

A New Family of Water-Soluble Sulfo-Fluoro Polyphosphazenes and their Assembly within Hemocompatible Nanocoatings

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ABSTRACT: In this work, novel sulfo-fluoro polyphosphazenes (PPzs) were synthesized *via* macromolecular substitution of polydichlorophosphazene utilizing “non-covalent protection” methodology by converting acid functionalities into hydrophobic alkylammonium salts. Resulting PPzs showed excellent solubility in aqueous solutions over a broad pH range and contained ~25% sulfo-groups and 20% of either trifluoroethoxy- (FESP) or trifluoromethylphenoxy- (FPSP) side groups, as determined by NMR spectroscopy. Their polyelectrolyte behavior was evaluated by binding with an oppositely charged polyion, branched polyethyleneimine (PEI), which resulted in the formation of interpolymer complexes as shown by dynamic light scattering (DLS). Contrary to a sulfonated, nonfluorinated PPz homopolymer (SP), fluorinated macromolecules effectively bound human serum albumin (HSA) as revealed by DLS and asymmetric flow field flow

fractionation (AF4) studies. Moreover, FESP and FPSP both displayed low hemolytic activity as evaluated in solution using porcine red blood cells. Using the layer-by-layer (LbL) technique, FESP and FPSP were assembled into nanocoatings with PEI. Both fluorinated and non-fluorinated, sulfo PPzs showed linear growth with PEI because of strong ionic pairing between sulfo and amino groups. However, films of fluorinated PPzs displayed higher hydrophobicity, lower swelling, and improved stability in high ionic strength environment when compared to coatings formed by a sulfonated, non-fluorinated SP, or a carbon-chain polymer poly(styrene sulfonic acid). Hemocompatibility of FESP and FPSP nanofilms was demonstrated *in vitro* using whole rabbit blood hemolysis tests, which showed less than 1% hemolysis. Altogether, the present study introduces a new class of hemocompatible, sulfo-fluoropolymers that show promise for life science applications.

KEYWORDS: polyphosphazenes, fluorinated polymers, polyelectrolytes, sulfonated polymers, nanocoatings, hemocompatible coatings, layer-by-layer assembly

INTRODUCTION

Fluorinated polymers and materials possess a range of unmatched properties for life science applications.¹ Outstanding biological inertness, stability, superhydrophobicity, and excellent biocompatibility are among their unique characteristics.¹⁻³ Taken together, these characteristics provide an abiotic tool for developing supramolecular constructs as well as a means to enable selective interactions with biological targets. Fluoropolymers find applications in medicine as coatings for cardiac stents,⁴ such as XIENCE V™ - poly(vinylidene fluoride-co-hexafluoropropylene)⁵ and Cobra PzF - poly[di(trifluoroethoxyphosphazene)],⁶ as surface modifiers to make infection resistant biomaterials,⁷ as nanoparticles for ¹⁹F MRI tracking,^{8,9} as

well as for various uses in ophthalmology and reconstructive surgery.¹ However, the non-ionic nature and poor solubility of the currently used fluorinated polymers restricts their processing to standard melt-processing techniques² and prevents their self-assembly into advanced functional coatings. Incorporation of ionic functionalities in these hydrophobic polymers results in ionomers and enables important new features, which so far have been mostly used to develop industrial proton-conducting membranes. Along with highly commercially successful Nafion,^{10,11} water insoluble fluorinated ionomers include various sulfonated block copolymers of poly(arylene ether),¹²⁻¹⁶ and a few polymers with inorganic backbones, produced through multi-step post-synthetic derivatization procedures.^{17,18}

Polyphosphazenes (PPzs) – hybrid inorganic-organic synthetic polymers with a phosphorus-nitrogen backbone and organic pendant groups - draw significant attention as macromolecules for biomedical applications.¹⁹⁻²² They are characterized by a highly flexible backbone, a unique repeat unit that enables high charge density, and “dial-in” biodegradability.¹⁹ We have recently introduced water-soluble, fluorinated PPz polyelectrolytes, which can be employed in all-aqueous assembly of hydrophobic fluorinated coatings.²³⁻²⁵ These copolymers were based on a combination of trifluoroethoxy- and carboxylatophenoxy- pendant groups.²⁵ However, the narrow pH range of their water-solubility, along with weak acidity of carboxyl groups may impose limitations on the utility of these important macromolecular systems. Therefore, in this work, we explore water-soluble, fluorinated and sulfonated PPzs in solution and as building blocks for macromolecular self-assembly into nanostructured coatings.

Self-assembly can be achieved through the layer-by-layer (LbL) assembly technique, which utilizes oppositely charged ionic macromolecules to create nanoscopic coatings on surfaces of a variety of shapes and chemistries.²⁶ In particular, the LbL method has been widely explored to

functionalize the surface of biomedical devices to control cellular adhesion and modulate delivery of bioactive molecules.²⁷⁻²⁹ The integration of LbL assembly with the unique material properties of fluorinated systems can open new prospects for the development of biosurfaces with highly sophisticated sets of characteristics, including controlled nanostructure, hydrophobicity, and/or biofunctionality.

The present paper describes the synthesis and characterization of a new family of fluorinated PPzs bearing sulfonic acid functionalities. The introduction of ionic groups in the polymer structure was achieved through a macromolecular substitution reaction of polydichlorophosphazene with a non-covalently protected sulfonic acid containing nucleophile - a method that avoids the use of harsh sulfonation conditions and eliminates the risk of introducing undesirable polymer irregularities.³⁰ Copolymers bearing either trifluoroethoxy or trifluoromethylphenoxy side groups displayed water-solubility in a broad pH range and demonstrated low cytotoxicity and high affinity for binding human serum albumin (HSA) – an important parameter in predicting potential biocompatibility of fluorinated materials.^{4,28} PPzs assembled in films with branched polyethyleneimine (PEI) through the LbL technique and the resulting hydrophobic nanocoatings display excellent stability in solutions with high ionic strength and high hemocompatibility when tested with whole rabbit blood.

MATERIALS AND METHODS

Materials. Diglyme, anhydrous; 2,2,2-trifluoroethanol, 4-trifluoromethylphenol, tetrahydrofuran (THF), anhydrous, 2-propanol, dimethyl sulfoxide (DMSO), methanol, sodium phosphate monobasic dihydrate, potassium chloride, branched polyethylene imine (PEI) of two molecular weights (M_w ~750 kDa and ~25 kDa), sodium hydride, chlorobenzene, human serum

albumin (Sigma-Aldrich, St. Louis, MO), dimethyldipalmitylammonium bromide (TCI America, Portland, OR), hexachlorocyclotriphosphazene (Fushimi Pharmaceutical, Kagawa, Japan), porcine red blood cells (RBCs) (Innovative Technology Inc., Novi, MI), and rabbit blood (Rockland Immunochemicals, Inc., Limerick, PA 19468) were used as received. Sodium 4-hydroxybenzenesulfonate (Sigma-Aldrich, St. Louis, MO) was vacuum dried at 80 °C. Polydichlorophosphazene (PDCP) was synthesized as described previously.³¹ Poly[di(phenoxyphosphazenesulfonic acid)] (SP) was synthesized using noncovalent protection of 4-hydroxybenzenesulfonic acid with tetraalkylammonium salts.³⁰ PPz containing 20% (mol/mol) of trifluoroethoxy groups and 80% (mol/mol) of carboxylatophenoxy groups (FP20) was synthesized using a previously described method.²⁵ Silicon wafers (100 orientation, P/B doped) were purchased from Wafer Pro Inc. Ultrapure Milli-Q water (Millipore, resistivity of 18.2 MΩ*cm) was used in all experiments.

Synthesis of a PPz containing trifluoroethoxy, p-sulfophenoxy, and ethylphenoxy side groups (FESP). All reactions were carried out under an atmosphere of dry nitrogen using MBraun Labstar Pro glove-box workstation or common air-free laboratory techniques. Sodium hydride (1.55 g; 0.065 mol) in THF (35 mL) was added slowly to 2,2,2-trifluoroethanol (6.47 g; 0.065 mol), and the resulting solution was then added dropwise to a reaction flask containing PDCP (10 g; 0.086 mol) in diglyme (80 mL) and THF (100 mL). The temperature was raised to 50 °C and the reaction was allowed to continue for 2 h while stirring. Diglyme (100 mL) and chlorobenzene (50 mL) were then added to the reaction mixture. Sodium 4-ethylphenoxide in diglyme (35 mL), which was prepared by mixing sodium hydride (1.55 g; 0.065 mol) with 4-ethylphenol (7.9 g; 0.065 mol), was added slowly to the reaction mixture. The temperature was increased to 70 °C and reaction continued under stirring for another 2 h. THF was let to evaporate at 70 °C.

Hydroxybenzenesulfonic acid, dimethyldipalmitylammonium salt, DPSA (54.9 g; 0.043 mol), which was prepared by reacting dimethyldipalmitylammonium bromide (DMDPA) with sodium salt of 4-hydroxybenzenesulfonic acid,³⁰ was dissolved in chlorobenzene (200 mL) and added slowly to the reaction mixture. The temperature was increased to 100 °C and the reaction continued under stirring for another 2 h. To ensure complete substitution, sodium 4-ethylphenoxide (4.97 g; 0.0345 mol) in diglyme (20 mL) was added, the temperature was increased to 115 °C, and the reaction continued under stirring for another 20 h. The flask was then brought to 95 °C and a mixture of 12.7 M aqueous potassium hydroxide (30 mL) and ethanol (25 mL) was added. The reaction continued under stirring for 1 h and then allowed to cool to ambient temperature. FESP was isolated by precipitating in isopropanol (1300 mL), re-dissolving in a mixture of 12.7 M aqueous potassium hydroxide (10 mL) and DMSO (500 mL) at 50 °C (1 h), and precipitating in isopropanol. The purification was repeated twice. The polymer was isolated in an acidic form by first dissolving in DMSO containing hydrochloric acid (8M, 20 mL) and precipitating with deionized water (2 L). FEPS was then vacuum dried. Yield - 8.1 g (27%).

¹H NMR (D₂O): 7.3 (br, 2H, C₆H₄); 6.5 (br, 6H, C₆H₄); 3.8 (br, 2H, O-CH₂-CF₃); 1.8 (br, 2H, Ar-CH₂-); 0.7 (br, 3H, -CH₃) ppm. ¹³C NMR (D₂O): 154 (br, 1C, OC); 149.5 (br, 1C, OC); 139 (br, 2C, C₆H₆, CSO₃); 128.3 (br, 2C, C₆H₆); 127.2 (br, 2C, C₆H₆); 124.2 (br, 1C, CF₃); 120.8 (br, 4C, C₆H₆); 62.5 (br, 1C, CCF₃) ppm. ³¹P NMR (D₂O): -16.2; -9.8; -8.0, -4.4; -2.2; 0.2; 1.7 ppm. ¹⁹F NMR (D₂O): -75 ppm. NMR spectra with assignments are shown in Fig. S1. The composition of FESP was calculated using the peak areas at 7.3 (sulfophenoxy- groups), 6.5 (sulfophenoxy- and ethylphenoxy- groups), and 3.8 ppm (trifluoroethoxy- groups).

Synthesis of PPz containing trifluoromethylphenoxy, p-sulfophenoxy, and ethylphenoxy side groups (FPSP). Sodium hydride (2.45 g; 0.097 mol) in diglyme (70 mL) was added slowly

to 4-(trifluoromethyl)phenol (15.8 g; 0.097 mol) and the mixture was then added dropwise to PDCP (15 g; 0.129 mol) solution in diglyme (125 mL). The temperature was increased to 70° C and sodium 4-ethylphenoxide (14 g; 0.097 mol) in diglyme (50 mL) was added with stirring. The mixture was diluted with chlorobenzene (50 mL), the temperature was raised to 90° C and the reaction was allowed to continue for 2 h. DPSA (115.3 g; 0.090 mol) in chlorobenzene (400 mL) was added slowly to the reaction mixture, the temperature was raised to 115 °C, and the reaction continued for another 2 h. To ensure complete substitution, sodium 4-ethylphenoxide (7.49 g; 0.052 mol) in diglyme (20 mL) was added to the reaction mixture and allowed to react at 115 °C for 20 h. The reaction mixture was brought to 90 °C, 143 mL of 12.7 M aqueous potassium hydroxide solution was added slowly, and the mixture was stirred at 90° C for 1 h. The polymer was isolated and purified using the same procedure as for FESP. The yield was 15 g (30 %).

¹H NMR (D₂O): 7.5 (br, 4H, C₆H₄); 6.7 (br, 4H, C₆H₄); 6.5 (br, 4H, C₆H₄); 2 (br, 2H, Ar-CH₂-); 1 (br, 3H, -CH₃) ppm. ¹³C NMR (D₂O): δ [ppm] 151 (br, 2C, OC)); 148 (br, 1C, OC)); 139.7 (br, 2C, C₆H₄, CSO₃); 130.5 (br, 2C, C₆H₄); 128.3 (br, 2C, C₆H₄); 127.4 (br, 2C, - C₆H₄); 124.3 (br, 2C, CCF₃, CCF₃); 120.5 (br, 6C, C₆H₄) ppm. ³¹P NMR (D₂O): -18.2; -15.4; -10.1, -8.0; -4.7; -2.5; -0.4 ppm. ¹⁹F NMR (D₂O): -63 ppm. NMR spectra with assignments are shown in Fig. S2. Due to the complexity of the structure of FPSP (three different types of phenoxy groups), it was not possible to determine the composition of FPSP based only on ¹H NMR spectra. Thus, polymer composition was estimated using ¹H NMR peak areas at 7.5 (sulfophenoxy- and trifluoromethylphenoxy- groups), 6.7-6.5 (sulfophenoxy-, ethylphenoxy-, and trifluoromethylphenoxy- groups), and ¹³C NMR peaks at 151 ppm (p-sulfophenoxy- and trifluoromethylphenoxy- groups), 148 ppm (ethylphenoxy groups), and 139.7 (sulfophenoxy- and ethylphenoxy- groups). Since in ¹³C NMR spectrum the area under the signal is not always

proportional to the number of carbons, an alternative method involving ^{13}C NMR peaks at 130.5 ppm (trifluoromethylphenoxy- groups) and 128.3-127.4 (sulfophenoxy- and ethylphenoxy- groups) was applied and the results were in agreement with those obtained using the first approach.

Characterization. NMR spectra were recorded using 400 MHz AscendTM Bruker NMR instrument (Bruker Biospin Corp, Billerica, MA).

Gel permeation chromatography (GPC) analysis was carried out using Hitachi La Chrome Elite system (Hitachi, San Jose CA) as described previously.³² Samples were prepared at a concentration of 0.5 mg/mL in PBS, pH 7.4 and were filtered using Millex 0.22 μm filters (EMD Millipore, Billerica, MA) prior to the analysis. Poly(ethylene oxide) molecular weight standards (American Polymer Standards Corporation, Mentor, OH) were used.

Malvern Zetasizer Nano series (Malvern Instruments Ltd., Worcestershire, UK) was employed for DLS measurements. Polymers were dissolved in PBS, pH 7.4 and filtered using Millex 0.22 μm filters prior to the analysis.

Asymmetric Flow Field Flow Fractionation, AF4 was carried out as described previously using a Postnova AF2000 MT instrument (Postnova Analytics GmbH, Landsberg, Germany).³² The percent of HSA binding was calculated on the basis of unbound protein in the formulation using equation $(A_a - A_c)/A_a \times 100$, where A_a and A_c correspond to protein peak areas in the fractograms for HSA and HSA-Ppz formulations, respectively.

SEM was performed on samples sputter coated with ~2 nm of Pt/Pd alloy using a JEOL JSM-7500F.

Hemocompatibility of Polymers. The cytotoxicity of FESP and FPSP was evaluated using a modified hemolysis test as described previously.³³⁻³⁶ Red blood cells, RBCs (1% suspension in PBS, 50 μ L) were added to polymer solutions in PBS (0.25 mg/mL, 950 μ L) containing 45 mg/mL of HSA and incubated at 37° C (3 h). Cells were separated by centrifugation (14,000 rpm; 5 min), and the absorbance of the supernatant (541 nm) was recorded. For comparison purposes, complete lysis of RBCs was achieved by ultra sonication (Model 450, Branson Ultrasonic, Danbury CT)). Experiments were carried out in triplicate.

Film Deposition. Layer-by-layer (LbL) films were assembled on silicon wafers that were cut into 1x1 cm² pieces. Prior to deposition, silicon wafers were cleaned *via* overnight treatment with UV light and followed by exposure to sulfuric acid for 40 min as described elsewhere.³⁷ Samples were primed with a monolayer of PEI (M_w of ~750 kDa, pH 9, 0.2 mg/ml) by soaking substrates for 15 min in BPEI solution, washing with 0.01 M phosphate buffer (PB), and drying with nitrogen. LbL films were dip deposited with 5 min dipping cycles in 0.2 mg/mL aqueous solutions of either PPz or PEI of M_w ~25 kDa both at pH 7.5 with a wash (0.01 M PB, pH 7.5) in between each dipping cycle and dried with a gentle flow of nitrogen after the desired bilayer number was reached.

Contact Angle Measurements. Contact angles were collected with an Imagine Source camera on a KSV Instruments Ltd. Setup. Contact angle measurements were performed with 5 μ L droplets of Milli-Q water and analyzed by One Attension software. Contact angle values were measured with ~100-nm thick films using three separate locations on each film.

Spectroscopic Ellipsometry Measurements. Spectroscopic ellipsometry was used to characterize film thickness and optical constants in both dry and swollen states using a M-2000 (J.

A. Woollam Co., Inc., Lincoln, NE, USA). Four angles of incidence: 45°, 55°, 65°, and 75° were used to analyze dry films while *in situ* measurements were carried out at 75°. For *in situ* measurements, samples were swollen in 0.01 M PB at pH 7.4 for 5 min before measurement. More details of fitting models used can be found in a previously published manuscript.³⁸

Salt Stability. Coatings of ~100-nm thickness deposited on silicon wafers were exposed to 5 mL of increasing concentrations of potassium chloride in 0.01 M PB at pH 7.4 for 1 h. After exposure, samples were washed with 0.01 M PB to remove excess salt adhering to the coating and dried with nitrogen. The thickness of coatings was measured in two spots with ellipsometry, and the results were averaged.

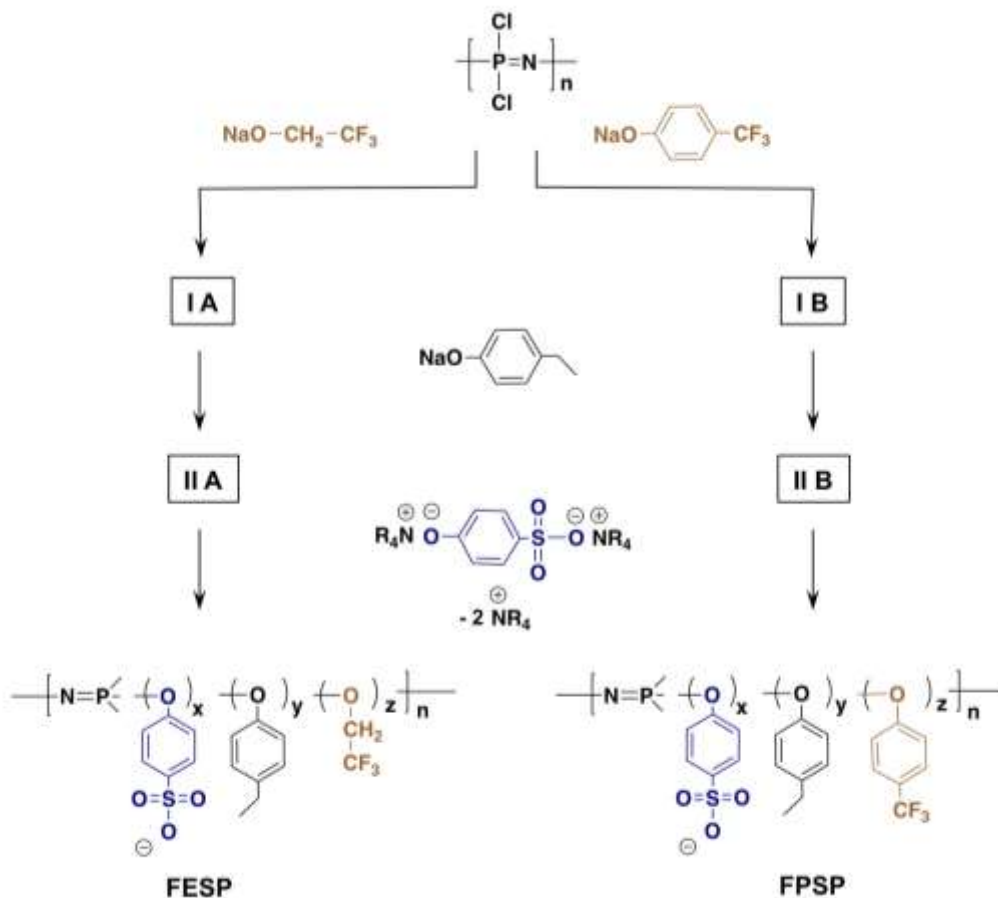
Hemocompatibility Studies of Polymer Coatings. Hemocompatibility tests of ~100-nm coatings were carried out using whole rabbit blood as described previously.^{38,39} Briefly, polymer coated silicon wafers (1x1 cm²; ~100-nm film thickness) were incubated (37 °C; 4 h) with diluted blood (1.0 mL, 2 mg/mL hemoglobin). Supernatant was isolated by centrifugation (14,000 rpm; 5 min) and the content of hemoglobin was measured by UV-VIS spectrophotometry. Analysis was conducted in duplicates. The difference between percent of free hemoglobin in the blood before and after incubation with coatings was reported as a percent of hemolysis.

RESULTS AND DISCUSSION

Synthesis of Trifluoroethoxy (FESP) and Trifluoromethylphenoxy (FPSP) Derivatives of Sulfonated PPzs and Their Physico-Chemical Characterization. PPzs were designed to have p-sulfophenoxy side groups and either trifluoroethoxy or trifluoromethylphenoxy groups as fluorine containing moieties (Scheme 1). Sulfonated groups were introduced using non-covalent protection of ionic functionalities using alkylammonium salts.³⁰ This method provides a mild,

single-step alternative to the more common technique that uses sulfuric acid or sulfur trioxide,⁴⁰⁻⁴² which may potentially result in undesirable modification or even degradation of pendant groups. Since many polymers that are used as biomaterials coatings have higher glass transition temperatures^{5,43,44} than those of typical fluorinated PPzs, such as poly[bis(trifluoroethoxyphosphazene)], a bulkier substituent – ethylphenoxy group, which is intended to decrease flexibility of the polymer chain, was also introduced.¹⁹ The reaction pathway included macromolecular substitution of polydichlorophosphazene (PDCP) with fluorinated nucleophile – either trifluoroethanol or trifluoromethylphenol, resulting in a partially substituted PPz IA and IB (Scheme 1), followed by the addition of ethylphenol (intermediates IIA and IIB). The sulfonic acid side groups were introduced by further modifying an incompletely substituted PPz with alkylammonium salt of p-hydroxybenzenesulfonic acid as described previously.³⁰

Scheme 1. The reaction pathway to the trifluoroethoxy (FESP) and trifluoromethylphenoxy (FPSP) derivatives of sulfonated PPzs and the schematic representation of their structures.



IA, IB, IIA, and IIB are intermediate products - partially substituted PPzs.

Table 1 shows data for molecular characteristics of PPzs. Based on ^1H NMR for FESP and ^1H NMR and ^{13}C NMR for FPSP, both polymers contained approximately 25 % (mol) of sulfonic acid groups and 20 % (mol) of fluorinated pendant groups. Despite the relatively high content of hydrophobic groups, both polymers demonstrated solubility in water in a broad range of pH between 1 and 14. GPC analysis showed single peak profiles for both polymers (Supporting information, Fig. S3), with weight average molar mass of approximately 70,000 g/mol and dispersity of less than 1.5.

Table 1. Physico-Chemical Characterization of Sulfonated PPzs.

Polyphosphazene		FESP	FPSP
Content of Pendant Groups* %, mol	p-Sulfophenoxy-	25	28
	Trifluoroethoxy- or Trifluoromethylphenoxy-	20	17
	Ethylphenoxy-	55	55
Molar Masses**	M _w , kg/mol	70.9	69.7
	M _n , kg/mol	44.3	43.4
	Đ	1.43	1.44
D _z ,*** nm		49	45

* Based on ¹H NMR for FESP and ¹H NMR and ¹³C NMR for FPSP; ** weight-average (M_w), number-average (M_n) molar masses, and dispersity (Đ) were calculated based on gel permeation chromatography (GPC) data; *** z-average hydrodynamic diameter (D_z) was determined by DLS in phosphate buffer (pH 7.4).

Ionic Interactions and Hemolysis Studies of FESP and FPSP in Aqueous Solutions.

Aqueous solution behavior of FESP and FPSP was investigated to better understand their potential as building block elements for LbL nanocoatings. DLS studies demonstrated that FESP and FPSP have hydrodynamic diameters of 49 and 45 nm, respectively, in phosphate buffer, pH 7.4 (Table 1). Upon addition of an oppositely charged polyion, PEI, to fluorinated polymers, a significant shift in their size distribution towards larger diameters occurred, indicating formation of polyelectrolyte complexes (Fig. 1A). The concentration threshold of PEI causing aggregation in the system was similar for both fluorinated polymers (Fig. 1B), and substantially lower than that for poly[di(phenoxyphosphazene sulfonic acid)] (SP), suggesting that higher hydrophobicity of fluorinated macromolecules strengthened binding between the polycation and fluorinated PPzs. Interestingly, the threshold of phase separation for FPSP was lowered in the presence of 4% (w/w)

sodium chloride (Fig. 1C). The addition of salt did not prevent the electrostatic interactions between the two oppositely charged polyelectrolytes, but appeared to decrease solubility of the system. Note that a similar decrease in solubility of PPzs in the presence of sodium chloride was already reported for a polymer containing hydroxybenzoic acid side groups, which are structurally similar to sulfophenoxy moieties.⁴⁵ This data suggests that hydrophobic interactions play an important role in stabilizing complexes in the solutions with high ionic strengths.

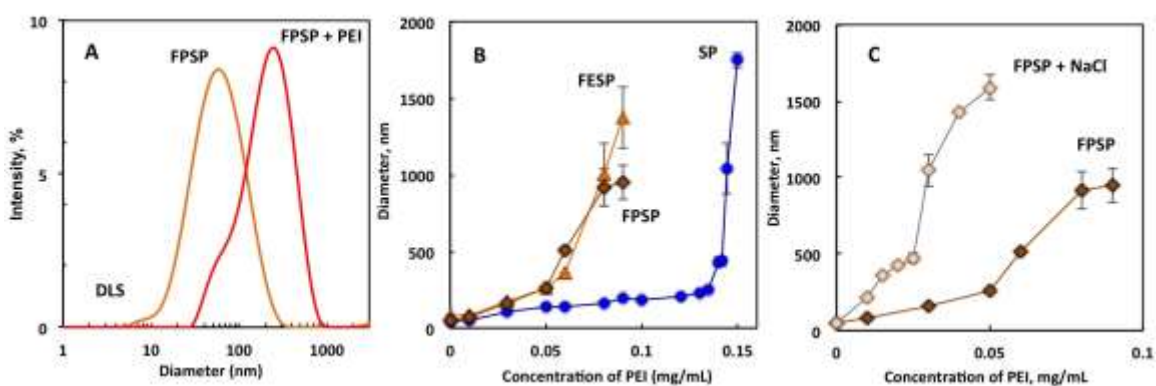


Fig. 1. (A) DLS profiles of FESP alone and with PEI (0.5 mg/mL FESP, 0.03 mg/mL PEI, PBS, pH 7.4); (B-C) z-average hydrodynamic diameters of (B) sulfonated PPzs versus concentration of PEI and (C) FPSP versus concentration of PEI in the presence and absence of sodium chloride (0.5 mg/mL polymers, phosphate buffer, 4% (w/w) sodium chloride, pH 7.4)

Biocompatibility of sulfonated PPzs was then investigated with respect to their interactions with the most abundant serum protein – human serum albumin (HSA) and porcine red blood cells. In general, biocompatibility of a synthetic material critically depends on the deposition of a protein layer on its surface.⁴⁶ It has been shown that adsorption of HSA, results in considerable decrease in platelet adhesion and activation.⁴⁶⁻⁴⁸ Overall, a high rate of adsorption of HSA on fluorine rich surfaces passivates the surface, contributes to thromboresistance, and prevents complement

activation, thus improving biocompatibility and hemocompatibility of the material.^{46,49-51} It has also been shown that superb, clinically proven hemocompatibility of poly[bis(trifluoroethoxy)phosphazene], PTFP,⁵²⁻⁵⁴ correlates with selective and irreversible adsorption of HSA from blood promoted by this material.²⁸ Therefore, both fluorinated PPzs were first evaluated for their ability to interact with HSA using AF4 and DLS methods. AF4 is an elution-based analytical technique, which separates macromolecules by their molecular dimensions and detects them by UV-VIS absorption. In contrast to other chromatographic techniques, AF4 can be applied to supramolecular assemblies with size as large as hundred micrometers.⁵⁵ The analysis is performed in a carrier liquid, while a cross-flow of the same liquid presses macromolecules against the semi-permeable membrane causing their size-based separation.⁵⁵ The fractograms of HSA or SP alone show clear, distinct peaks at elution times of ~7 min and ~14 min for HSA or SP, respectively (Fig. 2A). When their mixture is analyzed, peaks at both time points are observed, with only a 10% loss of area in the HSA peak, indicating that only 10% of protein was bound to the polymer. In contrast, fractograms for the mixture of HSA and FPSP (Fig. 2B), did not reveal any unbound protein, indicating complete binding of HSA to FPSP, while data for the mixture of HSA and FESP indicated approximately 70% protein binding (Fig. 2C). Higher rates of HSA binding observed for PPzs containing hydrophobic fluorinated groups correlate with previously established results on the importance of hydrophobic clefts of this protein for its interactions with various substrates.⁵⁶ Overall, the results suggest the importance of fluorinated side groups, as well as their structures, in modulating biologically relevant properties of PPzs.

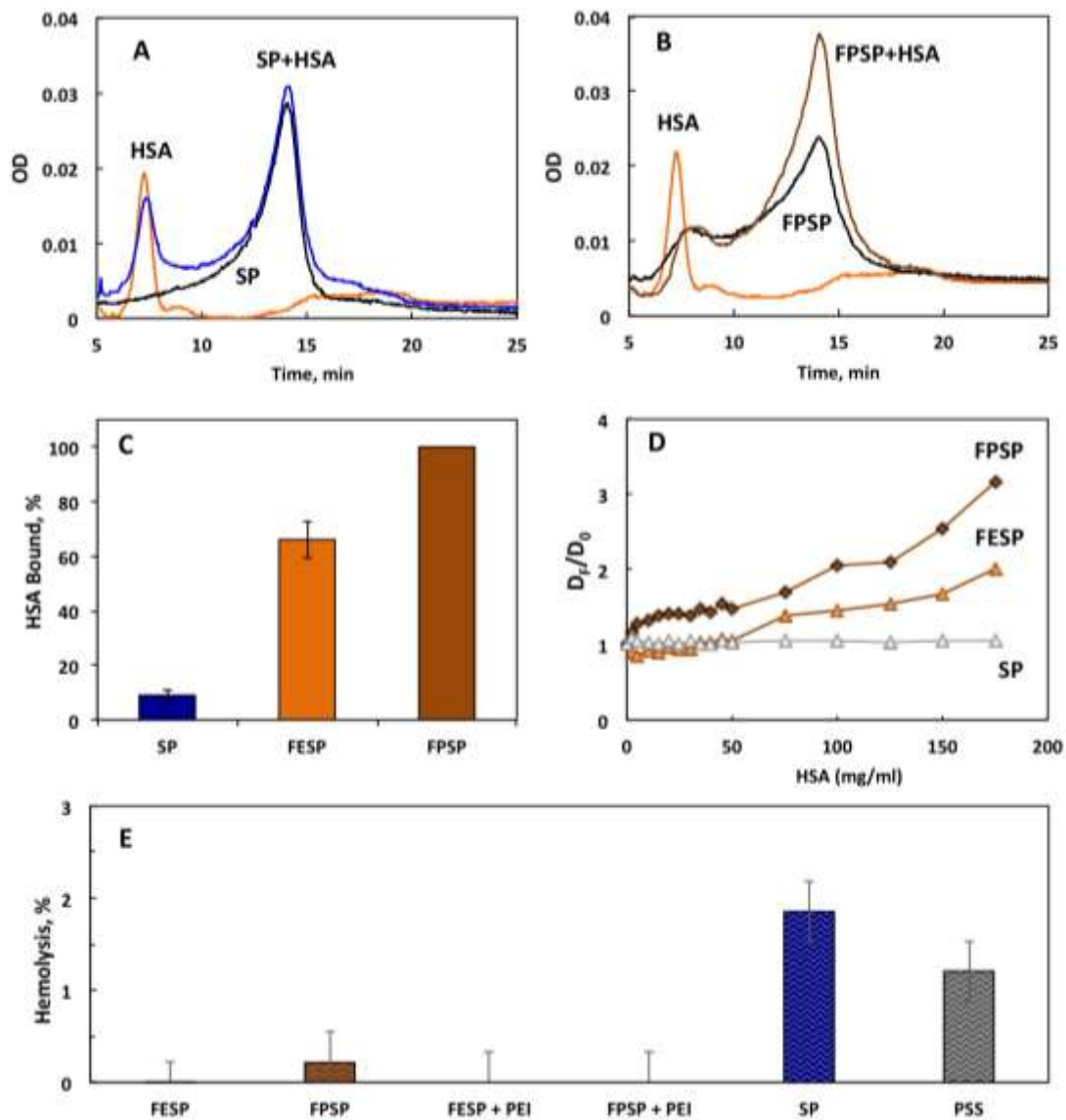


Fig. 2. AF4 fractograms of (A) HSA, SP, and their mixed formulation and (B) HSA, FPSP, and their mixed formulation (0.2 mg/mL polymers, 0.05 mg/mL HSA, pH 7.4); (C) percent of bound HSA for sulfonated PPzs as measured by AF4; (D) relative increase in hydrodynamic diameter of sulfonated PPzs (D_f/D_0) versus concentration of added HSA (0.5 mg/mL polymers, PBS, pH 7.4); (E) hemolysis of porcine red blood cells in the presence of various polymers and polymer complexes (concentration of polymers 0.25 mg/mL, PPz:PEI=12:1 (w/w); pH 7.4).

Self-assembly of fluorinated PPzs and HSA in aqueous solution was also independently confirmed by DLS (Fig. 2D). The aggregate size increased with HSA concentration, but complexes remained suspended in solution even at high protein concentrations. The larger sizes of FPSP+HSA complexes in comparison to FESP+HSA complexes detected by DLS is in good agreement with the higher efficiency of HSA binding by FPSP as determined by AF4 method. No changes in the hydrodynamic diameter of SP were observed upon the addition of HSA, confirming the AF4 results that there are no interactions between these components (Fig. 2D).

Cytotoxicity of PPzs was evaluated in a hemolysis assay using porcine red blood cells (RBCs). As seen in Fig. 2E, the percent of hemolysis for fluorinated polymers is significantly lower than the values for their hydrophilic sulfonated analogs – SP and poly(styrene sulfonic acid), PSS. This result agrees with an earlier report that within a certain concentration range PSS activates the serum complement system.⁵⁷ Additionally, while PEI of M_w 25 kDa has demonstrated significant toxicity in solution,^{58,59} Fig. 2E shows that, in complexes, its cytotoxicity was completely alleviated, likely due to shielding of its positive charge with associated negatively charged chains of fluorinated PPzs.

Nanoassembly and Physico-Chemical Properties of Polyphosphazene Coatings. Layer-by-layer (LbL) films were assembled in aqueous solutions using PEI as a polycation and silicon wafers as substrates. Fig. 3A compares deposition of LbL assemblies of PPzs that are fluorinated and non-fluorinated containing sulfonic acid groups (FESP and FPSP vs. SP) and a non-PPz polyelectrolyte, poly(styrene sulfonic acid) (PSS) (structures of SP and other polyanions used in coating experiments are shown in Supporting information, Fig. S4). The growth observed for all polymers containing sulfonic acid groups (FESP, FPSP, SP, and PSS) was linear with a constant increment of ~4.5-7.5 nm deposited per bilayer. Linear LbL film growth indicated strong ionic

pairing between sulfonated polymers and PEI, flattening of polymer chains upon adsorption, and suggested little to no influence of fluorination or backbone type on binding strength of polymer layers. The slight difference in the mass deposited for different system can likely be accounted for by the differences in the molecular mass of the polymer units. To understand the effect of chemistry of acidic units on polymer assembly, fluorinated PPzs with carboxylic acid (FP20) were deposited with PEI. In this case, significantly thicker films were formed, indicating weaker ionic pairing with positively charged PEI units, which is not surprising as the weaker ionic binding of carboxylic vs sulfo- groups is well known.^{60,61}

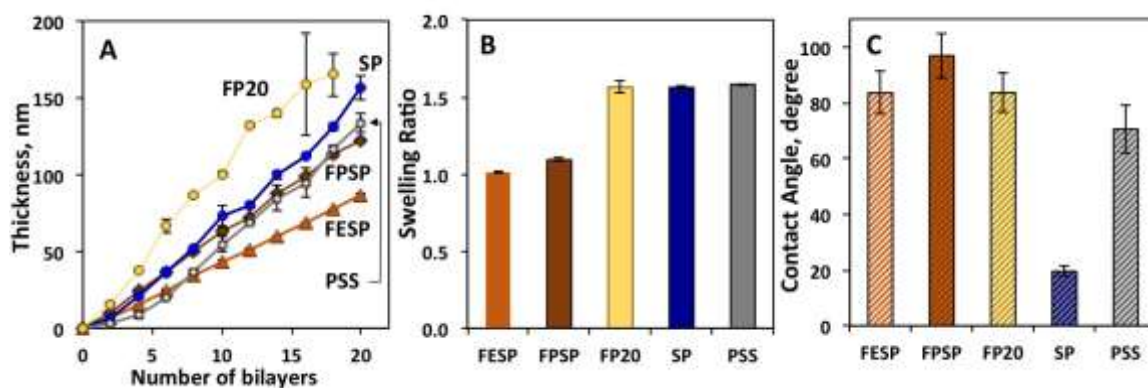


Fig. 3. (A) Thicknesses of LbL films of PPzs or carbon-chain PSS formed by deposition with PEI as measured by spectroscopic ellipsometry. (B) Swelling ratios of ~100-nm coatings of PPzs or PSS with PEI as measured in 0.01 M PB, pH 7.4. (C) Contact angles of ~100-nm coatings of PPzs or PSS with PEI as measured with Milli-Q water.

We then explored the effect of chemistry of ionic groups, degree of fluorination, and backbone chemical composition on the capability to swell and control hydrophobicity of the nanocoatings. Fig. 3B shows that films containing sulfo-fluoro PPzs (FESP and FPSP) did not swell (swelling degree ~1) when exposed to 0.01 M PB (pH 7.4), likely due to their strong binding and high hydrophobicity. In contrast, FP20, SP, and PSS coatings all displayed relatively high swelling

ratios of about 1.6. Significant uptake of water within films of PEI and FP20 probably results from its relatively weak ionic pairing with PEI. Unlike FP20, SP and PSS form strong ionic pairs with PEI, due to their sulfonated groups and high charge density.^{60,61} However, both SP and PSS lack fluorinated moieties which provide resistance to water uptake. The contact angles of water for fluorinated nanocoatings containing FESP, FPSP or FP20 were, as expected, much higher than for the coating assembled with non-fluorinated PPz, SP (Fig. 3C). At the same time, the contact angle on the PSS and PEI film was significantly higher than that for the SP and PEI film, indicating a possible contribution of the hydrophobic carbon-based polymer chain to film hydrophobicity.

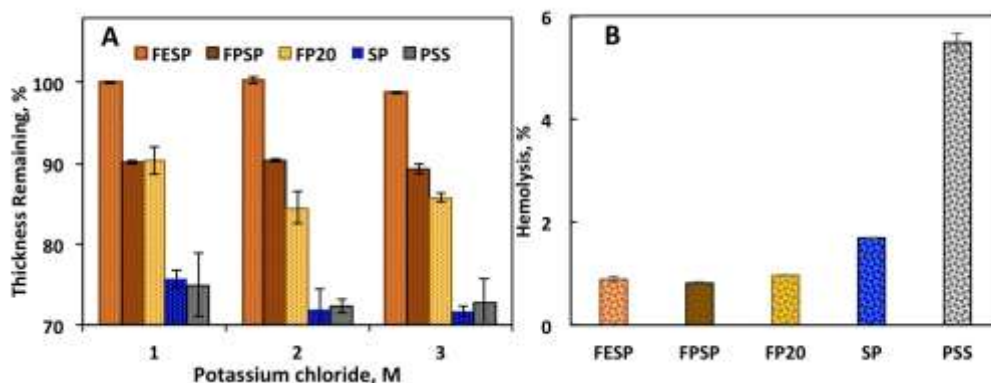


Fig. 4. (A) Remaining thickness of 100-nm films of PEI and PPzs or PSS after exposure to 1M, 2M, and 3M concentrations of KCl in 0.01 M PB at pH 7.4 for 1 h; (B) hemolysis percentage of dilute whole rabbit blood after 4 h exposure (37 °C) to ~100 nm films of PEI and PPzs or PSS.

Stability of LbL films in various environmental conditions (salt and pH) is important for their suitability for biomedical applications. All nanocoatings constructed using sulfonated polyelectrolytes were stable (showing <10% thickness loss) in solutions with pHs ranging from 7.5 down to 4 (SI Fig. S5). It is, however, well known that electrostatic pairing can be disrupted by small ions.⁶² Thus stability of the nanocoatings was investigated in concentrated solutions of potassium chloride. Sodium chloride was avoided due to previously reported unusual solubility

behavior of PPzs in this salt⁴⁵ and the observed decrease in solubility of FPSP as shown in Fig. 1C. Coatings formed by sulfo-fluoro PPzs showed minimal loss of their original thickness, with no mass loss for FESP/PEI and ~10% loss for FPSP/PEI films even in high ionic strength 3M KCl solutions (Fig. 4A) and little change in coating morphology (SI Fig. S6). Films of fluorinated carboxylic acid derivative FP20 with PEI were slightly less stable, losing about 15% of their original thickness in 3M KCl solutions. Interestingly, non-fluorinated films with sulfo groups in every polymer repeat unit but different backbone chemistry (SP and PSS, respectively) lost 20-25% of polymer mass (probably due to dissociation of the top layers of the film) even in 1M salt solutions. Additionally, for SP coatings there was a very distinct change in film morphology from very smooth to spinodal-like dewetting (SI Fig. S6).⁶³ These results demonstrate the importance of fluorinated groups in protecting nanocoatings against dissociation in concentrated salt solutions and also suggest that sulfonic acid functionalities may offer better stability in high ionic strength environment.

Hemocompatibility of Nanocoatings. Adequate biocompatibility is one of the main requirements for materials designed for life sciences applications.⁶⁴⁻⁶⁶ Therefore, PPz coatings were evaluated for hemolytic activity using dilute whole rabbit blood test (American Society for Testing and Materials).^{64,67} Fig. 4B presents the results of this test for coatings with matched thicknesses and PPzs as the top layer. Remarkably, both sulfonated PPzs with fluorinated side groups (FESP and FPSP) showed levels of hemolysis less than 1%. Low hemolytic activity was also observed for fluorinated PPz containing carboxylic acid groups - FP20 (1%). Sulfonated PPz containing only ionic groups (SP) display a slightly higher activity (~2%), whereas coatings formed using PSS were the most active inducing about 6% hemolysis. These results show the importance of fluorination for decreasing hemolysis, which correlates well with hemolysis studies

of water-soluble components observed earlier in this study (Fig. 2E), as well as with our previously published report on LbL with PPzs containing fluorinated and carboxylic groups.²³ Moreover, these PPzs demonstrate excellent biocompatibility for both LbL films and their macromolecular components in environments containing blood.

CONCLUSIONS

Two novel sulfo-fluoro polymers were synthesized using macromolecular substitution of polydichlorophosphazene with nucleophilic reagents, in which the sulfonic acid functionality was protected non-covalently by forming a salt with tertiary alkyl ammonium ions. Polyacids, which contained approximately 20% of either trifluoroethoxy or trifluoromethylphenoxy groups and about 25% of sulfonic acid functionalities, displayed excellent solubility in aqueous solutions over a broad pH range. Their polyelectrolyte behavior was confirmed by forming interpolymer complexes with oppositely charged PEI in aqueous solutions, which occurred even in the presence of 4% (w/w) of sodium chloride. Sulfo-fluoro PPzs and their water-soluble complexes with PEI showed low hemolytic activity and demonstrated the ability to bind HSA, which in the case of fluorinated macromolecules can serve as an indicator of their potential biocompatibility. Using the LbL technique, novel sulfo-fluoro PPzs were assembled with PEI into robust nanocoatings. The resulting nanofilms showed a range of properties, which make them promising candidates for further exploration as prospective biomaterials. These properties include higher hydrophobicity, lower swelling ratio, high stability in salt solutions, and superior hemocompatibility with whole rabbit blood as compared with control LbL films of non-fluorinated sulfonated PPzs or sulfonated carbon-chain polymers.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge. Polymer structures, NMR spectra, nanocoatings stability at various pH graph, GPC traces, SEM images of coating before and after exposure to 4 M KCl.

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Author Contributions

The manuscript was written through contributions from all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

PPz, polyphosphazene; PDCP, polydichlorophosphazene; DPSA, dimethyldipalmitylammonium salt of hydroxybenzenesulfonate; DMDPA, dimethyldipalmitylammonium bromide; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; FESP, polyphosphazene containing trifluoroethoxy, p-sulfophenoxy, and ethylphenoxy side groups; FPSP, polyphosphazene containing trifluoromethylphenoxy, p-sulfophenoxy, and ethylphenoxy side groups; SP, poly[di(sulfophenoxy)phosphazene]; FP20, polyphosphazene containing trifluoroethoxy and carboxylatophenoxy side groups; PEI, branched polyethyleneimine; PSS, poly(styrene sulfonic acid); PBS, phosphate buffered saline; HSA, human serum albumin; RBCs, red blood cells; DLS, dynamic light scattering; GPC, gel permeation chromatography; NMR, nuclear magnetic resonance; AF4, asymmetric flow field flow fractionation; M_w , weight-average molar mass; M_n , number-average molar mass; $\bar{D}$, dispersity; D_z , z-average hydrodynamic diameter; LbL, Layer-by-Layer; PB, phosphate buffer.

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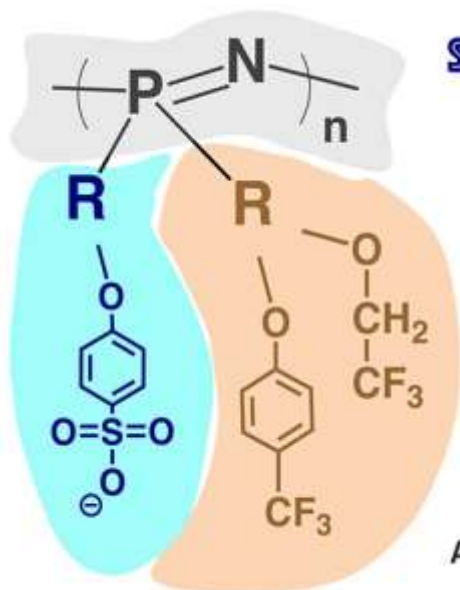
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SULFO-FLUORO POLYMERS

Hemocompatible Coatings

