooperativity coefficient, and methemoglobin (methHb) level compared to PEHs formed by the 1 ml volume small-scale manual extrusion method. Most notably, Hb encapsulation inside the polymer vesicles formed by the HF extrusion method increased two-fold compared to the manual extrusion method.

This work is important, since it will enable facile scale-up of homogeneous vesicle dispersions that are typically required for preclinical and clinical studies as well as industrial use.

INTRODUCTION

Vesicles are being exploited *in vivo* and *in vitro* in areas such as targeted drug delivery and tissue engineering.^{1–4} Vesicles can entrap macromolecules either in their aqueous core or membrane bilayer, depending on the hydrophilic/hydrophobic nature of the macromolecule. Vesicles encompass both liposomes and polymersomes. Liposomes are vesicles composed of phospholipid molecules, while polymersomes are vesicles composed of amphiphilic diblock copolymer molecules.

Various methods have been used to produce vesicles such as sonication, high shear homogenization, reverse phase hydration, ether injection technique, thin film hydration, direct

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Supporting Information Available

The method for large scale production of liposome encapsulated Hb dispersions using HF extrusion is provided. This information is available free of charge via the Internet at <http://pubs.acs.org>.

hydration and extrusion.^{5–8} Typically, all of these processes produce aqueous suspensions containing multilamellar vesicles (MLVs) that are not uniform in size. As a result, subsequent extrusion through a polycarbonate membrane with a defined pore size is performed to increase encapsulation of target macromolecules and form vesicles of uniform size.

Previously, Schneider et al.⁹ suggested the use of continuous high pressure gas extrusion processes for generating unilamellar vesicles. In this process, suspensions of MLVs are passed through successive filters with reducing pore size. In the process described by Schneider et al.,⁹ liposome suspensions were passed five times through two stacked polycarbonate membranes of the same pore size at pressures up to 10.5 MPa. This method facilitated easy generation of liposome dispersions; however, the encapsulation efficiency of macromolecules was found to be dependent on lipid composition and concentration as well as the final pore size of the membrane used during the extrusion process. Moreover, to obtain vesicles with mean diameters of 100 nm, five filters with decreasing pore size were used. Briefly, the MLV suspension was first passed through a 5.0 μm filter five times and then consecutively through 1.0, 0.4, 0.2 and 0.1 μm filters, five times each. Therefore, the process employed a total of 25 passes for one complete extrusion cycle. In addition, the encapsulation of macromolecules within the liposomes was highly dependent on the number of freeze-thaw cycles performed after passage through the 0.4 μm filter.

The conventional method for producing small (1 ml) volumes of vesicles relies on the use of small-scale manual extrusion. Suspensions of MLVs are mechanically broken down into smaller vesicles by repeatedly passing the suspension through polycarbonate membranes with a defined pore size. Therefore, the manual extrusion method generates smaller vesicles from large MLVs. Typically, the liposome suspension containing MLVs is loaded into a gas-tight syringe and placed into one end of the mini-extruder, while an empty gas-tight syringe is placed on the other end of the mini-extruder. The mini-extruder itself houses a polycarbonate membrane with a defined pore size. The MLV solution in the syringe is manually driven until the lipid/polymer solution is completely transferred to the alternate syringe. Then the plunger of the alternate syringe is manually driven to transfer the solution back to the original syringe. This cycle is repeated a total of 20 times to yield smaller vesicles of uniform size. The mini-extrusion system is closed to the environment and can be considered as a continuous recycling extrusion process.

In this work, we present a fully scalable extrusion method to prepare small vesicles (~ 200 nm in diameter) with uniform size distribution. In this extrusion technique, vesicles are extruded under low pressure through a hollow fiber (HF) membrane cartridge with a well-defined pore size. Figure 1, shows a schematic of experimental set up used in this study to generate vesicles. In this work, an aqueous sonicated copolymer mixture containing MLVs and hemoglobin (Hb) was continuously pumped and recycled through the HF membrane using a peristaltic pump to generate smaller vesicles with uniform size distribution. The experimental setup used for extrusion is the same as that employed for other membrane filtration applications such as protein concentration or diafiltration. The process can be scaled up simply by increasing the membrane surface area of the HFs.

Figure 2, shows a schematic of the underlying principle for the formation of vesicles using the HF membrane cartridge. The resultant vesicular dispersion was monodisperse and exhibited similar biophysical properties compared to vesicles produced by the small scale manual extrusion process. A nice feature of this extrusion process is that it operates at lower pressures (10–30 psi) compared to that of gas extrusion (1500 psi).⁹

MATERIALS AND METHODS

Materials

HF filter modules (part number: X12E-301-04S, rated pore size: 0.2 μm and surface area: 13 cm^2) were purchased from Spectrum Laboratories (Rancho Dominguez, CA). Polycarbonate membranes with a rated pore size of 200 nm were purchased from Avanti Polar Lipids (Alabaster, AL). Three biocompatible diblock copolymers composed of poly(butadiene-*b*-ethylene oxide) (PBD-*b*-PEO) were used in this study. All diblock copolymers were purchased from Polymer Source Inc. (Dorval QC, Canada). The physical properties of all three diblock copolymers are summarized in Table 1. For convenience, diblock copolymers will be referred to as PBD 1200, PBD 2500 and PBD 6500, where the numeric coefficient denotes the molecular weight of the PBD block in daltons.

Extraction and Purification of bovine Hb and human Hb from RBCs

Bovine and human Hb were purified from red blood cells via the method of Palmer et al.¹⁰

Polymersome/PEH Preparation

1.25 g of diblock copolymer was hydrated with 25 ml of 10 mM phosphate buffer (PB) at pH 7.3. Because of the low solubility of diblock copolymers in PB, the aqueous polymer mixture was sonicated (Branson Sonifier 450, VWR Scientific, West Chester, PA) for 8–10 hours at room temperature to yield the stock copolymer solution. The stock copolymer solution was then mixed with 25 ml of purified Hb (250–300 g/l), to yield a copolymer concentration of 12.5 mg/ml in the Hb-copolymer mixture. Empty polymersomes were prepared by diluting the stock copolymer solution in PB, instead of purified Hb solution, to yield a copolymer concentration of 12.5 mg/ml.

For the 1 ml volume manual extrusion method, the Hb-copolymer/copolymer mixture was extruded 20 times through 200 nm diameter polycarbonate membranes (Avanti Polar Lipids, Alabaster, AL). However, for the large-scale HF extrusion method (Figures 1 and 2), the Hb-copolymer/copolymer mixture was extruded through a 0.2 μm HF membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA).

For both extrusion methods, extruded PEH dispersions were dialyzed overnight using 300 kDa molecular weight cut off dialysis bags (Spectrum Laboratories Inc., Rancho Dominguez, CA) in PB at 4° C at a 1:1000 v/v (extruded PEH: PB) ratio to remove unencapsulated Hb from the vesicular dispersion.

Polymersome/PEH Size Distribution

An Eclipse[®] asymmetric flow field-flow fractionator (Wyatt Technology Corp., Santa Barbara, CA) coupled in series to an 18-angle Dawn Heleos[®] multi-angle static light scattering photometer (Wyatt Technology Corp., Santa Barbara, CA) was used to measure the size distribution of empty polymersomes and PEHs. The light scattering photometer is equipped with a 30 mW GaAs laser operating at a laser wavelength of 658 nm. Light scattering spectra were analyzed using the ASTRA software package (Wyatt Technology Corp., Santa Barbara, CA) to calculate the particle size distribution. The elution buffer consisted of 10 mM PB at pH 7.3.

Hb Encapsulation in PEH

To measure the amount of Hb that was encapsulated inside PEHs, dialyzed PEH dispersions were first lysed using 0.5% v/v Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PB. Lysed PEH samples were centrifuged at 14,000 rpm for 15 min and the supernatant collected for

analysis. The concentration of encapsulated Hb obtained after lysing the PEHs (mg/ml) was measured using the Bradford method via the Coomassie Plus protein assay kit (Pierce Biotechnology, Rockford, IL).

Oxygen-PEH/Hb Equilibria

Oxygen equilibrium binding curves of PEH dispersions and Hb were measured using a HemoxTM- Analyzer (TCS Scientific Corp, New Hope, PA, USA) at 37°C.¹⁰

Methemoglobin Level of PEH/Hb

The methemoglobin (metHb) level of Hb encapsulated inside PEH particles was assayed via UV-Vis spectroscopy using the cyanomethemoglobin method after lysis using 0.5% v/v Triton X-100.¹⁰

RESULTS AND DISCUSSION

Polymersome/PEH Size Distribution

Vesicle size plays an important role in regulating the clearance of vesicles *in vivo*.¹¹ Liposomes greater than 200 nm in diameter have short circulatory lifetimes as they are rapidly eliminated from the circulation by the reticular endothelial system (RES) through a complement mediated mechanism. On the other hand, very small liposomes (< 60 nm), circumvent RES uptake and accumulate in the liver.¹² Therefore liposomes with diameters ranging from 210–275 nm remain longest in the circulation.¹² It has been shown that above this size range (> 300 nm in diameter), the circulation half-life of vesicles drops off rapidly.¹³ As a result, this study focuses on the scale up of 200 nm diameter vesicles, which can be used in both industrial and clinical applications.

The absolute size distribution of empty polymersomes and PEHs extruded through the polycarbonate membrane and the HF membrane is shown in Figure 3. All polymersomes and PEHs exhibited monodisperse size distributions, which were Gaussian in shape. The average diameter of all polymersomes and PEHs in this study was less than 200 nm. The approximate distribution width ranged from 15–25 nm for extrusion through the polycarbonate membrane and 10–15 nm for extrusion through the HF membrane. Figure 3A and B compares the size of empty polymersomes extruded through the polycarbonate membrane and the HF membrane. As the molecular weight of the diblock copolymer increases, the size distribution of empty polymersomes extruded through the HF membrane became closer to the rated pore size of HF membrane. However, no such effect was seen in the case of polymersomes extruded through polycarbonate membranes. In the case of PEHs, extrusion through the HF membrane was more monodisperse and closer to the rated pore size of the HF membrane compared to PEHs extruded through the polycarbonate membrane (Figure 3C and D). Overall, the size distribution of PEHs extruded through the HF membrane was more similar to the rated membrane pore size compared to PEHs extruded through the polycarbonate membrane. This could be due to the ability of the HF membrane to withstand higher pressures (~ 30 psi) compared to the polycarbonate membrane (~ 10 psi).

The hydrophilic mass fraction (f), of the diblock copolymer serves as a good indicator of the type of colloid that will self-assemble from the aqueous copolymer solution.^{14, 15} A value of $f \approx 0.35 \pm 0.1$ indicates that polymersomes will most likely form in aqueous solution. Moreover, molecules with $f > 0.45$ are expected to form micelles, whereas molecules with $f < 0.25$ are expected to form inverted microstructures.^{14, 15} The aforementioned ranges of f and their associated microstructure are valid for diblock copolymers with molecular weights ranging from 2,000 to 20,000 Da.¹⁵ All of the diblock copolymers used in this study are within this molecular weight range. Previous experimental and molecular dynamics simulations studies

on polymersomes have established mathematical correlations between the vesicle membrane thickness (d) and the copolymer molecular weight.¹⁵

$$d \cong MW_h^b (b \cong 0.55) \quad (1)$$

Where d represent the thickness of the vesicle membrane, and MW_h represents the molecular weight of the hydrophobic block in the diblock copolymer. The calculated vesicle membrane thickness for polymersomes and PEHs in this study are extrapolated from Srinivas et al.'s¹⁵ data and shown in Table 1.

In the future, experiments that measure the membrane structure of these polymersomes will be performed. However, it should be noted that the molecular weight of PEO plays a big role in controlling vesicle porosity. Fusion of 2 to 3 polymersomes has been observed with low molecular weight PEO.¹⁶ However, large-scale aggregation occurs above a critical PEO molecular weight of 4000 Da.¹⁶ Due to this reason, the molecular weight of PEO blocks used in this study has been kept below 4000 Da. Above this molecular weight of PEO, aggregates which are highly porous with micrometer size pores are formed.¹⁶

Hb Encapsulation in PEH

Figure 4 shows the amount of Hb that was encapsulated in PEHs extruded in PB (pH 7.3). In both extrusion methods, PEHs encapsulating bovine Hb encapsulated less Hb versus PEHs encapsulating human Hb. The difference in the isoelectric points of the two Hbs is most likely the plausible reason for this observation. The pH of the PB buffer used in this study (pH 7.3) is closer to the isoelectric point of human Hb (~ 7.2).¹⁷ However, the isoelectric point of bHb is 6.8,¹⁸ which results in higher negative charge on bovine Hb molecules versus human Hb molecules. In addition, the PEO block of the polymersome bilayer is also negatively charged at pH 7.3.¹⁹ As a result, there is electrostatic repulsion between Hb and the negatively charged copolymer bilayer which results in lower Hb encapsulation. This effect is more pronounced in the case of bovine Hb due to the higher negative charge on the protein surface.

In this study, higher ionic strength buffer (phosphate buffered saline, pH 7.4) was used to shield the repulsion between the negatively charged lipid bilayer membrane and Hb. Higher Hb encapsulation was obtained in liposome encapsulated Hb dispersions (see supporting information).

The larger surface area (13 cm^2) of the HF membrane compared to the small scale polycarbonate membrane (1 cm^2) allows for more fluid resistance, which helps in mechanically breaking up MLVs to form smaller vesicles with Hb entrapped inside of them. Using a constant copolymer concentration of 12.5 mg/ml, the Hb/polymer mass ratio increased from 0.5 – 0.8 for manual extrusion through polycarbonate membranes to 0.9–1.3 for extrusion through HF membranes. The experimentally determined Hb/polymer mass ratios for the HF extrusion method are consistent with Hb/lipid mass ratios (0.9–1.6) found in the literature.^{19–23}

In general, the reduced Hb/polymer mass ratios observed in this study for PEHs may be attributed to the fact that all the aforementioned studies used liposomes to encapsulate Hb. Lipids have membrane thicknesses ranging from 3–4 nm. Therefore the high molecular weight diblock copolymers used in this study have thicker membranes,²⁴ which in turn provides less volume in the vesicle core that is available for Hb encapsulation as compared to that of liposomes. This was observed in the liposome formulation prepared using the large scale HF method which exhibited higher encapsulation of Hb (6 g/dl) (see supporting information).

However, Arifin and Palmer²⁵ reported Hb/polymer mass ratios ranging from 8–36. The authors used a 100 kDa MWCO dialysis membrane to remove unencapsulated Hb. However, while performing experiments with 100 kDa MWCO dialysis membranes, we observed that Hb does not pass through the 100 kDa membrane. This explains the artificially higher Hb/polymer mass ratios obtained by Arifin and Palmer.²⁵ The Hb concentration that is required for effective physiological oxygen delivery to tissues is still being debated.²⁶ Currently, we are successful removing unencapsulated Hb and concentrating PEH and liposome encapsulated Hb dispersions using HF diafiltration in our laboratory. In the future, PEH dispersions will be concentrated to make their oxygen carrying capacity comparable to that of blood.

Oxygen-PEH/Hb Equilibria

Equilibrium oxygen binding curves of PEH dispersions extruded through polycarbonate membranes and HF membranes are shown in Figure 5. The sigmoidal shape of the equilibrium oxygen binding curves exhibited by PEH dispersions qualitatively shows that the cooperativity of oxygen binding to Hb was not compromised by extrusion through both the polycarbonate membrane and the HF membrane. The oxygen affinity (P_{50}) (Figure 6) and cooperativity coefficient (n) (Figure 7) of PEH dispersions were regressed by fitting the equilibrium oxygen binding curves to the Hill equation.^{25, 27, 28} The P_{50} and n of all PEH dispersions did not vary much compared to a control of unencapsulated Hb.

The P_{50} for PEHs encapsulating human Hb was in the range of 12–14 mm Hg, and was comparable to that of human Hb ($P_{50} = 13.57$ mm Hg).¹⁰ The low P_{50} of these PEHs make them suitable for oxygenation of ischemic tissues and organs.^{29, 30} However, the P_{50} for PEHs encapsulating bovine Hb was in the range of 24–27 mm Hg, which was comparable to that of bovine Hb ($P_{50} = 26.03$ mm Hg).¹⁰ Since the P_{50} for PEHs encapsulating bovine Hb was similar to that of human RBCs¹⁰, these PEHs could be used in transfusion applications where there is no physiological stress exerted on the patient e.g. during routine surgery.

All PEH dispersions exhibited cooperativity coefficients ranging from 2.4 – 2.8, which suggests that the encapsulated Hb functions similarly to native Hb.¹⁰ In Table 3, the P_{50} and n of PEH dispersions prepared in this study are compared to published values for PEGylated liposome encapsulated Hbs (PEG-LEHs), PEGylated actin-containing liposome encapsulated Hbs (LEAcHbs) and PEHs.^{21, 25, 28, 31–39} The P_{50} for PEHs encapsulating bovine Hb was in the same range (23–26 mm Hg) compared to values measured for PEG-LEHs and LEAcHbs encapsulating bovine Hb (25–30 mm Hg). In contrast, the P_{50} for PEHs encapsulating human Hb was much lower (12–13 mm Hg) compared to values measured for PEG-LEHs encapsulating human Hb (30–50 mm Hg). The difference in the P_{50} values depends on the presence or absence of allosteric regulators for the two types of Hbs. The lower P_{50} values for PEHs encapsulating human Hb is due to the absence of 2, 3-diphosphoglycerate (DPG) in the purified Hb solution. DPG is a key allosteric regulator of oxygen binding to human Hb,⁴⁰ which is removed during the purification of Hb in this study.¹⁰ However, Cl^- ions, which is the allosteric regulator of oxygen binding to bHb,³⁷ is present in the buffer used for the oxygen-binding equilibria measurements. The higher P_{50} values for PEG-LEHs encapsulating human Hb reported by Tsuchida and coworkers^{21, 36, 37}, Kawaguchi et al³³, Nagomi et al³⁵ and Kaneda et al³⁸ is due to the presence of pyridoxal 5'-phosphate or inositol hexaphosphate in the PEG-LEH preparations. Pyridoxal 5'-phosphate or inositol hexaphosphate are phosphate analogues of DPG.⁴¹ DPG is chemically unstable and expensive which makes its utilization in commercial applications not practical.³⁷ For this reason, phosphate analogues of DPG are used. This explains why the P_{50} of PEHs encapsulating bovine Hb are similar to that of PEG-LEHs encapsulating bovine Hb, and the P_{50} of PEHs encapsulating human Hb is different from that of PEG-LEHs encapsulating human Hb.

Encapsulated MetHb Level

The metHb level (% of Hb in which the Fe atom is in the 3+ valence state instead of the 2+ valence state) of PEHs are shown in Figure 8. Most PEH dispersions had metHb levels as high as 6%. The metHb levels did not increase much with extrusion through the HF membranes as compared to the small scale extrusion through polycarbonate membranes.

In both extrusion methods discussed in this study, no reducing agents were used to suppress Hb oxidation. Nonetheless reducing agents like ascorbic acid could be easily incorporated into the aqueous core of the polymersome in order to further suppress Hb oxidation if desired. Ascorbic acid helps to maintain the stability of Hb against auto-oxidation.^{42, 43} Hence, vesicles that are more stable towards auto-oxidation can be prepared by adding ascorbic acid in physiological concentrations along with Hb.

Taken together, the results of this work demonstrate that the HF extrusion method is suitable for the large scale production of vesicles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Kim Y, Tewari M, Pajeroski JD, Cai S, Sen S, Williams J, Sirsi S, Lutz G, Discher DE. J Controlled Release 2009;134(2):132–140.
- Ahmed F, Pakunlu RI, Brannan A, Bates F, Minko T, Discher DE. J Controlled Release 2006;116(2):150–158.
- Christian N, Benencia F, Milone M, Li G, Frail P, Therien M, Coukos G, Hammer D. Molecular Imaging and Biology 2009;11(3):167–177. [PubMed: 19194761]
- Hearnden V, Lomas H, MacNeil S, Thornhill M, Murdoch C, Lewis A, Madsen J, Blanz A, Armes S, Battaglia G. Pharm Res 2009;26(7):1718–1728. [PubMed: 19387800]
- Mayer LD, Hope MJ, Cullis PR. Biochem Biophys Acta 1986;858(1):161–168. [PubMed: 3707960]
- Seltzer SE, Gregoriadis G, Dick R. Investigative Radiology 1988;23(2):131–138. [PubMed: 3343110]
- Szoka F, Olson F, Heath T, Vail W, Mayhew E, Papahadjopoulos D. Biochem Biophys Acta 1980;601:559–571. [PubMed: 6251878]
- Lee JC, Bermudez H, Discher BM, Sheehan MA, Won YY, Bates FS, Discher DE. Biotechnol Bioeng 2001;73(2):135–45. [PubMed: 11255161]
- Schneider T, Sachse A, Röling G, Brandl M. Int J Pharm 1995;117(1):1–12.
- Andre FP, Guoyong S, David RH. Biotechnol Bioeng 2009;25(1):189–199.
- Bradley AJ, Devine DV, Ansell SM, Janzen J, Brooks DE. Arch Biochem Biophys 1998;357(2):185–194. [PubMed: 9735159]
- Awasthi VD, Garcia D, Goins BA, Phillips WT. Int J Pharm 2003;253(1–2):121–32. [PubMed: 12593943]
- Maruyama K, Yuda T, Okamoto A, Kojima S, Suganaka A, Iwatsuru M. Biochem Biophys Acta 1992;1128(1):44–49. [PubMed: 1390877]
- Discher DE, Kamien RD. Nature 2004;430(6999):519–520. [PubMed: 15282592]
- Srinivas G, Discher DE, Klein ML. Nat Mater 2004;3(9):638–644. [PubMed: 15300242]
- Thomas PS, Christine F, Anthony JR, Giuseppe B. Macromol Rapid Commun 2008;29(23):1855–1860.
- Trong QN. Die Makromolekulare Chemie 1986;187(11):2567–2578.

18. De S, Girigoswami A. *J Colloid Interface Sci* 2006;296(1):324–331. [PubMed: 16225884]
19. Takeoka S, Ohgushi T, Terasa K, Ohmori T, Tsuchida E. *Langmuir* 1996;12(7):1755–1759.
20. Rabinovici R, Rudolph AS, Vernic J. *J Trauma* 1993;35:121–127. [PubMed: 8331701]
21. Sakai H, Hamada K, Takeoka S, Nishide H, Tsuchida E. *Biotechnol Prog* 1996;12(1):119–125. [PubMed: 8845102]
22. Takeoka S, Terasa K, Yokohama H, Sakai H, Nishide H, Tsuchida E. *J Macromol Sci Pure Appl Chem* 1994;A31:97–108.
23. Zheng S, Zheng Y, Beissinger R, Martin FJ. *Artif Cells, Blood Substitutes, Biotechnol* 1994;22(3):487–501.
24. Discher DE, Ahmed F. *Annu Rev Biomed Eng* 2006;8:323–341. [PubMed: 16834559]
25. Arifin DR, Palmer AF. *Biomacromolecules* 2005;6(4):2172–2181. [PubMed: 16004460]
26. Sakai H, Sato A, Takeoka S, Tsuchida E. *Langmuir* 2007;23(15):8121–8128. [PubMed: 17567054]
27. Arifin DR, Palmer AF. *Biotechnol Prog* 2003;19(6):1798–1811. [PubMed: 14656159]
28. Arifin DR, Palmer AF. *Artif Cells, Blood Substitutes, Biotechnol* 2005;33(2):137–162.
29. Sakai H, Cabrales P, Tsai AG, Tsuchida E, Intaglietta M. *Am J Physiol Heart Circ Physiol* 2005;288(6):H2897–2903. [PubMed: 15681705]
30. Cabrales P, Sakai H, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. *Am J Physiol Heart Circ Physiol* 2005;288(4):H1885–1892. [PubMed: 15563528]
31. Ahmed F, Discher DE. *J Controlled Release* 2004;96(1):37–53.
32. Awasthi VD, Garcia D, Klipper R, Phillips WT, Goins BA. *Int J Pharm* 2004;283(1–2):53–62. [PubMed: 15363501]
33. Kawaguchi AT, Fukumoto D, Haida M, Ogata Y, Yamano M, Tsukada H. *Stroke* 2007;38(5):1626–1632. [PubMed: 17395856]
34. Li S, Nickels J, Palmer AF. *Biomaterials* 2005;26(17):3759–3769. [PubMed: 15621266]
35. Nogami Y, Kinoshita M, Takase B, Ogata Y, Saitoh D, Kikuchi M, Ishihara M, Maehara T. *Annals of Surgery* 2008;248(2):310–319. [PubMed: 18650643]
36. Sakai H, Horinouchi H, Masada Y, Takeoka S, Ikeda E, Takaori M, Kobayashi K, Tsuchida E. *Biomaterials* 2004;25(18):4317–25. [PubMed: 15046922]
37. Sakai H, Masada Y, Takeoka S, Tsuchida E. *J Biochem* 2002;131(4):611–7. [PubMed: 11927000]
38. Shinichi K, Takanobu I, Hiroshi G, Tetsuhiro K, Katsutoshi I, Hiroaki K. *Artificial Organs* 33(2):146–152. [PubMed: 19178459]
39. Rameez S, Alost H, Palmer AF. *Bioconjug Chem* 2008;19(5):1025–32. [PubMed: 18442283]
40. Perutz MF, Imai K. *J Mol Biol* 1980;136(2):183–191. [PubMed: 7373649]
41. Lin W, Kazuya M, Satoru T, Tadashi S, Eishun T. *Polym Adv Technol* 1993;4(1):8–11.
42. Dunne J, Caron A, Menu P, Alayash AI, Buehler PW, Wilson TM, Dumitrescu R, Faivre B, Cooper CE. *J Biochem* 2006;399(Pt 3):513–524.
43. Buehler PW, Felice DA, Victoria H, Alayash AI. *J Pharmacol Exp Ther* 2007;323(1):49–60. [PubMed: 17622572]

Figure 1.
Schematic of the large scale HF extrusion system used in this study.

Figure 2. Schematic of the underlying principle for the formation of vesicles using the large scale HF extrusion system.

Figure 3.

Absolute size distribution of empty polymersomes and PEHs extruded through a 200 nm diameter polycarbonate membrane (SS) and 200 nm diameter HF membrane. (A) Represents empty polymersomes extruded on a SS. (B) Represents empty polymersomes extruded using a HF membrane. (C) Represents PEHs encapsulating bovine Hb. (D) Represents PEHs encapsulating human Hb.

Figure 4.
Comparison of Hb encapsulation in PEH dispersions extruded through a 200 nm diameter polycarbonate membrane (SS) and HF membrane. Experiments were done in duplicate.

Figure 5.

Comparison of O₂ equilibrium curves of PEH dispersions and Hbs extruded through a 200 nm diameter polycarbonate membrane (SS) and HF membrane: (A) represents human Hb and (B) represents bovine Hb. Here, Y is the fraction of Hb saturated with O₂ and pO_2 is the partial pressure of O₂.

Figure 6. Comparison of the oxygen affinity (P_{50}) of PEHs and Hbs extruded through a 200 nm diameter polycarbonate membrane (SS) and HF membrane. Experiments were done in duplicate.

Figure 7. Comparison of the cooperativity coefficient of PEHs and Hbs extruded through a 200 nm diameter polycarbonate membrane (SS) and HF membrane. Experiments were done in duplicate.

Figure 8. MethHb level of PEHs and Hbs extruded through a 200 nm diameter polycarbonate membrane (SS) and HF membrane. Experiments were done in duplicate.

Number-averaged molecular weight (M_n), polydispersity index (PDI), PEG length, hydrophilic mass fraction ($f_{hydrophilic}$), and hydrophobic membrane thickness (d) of the biocompatible and non-biodegradable poly(butadiene-*b*-ethylene oxide) copolymers used in this study.

Table 1

Diblock copolymer	M_n (Da)	M_w/M_n (PDI)	PEG length (Da)	$f_{hydrophilic}$	d (nm)
PBD(1200)- <i>b</i> -PEO(600)	1800	1.17	600	0.33	4–5
PBD(2500)- <i>b</i> -PEO(1300)	3800	1.04	1300	0.34	7–8
PBD(6500)- <i>b</i> -PEO(3900)	10400	1.10	3900	0.38	12–13

Table 2

Mean diameter, P_{50} , cooperativity coefficient (n), membrane thickness (d) and encapsulated methHb level of PEG-LEHs, LEAcHbs and PEHs

Type of Hb	Mean diameter (nm)	P_{50} (mm Hg)	n	d (nm)	MethHb level (%)
PEG-LEHs					
Human. ²¹	250	32	2.2	3–4	<5
Human. ³²	133	19.9	–	3–4	<10
Human. ³⁶	252	30	–	3–4	<5
Bovine. ³⁷	283	22	1.9	3–4	<5
Bovine. ²⁸	230	33.2	2.2	3–4	<5
Bovine. ²⁸	250	25.1	2.5	3–4	<5
Human. ³³	230	40	–	3–4	<10
Human. ³⁵	220	40–50	–	3–4	6.3
Human. ³⁸	230	40–50	–	3–4	<10
LEAcHb					
Bovine. ³⁴	272	28.5	2.1	3–4	<1
Bovine. ³⁴	281	27.5	2.3	3–4	7.4
PEHs					
Bovine. ²⁵	200	29.8	2.4	8–9	<1
Bovine. ²⁵	100	30	2.4	8–9	<1
Human. ³⁹	200	13	2.1	17	11
Bovine. ³⁹	200	20.81	1.8	7.5	<5
Bovine. ³⁹	200	24.2	1.9	17.5	<5
Human. [*]	170	12.46	2.75	4–5	<5
Human. [*]	170	13.01	2.64	4–5	<5
Bovine. [*]	170	23.82	2.41	4–5	<5
Bovine. [*]	170	25.51	2.74	4–5	<5

* This work