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### Novel method for the characterization of charged polymer self-assemblies: Multidetector thermal field-flow fractionation in aqueous mobile phase

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### ABSTRACT

Charged block copolymer self-assemblies, such as charged micelles, have attracted much attention as versatile drug delivery systems due to their readily tunable characteristics such as size and surface charge. However, current column-based analytical techniques are not suitable to fractionate and comprehensively characterize charged micelles in terms of size, molar mass, chemical composition and morphology. Multidetector thermal field-flow fractionation (ThFFF) is shown to be a unique characterization platform that can be used to characterize charged micelles in terms of size, molar mass, chemical composition and morphology in aqueous mobile phases with various ionic strengths and pH. This is demonstrated by the characterization of poly(methacrylic acid)-*b*-poly(methyl methacrylate) self-assemblies in high pH buffers as well as the characterization of cationic poly(2-vinyl pyridine)-*b*-polystyrene and poly(4vinyl pyridine)-*b*-polystyrene self-assemblies in low pH buffers. Moreover, it is shown that ThFFF is capable of separating charged micelles according to the corona composition. These investigations prove convincingly that ThFFF is broadly applicable to the comprehensive characterization of amphiphilic self-assemblies even when aqueous mobile phases are used.

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### 1. Introduction

Block copolymer self-assemblies such as micelles have received widespread research interest due to their relative ease of preparation and versatility in terms of tunable properties such as size, structure and surface charge [1–3]. Of the various types of self-assemblies, charged micelles have attracted significant attention in a variety of biomedical applications such as gene therapy and drug delivery [4,5]. It was shown that the surface charge greatly influences micelle stability where charged micelles accelerated enzymatic degradation compared the uncharged/neutral micelles. Positively charged micelles caused protein absorption and aggregation while negative charged or neutral micelles are stable in the presence of proteins such as BSA [5]. Moreover, it was shown that the readily tunable surface charges of micelles are ideal for the design of drug delivery systems that exhibit both long blood circulation and efficient cell uptake [1].

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In order to make full use of their potential, micelles must be extensively characterized not only in terms of size, aggregation number, morphology, corona composition and molar mass but also in terms of their respective distributions as these can also significantly influence the function and behavior of their applications [6-8]. However, although current bulk characterization techniques such as electron microscopy (SEM and TEM), atomic force microscopy (AFM) as well as dynamic (DLS) and static light scattering (SLS) are frequently used to determine particle size and its distribution, these techniques cannot be used to determine molar mass, chemical composition or their respective distributions [9]. Furthermore, although average corona composition and number average molar mass can be probed by NMR and fluorescence spectroscopy, these techniques cannot determine their distributions [10–12]. More importantly, it was shown that the suitability of current bulk characterization techniques is limited for samples exhibiting complex or multiple size, molar mass or composition distributions [7]. Thus, a single technique which can characterize the various properties as well as their distributions would be highly desirable [9,10].

To address this need, techniques such as field-flow fractionation (FFF) and size exclusion chromatography (SEC) have been

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used to separate and characterize self-assemblies such as micelles. However, SEC has some limitations regarding the characterization of both micelles and charged analytes. For micelles, SEC showed trapping in the column as well as disassembly and adsorption of micelles on the column packing during analysis [3,13]. With regard to charged analytes, the presence of hydrogen bonding as well as hydrophobic and ionic interactions need to be addressed in order to avoid non size exclusion phenomena or absorption onto the stationary phase [14]. In addition, SEC is not sensitive towards microstructure-based separations [15,16]. Microstructure is an important parameter as it can dramatically influence properties such as charge density [17].

To overcome the limitations of column-based techniques, FFF subtechniques such as flow field-flow fractionation (FIFFF), asymmetric flow field-flow fractionation (AF4) (which is a special form of FIFFF) and thermal field-flow fractionation (ThFFF) were developed and shown to be suitable techniques for the characterization of various self-assemblies such as micelles, vesicles and polymersomes [7,18,19]. It was shown that FIFFF, AF4 and ThFFF can determine properties such as size, molar mass (including their distributions) and morphology of neutral self-assemblies and that AF4 could reveal size distributions not observed by traditional bulk techniques such as DLS [7,18–20]. However, although FIFFF, AF4 and ThFFF can all separate self-assemblies according to size, only ThFFF has been shown to be capable of separating micelles according to corona composition [21–25]. Moreover, it has been shown that ThFFF can charged polymers in aqueous mobile phases [26].

In ThFFF, a temperature gradient is applied perpendicular to a solvent/mobile phase flowing freely through an open, thin, ribbonlike channel [21]. When analytes are introduced into the channel, they migrate away from the hot wall towards the cold wall through the mass transport process of thermal diffusion. Thermal diffusion is characterized by the thermal diffusion coefficient, D<sub>T</sub>, which is influenced by, amongst other factors, chemical composition and microstructure [21,27-29]. As a result of the increasing concentration of analytes at the cold wall, the analytes diffuse back towards the center of the channel through the mass transport process of diffusion. This translational diffusion is characterized by the diffusion coefficient, D, which is influenced by the analyte's size in solution. This interplay between D<sub>T</sub> and D forms the basis for the compositional (through D<sub>T</sub>) or size-based (through D) separation capabilities of ThFFF [21,30,31]. For a more in-depth explanation of the ThFFF principles, the reader is referred to several articles addressing the subject [21,23,30,31]. However, although ThFFF has been shown to be a promising platform to characterize neutral self-assemblies in organic mobile phases, the characterization of charged self-assemblies (such as charged micelles) has not yet been addressed.

This study shows for the first time that ThFFF is a powerful tool to characterize charged self-assemblies in terms of size, molar mass, morphology and composition in aqueous mobile phases of varying pH and ionic strength. This study addresses the characterization of anionic poly(methacrylic acid)-b-poly(methyl methacrylate) (PMAA-PMMA) self-assemblies in a high pH buffer as well as the characterization of cationic poly(2-vinyl pyridine)*b*-polystyrene (P2VP-PS) and poly(4-vinyl pyridine)-*b*-polystyrene (P4VP-PS) self-assemblies in a low pH buffer. Moreover, it is demonstrated that ThFFF can separate P2VP-PS and P4VP-PS micelles based on corona composition in aqueous mobile phases. This study describes the application of ThFFF coupled online to multiangle laser light scattering (MALLS), differential refractive index (dRI) and dynamic light scattering (DLS) detectors to separate and determine important characteristics such as size, molar mass, morphology and aggregation number of charged self-assemblies in aqueous mobile phases.

#### 2. Materials and methods

#### 2.1. Materials

Poly(2-vinyl pyridine)-polystyrene  $(60.8 \text{ kg mol}^{-1}, \text{ Sam-})$ ple code: P117-S2VP) and poly(4-vinyl pyridine)-polystyrene (59.0 kg mol<sup>-1</sup>, Sample code: P9849-S4VP) block copolymer standards were purchased from Polymer Source Inc. (Montreal, Canada). The poly(2-vinyl pyridine)-polystyrene ( $P2VP_{47}-PS_{13,8}$ ) block copolymer had a PS block with a molar mass of 13.8 kg mol<sup>-1</sup> and a P2VP block with a molar mass of 47 kg mol<sup>-1</sup> while the poly(4-vinyl pyridine)-polystyrene (P4VP<sub>40.5</sub>-PS<sub>18.5</sub>) block copolymer had a PS block with a molar mass of 18.5 kg mol<sup>-1</sup> and a P4VP block with a molar mass of 40.5 kg mol<sup>-1</sup>. In addition, two poly(t-butyl methacrylate)-poly(methyl methacrylate) (PtBMA-PMMA) block copolymer standards with molar masses of 185.0 and 155.5 kg mol<sup>-1</sup> (Sample code: P4986-MMAtBuA and P330-MMAtBuMA, respectively) were also purchased from Polymer Source Inc. (Montreal, Canada). The 185.0 kg mol<sup>-1</sup> PtBMA-PMMA copolymer (PtBMA<sub>84.8</sub>-PMMA<sub>70.7</sub>) had a PtBMA block with a molar mass of  $84.8 \text{ kg mol}^{-1}$  and a PMMA block with a molar mass of  $70.7 \text{ kg mol}^{-1}$  while the  $155.5 \text{ kg mol}^{-1}$  PtBMA-PMMA copolymer (PtBMA<sub>120</sub>-PMMA<sub>65</sub>) had a PtBMA block with a molar mass of  $120 \text{ kg mol}^{-1}$  and a PMMA block with a molar mass of  $65 \text{ kg mol}^{-1}$ . Milli-Q water (Millipore, filtered through a 0.2 µm filter), borax (Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>·10H<sub>2</sub>O), sodium hydroxide (NaOH), sodium chloride (NaCl), acetic acid and sodium acetate were purchased from Sigma-Aldrich (South Africa).

### 2.2. Hydrolysis of poly(t-butyl methacrylate)-poly(methyl methacrylate) block copolymers

The two PtBMA-PMMA block copolymer standards were hydrolysed to the corresponding poly(methacrylic acid)-poly(methyl methacrylate) block copolymers in a dichloromethane/trifluoroacetic acid solution. The full experimental details are reported in the supplementary material. The resulting PMAA<sub>51.4</sub>-PMMA<sub>70.7</sub> and PMAA<sub>72.7</sub>-PMMA<sub>65</sub> block copolymers (subscripts indicate block molar masses) were characterized by FTIR and it was found that complete hydrolysis was achieved as evident by the disappearance of the characteristic absorbance of the t-butyl group at 1365 cm<sup>-1</sup>. The FTIR spectra of the two PMAA-PMMA block copolymers used in this study are shown in the Supplementary material as Fig. S1.

#### 2.3. Micelle preparation

The block copolymer micelles were prepared in buffer solutions of various pH and ionic strengths by the co-solvent method reported in the supplementary material. Preparation of the high pH (NaOH/Borax, pH = 10-11) and low pH (acetic acid/sodium acetate, pH = 2-3) buffers are also reported in the Supplementary material.

#### 2.4. Thermal field-flow fractionation

The thermal FFF system TF2000 (Postnova Analytics, Landsberg, Germany) was coupled online to MALLS (PN 3070, Postnova Analytics), dRI (PN 3150, Postnova Analytics) and DLS detectors (Zen 1600, Malvern Instruments, Worcestershire, UK). The TF2000 channel had a tip-to-tip length of 45.6 cm, breadth of 2 cm, thickness of 127  $\mu$ m and void volume of 1.14 mL. The temperature of the cold wall was 30 °C and a constant  $\Delta$ T of 40 degrees was used to achieve fractionation. The analytes were introduced into the channel via a Rheodyne manual injection valve with a 100  $\mu$ L capillary sample loop while the carrier flow of 0.3 mLmin<sup>-1</sup> was generated by an isocratic pump (PN 1130, Postnova Analytics). Diffusion (D) (and

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