Analytical Biochemistry **302,** 75–80 (2002)
doi:10.1006/abio.2001.5507, available online at http://www.idealibrary.com on IDEAL®

Methodologies for Monitoring Nanoparticle Formation by Self-Assembly of DNA with Poly(L-lysine)

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Received July 12, 2001; published online January 23, 2002

DNA self-assembly with polycations produces nanoparticles suitable for gene delivery, although there is no standard methodology to measure particle formation and stability. Here we have compared three commonly used assays, namely, light scattering, inhibition of ethidium bromide fluorescence, and modified electrophoretic mobility of DNA. Analysis by light scattering and loss of ethidium bromide fluorescence both showed poly(L-lysine) (pLL)/DNA nanoparticles form over the lysine/phosphate ratio range 0.6–1.0, although retardation of DNA electrophoretic mobility commenced at lower lysine/phosphate ratios. This probably indicates that the first two assays monitor DNA collapse into particles, while the electrophoresis assay measures neutralization of the charge on DNA. Gel analysis of the complexes showed disproportionation during nanoparticle formation, probably reflecting cooperative binding of the polycation. The assays were used to examine stability of complexes to dilution in water and physiological salts. Whereas all pLL/DNA nanoparticles were stable to dilution in water, the presence of physiological salts provoked selective disruption of complexes based on low-molecular-weight pLL. Polyelectrolyte complexes for targeted application *in vivo* should therefore be based on high-molecular-weight polycations, or should be stabilized to prevent their dissociation under physiological salt conditions.

Key Words: **gene therapy; poly(L-lysine); condensation; ethidium bromide.**

Polyelectrolyte complexes provide a versatile nonviral vector for delivery of nucleic acids in genetic therapies (1). Polyanionic DNA can be complexed with simple cationic polymers, undergoing a self-assembly reaction to produce discrete nanoparticles suitable as gene delivery vectors. Previous studies have examined the influence of the cationic polymer on the biophysical properties of the complex produced with DNA, and rationally designed or ligand-modified polymers are being increasingly used in sophisticated vectors developed to meet specific biological objectives (2–4).

A range of techniques have been employed to monitor the self-assembly process and to characterize properties of the polyelectrolyte complexes produced. Gel retardation is a method that monitors the loss of electrophoretic mobility of DNA when it binds a critical amount of cationic polymer (5). However, the mechanistic basis of the assay is unclear, specifically whether it represents nanoparticle formation or simply neutralization of DNA. Similarly, inhibition of ethidium bromide (EtBr)[2]/DNA fluorescence resulting from DNA condensation by cationic polymers is a convenient method to monitor the polyelectrolyte interaction (6); again, however, it is not known whether the assay measures particle formation or simply neutralization of the DNA. In addition, possible influences of EtBr concentration have not been characterized; neither is it known whether EtBr is expelled from the DNA structure during condensation or merely quenched.

In this study we have assessed fundamental aspects of the interpolyelectrolyte self-assembly reaction of plasmid DNA with poly(L-lysine) (pLL), and have compared the analytical techniques commonly used to monitor particle formation. The two preferred techniques—light scattering (7) and EtBr/DNA fluorescence—were then used to characterize the stability of nanoparticles formed using polycations of varying molecular weight, to address the possibility of differential

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[2] Abbreviations used: EtBr, ethidium bromide; pLL, poly(L-lysine); pLAA, poly(L-aspartic acid); PBS, phosphate-buffered saline; pHPMA, poly(*N*-[2-hydroxypropyl]methacrylamide.

0003-2697/02 $35.00

stability under physiological conditions, enabling more informed design of vectors for gene delivery *in vivo*.

MATERIALS AND METHODS

Sources of DNA. The 6-kb expression vector pSV2BCL2 containing the BCL2 gene driven by the SV40 promoter (8) was grown in *Escherichia coli* and purified using Qiagen Gigaprep Kits (Crawley, West Sussex, UK). Concentration and purity of the DNA were checked on a spectrophotometer at A_{260}/A_{280} absorbance wavelengths.

Formation of pLL/DNA complexes. DNA (20 μg/ml) was added in ultrapure water to a polypropylene Eppendorf and mixed thoroughly. pLL (2.5 mg/ml stock in water) was added to give an N:P ratio of 2.0 (defined as the molar ratio of amino groups in the pLL to phosphate groups in the DNA), unless otherwise stated, and gently mixed. Complexes were allowed to form for 30 min prior to use. The N:P ratio of 2.0 was selected to ensure efficient complex formation without a great excess of free pLL. Although some pLL will remain unbound following complex formation, this is unlikely to interfere with the assays performed.

Cy-3 labeling of DNA expression vectors. The Cy-3 labeling kit was purchased from Panvera (Madison, WI), and 10 μg of plasmid DNA labeled using the standard protocol. Briefly, DNA (0.1 mg/ml) was reacted with 10 μl of Cy-3 in appropriate buffer at room temperature for 1 h. Unreacted dye was removed by centrifugation through a G-50 microspin purification column. Cy-3-labeled DNA was analyzed following agarose gel electrophoresis using a Typhoon Fluorimager (Molecular Dynamics, High Wycombe, UK) (λ_{ex} 550 nm, λ_{em} 570 nm).

Agarose gel electrophoresis. pLL/DNA complexes were prepared in water (N:P ratios 0.2–2.0) and electrophoresed on agarose gels (1% w/v, 120 min, 100 V). In some studies the gels were incubated, postelectrophoresis, in heparin sulfate solution to disturb the DNA–cationic polymer complexes, and restore access of EtBr.

Measurement of complex formation by light scattering. Complex formation was investigated by measuring changes in light scattering intensity using a fluorometer. Plasmid DNA (20 μg/ml) in Hepes (10 mM, pH 7.4) was incubated in a cuvette and the intensity of scattered light (λ_{ex} 600 nm, λ_{em} 600 nm) was set to zero. pLL (111 kDa) was added at 0.2 N:P increments and the changes in scattering intensity monitored. Data are presented as the relative scattered intensity, with the maximum signal detected at any N:P ratio set to 1.0.

Inhibition of EtBr/DNA fluorescence by poly(L-lysine). The EtBr/DNA fluorescence (λ_{ex} 510 nm, λ_{em} 590 nm) of plasmid DNA (20 μg/ml) in ultrapure water containing EtBr (400 ng/ml–3 μg/ml) was measured and set to 100%. Aliquots of pLL (2.5 mg/ml stock in water, 111 kDa) were added sequentially and the fluorescence was measured after each addition, until the N:P ratio reached 2.5. In some experiments poly(L-aspartic acid) (pLAA) was added incrementally to the complexes to sequester the pLL and restore EtBr/DNA fluorescence.

Determination of fluorescence spectra. Fluorescence excitation spectra for EtBr (0.4 μg/ml), DNA (20 μg/ml)/EtBr and pLL/DNA/EtBr (N:P = 2) (all in water) were determined using 2 ml solution and scanning excitation (λ_{ex} 350–560 nm) with λ_{em} fixed at 590 nm.

Evaluation of stability of complexes to dilution. Stability of pLL/DNA complexes was assessed by measuring restoration of EtBr/DNA fluorescence on dilution. Complexes were formed by sequential addition of pLL (4.0, 29.3, or 111 kDa) to DNA (20 μg/ml) in water containing EtBr (400 ng/ml), to a final N:P ratio of 2.8. EtBr/DNA fluorescence was measured after each addition. Ten percent (v/v) 10× PBS solution (without EtBr) was then added. After 15 min the change in fluorescence was measured and the complexes were subject to a series of dilutions in PBS (without EtBr). Alternatively, complexes were diluted in pure water. Fluorescence was measured after each dilution and corrected for volume changes and for salt-mediated inhibition of EtBr/DNA fluorescence (using matched EtBr/DNA controls without pLL).

RESULTS

Influence of Charge Ratio on Electrophoretic Mobility of DNA

Self-assembly of pLL/DNA complexes was examined by agarose electrophoresis, using plasmid DNA trace labeled with Cy-3. Complexes were formed in water, using the single-step procedure, allowed to stabilize for 1 h, and then subjected to agarose gel electrophoresis (Fig. 1A). At N:P ratios of 0.2 and 0.4, more than 92% of the total DNA showed bands corresponding to supercoiled and relaxed forms that comigrated with free Cy-3-labeled DNA. In contrast, complexes formed at N:P ratios of 1.0–1.4 all showed fluorescence that was almost entirely retained in the well (more than 93% in each case, Fig. 1B). Complexes formed at N:P ratios of 0.6 and 0.8 showed intermediate behavior, with 74.1 ± 13.3 and 33.5 ± 6.5% behaving as free DNA, respectively, and 25.9 ± 13.3 and 66.5 ± 6.5% retained in the well. Complexes formed at such N:P ratios showed no obvious signs of banding, rather evidence of smearing suggesting that nonneutralizing binding of pLL to the Cy-3-DNA can affect its migratory capacity. At certain N:P ratios, e.g., 0.4 and 0.6, there was also evidence for DNA migrating in the gel even faster than supercoiled

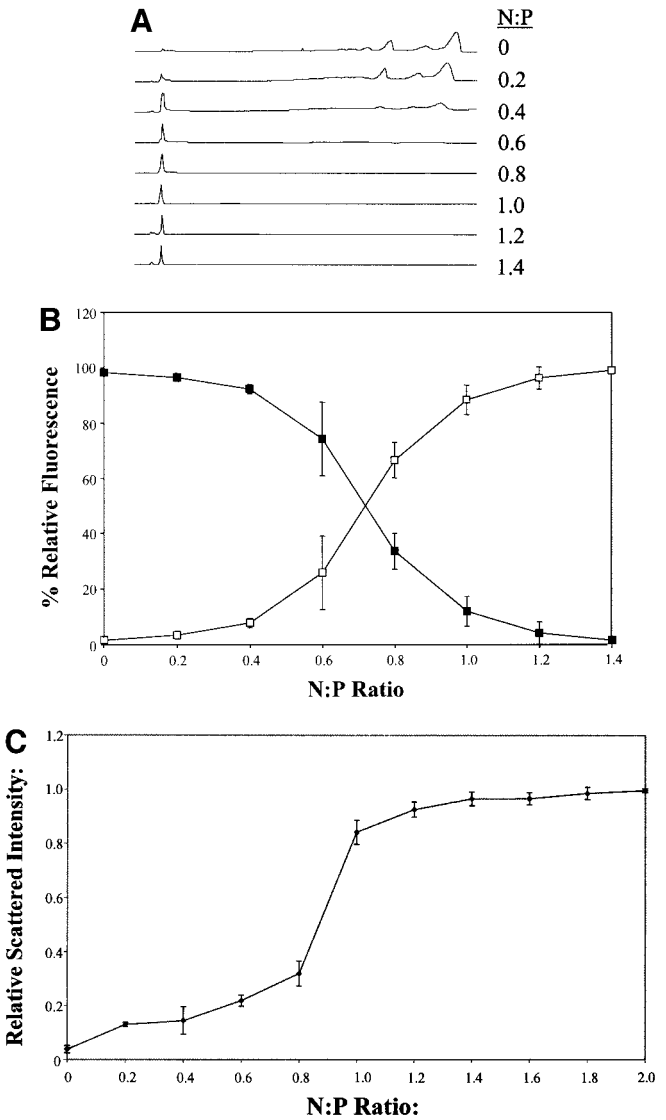

FIG. 1. Cy3-DNA (10 μg) was complexed with pLL (111 KDa, N:P ratio 0–1.4) and electrophoresed, and the signal in individual lanes was quantified using a Typhoon Fluorimager (A). The distribution of DNA retained in the well (□) or entering the gel (■) is shown in (B). Complex formation was also monitored by light scattering (C), with polycation titrated into a DNA solution (20 μg/ml) in pure water, and the intensity of scattered light measured.

DNA (so called "gel acceleration"). This is thought to represent semicondensed DNA, still bearing a strong net negative charge but with a more compact structure that enables easier penetration through agarose.

Some preparations (e.g., N:P 0.4) contain both DNA with full electrophoretic mobility and DNA that is retained in the well, suggesting that disproportionation occurs during complex formation. Despite this possibility for disproportionate binding, there was very little evidence for DNA condensation at very low N:P ratios; for example, at N:P 0.2, only 2% of the DNA shows decreased mobility. If pLL does interact electrostatically with DNA at these low N:P ratios it appears to be easily released under electrophoresis conditions.

Influence of Charge Ratio on Intensity of Light Scattering

Plasmid DNA was mixed with pLL (111 kDa) by single-step additions, at N:P ratios 0–2. The intensity of scattered light was measured and found to increase up to N:P 1.0, with particularly steep rise between N:P 0.6 and 1.0 (Fig. 1C). Above N:P 1.0 there was minimal further increase. These data suggest few changes in DNA morphology at low N:P values, with self-assembly of discrete nanoparticles occurring between N:P 0.8 and 1.0.

Effect of EtBr Concentration on the Fluorescence Observed with pLL/DNA Complexes

The profile of EtBr/DNA fluorescence observed during pLL binding to DNA was dependent on the concentration of EtBr used. Low EtBr concentrations (400 ng/ml) showed a sigmoidal curve, falling sharply over the N:P ratios 0.75–1.0. In contrast, high EtBr concentrations (3 μg/ml) produced a more linear fall across the entire N:P range. Intermediate EtBr concentrations showed a gradual shift from sigmoidal to linear profile (Fig. 2A). These data are compatible with a finite number of EtBr binding sites in DNA that become gradually unavailable with increasing pLL binding. For high EtBr concentrations (where most of the sites are occupied in free DNA) this results in a linear fall in fluorescence. In contrast, for low EtBr concentrations (where 100% fluorescence corresponds to occupation of only a fraction of the sites available in DNA) fluorescence remains high during the early part of the condensation but is lost rapidly toward the end of condensation.

Fluorescence observed using the highest concentrations of EtBr (2.5–3 μg/ml) never fell as low (in % terms) as those obtained using low EtBr concentrations. This suggests that high levels of intercalated EtBr stabilize the DNA structure against complete inhibition of fluorescence by pLL. Addition of the polyanion pLAA restored fluorescence to 100% when a low EtBr concentration was used, although higher EtBr concentrations allowed a lower maximum percentage restoration. This supports the model of a finite number of EtBr/DNA sites and suggests that pLAA is able to release only a fraction of them, presumably resulting in the formation of a ternary pLAA/pLL/DNA complex wherein some of the DNA is available for EtBr binding. The formation of a ternary complex was confirmed by agarose gel analysis, where addition of pLAA to pLL/DNA complexes led to a restoration of fluorescence,

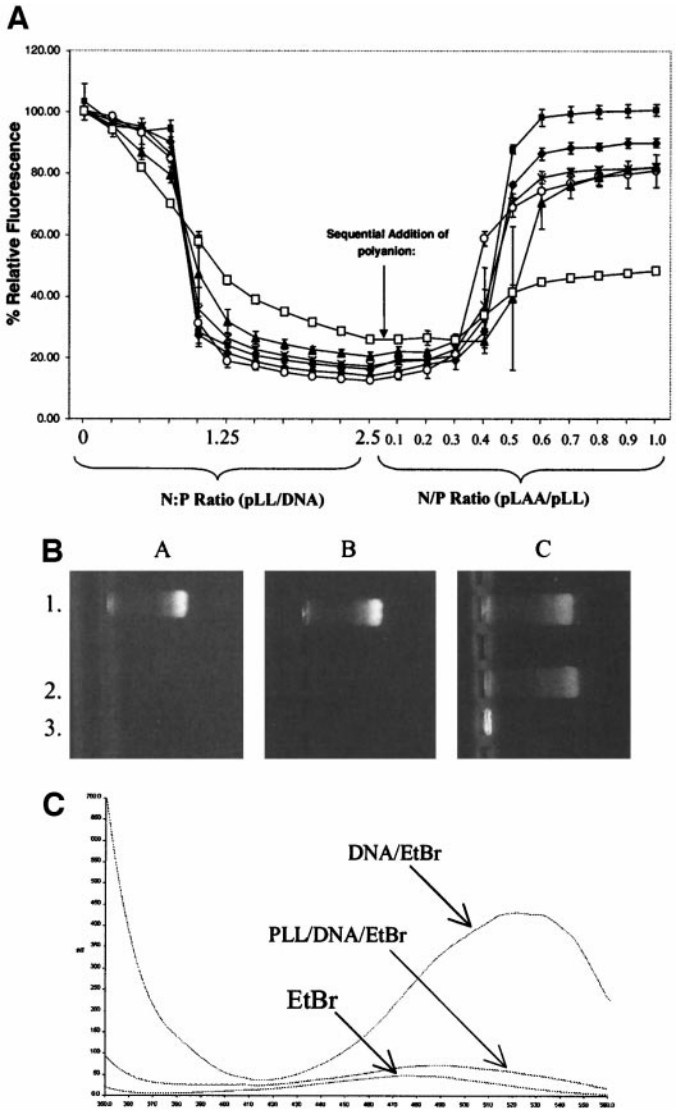

FIG. 2. Complexes were formed (N:P 2.5) as described in the text, in the presence of EtBr, monitoring inhibition of EtBr fluorescence to indicate particle formation (A). EtBr concentrations were 0.4 (■), 1.0 (◆), 1.5 (○), 2.0 (×), 2.5 (▲), and 3.0 (□) μg/ml. Subsequently, complexes were destabilized by the sequential addition of pLAA, and restoration of fluorescence was monitored. Expulsion of EtBr from DNA during particle formation was demonstrated by electrophoretic analysis (B(A)) of DNA/EtBr (lane 1), DNA alone (lane 2), and pLL/DNA/EtBr (lane 3) on an EtBr-free agarose gel. The gel was then incubated in heparin solution (B(B)), and DNA in the gel visualized with EtBr (B(C)). Excitation spectra for EtBr, DNA/EtBr, and pLL/DNA/EtBr were also analyzed (C) using a fixed emission wavelength (590 nm).

although the DNA was unable to enter the agarose gel (data not shown).

Evaluation of the Mechanism of Inhibition of EtBr/DNA Fluorescence

Two assays were used to determine whether pLL-mediated inhibition of EtBr/DNA fluorescence was caused by fluorescence quenching or by expulsion of EtBr from the DNA structure. In the first study, DNA was preincubated with EtBr (400 ng/ml), complexed with pLL (111 kDa, N:P 2.0), and electrophoresed on an EtBr-free agarose gel (1%, 45 min, 90 V). Controls included plasmid DNA alone, with and without EtBr. The only fluorescence visible on the gel was from the free plasmid with EtBr (Fig. 2B). This did not change following dissociation of the complexes *in situ* by incubation of the gel, postelectrophoresis, in heparin solution. Subsequent incubation of the gel in a solution containing EtBr produced fluorescence from all DNA samples. Free plasmid DNA showed mobility unaffected by preincubation in EtBr, whereas the pLL/DNA complexes showed fluorescence only in the well, as expected.

Since EtBr is not removed from free DNA during electrophoresis (Fig. 2B), and complexes formed in the presence of EtBr did not fluoresce even following disruption with heparin, this indicates that EtBr is expelled during complexation.

The fluorescence excitation spectra for EtBr are also consistent with its expulsion from DNA during condensation (Fig. 2C). EtBr in free solution shows an excitation peak at 480 nm approximately, shifting to 520 nm following intercalation into DNA. Following pLL complexation the maximum shifts back to about 490 nm, suggesting loss of the intercalated species. Low-level fluorescence from residual intercalated EtBr (15% approx, Fig. 2A) is thought to combine with the fluorescence of EtBr free in solution to give the intermediate spectrum observed.

Determination of the Stability of pLL/DNA Complexes at High Dilution

pLL/DNA complexes were formed (N:P ratio of 2.8, pLL molecular weights 4.0K, 29.3K, and 111K) in the presence of EtBr (400 ng/ml). All complexes were found to be stable to serial dilutions in water, judged by a lack of restoration of EtBr fluorescence. Analysis using photon correlation spectroscopy of the sizes of complexes based on pLL 29.3 and 111 kDa showed no changes during dilution of complexes (data not shown), with hydrodynamic radius unaffected after up to 16-fold dilution. These dilutions represent the highest dilutions that could be tested subject to the sensitivity limitations of the analytical techniques.

To determine stability in physiological salts, complexes were formed in water (as above) and then the salt concentration was raised to physiological levels. EtBr/DNA fluorescence was measured and corrected for the slight quenching effect of PBS. Complexes formed between medium (29.3K)- and high (111K)-molecular-weight pLL showed a relative increase in fluorescence to 50% of the initial value (Fig. 3),

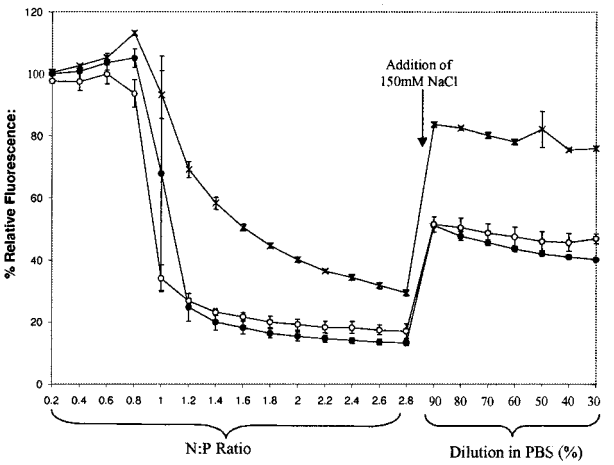

FIG. 3. To investigate the effect of dilution in physiological salts, complexes were formed by sequential addition of pLL to DNA in water containing EtBr (0.4 μg/ml), using pLL of molecular weight average 4K (×), 29.3K (◆), and 111K (○). Ten percent (v/v) of 10× PBS was then added. After 30 min the change in EtBr/DNA fluorescence was measured and complexes were subjected to serial dilutions in PBS. Changes in fluorescence were monitored and corrected for the dilution factor and the slight quenching effect of PBS on EtBr/DNA fluorescence.

whereas complexes formed using low-molecular-weight pLL (4.0K) were more susceptible to destabilization, with fluorescence increasing to around 80% of the value for DNA/EtBr alone. Effects of further dilution in PBS showed no progressive destabilization of the complexes. The slight fall in fluorescence at higher dilutions is thought to be an artifact resulting from loss of signal linearity due to proximity to the machine limit of fluorescence detection. These data suggest that pLL/DNA complexes are generally stable to dilution in water or 10 mM Hepes buffer; however, complexes formed using lower-molecular-weight polycations are more prone to destabilization when introduced into physiological concentrations of salt.

DISCUSSION

In this study we have compared three different assays that are often used to study self-assembly of DNA with polycations, namely, particle formation by light scattering, loss of EtBr fluorescence, and changed electrophoretic mobility of DNA on agarose gels. Probably the simplest measure is the light scattering approach, which indicates physical formation of nanoparticles capable of reflecting light. However, this approach requires relatively pure samples, and it is difficult to employ it productively in typical biological samples. Information gained using light scattering was therefore compared with that obtained using the other, more robust, approaches to determine which is a better indicator of particle formation.

Analysis of DNA mobility changes showed evidence for disproportionation of pLL binding to plasmid DNA, with some plasmids binding large amounts of pLL and losing electrophoretic mobility, and others binding little or no pLL and behaving as free plasmid on electrophoresis. Such disproportionate behavior of plasmid DNA when mixed with polycations at low N:P ratios is in concordance with recently published articles (9). This effect could result from cooperative binding of cationic polymers to plasmids, possibly promoted by the formation of locally charge-neutral, relatively hydrophobic, regions that facilitate the binding of further cationic polymers to adjacent regions of the DNA. Considering the gel shift information itself, substantial amounts of DNA could be retained in the well at relatively low N/P ratios (e.g., 25% at N/P 0.6) when particle formation (as gauged by light scattering) was very limited; this suggests that the gel shift assay determines formation of plasmids that bind a certain critical amount of polycation, assuming modified charge or structural properties that change their electrophoretic behavior, but may not actually reflect formation of discrete nanoparticles.

Monitoring loss of EtBr/DNA fluorescence can also be used to determine DNA condensation; however, the fluorescence profile obtained is strongly dependent on the concentration of EtBr used. This partly reflects the ability of EtBr to stabilize DNA against polyelectrolyte condensation, but also results from the ability of small EtBr concentrations to give 100% fluorescence signals even when the majority of binding sites are no longer available. The assay must therefore be used with caution, including when it is used to monitor destabilization of complexes since, at low EtBr concentrations, 100% restoration of fluorescence may represent availability of only a fraction of the binding sites in DNA. Nevertheless, carefully interpreted, this assay generates results that are similar to those from light scattering, and provides a simple and sensitive means to monitor particle formation. Each of the assays reported was also performed using linear calf thymus DNA, with the results generated being virtually identical; hence any role played by the constrained topology of circular DNA on influencing condensation properties is thought to be minor.

A range of techniques show that loss of fluorescence coincides with expulsion of EtBr from the DNA, rather than quenching. In addition to the two assays shown, we have also formed pLL/DNA complexes in the presence of limiting amounts of EtBr and surface modified them with a multivalent reactive polymer based on poly(*N*-[2-hydroxypropyl]methacrylamide) (pHPMA), as described elsewhere (10). The resulting polymer-coated complexes are stable to disruption by polyanions, and show no increase in EtBr/DNA fluorescence following addition of pLAA. However, addition of free

DNA results in return of fluorescence, indicating that the EtBr is free in solution and not trapped within the coated complex.

Finally, previous studies using atomic force microscopy showed that low-molecular-weight pLL produced very small (25-nm) complexes at high N/P ratio with DNA (11), spawning the hypothesis that the small size reflected a dynamic structure capable of continual rearrangement. We therefore evaluated the ability of salts to destabilize pLL/DNA complexes, measured by return of EtBr fluorescence, and found, as predicted, that complexes formed using small pLL are more easily disrupted by physiological salts. Small complexes such as these are attractive for size-restricted gene delivery applications, for example, where vectors must extravasate through small interendothelial cell fenestrations to gain access to target tissues. However, to apply these small complexes in biological environments it will be necessary to stabilize them against disruption by salts. Several techniques are available to achieve this, including surface modification with multivalent pHPMA, as described above. The use of such complexes is particularly enticing following arrival within the cell, since removal of the stabilizing polymer should enable salt-assisted dissociation of the complex, providing the DNA in a form suitable for efficient transcription. In addition, such systems would be ideally suited for delivery of mRNA, where it is known that the nucleic acid must be released within the cytoplasm in a relatively free form, to undergo efficient ribosomal translation (12).

ACKNOWLEDGMENTS

We are grateful to the Cancer Research Campaign and the Biotechnological and Biological Sciences Research Council for support.

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