Development of Improved Nanoparticulate Polyelectrolyte Complex Physicochemistry by Nonstoichiometric Mixing of Polyions with Similar Molecular Weights

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Water-based, biodegradable polyelectrolyte complex dispersions (PECs) prepared by mixing oppositely charged polyions are advantageous drug delivery systems due to constituent biocompatibility and nanoparticulate architectures. Reaction phase environmental parameters dictate PEC physicochemical properties, and specifically, complexation between polyelectrolytes having significantly different molecular weights leads to formation of water-insoluble aggregates. Starting with this fact, four-component similar and dissimilar molecular weight PEC chemistries were applied and compared with and without frequency-induced dispergation. The goal was to define nanoparticulate PEC systems with desirable characteristics for use in biological systems. Results show PEC formulations from precursors with similar low molecular weights yielded dispersions with suitable physicochemical characteristics, as verified by photon correlation spectroscopy and TEM, presumably due to efficient ion pairing. Similar low molecular weight PECs fabricated with dispergation exhibited pH-independent stability, as validated by charge and size measurements. These physicochemical advantages lead to an ideal delivery platform.

Introduction

Efficient nanoscale and microscale therapeutic vehicles are ideally nontoxic, nonimmunogenic, and made from versatile building blocks that allow optimal delivery to specific cells and tissues. The potential of polymer nanostructures as targeted drug delivery vehicles has led to the creation of multitudes of colloidal formulations. This technology results from a collaboration of medicine and engineering for the delivery of macromolecular drugs that cannot be efficiently administered systemically.[1] Integration of pharmacological agents, like peptide segments, proteins, and DNA vectors, into nanoparticulate polymer matrices, together, with both targeting and therapeutic abilities offers many benefits, including controlled drug release and protection, prolonged blood circulation times, and other tunable characteristics.[2,3] The advantages of using polymeric nanoparticles result from their two basic properties. First, nanoparticles, due to their small size, can perfuse capillaries and be internalized by cells, which allows for effective drug distribution to various sites in the body.[4,5] Second, the use of biodegradable materials for nanoparticle preparation allows sustained drug release within a target site over a period of days or even weeks after administration.[6]

Many current polymeric strategies are limited by the formation of potentially toxic byproducts due to the use of organic solvents or mineral oils as reaction environments. Remnants, in the form of unreacted monomers, initiators, and surfactants, present safety issues in the final product. For example, polylactide−glycolide copolymer, a widely used polymer for nanoparticulate systems, uses a toxic organic solvent (methylene chloride in most cases), which adds to regulatory approval issues. Alkylcyanoacrylate nanotechnology is another polymeric approach that suffers from toxic breakdown products.[7]

Water-soluble, biodegradable, polymeric polyelectrolyte complex dispersions (PECs) have evolved because of the limitations of the currently available systems. The nanoparticulate architecture of PECs permits the environmentally attractive use of water as a solvent, a major advantage for products that may be applied as drug delivery systems in humans. PECs result from strong electrostatic interactions between charged microdomains of at least two oppositely charged polyelectrolytes.[8] The most predominant forces for PEC assembly are strong electrostatic interactions, but hydrogen bonding, hydrophobic interactions, and van der Waals forces complement PEC formation, and they are related to physical considerations presented previously.[9] Two major steps dictate PEC complexation: (1) the kinetic diffusion process of mutual entanglement between polymers, which is rapid and depends on molar size differences, and (2) thermodynamic rearrangement of the initial simplex aggregate due to conformational changes and disentanglement. The latter process occurs more slowly, leading to PEC instability as a consequence of phase separation in aqueous media.

The mixing of polyanions and polycations leads to spontaneous formation of insoluble PECs under certain conditions governed by the strength and location of ionic sites, polymer chain rigidity, precursor chemistries, pH, temperature, and salt concentration. In particular, complexation between polyelectrolytes having significantly different molecular weights, weak ionic groups, or nonstoichiometric mixing ratios leads to the formation of water-insoluble aggregates.[10−12] These types of particles consist of a long host molecule sequentially complexed with shorter guest polyions of opposite charge according to a "zip" mechanism where there is often insufficient ion pairing. As the stoichiometry and polymer size is adjusted under dilute

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Table 1. Components of Similar (LMW) and Dissimilar (HMW) Molecular Weight PEC Chemistries[a]

precursor		component	MW (Da)
anion	HMW	HMW sodium alginate	460 000
		cellulose sulfate	1 200 000
	LMW	LMW sodium alginate	12 000
		chondroitin sulfate	15 000
cation		spermine tetrahydrochloride	384
		calcium chloride	111
		poly(methylene-*co*-guanidine) (PMCG)	5 000
		Pluronic F-68	8 400

[a] PECs are prepared with and without the use of frequency dispergation to determine the effect of polyion molecular weight on efficiency of complexation determined by physicochemical observations. The cationic baths for both LMW and HMW PEC formulations contained 1% m/v Pluronic F-68.

conditions (10^{-4} g/mL), the reaction mixture develops a colloidal, Tyndall effect due to the assembly of PECs consisting of a neutral and stoichiometric core. The internal phase is surrounded by excess binding polyelectrolyte, which stabilizes PECs against aggregation to provide practical nanoscale and microscale products.[8,13] The excess polyelectrolyte dictates the condition for stability in different medium conditions;[14] i.e., surplus cation bound to a neutralized anionic core leads to the PEC stability. The creation of large, water-insoluble aggregates is undesirable because particles greater than 1000 nm can cause blockage of blood capillaries and inflammatory tissue responses.[15−17] Conversely, particles that are too small quickly leave systemic circulation without reaching target tissues.[18]

Using these facts as a starting point, this study compares PEC systems with similar and dissimilar molecular weights to identify a suitable and controllable product for biological use. Several PEC characteristics are favorable for colloidal stability and provide the benchmarks for definition of an advantageous PEC system, including hydrodynamic diameter less than 200 nm,[17,19] empirical surface charge of greater than 30 mV or less than −30 mV, spherical morphology, and a low polydispersity index indicative of a homogeneous distribution.[20−22] Maintenance of these properties, particularly size and shape, is critical for cellular uptake.[23,24] The current technology applied herein has utilized a water-based approach for producing PECs under the prevailing assembly and complexation theory. These biocompatible, nontoxic PECs are produced using a multipolymeric mixture with a minimum of two polyion pairs for enhancement of thermodynamic stability and controlled incorporation of molecules such as proteins or targeting peptides.

Materials and Methods

PEC Chemistries. Anionic solutions contain 0.5 mg/mL low molecular weight (LMW) or high molecular weight (HMW) polyions dissolved in type I distilled water (18.2 MΩ cm resistivity). The components and their respective molecular weights are listed in Table 1. The HMW anionic solution contained HMW sodium alginate (Kelco, San Diego, CA), and cellulose sulfate (Janssen Chimica, Geel, Belgium). For LMW formulations, LMW alginate (FMC Biopolymer, Drammen, Norway), and chondroitin sulfate (Sigma Chemical Co., St. Louis, MO), were applied. The cationic solution contained 0.5 mg/mL spermine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO); poly(methylene-*co*-guanidine) hydrochloride (PMCG), (Scientific Polymer Producs, Ontario, NY); calcium chloride (Sigma Chemical Co., St. Louis, MO); and 1% m/v Pluronic F-68 (Sigma Chemical Co., St. Louis, MO). All solutions were filtered through 0.22 μm nylon filters (Nalgene, Rochester, NY). With the exception of PMCG and Pluronic F-68, all components were derived from biological systems. Table 2 describes the polymeric sources and current applications.

PEC Production Process. A batchwise, nonstoichiometric process was used to create the PECs. The solution of two anionic polyions (2 mL) was titrated into a cationic bath (20 mL), containing Pluronic F-68, with or without 20 kHz (maximum) frequency dispergation under conditions of mild mechanical stirring. The molar anion/cation charge ratio for each preparation was 0.168. The system consisted of a needle (#26 gauge) connected to a 5 mL syringe, which was inserted into an ultrasonic, hollow, titanium probe with a 1.85 mm i.d. conical tip. The probe was connected to a transducer and power generator (Misonix, Farmingdale, NY). Anionic solution was slowly extruded via controlled air pressure (3 psig) at 1 mL/min. The complexes formed instantaneously.[1,19,34]

Colloidal Stability. In preparation for biological study, stability of PEC systems was evaluated by resuspending centrifuged preparations in various pH media and measuring their physicochemical response. After preparation of LMW and HMW PECs with and without frequency dispergation, the 22-mL reaction suspension was transferred to 50-mL polypropylene ultracentrifuge tubes (Nalgene, Rochester, NY), the pH was measured (Fisher Accumet, Fairlawn, NJ), and the colloidal suspension was pelleted three times at 35 000*g* at 4 °C for 10 min (Beckman, Model L5-50, Rotor Type 60 Ti). Subsequent to the first two centrifugations, the pellet was resuspended in 1 mM sodium acetate buffer, pH 4.2. The final centrifugation was followed by dispersion in 10 mL of 1 mM buffer: pH 4.2 and 5.2 (sodium acetate/acetic acid), pH 6.2 (sodium citrate/citric acid), pH 7.2 and 8.2 (Trizma/HCl), or pH 9.2 (carbonate-bicarbonate). Each buffer was prepared in type I distilled water (18.2 MΩ-cm resistivity) and filtered with a nylon 0.22 μm filter.

PEC Size and ζ-Potential. Following colloidal preparations, washing and dispersion at varying pH, a 1-mL sample was removed for size and ζ-potential measurement with the Malvern ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK). Each measurement was performed in triplicate. The particle-sizing device uses noninvasive back-scattering with photon correlation spectroscopy (PCS), which has a particle sensitivity in the range from 0.6 nm to 10 μm. Size-related measurements are reported as *z*-average mean, hydrodynamic diameter, and polydispersity index (PDI). The *z*-average mean is classically the parameter most comparable to diameters measured by transmission electron microscopy.[35] PDI is a dimensionless number that describes the heterogeneity of the sample, and it is scaled such that values less than 0.05 are rarely seen. The maximum value is 1.000. Values greater than 0.700 indicate a very broad size distribution and a lack of sample homogeneity.

Transmission Electron Microscopy (TEM). Reaction mixture preparations were analyzed for morphology and ZetaSizer validation by pipetting a 20 μL sample volume onto a dry, Formvar-coated, 400-mesh copper grid (Electron Microscopy Sciences, Hatfield, PA). The volume was allowed to adsorb onto the grid surface for 30 s, after which the excess liquid was blotted carefully with filter paper. The specimen was then viewed with a Philips CM-12, 120 keV electron microscope equipped with a CCD camera. PEC diameter was evaluated by using point-to-point pixel-based measurements for a minimum of 280 individual observations using software complementary to the TEM CCD camera (Advanced Microscopy Techniques, Danvers, MA).

Statistical Analysis. All statistical analyses were performed using JMP-IN 5.1.2 (SAS, Cary, NC). Reaction mixture formulations with and without dispergation were compared by two-sample *t*-test to evaluate the differences between sizes, ζ-potentials, and polydispersity indices within and between PEC systems. Hydrodynamic diameter and surface charge was tested for significant differences from 200 nm and +30 mV by a one-tailed *t*-test. TEM size distributions were tested using the Kolmolgorov−Smirnov two-sample test to determine whether

Table 2. Polyion Sources and Common Uses

polymer	source	current application
sodium alginate	algal cell walls	controlled release and bioadhesive systems[25]
cellulose sulfate	plant-derived	clinical trials as anti-HIV agent[26]
chondroitin sulfate	animal cartilage, ligaments, tendons	osteoarthritis management[27]
spermine tetrahydrochloride	mammalian sperm	cancer diagnosis and treatment[28]
calcium chloride	ubiquitous salt in all organisms	cell, tissue polyelectrolyte maintenance[29]
PMCG	synthetic	microencapsulation[30−33]

the PEC populations were distributed identically as a function of frequency dispergation. Kurtosis and skewness for the TEM distributions were evaluated by one-tail *t*-test to evaluate deviations from normality. Colloidal stability for hydrodynamic diameter was first tested by one-way ANOVA to determine variations in the means as a function of pH. The one-way ANOVA was followed by Dunnet's test, which compared each mean to the reaction mixture. A one-tailed *t*-test was applied to determine at which pH instabilities in preparations, as measured by ζ-potential, deviated from the empirical standard for stability, ±30 mV.[22] Each analysis was evaluated at the 95% confidence level.

Results and Discussion

PEC Physicochemical Aspects. The components of the anionic solution were altered to determine the effect of low molecular weight polymers on the hydrodynamic diameter, surface charge, and PDI of polyelectrolyte complexes. Polyelectrolyte concentrations for nonstoichiometric addition of HMW anions into cations were established previously.[1,19] Increased polymer concentration and further anion titration led to PEC size increase, aggregation, and precipitation as the overall complex charge neared neutrality. Lower polymer concentrations led to significantly lower PEC yields. Thus, the chosen concentrations represented a practical compromise.[1,36] In both LMW and HMW formulations, the molar anion/cation charge ratio, 0.168, was constant, as calculated from the structures and molecular weights provided by the manufacturers. The criteria for acceptance of PEC systems for biological testing were hydrodynamic diameter statistically less than or equal to 200 nm,[17,19] surface charge $>|\pm 30$ mV$|$,[20−22] and a low PDI, indicative of a narrow size distribution. The physicochemical characteristics of both LMW and HMW PECs were studied by titrating, nonstoichiometrically, anions into a cationic bath without or with (20 kHz) frequency dispergation. The spontaneous complexation resulted in cationic particles, regardless of PEC formulation, that exhibited a colloidal, Tyndall effect. PECs, as prepared in their native state, had a measured pH of 4.2, due to excess cations present in the reaction mixture. The complexation led to a core-shell morphology, with the excess cation dominating the corona surface and a neutralized inner phase of oppositely charged polyions.

The *z*-average size, PDI, and ζ-potential for PEC preparations with LMW and HMW polyions are shown in Figure 1. LMW formulations provided PECs with superior characteristics independent of frequency dispergation, an outcome related to polymer molecular weight. Statistical differences in PDI, ζ-potential, and hydrodynamic diameter of PECs were observed among the two titration conditions and chemistries. Both LMW titration conditions provided PECs with hydrodynamic diameters averaging statistically less than 200 nm, while the dispergated HMW formulation was the only dissimilar molecular weight chemistry with appropriate physicochemical properties. Frequency dispergation reduced HMW and LMW PEC hydrodynamic diameter by 25% and 12%, respectively. When comparing

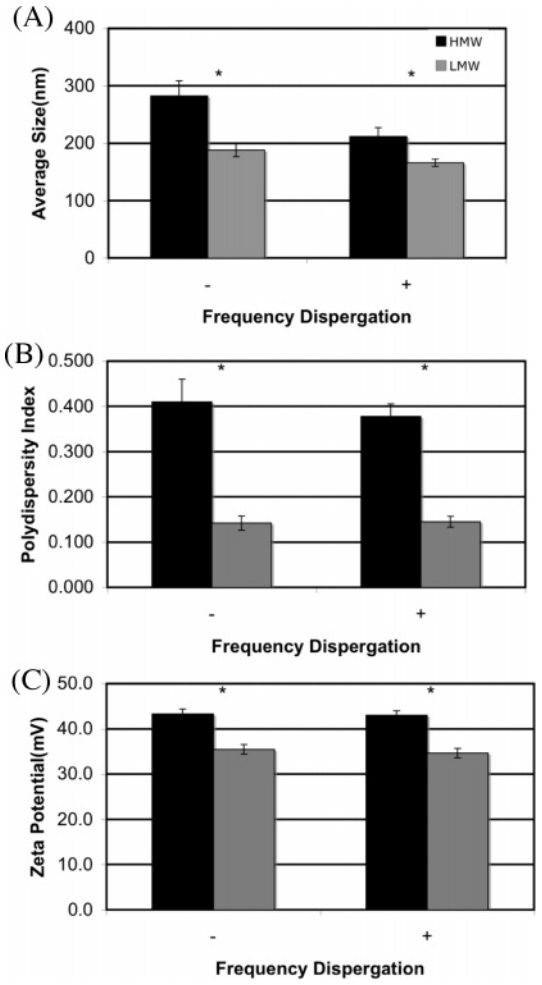


Figure 1. Size and ζ-potential measurements for similar (LMW) and dissimilar (HMW) weight components. (A and B) Measurements of *z*-average diameter and polydispersity index for PECs obtained with (+) and without (−) ultrasonic dispergation for similar and dissimilar molecular weight components. (C) The ζ-potential for dissimilar (HMW) and similar (LMW) molecular weight components. Asterisks indicate that pairs of means differ statistically by two-sample *t*-test at the 95% confidence level for 10 replicates.

titration frequencies between systems, LMW PECs resulted in a 33% and 21% decrease in size for formulations without and with frequency dispergation, respectively. The PDIs measured for HMW PECs, 0.410 and 0.378, indicated very heterogeneous populations of particles within the desired size range, as well as aggregate structures with much greater diameter, while the reduced PDI for LMW PECs, 0.142 and 0.145, indicated more homogeneous preparations. For reference, a monodisperse distribution of standard latex beads yields a PDI of 0.05.[37] This reduction in polydispersity helps to minimize the possibility of in vivo circulatory limitations. ζ-potential was reduced for LMW PECs, but the systems were stable. In fact, a decreased positive surface charge has reduced toxicity at cellular and systemic levels.[38]

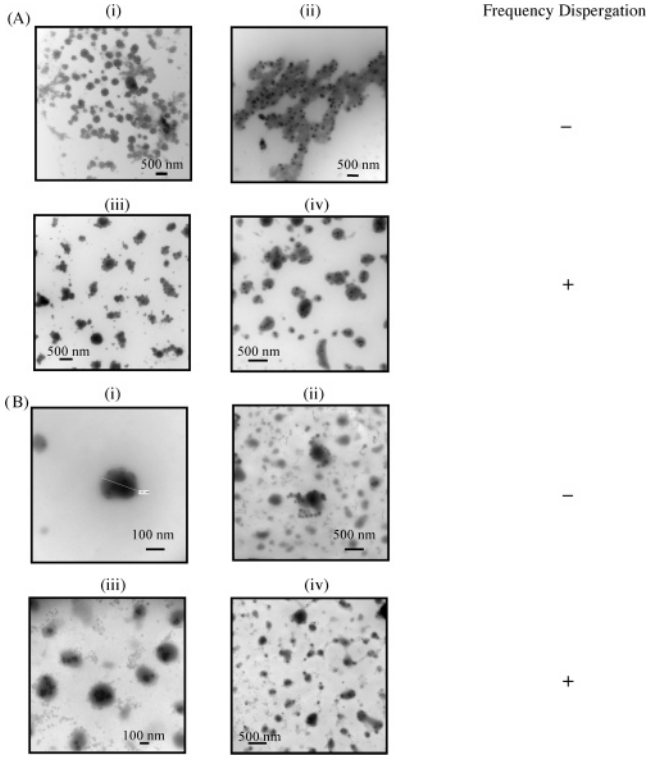


Figure 2. TEM micrographs for PECs prepared with and without frequency dispergation for HMW (A) and LMW (B) precursors. Images assigned i and ii are PECs created without dispergation, and iii and iv represent PECs fabricated with dispergation.

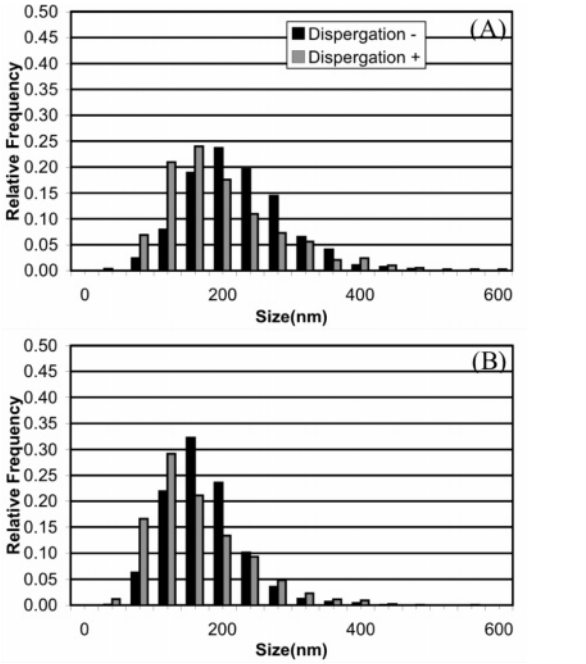


Figure 3. TEM PEC diameter evaluation. PEC diameter measured using TEM CCD camera software for suspensions prepared from HMW (A) and LMW (B) precursors. For HMW, 784 and 284 events were tabulated with and without dispergation, respectively; 1486 and 1860 observations were recorded for LMW PECs created with and without dispergation, respectively. Distribution shifts are statistically evaluated by the Kolmogorov−Smirnov test.

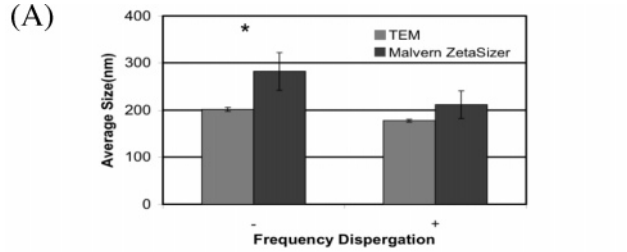
According to these results, the complexation of oppositely charged polymers was possibly enhanced because of more efficient interactions between charged regions. The particle-forming process depends on the availability of charges on the polyelectrolytes. For LMW polyions, the decreased polymer chain length could lead to more well-organized ion pairing and charge neutralization, while also allowing a sufficient number of unpaired positive charges for colloidal stability. In addition, the complexation was likely better controlled due to less bulky anions and decreased potential for polyanion charge shielding. The size and length of HMW polymers in aqueous solution likely caused a conformation that protected their complexation sites.[39] Therefore, cations could not diffuse into and align charges to condense into more homogeneous PEC populations.

The improved physicochemical properties observed with and without frequency dispergation may be due to the difference in sequential addition of anion to cation. Frequency dispergation provides the anionic solution as an aerosol, upon yielding a smaller core template for subsequent cationic interactions and nucleation of the complexes. However, LMW PECs suitable biological preparations were formed independent of frequency dispergation. Therefore, LMW anions needed no modified titration to provide polyions in the appropriate conformation for efficient PEC creation.

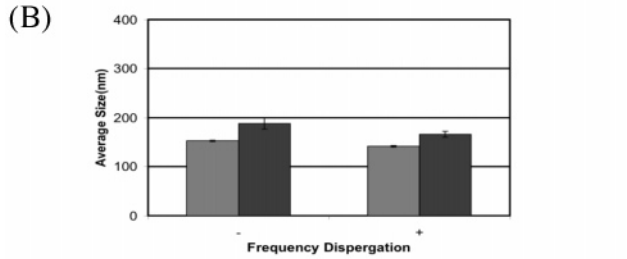
PEC Morphology by TEM. TEM micrographs presented in Figure 2 provided verification of LMW and HMW PEC size with and without frequency dispergation. Unstained TEM specimens were used, since the PECs had enough contrast and superstructure for visualization. Observations of PEC morphology were followed by point-to-point, pixel-based diameter evaluation to develop a size distribution to compare with PCS measurements. Similar structures and size for PEC structures have been reported in the literature.[2,40]

Figure 2Ai−iv showed the heterogeneity of populations created under both dispergation conditions for the HMW preparations. These images provided a physical illustration of classic PEC models dictated by the characteristics of the polyion groups, stoichiometry, and molecular weights: (1) the ladderlike structure, where complex formation takes place on a molecular level via conformational adaptation; and (2) the scrambled-egg model, where a high number of chains are incorporated into the particle architecture.[41] Figure 2Aii showed the ladderlike structures formed as a result of mixing polyelectrolytes having weak ionic groups, large differences in molecular dimensions, polymer shielding, and zipperlike assembly due to adjacent polyions.[41,42] HMW PECs prepared with frequency dispergation, (Figure 2Aiii,iv) showed compact, scrambled-egg architectures, which appeared to be highly aggregated. This morphology is typical for PECs formed between polyelectrolytes having different molecular weights.[12] Figure 2Bi−iv, for complexes formed with low molecular weight constituents, exhibits more homogeneous populations of condensed, compact structures, which only displayed scrambled-egg behavior. The more uniform morphology was consistent with their lowered PDI. Again, this was likely due to more efficient interactions between polyions. The particles exhibited a condensed, opaque core, surrounded by a thick, fluffy coat. This opacity may be due to the presence of divalent cations in the formulations. Similar behavior has been previously observed for chitosan/PEO−PPO nanoparticles.[43]

Instrumentation that is complementary to the TEM CCD camera was used to measure manually and estimate the diameter of the PECs for validation of the *z*-average mean diameter. Only spherical structures were sized, since the aggregate/filament structures provided no discernible points of reference for accurate diameter evaluation. Figure 3 displays the histograms for LMW and HMW PECs created with and without frequency dispergation. Number average sizes were 152.4 and 141.5 nm for LMW PECs without and with frequency dispergation, respectively, while HMW PECs had mean diameters of 201.5



dispergation	TEM (nm)	Malvern (nm)	% TEM/Malvern
-	201.5	282.1	71.4
+	177.5	211.3	84.0



dispergation	TEM (nm)	Malvern (nm)	% TEM/Malvern
-	152.5	188.2	81.0
+	141.5	165.8	85.4

Figure 4. Comparison of PEC diameter measured by Malvern ZetaSizer Nano ZS and TEM for HMW (A) and LMW (B) polymer PECs. The above figure details percent agreement between the two techniques along with a side-by-side graphical comparison. Asterisks indicate pairs of means that differed according to a two-sample *t*-test at the 95% confidence interval, but not at 90%, indicating an equivalence among means for the two physicochemical characterization methods.

(without) and 177.5 (with). The results correlated to measurements performed using PCS, insofar as the effect of both precursor chemistry and dispergation according to two-sample Student's *t*-tests at the 95% confidence level. None of the distributions were normally distributed as determined by statistically testing the skewness and kurtosis of each distribution. The PDI measured by the ZetaSizer was qualitatively verified by coefficient of variance, an indication of the variabilities of the populations. Calculations performed on the distributions ranged from 34.9% (LMW without) to 49.0% (HMW with). Conversely, Kolmogorov−Smirnov tests showed that HMW and LMW size distributions were significantly different for the two frequency dispergation conditions. This result detected differences between formulations, with and without dispergation, while this difference could not be detected by two-sample *t*-tests using PCS data. Although, the two statistical tests yielded different interpretations, the algorithms for evaluation of PEC diameter are quite different. PCS uses a combination of light scattering as a function of Brownian motion to derive diffusion coefficients, which can then be used to calculate hydrodynamic diameter from the Stokes−Einstein equation. The measurement of diameter via TEM was a more direct method for evaluating diameter not convoluted by the aqueous parameters that affect PCS measurements, as discussed below.

Figure 4 shows the agreement between PCS measurements and TEM diameter evaluation. TEM estimates were consistently 71%−85% lower than PCS measurements. The ZetaSizer algorithms assume that the particle population only contains spherical structures; therefore, microfilaments are assumed to be large, round objects. Besides the assumption of uniform morphology, the ZetaSizer values include surface structure and electrical double layers surrounding the particle that contributed to the increased diameter measured by PCS. Previous studies on polymeric nanoparticles show similar TEM/PCS ratios.[2,44,45] In conclusion, TEM was suitable for discerning PEC morphology and quantitative diameters, but the ZetaSizer provided a higher throughput, unbiased method. Both types of analysis need to be considered when characterizing these types of physicochemical phenomena.

Colloidal Stabilty as a Function of pH. The addition of electrolytes or the change in the pH affects the colloidal stability of colloidal dispersions. These modifications are significant considerations for further use of any nanoparticulate system in biological media.[46] The alteration of surface groups in electrostatic complexes between anions and cations can lead to a large size change, dependent upon the extent of ionization.[47] Any ionizable groups in the charged domains presented at the nanovehicle periphery can act as electrostatic stabilizers, which are generated by repulsive Coulombic interactions between particle surface charges.[48,49] As shown in Figure 1, reaction mixture PECs yielded stable, positively charged structures with ζ-potentials greater than +30 mV. However, this condition occurred at low pH (~4.2) and may not be a suitable suspension for in vivo or in vitro use. The cationic charge of the PECs indicated that the surface-exposed polymeric groups were in a highly protonated state, causing repulsive forces to propagate and prevent particle coalescence in solution. In addition, the delicate balance between electrostatic double layers and Van der Waals forces likely contributed to the maintenance of colloidal stability, signified by ζ-potential.[50]

The stability of the four PEC systems was evaluated by PEC collection and resuspension at various pH in low salt and ionic strength media. Salts can cause secondary aggregation and flocculation, as well as a disintegration of the complexes.[51] Therefore, if the salt concentration is low, the effect of pH on PEC physicochemistry is isolated. Because of the presence of positively charged, pH-ionizable, primary amino groups that were present as a result of excess PMCG and spermine of the cationic shell, a pH variation should modify the electrical state and thus the stability of the complexes. Hydrodynamic diameter (*z*-average size) and ζ-potential were used as stability indices.

Figures 5 and 6 show the variation of PEC diameter and ζ-potential, respectively, as a function of pH. The two properties were directly related, and they were consecutively measured by the ZetaSizer immediately after colloidal resuspension and pH measurement. Surface charge stabilities were empirically defined as values $>|\pm 30$ mV|. For *z*-average, statistical instability was defined by one-way ANOVA and then by comparison of means to the reaction mixture by Dunnet's test. Figure 5 shows a progressive reduction in statistical instabilities moving from HMW without dispergation to HMW with dispergation, LMW without dispergation, and LMW with dispergation. Figure 6 shows a similar trend, but LMW systems provided greater colloidal stability as a function of pH. In general, the behavior for Figures 5A−C and 6A−C, showed a clear tendency toward aggregation as pH approached neutrality, corresponding to changes in surface charge. Therefore, the maintenance of ideal dimensions was governed by electrostatic contributions. For these particular systems, PECs coalesced, rather than swelled, and the effect was irreversible. If particles aggregate, then they have reached an endpoint state. The increase in pH from 4 to 7 brought about a rapid decrease in ζ-potential and colloidal stability, as shown by fusion of PECs as the repulsive surface charges were reduced. As the resuspension pH was increased

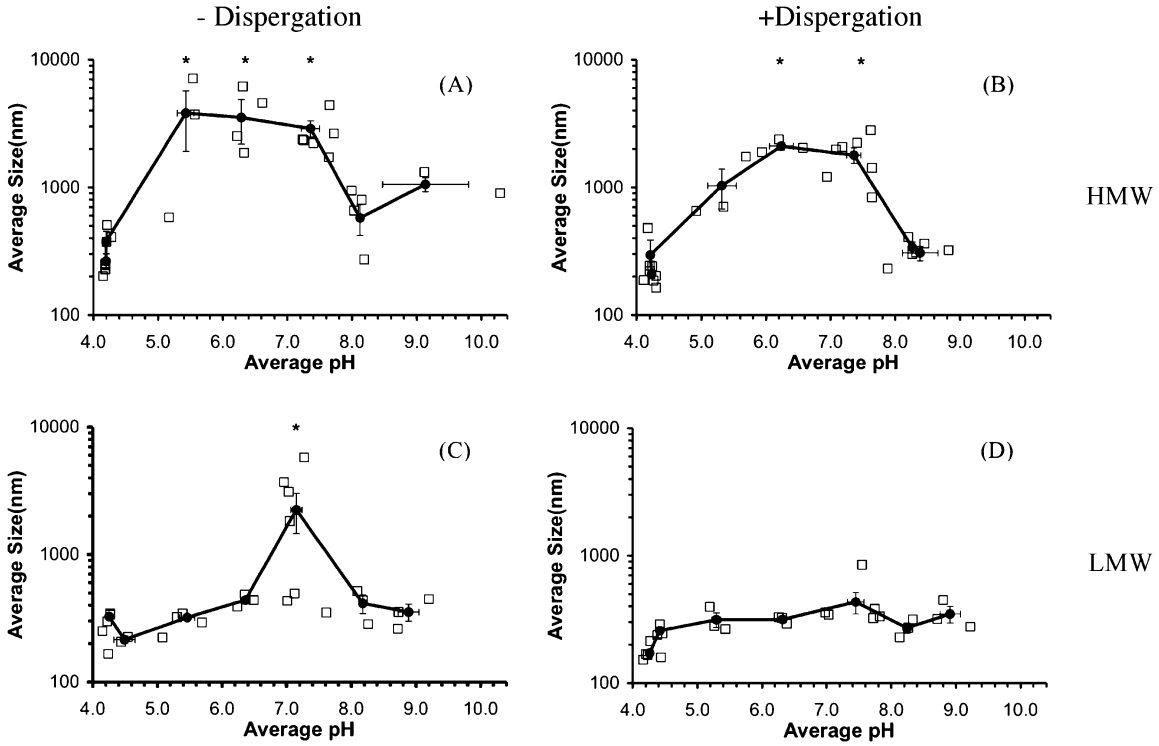


Figure 5. Response of PEC diameter in varied pH, low salt environments. (A and B) The effect of pH on HMW PECs prepared without and with frequency dispergation, respectively. (C and D) The response of LMW PECs. Raw data are represented by squares, while the average of at least three replicates is shown with circles and error bars corresponding to standard error. The asterisks indicate means that differ from the reaction mixture at the 95% confidence interval using Dunnet's test.

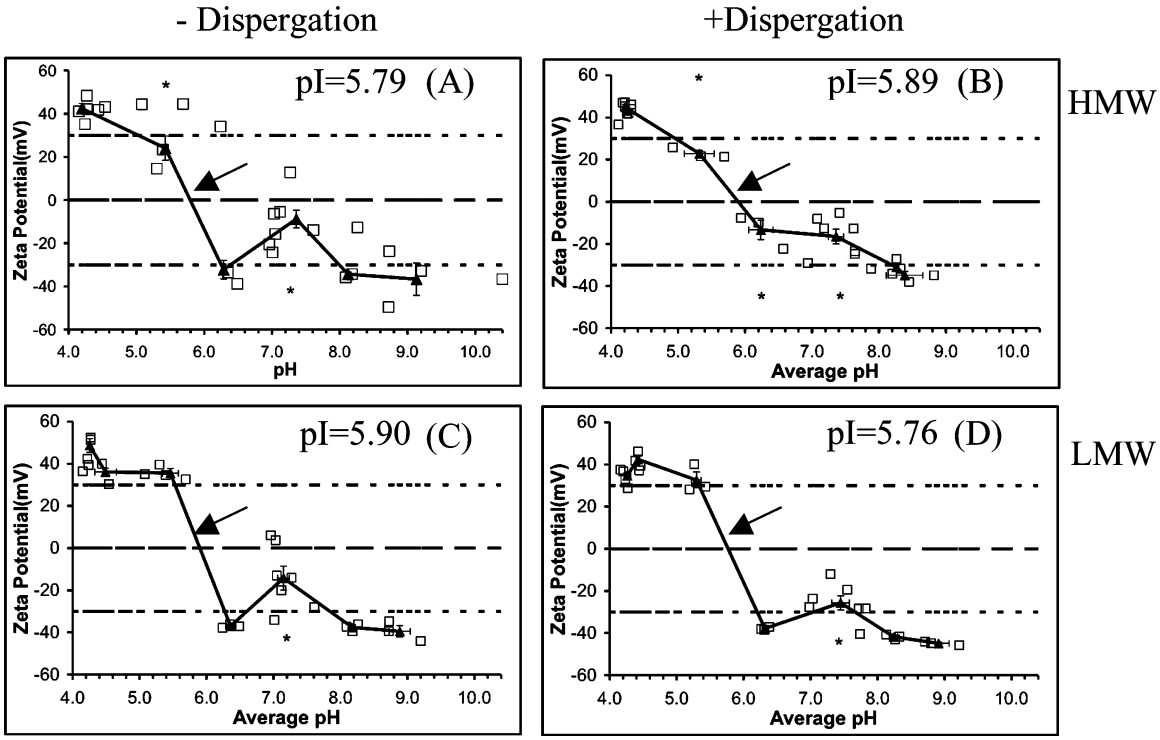


Figure 6. Response of PEC surface charge, ζ-potential, in varied pH, low salt environments. (A and B) The effect of pH on HMW PECs prepared without and with frequency dispergation, respectively. (C and D) The response of LMW PECs. Raw data are represented by squares, while the average of at least three replicates is shown by circles and error bars corresponding to standard error. Asterisks denote ζ-potentials that deviate, statistically, from the empirical stability criterion of |±30 mV|, defined by dashed lines, according to a one-tail *t*-test at the 95% confidence interval. Arrows indicate experimental p*I*s.

beyond a transition point (∼7.8), the suspension appeared to become stable, as shown by preservation of the Tyndall effect and negative ζ-potential less than −30 mV. This type of behavior, with ζ-potential shoulders on either side of neutrality, indicated the possibility that these PECs exhibit zwitterionic behavior. Therefore, PECs upon assembly contain both positive and negative ionizable groups and behave as acidic or basic groups of monoacids or monobases. At low pH, pendent amino

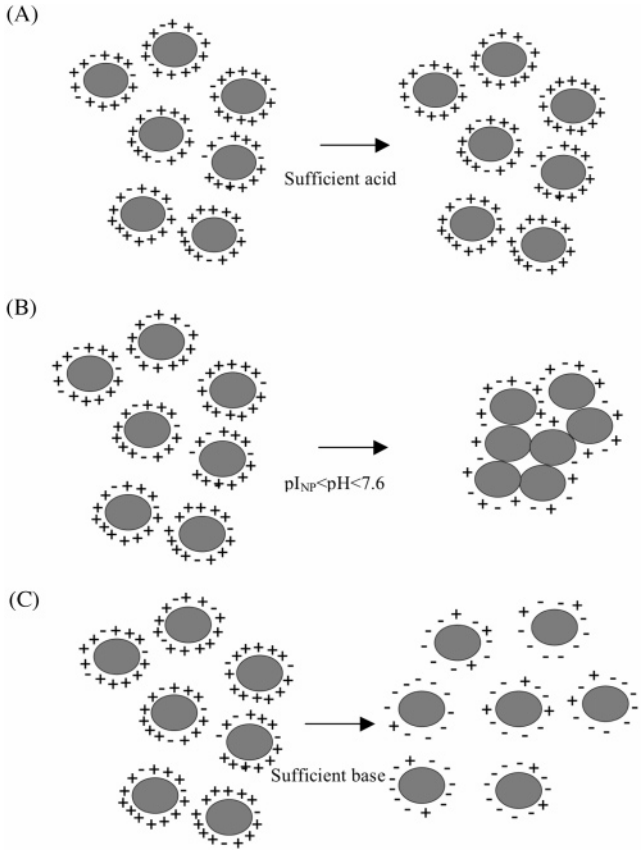


Figure 7. Schematic representation of PEC charge modification. The left figures are the PEC suspensions immediately after preparation. The native state has a surface dominated by excess cationic charge, as shown by the significantly positive ζ-potential. The presence of sufficient acid maintains a stable protonation state as shown in A. As pH changes and nears the p*I* and neutrality, the ionizable states of the complexes are modified. The decreasing total surface charge depletes the complexes' ability to repel each other and they effectively coalesce, as displayed in B. But, if adequate base is present, the carboxyl groups become ionized and the colloid remains stable, as denoted in C.

and carboxyl groups remain in a protonated state, leading to a predominance of positive charges. Following resuspension at increasing pH, the protons from the charged groups dissociate. The decreasing total surface charge depletes the PECs' ability to repel each other and they effectively coalesce. However, adequate negative charge appeared to be generated at basic conditions when sufficient protrusion and ionization of the carboxyl groups from anions occurs.[52] Figure 7A−C schematizes this phenomenon. In all cases, an experimental PEC isoelectric point (p*I*)—the pH where dispersions carry and are in their least stable state—was determined by fitting a line through the most linear portion of the ζ-potential versus pH curve (Figure 6). The calculated *x*-intercept was designated as the experimental p*I*. p*I*s for the systems ranged from 5.76 (LMW without dispergation) to 5.90 (LMW with dispergation) and from 5.79 (HMW without dispergation) to 5.89 (HMW with dispergation). The variability in p*I*s between systems and/or titration conditions, including possibly two regions of instability exhibited in Figure 6A,C, may be directly due to the assembly mechanisms and presentation of charged groups for complexation.

The most interesting results were obtained for LMW PECs prepared with dispergation, as shown in Figures 5D and 6D. Even though the ζ-potential−pH curve exhibited a typical behavior, there appeared to be some intraparticle forces that allowed the complexes to retain their dimensions even when the ζ-potential neared neutrality. The maintenance of hydrodynamic diameter may also be related to the presence and incorporation of Pluronic F-68, assumed to be a steric stabilizer with no net charge.[53] The long chains of flexible, triblock copolymer polyethylene oxide/polypropylene oxide (PEO/PPO), when adsorbed to the surface, could create an osmotic and entropic barrier to particle−particle interactions induced by pH alterations.[34] Pluronic F-68 may discourage surface adhesion and conceivably interparticle interactions. Intimate blending of PEO/PPO polymers into core−shell nanomatrices has been shown to attenuate hydrodynamic diameter and enhance structural integrity.[54,55] The electrically neutral copolymer, composed of hydrophilic and bydrophobic segments, is mechanically entrapped when present in a cationic bath, as recently observed by NMR (unpublished data). The incorporation of PEO/PPO was possibly facilitated by the intermolecular bonding between electropositive amino hydrogens of the constitutive cationic polymers and the electronegative oxygens of PEO/PPO.[56] The interaction between the oxygen atom of PEO/PPO and the amino groups of the corona solution is weak but could still have an effect on PEC stability and formation. The retention of ideal dimensions denotes the LMW dispergated formulation for biological application. Preliminary in vitro testing of PEC stability has shown serum and media stability supporting their use for specific cell binding and internalization applications. The hydrodynamic diameter for this LMW nanoparticulate system was 296.8 ± 16.8 nm (mean ± standard error). Although the size falls outside of the benchmark of less than 200 nm, it is still expected to efficiently interact with biological systems. This increase in size is most likely due to interactions with media components.[57]

Conclusion

This work, through the use of TEM and PCS technology, shows the effect of polyanion size reduction on PEC physicochemical properties that are essential for developing a biocompatible system for targeted drug delivery. As shown, the titration of nonstoichiometric amounts of HMW anions, compared to LMW anions for equivalent total molar charges, into cations formed larger complexes with marked size heterogeneity. In addition, HMW formulations resulted in decreased physicochemical stability. A system has been further optimized for size and charge properties that are pH insensitive, making it a useful system for optimal delivery to a range of tissues. The surface density of PEO/PPO may ensure sufficient steric stabilization, phagocytic resistance, and prolonged systemic circulation.[54,55,58,59] This new formulation is an improvement over a previous nontoxic system[33,60,61] that has been shown to effectively deliver genes to cells of hematopoietic origin.[19]

The pH and serum insensitivity, self-assembly, modular nature, and unique polymeric architecture of dispergated LMW PECs allows for surface groups to be independently modified to impart desired characteristics without any substantial alteration of existing properties. The polycationic nature of the PEC periphery, predominantly polyamine and guanidinium moieties, may also facilitate their cellular uptake and transport via cell surface heparan sulfate proteoglycans or polyamine-based transport.[62−64] In particular, current and future work involves serial modifications of the outer shell of amine groups with specific targeting ligands for directed specificity to surface receptors with low toxicity and high efficiency. Taken together, the favorable characteristics of this system suggest that this PEC may find utility as safe and highly efficient, bioactive vehicles.

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