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Polyphosphate Poly(amine) Nanoparticles: Self-Assembly, Thermodynamics and Stability Studies.

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ABSTRACT. The interaction of the polyamine poly(allylamine hydrochloride) (PAH) with Na_3PO_4 , $\text{Na}_4\text{P}_2\text{O}_7$, $\text{Na}_5\text{P}_3\text{O}_{10}$, $\text{Na}_6\text{P}_6\text{O}_{18}$ and $(\text{NaPO}_3)_{26}$ salts, and the formation of polyamine phosphate nanoparticles (PANs) are studied here. Dynamic light scattering, isothermal titration calorimetry (ITC), electrophoretical mobility measurements, atomic force microscopy and transmission electron microscopy are used to explore the formation, stability and pH sensitivity of PANs. An optimal concentration for PAN formation is found for each phosphate salt in terms of most stable size and lowest polydispersity index of the nanoparticles. The minimal concentration of phosphate ions for PAN formation decreases with the increasing number of phosphate groups per phosphate salt. ITC measurements show that all polyphosphates display a characteristic endothermic peak, which is not present when monophosphates are used for PAN formation. PH stability of PANs depends on the type of phosphate salt. PANs formed with small phosphates show a small window of stability with pH, from 8 to 9, while those formed with long phosphates are stable in more acidic pH environments. Our findings open multiple possibilities for fine tuning the pH sensitivity of PANs by varying phosphate salts for potential applications in drug delivery.

Introduction

Polyamines interact with phosphate ions through electrostatic and hydrogen bonding¹⁻³ leading to supramolecular structures,⁴ where the phosphate ions link different polyamine chains together,^{5,6} Colloidal particles^{7,8} and thin films^{9,10} can be easily fabricated through the complexation of polyamines and inorganic¹¹ phosphates. Polyamine phosphate interactions have also been extensively applied for the complexation of nucleic acids for gene therapy applications.¹² In a recent paper we have explored the use of polyamine phosphate nanoparticles (PANs) for siRNA delivery. Nanoparticles (NPs) were fabricated with the polyamine poly(allylamine hydrochloride)

(PAH) and phosphate buffer (PB) prepared with a 5:2 molar ratio of Na_2HPO_4 and KH_2PO_4 . PANs are stable in a narrow pH range from 6 to 8. Outside this pH range PANs disassemble.¹³ This narrow stability window can be explained by the sensitivity of the amines of PAH and the phosphate ions to pH. At acidic pH, phosphate ions lose their charge, and the interaction with the amines is lost. At basic pH, PANs disassemble because the amines of PAH deprotonate.¹⁴ This characteristic of the PANs could be applied for endosomal delivery as the NPs will disassemble at pH ~ 5.5 ,¹⁵ the endosomal pH, liberating encapsulated cargo.¹³ Most works related to PANs have used either PB or the Na_2HPO_4 salt for NP fabrication. However, there are several other phosphate molecules with more than one phosphate group, including lineal or cyclic chains with up to 26 phosphate units, that could be used for particle formation. Polyphosphates have been shown to have an important biological role acting as pro-coagulant in the human body for $(\text{NaPO}_3)_n$ where $n \geq 60$.^{16,11} In addition, polyphosphates also act as chelating agents for calcium¹⁷ thus being of utmost importance in cellular metabolism. Polyphosphates usually have pK values below those of Na_2HPO_4 and KH_2PO_4 and could retain their charges at pH where the monophosphate salts are not charged.¹⁸ The use of polyphosphates with polyamines for NP fabrication could increase the range of pH where PANs are stable. It is also likely that there would be more amine groups interacting per phosphate ion than for monophosphates, which also can impact on the minimum amount of phosphate ions needed for the formation of the PANs and on their overall stability. Motivated by the interest in designing PANs with novel properties, we have prepared complexes of PAH with five different phosphate salts: monophosphate salt (H_3PO_4), diphosphate salt ($\text{Na}_4\text{P}_2\text{O}_7$), linear triphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$), cyclic hexaphosphate ($\text{Na}_6\text{P}_6\text{O}_{18}$) and Graham's salt $(\text{NaPO}_3)_{26}$, a lineal and long polyphosphate chain. We studied the formation of NPs, and their pH and salt stability by dynamic light scattering (DLS), electrophoretical mobility measurements, atomic force

microscopy (AFM), and transmission electron microscopy (TEM). The thermodynamics of nanoparticle formation was studied by isothermal titration calorimetry (ITC). Our results bring insight into the interaction of phosphate and amines, and pave the way for development and use of novel polyamine phosphate NPs with tunable physical and chemical properties.

Materials and Methods

Chemicals: Poly(allylamine hydrochloride) salt (PAH) (MW: 1.5×10^4 g/mol), trisodium phosphate (Na_3PO_4), tetrasodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$), sodium tripolyphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$), sodium trimetaphosphate ($\text{Na}_6\text{P}_6\text{O}_{18}$), Graham's salt (NaPO_3)₂₆, hydrochloric acid (HCl), sodium hydroxide (NaOH) and sodium chloride (NaCl) were all purchased from Sigma-Aldrich with purity >99% and used without further purification. Carbon-coated copper grids and ammonium molybdate were purchased from Electron Microscopy Sciences (USA). Water used for all experiments was ultrapure MilliQ water.

Preparation of polyamine phosphate nanoparticles (PANs): PANs were prepared by mixing a stock solution of 100 mg/mL PAH (6.7×10^{-3} M) to a final concentration of 1 mg/mL (6.7×10^{-5} M) in phosphate solution. All samples were prepared at room temperature and left equilibrate for 10 minutes before being analyzed. The addition of PAH to the phosphate solution rapidly results in the formation of PANs. The pH responsiveness of PANs was studied by adding different amounts of 2 M HCl (or 2 M NaOH) to the NP solution and leaving the solution to equilibrate for 5 minutes before measurements. The molar ratio between phosphate salts and PAH¹⁹ is expressed by

$$R = \frac{[anion] \times |Z^-|}{[PAH] \times |Z^+|}$$

Where $|Z^-|$ indicates the number of negative charges per anion molecule, and $|Z^+|$ indicates the number of positive charges per PAH molecule, around 160 positive charges. Both $|Z^+|$ and $|Z^-|$ will vary with pH and $|Z^-|$ will vary depending on the phosphate ion. However, we assume that PAH will be fully protonated under the conditions used for PAN formation and that phosphate ions display their maximal charge for the preparation of PANs.

Dynamic light scattering (DLS) and electrophoretic mobility measurements: DLS measurements were performed in a Zetasizer Nano ZS from Malvern using 1 mL semi-micro PMMA disposable cuvettes at a fixed angle of 173° (backscattering mode) with laser position and attenuation automatically selected. Short time measurements of 15 runs with a duration of 5 seconds each were carried out for a total of 10 minutes, with 5 consecutive measurements for each sample. From the autocorrelation function of these measurements, the hydrodynamic diameter (D_h) was obtained.²⁰ The concentration of PAH and phosphate salts used were diluted enough to ensure that particle-particle interactions among PANs do not affect DLS measurements, which was verified by measuring the hydrodynamic diameter after diluting 10 times. and obtaining the same D_h values, data not shown.²¹ Cumulant analysis was used to extract the z average diffusion coefficient and PDI from the autocorrelation function.²² The stability of PANs was evaluated by measuring with DLS different NP preparations for up to 24 hours. The pH stability range of the formed PANs was obtained by adding a few μL of 2 M HCl for $\text{pH} < 7$, or 2 M NaOH for $\text{pH} > 7$. A volume of 10 mL PANs solution was used for each phosphate salt studied thus dilution from the small amount of acid or basic solution added can be considered negligible. All experiments were performed at 25°C . Electrophoretic mobility of PANs was measured in the presence of 10 mM NaCl. Measurements were performed with the Zetasizer Nano, in auto-mode at 25°C , with 5

consecutive measurements for each sample. Voltage and attenuation were automatically selected by the software.

Atomic force microscopy (AFM): For morphological characterization of NPs, AFM images were obtained with a Veeco Multimode atomic force microscope attached to a Nanoscope V controller. AFM tips were purchased from Bruker, TESP-V2 model. Samples were drop casted over a freshly cleaved mica surface, washed twice with MilliQ water and left to evaporate for at least 24 hours under vacuum at room temperature before measuring in dry state. To observe PAN morphology after an acid shock, PAN solutions were prepared with 1 mM $\text{Na}_5\text{P}_3\text{O}_{10}$ and 0.01 mM $(\text{NaPO}_3)_{26}$, and PAH at 1 mg/mL. Two identical samples for each phosphate salt were prepared by drop casting. For each pair of samples, one was prepared with 10 μL MilliQ water and the other with 10 μL HCl pH = 3, and then with 10 μL MilliQ water.

Transmission electron microscopy (TEM): Samples were prepared on normal and ultra-thin plasma coated carbon films. 2 μL of stable PAN solutions prepared with 4 mM, 1.5 mM, 2 mM, 0.5 mM and 0.1 mM of Na_3PO_4 , $\text{Na}_4\text{P}_2\text{O}_7$, $\text{Na}_5\text{P}_3\text{O}_{10}$, $\text{Na}_6\text{P}_6\text{O}_{18}$, and $(\text{NaPO}_3)_{26}$ in the conditions described above were transferred to the plasma coated grids. Samples were allowed to deposit to the grid for 1 min, then were washed with MilliQ water, and finally incubated with 3 μL of 20 mg/mL ammonium molybdate for 1 min and washed twice with Nanopure MilliQ water. Transmission electron microscopy was performed with a JEOL JEM 1010 microscope operating at an acceleration voltage of 100 kV.

Isothermal titration calorimetry (ITC): Calorimetric experiments were performed with a MicroCalorimeter VP-ITC. Experiments were carried out at 24 °C under continuous stirring of the sample cell at 307 rpm. 1.456 mL of 1 mg/mL PAH solution was titrated with a stock solution of polyphosphates. The concentration of phosphate salts was selected to cover the concentration range at which PANs are formed. Prior to the experiment the pH of the solution was fixed at 7 by adding a few μL of 2 M NaOH, except for Na_3PO_4 for which the pH was fixed at 8.1, as $\text{pH} = 7$ is not within the stability window of the PANs formed with this salt. The addition of phosphate solution was stopped before reaching the concentration at which PANs precipitate. The titration consisted of 31 injections, the first one of 2 μL (discarded), and the following ones of 4 μL at intervals of 600 seconds each. Data were corrected by taking into account the dilution heat of each polyphosphate solution measured with polyamine free solution the same day. Raw data are reported as a function of the polyphosphate concentration as well as the R charge ratio.

Results and Discussion

Nanoparticle preparation and characterization

PAN solutions were prepared by mixing PAH and phosphate salt solutions as described in materials and methods.^{23,24} PANs were prepared with the following phosphate salts: monophosphate salt (Na_3PO_4), diphosphate salt ($\text{Na}_4\text{P}_2\text{O}_7$), linear triphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$), cyclic hexaphosphate ($\text{Na}_6\text{P}_6\text{O}_{18}$) and Graham's salt, (NaPO_3)₂₆ (Table 1). The phosphate salts chosen allow us to study the role of the number of phosphate groups and charges on PAN formation, starting with a phosphate salt with one phosphate group (Na_3PO_4) and reaching the large Graham's salt with 26 phosphate groups, (NaPO_3)₂₆. The concentration of phosphate salts necessary for PAN formation was determined by DLS, both by measuring the increase in scattered intensity expressed

as derived counts rate (kcounts/s) and analyzing the corresponding autocorrelation function ($G^1(\tau)$). DLS provides the critical ratio of phosphate and amines below which PAN formation is not detectable due to a low scattering intensity, which is comparable to the scattered intensity of free PAH in bulk. The derived count rate is used to compare the scattering from different samples. The derived count rate is defined as the measured kcount rate corrected by an attenuation factor. The attenuation factor is automatically adjusted by the software of the Zetasizer instrument to ensure that the scattering intensity remains within the optimal range of the Avalanche Photo Diode (APD) detector in the Zetasizer Nano instrument.²⁵

A general trend is reported of the derived count rate of PAN formation for a constant concentration of 1mg/mL PAH (6.67×10^{-3} mM, with around 160 monomers not necessarily all charged), in the presence of increasing amounts of $\text{Na}_5\text{P}_3\text{O}_{10}$, with 5 negative charges per molecule (Figure 1a). The light scattered by the solution increases from 60 to 70000 kcounts/s as the phosphate concentration increases from 0.1 mM to 50 mM $\text{Na}_5\text{P}_3\text{O}_{10}$. PAN formation is observed at 0.3 mM $\text{Na}_5\text{P}_3\text{O}_{10}$ as revealed by the change in the autocorrelation function (Figure 1b), where the curve corresponding to 0.2 mM (black curve) shows a similar autocorrelation as to free PAH while the curve corresponding to 0.3 mM (red) has a well defined autocorrelation curve from which particle D_h can be confidently extracted. Nevertheless, the low count rate (around 500 kcounts/s) and the high polydispersity index ($\text{PDI} > 0.3$) indicate that the NPs are not homogeneously formed. At 50 mM $\text{Na}_5\text{P}_3\text{O}_{10}$ the polyamine phosphate complexes start to precipitate resulting in a solid phase that can be observed by eye and in the respective autocorrelation function obtained by DLS in Figure 1b (blue), presenting long correlation times with multiple characteristic decay times, most probably belonging to the precipitating particles. Thus, we define the optimum concentration of phosphate salt for NP formation as the concentration with the lowest PDI and highest count rate.

For PANs formed from $\text{Na}_5\text{P}_3\text{O}_{10}$ the optimum concentration is observed at 2 mM $\text{Na}_5\text{P}_3\text{O}_{10}$. At this concentration, the PAN diffusion coefficient is $3.93 \pm 0.02 \mu^2 \cdot \text{s}^{-1}$ corresponding to a D_h of 125 ± 1 nm (Figure 1c), with $\text{PDI} = 0.08 \pm 0.03$. TEM images corroborate these results as spherical NPs can be observed (Figure 1d). The average size of PANs measured by TEM is 86 ± 36 nm.

Table 1. Molecular structure, formula, minimum and optimal concentrations, D_h , PDI and electrophoretic mobility (EM) of PANs.*

Molecular structure	Molecular formula	Optimal concentration (mM)	D_h (nm)	PDI	EM ($\mu\text{m} \cdot \text{cm} / \text{V} \cdot \text{s}$)
Na_3PO_4	4	123 ± 2	0.06 ± 0.02	$2,3 \pm 0,4$
$\text{Na}_4\text{P}_2\text{O}_7$	2	128 ± 2	0.03 ± 0.02	$-0,75 \pm 0,05$
$\text{Na}_5\text{P}_3\text{O}_{10}$	2	125 ± 1	0.08 ± 0.03	$-0,41 \pm 0,06$
$\text{Na}_6\text{P}_6\text{O}_{18}$	1	113 ± 6	0.22 ± 0.01	$6,2 \pm 0,3$
$(\text{NPO}_3)_{26}$	0.2	136 ± 5	0.24 ± 0.03	$5,9 \pm 0,4$

*PANs prepared with 1 mg/mL PAH and phosphate salts at reported concentrations. Measurements were performed at 25°C.

Figure 1. DLS and TEM characterization of PAN formation with $\text{Na}_5\text{P}_3\text{O}_{10}$. (a) Derived count rate (kcounts) of PAH vs different concentrations of $\text{Na}_5\text{P}_3\text{O}_{10}$. Red dashed line indicates the minimum phosphate salt concentration for PAN formation. Shaded zone indicates PANs precipitation (b) Autocorrelation functions of solutions made from PAH with (●) 0.2 mM (●) 0.3 mM and (●) 50 mM $\text{Na}_5\text{P}_3\text{O}_{10}$. (c) Hydrodynamic diameter (nm) of PANs made from PAH and 2 mM $\text{Na}_5\text{P}_3\text{O}_{10}$. (d) TEM image of PANs prepared with 2 mM $\text{Na}_5\text{P}_3\text{O}_{10}$. Scale bar corresponds to 100 nm. All solutions were prepared with 1 mg/mL PAH.

DLS curves for the formation of PANs with the other polyphosphates studied had features similar to those for $\text{Na}_5\text{P}_3\text{O}_{10}$, showing an increase in derived count rate with increasing phosphate

concentration (Supporting information, Figures S1a-c). For all polyphosphates, PANs start to precipitate in a solid phase, visible to the eye, once a critical concentration of polyphosphates is reached. Maechling et al.²⁶ have already reported precipitation of PAH induced by $\text{Na}_6\text{P}_6\text{O}_{18}$. In our previous work on PAN formation with phosphate buffer no precipitates were observed.²⁷ The absence of precipitates in case of phosphate buffer could probably be because the concentration of monophosphate salts needed to induce precipitation is very high and was not reached in our experiments. Also, no precipitation is observed in case of Na_3PO_4 (Figure S1). Moreover, DLS curves of NP formation with Na_3PO_4 exhibit a unique dependence on phosphate concentration among all studied salts. NPs start to grow very slowly until 3 mM Na_3PO_4 where there is an abrupt increase in derived count rate (Figure S1). Increasing Na_3PO_4 concentration above 5 mM leads to a rapid decrease in derived count rate. The decrease in scattered light and autocorrelation function similar to free PAH indicates that PANs are disassembled and are no longer present in solution. This peculiar behavior could be explained by the strong basicity of solutions of this salt. Phosphate salts can act as weak bases subtracting protons from the medium,²⁸ thus an increase in Na_3PO_4 concentration may lead to a deprotonation of the amine groups in the polymer. Probably, at values over the critical concentration for NP formation, the solution is basic enough ($\text{pH} = 9.17$) due to Na_3PO_4 high pK_a ²⁹ to deprotonate the PAH hindering particle formation.

For comparison, all DLS curves of NP formation as a function of phosphate salts were plotted together as derived count rate vs the concentration of phosphate salts (Figure 2). Curves are shown at phosphate concentrations below the critical concentration for precipitation. In all cases, an increase in derived count rate is found by increasing polyphosphate salt concentration. Interestingly, when increasing the concentration of $\text{Na}_4\text{P}_2\text{O}_7$ and $\text{Na}_5\text{P}_3\text{O}_{10}$ beyond 1 mM, the derived count rate presents a small plateau before precipitation occurs indicating that the addition

of more of these polyphosphates is not inducing the formation of NPs. The analysis of the autocorrelation functions shows a clear difference between phosphate salts concentration at which only free PAH polymer is present in solution and concentrations at which PANs are formed. Red dashed lines in Figures S1a-d indicate the concentration of polyphosphate at which PANs' formation takes place as observed by the change in the autocorrelation function. It can be noticed that the larger the number of charges in the polyphosphates, the smaller the concentration of the polyphosphate at which NPs are formed. Indeed, polyphosphates produce NPs at concentrations 5 times smaller than the monophosphate. PAN formation with polyphosphates may be the result of the association of multiple amines from different PAH chains linked with a single polyphosphate ion. On the other hand for monophosphates a larger number of phosphate ions is needed to link the polyamines.

Figure 2. Comparison of derived count rate of solutions of 1 mg/mL PAH with increasing concentrations of (●) Na₃PO₄; (●) Na₄P₂O₇; (●) Na₅P₃O₁₀; (●) Na₆P₆O₁₈; and (●) (NaPO₃)₂₆. All

points are below the PAN precipitation concentration for the corresponding phosphate. Measurements were performed at 25°C. Error bars in some points are smaller than the symbol. Connecting lines serve as guides.

The presence of a well defined autocorrelation function obtained at low phosphate salt concentrations allows determination of a minimum concentration of polyphosphate at which NPs are formed, however with high polydispersity ($PDI > 0.3$, data not shown). The minimum concentration of phosphate that produces PANs decreases with the number of phosphate groups in the phosphate salt from 3 mM for Na_3PO_4 to 0.02 mM for $(NPO_3)_{26}$ (Table 1).

We define the optimal concentration as the concentration of phosphate that induces the formation of PANs with $PDI < 0.2$ at the highest derived count rate value. Optimal phosphate concentrations for PANs formation range from 4 mM for Na_3PO_4 to 0.2 mM for $Na(NPO_3)_{26}$ with 1 mg/mL PAH (Table 1). PANs formed with these phosphate concentrations displayed a $D_h = 125 \pm 8$ nm independent of the phosphate used (Table 1). This result supports the hypothesis that polyphosphate molecules with more binding sites require a lower concentration to form similar sized PANs. TEM images of PANs reveal spherical NPs and confirm the size obtained by DLS (Figures S2a-d). Stability of NPs in solution was measured by DLS 24 hours after preparation to evaluate the aggregation or degradation of NPs. The size and PDI of PANs were constant during the evaluated period for all polyphosphates studied, and no precipitation was observed (Figures S3a-c).

In parallel, electrophoretic mobility measurements were performed to evaluate the effect of the different phosphate salts on the surface charge of the resulting PANs. The Smoluchowski and Guy Chapman equation used to describe the electrophoretic mobility and to obtain a value for ζ -potential are valid for smooth and hard surfaces.³⁰ For soft or hairy particles it is not known to

what extent the charged groups and counterions confined in the particles contribute to the ζ -potential. It may well be that the PANs, especially those fabricated upon the larger polyphosphate ions are structures open enough to allow the electroosmotic field to penetrate inside the particles, giving the Schmolkoski equation limited validity.³¹ However, electrophoretic mobility values are independent of the model applied for obtaining the ζ -potential and show that the PANs of polyphosphates are highly positively charged with charges coming from amines not compensated by the phosphate ions.

The electrophoretic mobility of PANs in the optimum concentrations for PAN formation with an ionic strength of 10 mM NaCl was determined. For PANs formed with polyphosphates with a high number of phosphate units an extremely high positive electrophoretic mobility was found, with values of $+5.9 \pm 0.4 \text{ } \mu\text{m.cm/V.s}$ for 1 mM $\text{Na}_6\text{P}_6\text{O}_{18}$ and $+6.2 \pm 0.3 \text{ } \mu\text{m.cm/V.s}$ for 0.2 mM $(\text{NaPO}_3)_{26}$ (Table 1). Similarly, Adamczyk et al.³² have reported a positive ζ potential of +63 mV (corresponding to an electrophoretic mobility of $4.9 \text{ } \mu\text{m.cm/V.s}$) for PAH without phosphates at an ionic strength of 5 mM NaCl. The high positive electrophoretic mobility values of the PANs are probably associated with the fact that only a few polyphosphate ions are needed to form NPs. Longer polyphosphates have multiple negative charges in their chains where the positively charged amines from PAH can bind by electrostatic forces. A single polyphosphate molecule can link several amines from different PAH chains, leaving a large number of amines with uncompensated charges.

The electrophoretic mobility was measured for PANs prepared with different concentrations of phosphate salts with an ionic strength of 10 mM NaCl (Figure 3). PANs formed in the presence 1 mM $\text{Na}_4\text{P}_2\text{O}_7$ display a positive electrophoretic mobility of $+3.92 \pm 0.06 \text{ } \mu\text{m.cm/V.s}$ and those formed with 1 mM $\text{Na}_5\text{P}_3\text{O}_{10}$ display a value of $+5.38 \pm 0.09 \text{ } \mu\text{m.cm/V.s}$. Increasing the

concentration of phosphate up to 2 mM causes electrophoretic mobility values of PANs to become close to zero. This behavior suggests that the charge compensation between PAH and the polyphosphates has been reached. Beyond this concentration, charge overcompensation occurs, resulting in negatively charged NPs with electrophoretic mobility values of $-1.1 \pm 0.1 \mu\text{m.cm/V.s}$ for PANs formed with $\text{Na}_4\text{P}_2\text{O}_7$ and $-2.1 \pm 0.1 \mu\text{m.cm/V.s}$ for those formed with $\text{Na}_5\text{P}_3\text{O}_{10}$ at 3 mM phosphate salt. In contrast, PANs formed with $\text{Na}_6\text{P}_6\text{O}_{18}$ and $(\text{NaPO}_3)_{26}$ only display positive electrophoretic mobility values. For $(\text{NaPO}_3)_{26}$ the mobility increases from $+1.6 \pm 0.4 \mu\text{m.cm/V.s}$ to $+5.9 \pm 0.4 \mu\text{m.cm/V.s}$ with increasing phosphate concentration. For $\text{Na}_6\text{P}_6\text{O}_{18}$ the mobility remains practically constant over the whole range of concentrations studied, $+6.2 \pm 0.1 \mu\text{m.cm/V.s}$. This observation supports the idea that multiple PAH chains can interact with polyphosphate ions or several amines from a chain, gaining enough stability for NP formation with a small number of phosphate ions, but without compensating as many positive charges as the small phosphates do. The arrangement of PAH chains in PANS according to the size of the phosphate salt is sketched in Scheme 1. This interaction results in a high density of charged amines per NP and in a highly positive electrophoretic mobility for the formed PANs. The increase in positive values of the electrophoretic mobility as the concentration of polyphosphate increases hints at a different organization of the PAH compared with the monophosphate with several amines linked to the phosphate group but the rest of them free, not interacting with the phosphates. As the polyphosphate size increases, we can expect more free amines since the number of phosphate ions needed for PAN formation decreases by more than an order of magnitude when changing from $\text{Na}_6\text{P}_6\text{O}_{18}$ to $(\text{NPO}_3)_{26}$ as a linker (from 0.2 to 0.02 mM respectively), suggesting that the organization of the PAH chains in the PANs can be quite different.

Scheme 1. Scheme of the arrangement of polyamines in PANs for the three sizes of phosphate salts used: monophosphates, middle size or short polyphosphate, and long polyphosphates. For monophosphate denser packing of PAH chains can be expected with large number of phosphate ions in the PANs. For short and long polyphosphate a smaller number of phosphate ions are need for PAN formation and the chains are less packed as they are linked by fewer ions.

The electrophoretic mobility of PANs is strongly influenced by the ionic strength of the medium in which they are formed. When increasing the ionic strength of the solution, a monotonic decrease in the electrophoretic mobility of PANs was found (Figure S4a). In the presence of 10 mM NaCl, the electrophoretic mobility is $+5.9 \pm 0.2 \mu\text{m.cm/V.s}$, which decreases to $+3.6 \pm 0.1 \mu\text{m.cm/V.s}$ at 100 mM NaCl, as expected by charge screening. Increasing the ionic strength from 10 to 100, i.e. increasing one order of magnitude the ionic strength, reduces electrophoretic mobility only to approximately a half, hinting of highly charged particles. Conversely, the size of the PANs remains stable when increasing the ionic strength from 10 to 100 NPs formed with $\text{Na}_6\text{P}_6\text{O}_{18}$ and $(\text{NaPO}_3)_{26}$. An apparent increase in size from $93 \pm 1 \text{ nm}$ to $152 \pm 1 \text{ nm}$ was found for $\text{Na}_5\text{P}_3\text{O}_{10}$, which may be due to charge screening (Figure S4b). However, this diminution of electrophoretic mobility and change in size did not induce precipitation of the PANs.

Figure 3. Electrophoretal mobility of PANs for different polyphosphates and three concentrations for each phosphate salt. Small polyphosphates have negative values below the precipitation concentration. Measurements were made at 25°C and with an ionic strength of 10 mM NaCl.

Isothermal titration calorimetry

ITC measurements were performed to gain insight into the thermodynamics of PAN formation. Raw data from Na_3PO_4 and $\text{Na}_6\text{P}_6\text{O}_{18}$ PANs is presented in Figure 4a and 4b respectively. A linear baseline joining the points just before the injections was used. Data were processed measuring the dilution heat of each phosphate salt and subtracting it from the heat of PAN formation (Figure 4a,b). A plot of the binding isotherm of PAH as a function of phosphate concentration was obtained performing the curve integration between injection points (Figure 4c,d).

Figure 4. Representative (a) raw and (c) integrated ITC data obtained by titrating 1 mg/mL PAH with 20 mM Na_3PO_4 . Representative (b) raw and (d) integrated ITC data obtained by titrating 1 mg/mL PAH with 10 mM $\text{Na}_6\text{P}_6\text{O}_{18}$. Raw data have been transformed to relate the phosphate salt concentration to enthalpy changes. The red line through the raw data is the integration baseline. Measurements were made at 24°C.

The ITC titration curve of the PAH solution with Na_3PO_4 in Figure 4a shows a small exothermic region between 1 mM and 1.8 mM Na_3PO_4 , reaching a value of -0.55 kcal/mol Na_3PO_4 at 1.5 mM Na_3PO_4 , associated with the nucleation of the particles. However, increasing the phosphate concentration, we observe a broad endothermic region between 1.8 mM and 3.5 mM that reaches a maximum value of +0.38 kcal/mol Na_3PO_4 , followed by an exothermic region. For Na_3PO_4 , the exothermic region starting at 1 mM indicates the enthalpy gain from the formation of the amine-phosphate complex overcoming the endothermic heat associated with the change in the hydration

around the phosphate. When the phosphate concentration is increased above 1.8 mM Na_3PO_4 only little enthalpy is gained by amine-phosphate interactions as most amines have been linked through the phosphate, or because the PAN structure sterically prevents further interactions between amines and phosphate ions. This profile is similar to the one found for PAH-phosphate buffer NPs.²⁷

ITC curves for the titration with $\text{Na}_6\text{P}_6\text{O}_{18}$ show an enthalpy gain close to 0 in the region between 0.15 mM and 0.4 mM followed by an endothermic peak between 0.4 mM to 0.6 mM reaching +10 kcal/mol when 0.48 mM $\text{Na}_6\text{P}_6\text{O}_{18}$ has been added. For $(\text{NaPO}_3)_6$ a similar regime is observed, just with higher endothermic values present at lower salt concentrations. The ITC curve for $\text{Na}_4\text{P}_2\text{O}_7$ only shows an exothermic region at phosphate concentrations at which PANs are formed with a value of -3.6 kcal/mol $\text{Na}_4\text{P}_2\text{O}_7$ at 1 mM $\text{Na}_4\text{P}_2\text{O}_7$. Surprisingly, this phosphate salt shows a small endothermic increase at 0.75 mM $\text{Na}_4\text{P}_2\text{O}_7$. For $\text{Na}_5\text{P}_3\text{O}_{10}$ there is a range of phosphate additions between 0.5 to 0.8 mM $\text{Na}_5\text{P}_3\text{O}_{10}$ where a broad endothermic peak can be observed. After this endothermic peak, the addition of more phosphate salt generates almost no additional heat. Formation of PANs with all polyphosphates studied displays a similar enthalpy profile (Figure 5a). An endothermic peak was found for all polyphosphate salts in the concentration range studied. This behavior has been reported for the interaction of PAH with $\text{Na}_6\text{P}_6\text{O}_{18}$.²³ The endothermic peak found in polyphosphate systems takes place at concentrations where PANs are already formed, as confirmed by DLS. Moreover, if heat is normalized by the amount of charges per polyphosphate and then plotted against charge ratio $R = [\text{anion}] \times |Z^-| / [\text{PAH}] \times |Z^+|$ all endothermic peaks fall in the same region (Figure 5b). The overlapping of the corrected enthalpy profiles suggests that the total heat is directly associated with the number of phosphate groups and charges in the polyphosphate.

Figure 5. (a) Enthalpy isotherms obtained by titrating 1 mg/mL PAH solution with phosphate stock solutions vs. phosphate concentration: (●) Na_3PO_4 ; (●) $\text{Na}_4\text{P}_2\text{O}_7$; (●) $\text{Na}_5\text{P}_3\text{O}_{10}$; (●) $\text{Na}_6\text{P}_6\text{O}_{18}$; (●) $(\text{NaPO}_3)_{26}$. (b) Same enthalpy isotherms corrected for the charge number of each phosphate salt vs. R as defined in Materials and Methods.

The endothermic heat can be attributed to the reorganization of water associated with the phosphate groups through the formation of complexes of phosphates and amines. The interaction of the positively charged amines of PAH, and the negatively charged phosphates, is exothermic. The endothermic peak observed in polyphosphates tells us that the gain through the formation of the

complex is not large enough to compensate for the loss of water molecules solvating the phosphate ions. The formation of the amine phosphate complexes must be then entropically controlled by the release of counter ions from both PAH and phosphate salts, and by a more disordered water configuration as the hydration of the phosphate ions is altered. Counterion release is considered a major contributing factor to the formation of layer-by-layer film and polyelectrolyte complexes.³³ For each polyelectrolyte chain, several counterions are released during complexation, increasing the number of free species in solution. For polyphosphates we can assume as well that several, and in some cases all, counterions may be released during complexation with amines, increasing the number of free species and the overall entropy of the system.³⁴ At higher concentrations of phosphate we observe practically no heat gain or loss, meaning that endothermic and exothermic heats are compensating, or that the phosphate salts added are not interacting or only interacting weakly with the amines.

pH stability of PANs

The degree of protonation of PAH, $pK_a = 8-9$ ³⁵, and the polyphosphate is considered determinant for PAN formation and stability,¹⁶ as PANs are held together by electrostatic interactions between the negatively charged polyphosphates and the positive charges from the amines of PAH. Therefore, pH environments at which the polyamines or the phosphate are not charged may lead to NP disassembly.

PANs formed at optimal concentrations of polyphosphates, low PDI and high derived count rate, were exposed to different pH. DLS measurements were performed to study the stability of PANs over a wide range of pH, from 2 to 11. Figure 6a shows derived count rate plotted against pH for a solution of PANs prepared with $Na_4P_2O_7$ at different pH values. The derived count rate value

remains constant between pH 5.5 and 10. Outside of this pH range there is a decrease in the scattered light (Figure 6a), and an increase in PDI (data not shown) indicating that PANs are dissolving. For $\text{pH} > 10$ the derived count rate decrease sharply compared to the trend at $\text{pH} < 5$. DLS data for the other salts are presented in Figures S5a-e. We observed that while PANs prepared from Na_3PO_4 are stable in a narrow pH range of 6 to 8, PANs from polyphosphates have a larger stability range, which spans from pH 2 to 10 for $\text{Na}_6\text{P}_6\text{O}_{18}$ (Figure 6b). PANs made from polyphosphates appear to be more resistant to acidic protonation and the longer the phosphate chain, the lower the pH for particle dissociation. The longest polyphosphate, $(\text{NaPO}_3)_{26}$, is still stable at pH 2. In the basic regime, the stability of PANs extends to higher pH, up to 11. At pH values beyond 12, PANs are fully dissolved.

Figure 6. (a) Derived count rate of 1 mg/mL PAH in 2 mM $\text{Na}_4\text{P}_2\text{O}_7$ at different pH. Region between red dashed lines is considered to be the pH stability range of PANs (b) pH stability range of PAH-polyphosphate complexes: (●) Na_3PO_4 ; (●) $\text{Na}_4\text{P}_2\text{O}_7$; (●) $\text{Na}_5\text{P}_3\text{O}_{10}$; (●) $\text{Na}_6\text{P}_6\text{O}_{18}$; (●) $(\text{NaPO}_3)_{26}$. Ranges were determined by analyzing autocorrelation functions obtained by DLS. Measurements were performed at 25°C

Stability of PANs at lower pH values could be associated with the capacity of the phosphates to retain negative charge at these pH, while at basic pH disassembly results from PAH deprotonation. The fact that the polyphosphates extend the pH stability of PANs towards more basic values compared with the monophosphates can be attributed to the capability of polyphosphate to interact with a larger number of monomer intra and inter chains. To illustrate this behavior, we have sketched the degradation of PANs with pH depicting that at high pH values PAH loses positive charges while at low pH the polyphosphates lose the negative charges (Scheme 2).

Scheme 2. Scheme of PAN formation and degradation in different pH environments. Basic media deprotonate the amines in the PAH and acidic media protonate the phosphate groups; both extremes produce the disaggregation of the PANs.

To confirm the degradation of PANs made from short polyphosphates and the resistance of PANs from polyphosphates to an acidic environment, we used AFM to trace changes in the size, shape and structure of PANs subjected to a wash with pH = 3 solution and to a solution of neutral pH (Figures 7a,c). PANs scanned by AFM were found to have a similar diameter as those observed with TEM, however their height was approximately 12 ± 5 nm, indicating NPs with a disc shape. The different shapes found in TEM (globular) and AFM (disc shape) of the NPs would indicate that the PANs undergo a deformation during sample preparation for AFM measurement, most

probably due to the interaction with the supporting material during evaporation. It is also worth noting that horizontal interaction with the tip of the cantilever may overestimate the diameter of the particle.

PANs were subjected to an acidic wash with $\text{pH} = 3$, a pH low enough to disassemble the particles formed with $\text{Na}_5\text{P}_3\text{O}_{10}$, but not enough to disassemble the PANs formed with $(\text{NaPO}_3)_6$. For $\text{Na}_5\text{P}_3\text{O}_{10}$ PANs treated with acid, AFM images showed large portions of mica are covered with free polymer islands and no NPs were found (Figure 7b). Meanwhile, PANs formed with $(\text{NaPO}_3)_6$ resisted the acidic treatment and images revealed NPs similar in size and quantity to those exposed to only water (Figure 7d).

Figure 7. Dry mode AFM height images 500 nm x 500 nm for (a) PANs formed from 1 mg/mL PAH in 2 mM $\text{Na}_5\text{P}_3\text{O}_{10}$; (b) same PANs treated with HCl, at $\text{pH} \sim 3$ (c) PANs formed from 1

mg/ml PAH in 0.2 mM $(\text{NaPO}_3)_{26}$ and (d) same PANs treated with HCl, at pH ~ 3 . All samples were drop casted over a freshly cleaved mica surface.

Conclusions

We explored here the formation of polyamine phosphate particles based on multiple phosphate salts, mainly polyphosphates. We observed that the formation of particles is possible in all cases studied but the conditions and concentration range of phosphate ions leading to particle assembly are different for each salt. Increasing the number of phosphate units in the polyphosphate decreases the minimal concentration of phosphate ions needed for particle formation. Differently from monophosphate salts, polyphosphates form precipitates at low concentrations and this tendency is more pronounced as the number of phosphate units increases. The long polyphosphate salt, $(\text{NaPO}_3)_{26}$, forms particles stable at pH values between 2 and 11. This enhanced pH stability is due to the capacity of the polyphosphate to retain charges at low pH and the ability to link multiple chains of PAH or monomers in a chain. Particles based on polyphosphates also have high electrophoretic mobility values, up to $+5.9 \pm 0.4 \mu\text{m.cm/V.s}$ for $(\text{NaPO}_3)_{26}$, which indicates a large number of non-overcompensated amines. ITC measurements reveal that the interaction of polyphosphates with the amines of PAH results in an endothermic heat, probably related to changes in water hydration of the phosphate ions during amine phosphate complexation and suggests that PAN formation is entropically driven at low phosphate concentration.

Overall, we have advanced our knowledge on the formation of particles by complexation of polyamines with phosphate salts. We have proved that it is possible to fine tune particle properties and characteristics by choosing the proper phosphate salt, which can be beneficial for drug delivery applications.

ASSOCIATED CONTENT

Supporting Information: Supporting Information and additional data are available. This material is available free of charge via the Internet at <https://pubs.acs.org>. Figure S1. Derived count rate of PAH plotted against polyphosphate molar concentration. Figure S2. Derived count rate, hydrodynamic diameter (nm), and PDI of PANs studied at 1 hour and 24 hours after preparation. Figure S3. TEM images and corresponding autocorrelation functions of PANs formed with PAH and different phosphate salts. Figure S4. Electrophoretical mobility and hydrodynamic diameter of PANs formed at different ionic strength with various phosphate salts. Figure S5. Derived count rate intensity for PANs formed by the interaction of PAH with various phosphate salts at different pH.

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Author Contributions

All author contributed to the experimental design and work, and to the analysis and interpretation of result. The manuscript was written through contributions of 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

PAN, polyamine phosphate nanoparticle; AFM, atomic force microscopy; NP, nanoparticle; ITC, isothermal titration calorimetry; TEM, transmission electron microscopy; PAH, poly(allylamine hydrochloride) salt; DLS, dynamic light scattering; PDI, polydispersity index.

REFERENCES

1. Agazzi, M. L.; Herrera, S. E.; Cortez, M. L.; Marmisollé, W. A.; von Bilderling, C.; Pietrasanta, L. I.; Azzaroni, O., Continuous assembly of supramolecular polyamine–phosphate networks on surfaces: preparation and permeability properties of nanofilms. *Soft Matter* **2019**, *15* (7), 1640-1650.
2. Liquori, A. M.; Costantino, L.; Crescenzi, V.; Elia, V.; Giglio, E.; Puliti, R.; De Santis Savino, M.; Vitagliano, V., Complexes between DNA and polyamines: a molecular model. *J. Mol. Biol.* **1967**, *24* (1), 113-122.
3. Kimura, E.; Kodama, M.; Yatsunami, T., Macromonocyclic polyamines as biological polyanion complexons. 2. Ion-pair association with phosphate and nucleotides. *J. Am. Chem. Soc.* **1982**, *104* (11), 3182-3187.

4. Marmisollé, W. A.; Irigoyen, J.; Gregurec, D.; Moya, S.; Azzaroni, O., Supramolecular Surface Chemistry: Substrate-Independent, Phosphate-Driven Growth of Polyamine-Based Multifunctional Thin Films. *Adv. Funct. Mater.* **2015**, *25*, 4144-4152.
5. Mi, F.-L.; Shyu, S.-S.; Wong, T.-B.; Jang, S.-F.; Lee, S.-T.; Lu, K.-T., Chitosan–polyelectrolyte complexation for the preparation of gel beads and controlled release of anticancer drug. II. Effect of pH-dependent ionic crosslinking or interpolymer complex using tripolyphosphate or polyphosphate as reagent. *J. Appl. Polym. Sci.* **1999**, *74* (5), 1093-1107.
6. Park, J.-J.; Choi, Y.-W.; Kim, K.-B.; Chung, H.-I.; Sohn, D.-W., Aggregation Processes of a Weak Polyelectrolyte, Poly(allylamine) Hydrochloride. *Bull. Korean Chem. Soc.* **2008**, *29* (1), 104-110.
7. Chopra, A.; Krishnan, S.; Simmel, F. C., Electrotransfection of Polyamine Folded DNA Origami Structures. *Nano Lett.* **2016**, *16* (10), 6683-6690.
8. Thomas, T. J.; Tajmir-Riahi, H. A.; Thomas, T., Polyamine–DNA interactions and development of gene delivery vehicles. *Amino Acids* **2016**, *48* (10), 2423-2431.
9. Holder, K. M.; Smith, R. J.; Grunlan, J. C., A review of flame retardant nanocoatings prepared using layer-by-layer assembly of polyelectrolytes. *J. Mater. Sci.* **2017**, *52* (22), 12923-12959.
10. Apaydin, K.; Laachachi, A.; Ball, V.; Jimenez, M.; Bourbigot, S.; Toniazzi, V.; Ruch, D., Intumescent coating of (polyallylamine-polyphosphates) deposited on polyamide fabrics via layer-by-layer technique. *Polym. Degrad. Stab.* **2014**, *106*, 158-164.
11. Smith, S. A.; Choi, S. H.; Collins, J. N. R.; Travers, R. J.; Cooley, B. C.; Morrissey, J. H., Inhibition of polyphosphate as a novel strategy for preventing thrombosis and inflammation. *Blood* **2012**, *120* (26), 5103.
12. Kondinskaia, D. A.; Kostritskii, A. Y.; Nesterenko, A. M.; Antipina, A. Y.; Gurtovenko, A. A., Atomic-Scale Molecular Dynamics Simulations of DNA–Polycation Complexes: Two Distinct Binding Patterns. *J. Phys. Chem. B* **2016**, *120* (27), 6546-6554.
13. Andreozzi, P.; Diamanti, E.; Py-Daniel, K. R.; Cáceres-Vélez, P. R.; Martinelli, C.; Politakos, N.; Escobar, A.; Muzi-Falconi, M.; Azevedo, R.; Moya, S. E., Exploring the pH Sensitivity of Poly(allylamine) Phosphate Supramolecular Nanocarriers for Intracellular siRNA Delivery. *ACS Appl. Mater. Interfaces* **2017**, *9* (44), 38242-38254.
14. Di Silvio D.; Martínez-Moro M.; Salvador C.; Ramirez M.A.; Cáceres-Velez P. R.; . Ortore M. G.; Dupin D.; Andreozzi P.; Moya S.E. Self-assembly of poly(allylamine)/siRNA nanoparticles, their intracellular fate and siRNA delivery *Journal of Colloid and Interface Science* (online early access) DOI: 10.1016/j.jcis.2019.09.082. Published online: Sep 23 2019.
15. Bareford, L. M.; Swaan, P. W., Endocytic mechanisms for targeted drug delivery. *Adv. Drug Delivery Rev.* **2007**, *59* (8), 748-758.
16. Müller, F.; Mutch, N. J.; Schenk, W. A.; Smith, S. A.; Esterl, L.; Spronk, H. M.; Schmidbauer, S.; Gahl, W. A.; Morrissey, J. H.; Renné, T., Platelet Polyphosphates Are Proinflammatory and Procoagulant Mediators In Vivo. *Cell* **2009**, *139* (6), 1143-1156.
17. Gao, R.; van Halsema, F. E. D.; Temminghoff, E. J. M.; van Leeuwen, H. P.; van Valenberg, H. J. F.; Eisner, M. D.; Giesbers, M.; van Boekel, M. A. J. S., Modelling ion composition in simulated milk ultrafiltrate (SMUF). I: Influence of calcium phosphate precipitation. *Food Chem.* **2010**, *122* (3), 700-709.
18. Akhmetova, T. I.; Vdovina, S. V.; Mukhutdinov, E. A., Features of calculating the titration curve of sodium (potassium) decaoxotriphosphate (v). *Bull. Kazan Tech. Univ.* **2015**, *18* (9), 134-136.

19. Rana, R. K.; Murthy, V. S.; Yu, J.; Wong, M. S., Nanoparticle Self-Assembly of Hierarchically Ordered Microcapsule Structures. *Adv. Mater.* **2005**, *17* (9), 1145-1150.
20. Pusey P.N.; Tough R.J.A. Particles Interactions. In *Dynamic Light Scattering*; Pecora R. Ed.; Springer US, New York, **1985**; pp 96-101.
21. Stetefeld, J.; McKenna, S. A.; Patel, T. R., Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophys Rev* **2016**, *8* (4), 409–427.
22. Tscharnuter, W., Photon Correlation Spectroscopy in Particle Sizing. In *Encyclopedia of Analytical Chemistry*, Meyers, R. A., Ed. John Wiley & Sons Ltd: Chichester, 2006; pp 5469–5485.
23. Mouslmani, M.; Rosenholm, J. M.; Prabhakar, N.; Peurla, M.; Baydoun, E.; Patra, D., Curcumin associated poly(allylamine hydrochloride)-phosphate self-assembled hierarchically ordered nanocapsules: size dependent investigation on release and DPPH scavenging activity of curcumin. *RSC Adv.* **2015**, *5* (24), 18740-18750.
24. Ulbrich, K.; Holá, K.; Šubr, V.; Bakandritsos, A.; Tuček, J.; Zbořil, R., Targeted Drug Delivery with Polymers and Magnetic Nanoparticles: Covalent and Noncovalent Approaches, Release Control, and Clinical Studies. *Chem. Rev.* **2016**, *116* (9), 5338-5431.
25. *Zetasizer nano series user manual MAN0485 Issue 1.1*. Malvern Instruments Ltd.: England, 2004.
26. Maechling, C.; Ball, V., Exothermic–Endothermic Transition in the Titration of Poly(allylamine chloride) with Sodium Hexametaphosphate Associated with a Change in the Proton Release Regime. *J. Phys. Chem. B* **2016**, *120* (20), 4732-4741.
27. Andreozzi, P.; Ricci, C.; Porcel, J. E. M.; Moretti, P.; Di Silvio, D.; Amenitsch, H.; Ortore, M. G.; Moya, S. E., Mechanistic study of the nucleation and conformational changes of polyamines in presence of phosphate ions. *J. Colloid Interface Sci.* **2019**, *543*, 335-342.
28. Storer, A. C.; Cornish-Bowden, A., Concentration of MgATP^{2-} and other ions in solution. Calculation of the true concentrations of species present in mixtures of associating ions. *Biochem. J.* **1976**, *159* (1), 1-5.
29. Powell Kipton, J.; Brown Paul, L.; Byrne Robert, H.; Gajda, T.; Hefter, G.; Sjöberg, S.; Wanner, H., Chemical speciation of environmentally significant heavy metals with inorganic ligands. Part 1: The Hg^{2+} – Cl^- , OH^- , CO_3^{2-} , SO_4^{2-} , and PO_4^{3-} aqueous systems (IUPAC Technical Report). *Pure Appl. Chem.* **2005**, *77* (4), 739.
30. Hunter, R. J., *Zeta Potential in Colloid Science*. Academic Press: 1981.
31. Irigoyen, J.; Arekalyan, V. B.; Navoyan, Z.; Iturri, J.; Moya, S. E.; Donath, E., Spherical polyelectrolyte brushes' constant zeta potential with varying ionic strength: an electrophoretic study using a hairy layer approach. *Soft Matter* **2013**, *9* (48), 11609-11617.
32. Adamczyk, Z.; Zembala, M.; Warszyński, P.; Jachimska, B., Characterization of Polyelectrolyte Multilayers by the Streaming Potential Method. *Langmuir* **2004**, *20* (24), 10517-10525.
33. Márquez-Beltrán, C.; Castañeda, L.; Enciso-Aguilar, M.; Paredes-Quijada, G.; Acuña-Campa, H.; Maldonado-Arce, A.; Argillier, J.-F., Structure and mechanism formation of polyelectrolyte complex obtained from PSS/PAH system: effect of molar mixing ratio, base–acid conditions, and ionic strength. *Colloid Polym. Sci.* **2013**, *291* (3), 683-690.
34. Masson, J. F., Brief Review of the Chemistry of Polyphosphoric Acid (PPA) and Bitumen. *Energy Fuels* **2008**, *22* (4), 2637-2640.
35. Dickhaus, B. N.; Priefer, R., Determination of polyelectrolyte pKa values using surface-to-air tension measurements. *Colloids Surf., A* **2016**, *488*, 15-19.

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