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The Molecular Structures of Poly(ethylene glycol)-Modified Nonviral Gene Delivery Polyplexes

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Abstract: Polycations can complex with DNA and form compact nanoparticles (polyplexes) to facilitate gene transfection. Recently, poly(ethylene glycol) (PEG) was incorporated in the polyplexes to improve their in vivo stability and defer body clearance. This work provided a direct look using atomic force microscopy at the molecular conformation of PEG molecules on the polyplex surfaces. Individual PEG strands were seen to extend from the compact cores and intertwined with each other to form the protective surface layer.

Keywords: Polyplex; poly(ethylene glycol); atomic force microscopy; photon correlation spectroscopy; morphology

For successful gene therapy applications, the development of safe and effective gene delivery systems is essential.[1] Several cationic polymers including polylysine (PLL), polyethylenimine (PEI), and polyamidoamine (PAMAM) dendrimer have been shown to be able to form complexes with plasmid DNA and facilitate gene transfection both in vitro and in vivo.[2,3] The complexation process was believed to be based on polyelectrolyte interaction and has been studied extensively.[4,5] Upon a critical extent of charge neutralization, the extended DNA chains would bend or undergo secondary structural changes to collapse into compact nanoparticles such as rods, toroids, or spheroids with sizes ranging from 50 to 300 nm. The so-called condensation or compaction effect is considered essential for facilitating gene delivery, and the molecular structure of polycation/DNA complexes is critical for the mechanism of gene transfection in vitro.

But for in vivo gene therapy applications, especially by systemic administration, there seem to be more challenges. During circulation and distribution in a biofluid, the polyplexes would encounter many disruptive events, such as aggregation with serum proteins, attack from the immune system, and destabilization by extracellular matrixes, before they reached the target cells.[6] For protection of the polyplexes' stability and for avoidance of the clearance in the body, surface modifications using hydrophilic polymers (mainly PEGs) were proposed.[7,8] Such strategies have been very successful for several other biological nanoparticles, including the "stealth" liposome formulation and the protein pegylation products.[9−11] It is generally accepted that PEG molecules that are grafted onto the surface of the particles would form a flexible but protective layer along the surface to render the steric stabilization effect.

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There have been increasingly more studies reported taking this approach to improve in vitro and in vivo gene delivery.[7,12−15] PEG-polycation block copolymers or PEG-grafted polycations with various molecular structures and molecular weights were synthesized and tested.[16−19] Reduction of serum protein binding and extension of circulation time were observed. The in vivo behavior is believed to be highly dependent on the surface molecular properties of the polyplexes, especially the size, shape, surface density, and molecular conformation of the surface PEG molecules.

Therefore, we provide in this study a close-up look at PEG-*g*-PLL/DNA polyplexes formed under various conditions using atomic force microscopy (AFM). Most interestingly, we were able to see for the first time the detailed molecular morphology of PEG molecules at the surface of the polyplexes. The PEG chains were shown to extrude from the condensed core and form specific organization patterns. These micrographs provide the first visual evidence of the "stealth effect" of surface PEG modifications.

We used high molecular weight (MW) polylysine hydrochloride (PLL, MW = 28500) purchased from Sigma Chemical (St. Louis, MO) as the polycation backbone in our studies. PLL is a commonly used and the most extensively studied polycation for DNA compaction. Two different-sized PEG molecules with MW 7000 and 20000 were used to conjugate with PLL. mPEG-NHS molecules were kindly provided by Professor Huang Junlian. PEG-*g*-PLLs were synthesized by coupling mPEG-NHS to the amino groups of PLL by mixing them at specific molar ratios ($mPEG_{7000}$-NHS:PLL = 6:1; $mPEG_{20000}$-NHS:PLL = 4:1) in phosphate buffer (pH 8.0) and incubating for 12 h. The PEG grafting was confirmed by capillary electrophoresis (CE) analysis (100 mM pH 2.0 phosphate buffer, UV detection at 200 nm), which showed that 77(±5)% of the PLL were coupled with PEG_{7000}, and 45(±9)% of the PLL were coupled with PEG_{20000}. The CE analysis also showed there were one, two, or three PEG chains per PEG_{7000}-*g*-PLL molecule and one or two PEG chains per PEG_{20000}-*g*-PLL molecule.

We used the commonly employed gel band shift assay to study the DNA compaction property of these PEG-*g*-PLL molecules. Polyplexes were prepared at various weight ratios of polycation to DNA by quickly injecting the plasmid solution into the polymer solution using a wide end pipet tip. The resulting polyplexes were examined by gel band shift assay as shown in Figure 1. Complete polyplex formations were observed at 1:1, 6:1, and 10:1 (w/w) for PLL (Figure 1A), PEG_{7000}-*g*-PLL (Figure 1B), and PEG_{20000}-*g*-PLL (Figure 1C), respectively. These points were used as the approximate charge neutralization points when we estimated PLL/DNA charge ratio in later experiments.

The resulting polyplex sizes were examined using photon correlation spectroscopy (PCS) on a Malvern ZetaSizer 3000HS (Malvern Instruments Ltd., Worcestershire, U.K.). Figure 2 shows the mean diameters (*Z*-average) of the various polyplexes formed at different polycation/DNA weight ratios. Both PLL and PEG-*g*-PLL can condense DNA molecules into discrete particles with sizes of around 100−200 nm. But when the PLL/DNA ratio was approaching the charge neutralization point (weight ratio 1:1), the polyplexes formed were prone to aggregation, resulting in drastically increased particle sizes. Such instability was commonly reported by many other studies and considered as one of the major limitations of the polycation gene delivery system.[2] But indeed, the incorporation of PEG significantly improved the polyplex size stability. The PEG-*g*-PLL/DNA polyplexes were all about 100−200 nm across all the polycation/DNA weight ratios examined. There was no detectable particle aggregation under both charge neutralization conditions. The mean diameter of the PEG_{20000}-*g*-PLL polyplexes was a little bigger than that of the PEG_{7000}-*g*-PLL polyplexes, indicating the effect of the longer PEG chain.

Because the condensation status of the PLL/DNA complexes was considered essential for their activities, we thus examined the microscopic structure of PEG-*g*-PLL/DNA

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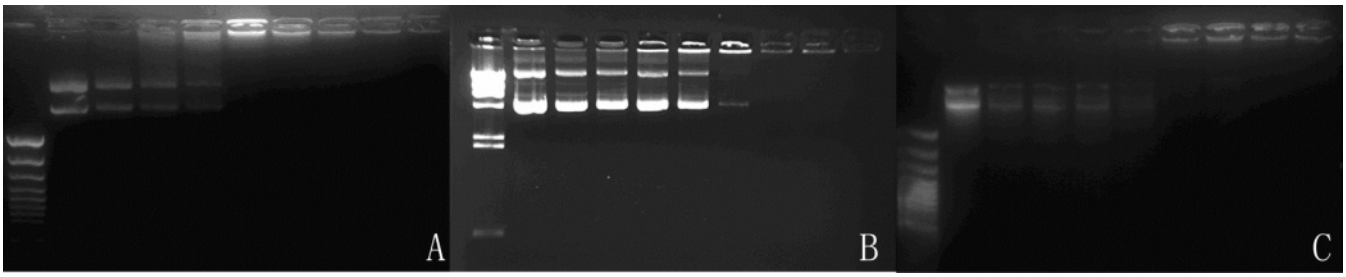

Figure 1. Gel band shift assay of PLL/DNA, PEG_{7000}-*g*-PLL/DNA, and PEG_{20000}-*g*-PLL/DNA polyplexes. (A) Lane 1: DNA ladder. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10: weight ratio of PLL/DNA = 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 10, respectively. (B) Lane 1: DNA ladder. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10: weight ratio of PEG_{7000}-*g*-PLL/DNA = 0, 0.5, 1, 2, 4, 6, 9, 12, 20, respectively. (C) Lane 1: DNA ladder. Lanes 2, 3, 4, 5, 6, 7, 8, 9, 10: weight ratio of PEG_{20000}-*g*-PLL/DNA = 0, 1, 4, 6, 8, 10, 12, 15, 20 respectively.

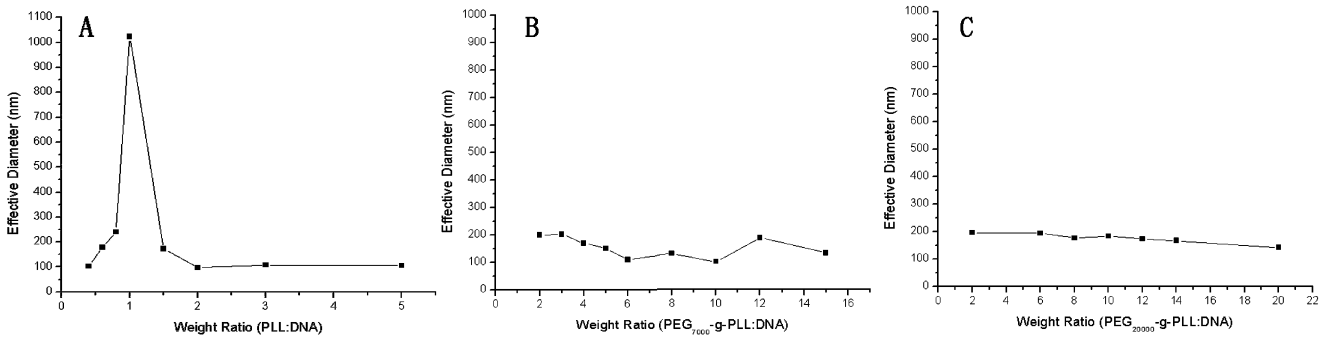

Figure 2. Particle sizes *Z*-averages of polyplexes at different weight ratios in HEPES (4 mM, pH 7.2) by photon correlation spectroscopy: (A) PLL/DNA polyplexes; (B) PEG_{7000}-*g*-PLL/DNA polyplexes; (C) PEG_{20000}-*g*-PLL/DNA polyplexes.

complexes. There have been extensive studies published concerning the theory as well as experimental observations of the DNA condensation process resulting from various polycation binding including PLL.[4,20−22] It is generally accepted that upon a critical extent of charge neutralization, the extended and flexible DNA chains will fold into rigid and specific conformations. Electron microscopy (EM) was generally used to study DNA condensation structures. Nanoscopic particles that looked like "toroids" and "rods" were found to be the characteristic structures after condensation. Recently, newer techniques such as scanning force microscopy (SFM) and AFM have been developed as better systems to study biological samples, and several studies have provided more insights into the condensation process.[7,23,24] Using SFM, Dunlap and co-workers first showed the possibility to visualize individual DNA molecules. They coated mica with poly-L-ornithine to make the surface positively charged, so that complexes with excess negative charges could stick to the mica surface. They used this technique to follow the DNA condensation process with cationic lipid dioctadecylamidoglycylspermine (DOGS) and polycation PEI.[23] But since only complexes with excess DNA can be seen, what they observed were mostly intermediates which contained partial condensed structure. Vijayanathan et al. examined the polyamine/DNA complexes in solution using AFM.[24] They also found various partially formed toroids mixed with DNA strands and loops.

In our study, we examined polyplexes with complete charge neutralization. The polyplexes were prepared at different weight ratios, and about half an hour elapsed before AFM study. A drop of the sample (5 μL each) was deposited onto a freshly cleaved mica surface (Agar Scientific Ltd., U.K.), followed by a 5 min wait for adsorption. Because the bare mica surface is negatively charged, polyplexes with excess positive charges can stick easily. Excess fluid was then removed with a filter paper, and the samples were then dried under a vacuum dryer for at least 6 h. All the imaging was taken with the tapping mode in the Nanoscope IIIa system (Digital Instruments, Santa Barbara, CA). A 200 ±

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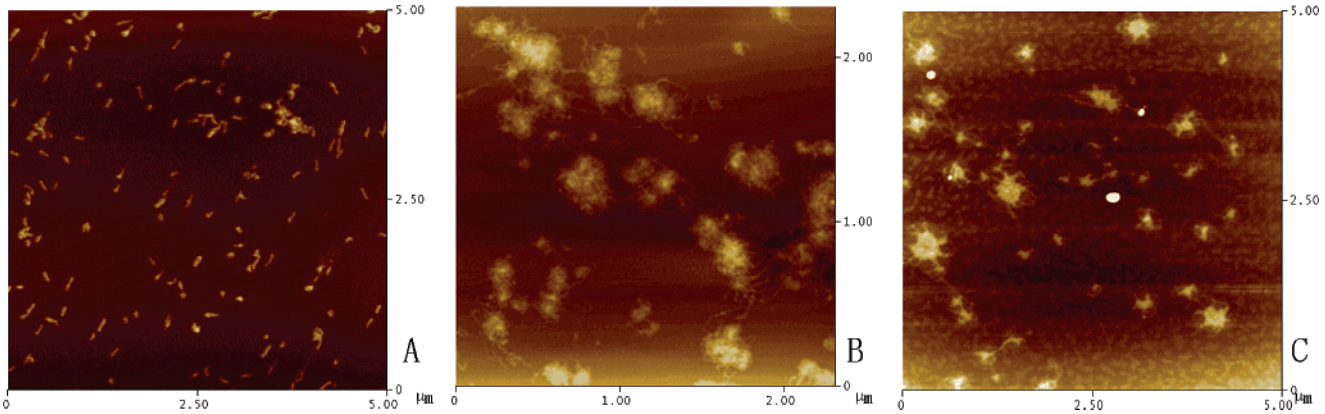

Figure 3. Atomic force microscopic pictures of polyplexes: (A) PLL/DNA polyplexes (PLL:DNA = 2 w/w); (B) PEG_{7000}-*g*-PLL/DNA polyplexes (PEG_{7000}-*g*-PLL:DNA = 10 w/w); (C) PEG_{20000}-*g*-PLL/DNA polyplexes (PEG_{20000}-*g*-PLL:DNA = 15 w/w).

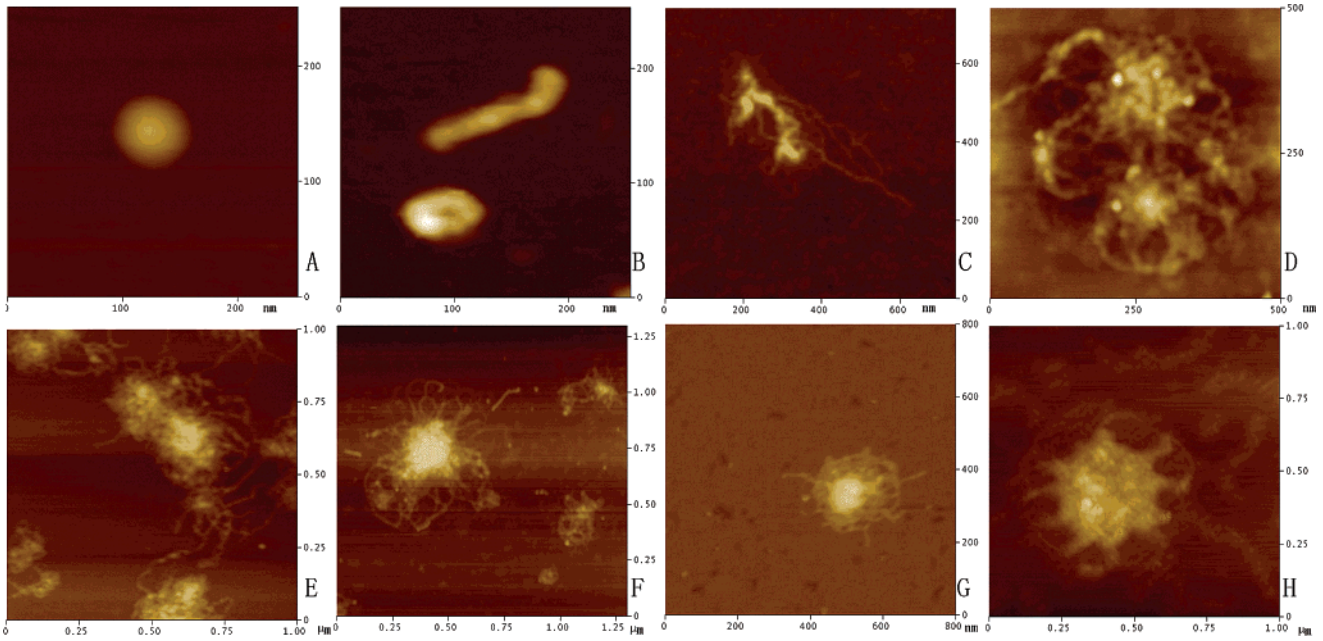

Figure 4. Close-up atomic force microscopic pictures of various polyplex structures: (A, B) PLL/DNA polyplexes (PLL:DNA = 2 w/w); (C) PEG_{7000}-*g*-PLL/DNA polyplexes (PEG_{7000}-*g*-PLL:DNA = 6 w/w); (D, E, F) PEG_{7000}-*g*-PLL/DNA polyplexes (PEG_{7000}-*g*-PLL:DNA = 10 w/w); (G) PEG_{20000}-*g*-PLL/DNA polyplexes (PEG_{20000}-*g*-PLL:DNA = 10 w/w); (H) PEG_{20000}-*g*-PLL/DNA polyplexes (PEG_{20000}-*g*-PLL:DNA = 15 w/w).

5 μm long nanocontact silicon cantilever (NSC11/50, Silicon-MDT Ltd., Moscow, Russia) was used.

Figure 3 showed the most typical polyplex structures observed when PLL, PEG_{7000}-*g*-PLL, and PEG_{20000}-*g*-PLL were used to compact DNA. The PLL/DNA complexes were about the same as what have been described in the literature: spherical particles, toroids, and rods (Figures 3A and 4A,B). But the PEG-*g*-PLL/DNA polyplex images were surprisingly sophisticated, containing specific surface molecular features as shown in Figure 3B,C.

For PEG_{7000}-*g*-PLL/DNA polyplexes, there were many flexible filamentous structures visible on the surface of the compacted particles (Figures 3B and 4C−F). Compared to the PLL/DNA polyplex structure, we believe the filaments are individual PEG chains. With increasing PEG_{7000}-*g*-PLL:DNA ratio, more strings were visible on the surface. These strings seem flexible, but sometimes they would also loop back and tangle with other strings. All these surface features combined indeed made it look like there was a protective shell around the compact core, as suggested by the initial hypothesis, and should account for the increased complex stability of PEG-grafted polyplexes. In fact, Figure 4D provided a clear visualization of two neighboring complexes with contacting PEG filaments but having enough space between them to prevent aggregation.

The surface shell structure was again different when longer PEG chains were used. In PEG_{20000}-*g*-PLL/DNA polyplexes formed at a 10:1 weight ratio, the surface strings can be seen to organize into cobweblike networks (Figure 4G). There were also some loose ends hanging out of the weblike structure. But when more PEG_{20000}-*g*-PLL were used in PEG_{20000}-*g*-PLL/DNA polyplexes (15:1 weight ratio), the

surface surrounding web structure became even denser and more angular, and fewer loose ends were visible. The complexes looked a bit more like starfish (Figures 3C and 4H). The polyplex size distributions agreed well with the PCS results, although the particles observed under AFM were a little bigger than the PCS measurements, due to a "flattening effect" commonly existing in AFM studies when 3-dimensional particles spread out on the surface under forces.

To our knowledge, Figures 3 and 4 provided the first clear images of filamentous molecular structures on the surface of nanoscopic particles. There had been some attempts to look into the molecular structure of PEG modifications on lipid monolayer or other surfaces using AFM.[19,25] Both studies observed surface roughness changes after PEG grafting, and the PEG layer height was estimated to be around 5 nm for PEG chains with a MW of 5000. But no individual chain structures were visible. When the microscopic structures of PEG-modified polyplexes were examined in several early studies, the morphology reported was similar to that of simple PLL/DNA polyplexes. Toroids and rods were seen in the EM studies by Mannisto and co-workers,[26] while generally round and discrete particles were commonly observed in the works by Seymour and colleagues using either EM or AFM.[7,16,20] But most of these works used PEG molecules with a MW of less than 3000, which might be the reason why surface PEG chains were not visible in their studies. Only Peterson et al. in their comprehensive studies used grafted PEG chains of MW 20000.[18] AFM images of their polyplexes had some hint of surface roughness, but no clear features were visible. Compared to all those studies, we were using PEG-*g*-PLL molecules with a rather small grafting density, but rather long PEG chain. The resulting polyplexes contained excess PEG-grafted PLL molecules and were positively charged. In addition, we performed the AFM imaging on dried samples using the tapping mode. All these may contribute to the fact that we were able to see the finer structure of PEG-modified polyplexes.

It is also possible that the strings could be DNA, PLL, or free PEG fibers that were uncompacted from the polyplexes. Therefore we performed several control studies. First we tried to visualize individual DNA molecules and excess DNA contained in PLL/DNA polyplexes (PLL:DNA = 0.5 w/w). But since the mica surface is negatively charged, DNA molecules and negative PLL/DNA polyplexes will not stick unless certain surface coating was done as suggested by Dunlap et al.[23] Then, polyplexes with higher weight ratios of PLL to DNA (weight ratio 3:1, 5:1, and 10:1) were also examined. The polyplexes should contain a great excess of PLL molecules, but the polyplexes all had very smooth surfaces. So the abundant filamentous surface structures we observed on PEG-*g*-PLL/DNA polyplexes were probably not PLL. Furthermore, nothing can be seen with pure PEG samples. We hypothesized that the individual PEG chains randomly dispersed in the solution may be too flexible and too small to be seen, or they may get lost during the drying step.

On the basis of the various control studies, we are convinced that the filamentous surface structures were not PLL chains or individual DNA molecules. There was a possibility that the strings were PLL/DNA complexed fibers uncompacted in the presence of PEG. However, we tend to think it is unlikely because the strings we observed organized in different patterns with different PEG chain lengths. It was the PEG chains that apparently played the most crucial role in the molecular shell structure. Therefore, we are confident to believe that the "compact core−molecular shell" structure observed is indeed a specific feature of PEG-containing polyplexes.

It is interesting to observe the complex molecular pattern of the PEG shells on the surface. It was usually believed that PEG molecules on the surface took random and highly flexible conformations. We showed here, however, that when there were enough PEG chains on the surface and when the PEG chains reached a certain length, they may organize into various structural patterns. These surface features were surprisingly clear and distinctive in AFM images. Considering the fact that we failed to see dispersed PEG and PEG-*g*-PLL molecules in solution, we would like to hypothesize that it was because there were a lot of PEG molecules concentrated and confined on the polyplex surface that they could interact and tangle with each other to form specific and visible features. The PEG_{7000} strings were mostly extended. They sometimes tangled together to form elongated filaments, folds, and loops. The PEG_{20000} chains would, however, wind around and form weblike structures. Notably, the surface web conformation was quite regular, especially when the weight ratio of PEG_{20000}-*g*-PLL to DNA increased to 15, which indicated some kinds of periodical arrangement. The detailed mechanism for the formation of these different features is not clear. It would be interesting to find out in further studies whether these distinctive structures would have implications in gene delivery.

The visualization of PEG molecules on the surface also shed some light into a long-standing question of PEG-PLL/DNA compaction. Since DNA compaction by polycations is believed to be highly dependent on the polycation charge density, there has been always a concern that the bulky hydrophilic chain in the middle of the polycation would decrease charge density, interfere with DNA condensation, and destabilize polyplex structure. Several studies promote the so-called post-PEG modification strategy so the PEG modification could be done after the particle formation and only on the surfaces.[8] In our studies, we used the pre-PEG modification approach in which PEGs were directly conjugated to the polycation backbone before interaction with

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DNA. On the basis of our AFM micrographs, both PEG_{7000}-*g*-PLL and PEG_{20000}-*g*-PLL can condense DNA well into discrete, spherical particles. The core of the particles looked all compact, with sizes and densities similar to those of PLL/DNA particles. So it seems that, during complex formation, the charge neutralization effect was sufficient to cause DNA condensation to form a compact structure, while the PEG-*g*-PLL backbone was flexible enough to reorganize without trapping too many extended PEG chains inside the core. The pre-PEG modification method is certainly more economical, and it would enable better quality control in future gene therapy applications.

In summary, we present in this paper a study of the effect of PEG modifications of the traditional PLL/DNA polyplexes. AFM micrographs show, for the first time, the detailed morphology of PEG molecules on polyplex surfaces. These specific PEG features on the surface of polyplexes are believed to contribute to the in vivo stability and transfection activities of PEG-modified polyplexes.

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