論文の内容の要旨
Abstract of Dissertation

論文題目　Engineering of tethered PEG density on polyplex micelle to optimize its structure for systemic application
（全身投与型遺伝子内包高分子ミセルの構造最適化のための表面ポリエチレングリコール密度の設計）

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（本文）(Abstract)

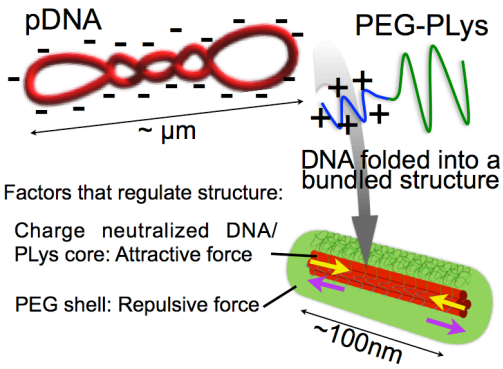
1. Introduction

Non-viral gene therapy attracts much attention as a relatively safe approach in the on-going quest to treat not only acquired, but also inherited intractable diseases. Nonetheless, putting this concept into systemic application requires designing an effective nanotechnology-based DNA delivery system having adequately high retention in blood circulation so as to maximize the possibility for its fragile DNA cargo to contact target sites. This is a huge challenge because foreign particles are easily detected by opsonins (proteins, which recognize and mark foreign particles), which subsequently encourage their removal from the blood compartment through reticuloendothelium system (RES) uptake.

To this point, surface modification by tethering poly(ethylene glycol) (PEG) onto nanoparticles is widely acknowledged as an effective strategy to improve retention in blood compartment, as a layer of crowded tethered PEG can suppress adsorption of opsonins and make PEGylated nanoparticles less palatable for RES uptake. This strategy is also applicable to DNA delivery system, but PEG crowdedness of this system has not been identified although it should be a crucial factor affecting delivery aspect. This limits the understanding of relationship between tethered PEG crowdedness and systemic circulation. Focusing on polyplex micelle gene carriers prepared from polyion complexation of DNA and PEG-polycation block copolymers (Scheme 1), this study seeks to (1) identify achievable range of PEG crowdedness for typical rod-shaped structures of this system, (2) establish their relationship to biological performance in systemic application, (3) provide governing principles in engineering tethered PEG to optimize biological performance, and (4) ultimately present a revolutionary new polyplex micelle system according to the elucidated principles.

2. PEG crowdedness of polyplex micelles

To investigate achievable range of PEG crowdedness for polyplex micelle gene carriers, PEG tethering density (σ) was examined for polyplex micelles based on PEG-poly(L-lysine) block copolymers (PEG-PLys) (PEG M_w 12,000g/mol (PEG 12K)) prepared from varying PLys segment length (degree of polymerization, DP 19, 39, and 70), because PLys DP may affect electrostatically-driven tethering number of PEG-PLys to pDNA, and thus possibly σ. Note that σ evaluation is difficult for most gene delivery system because the two requirements (core surface area and tethering number) are missing, but rod-shaped polyplex micelles have been an exception because detailed packaging structure of pDNA were revealed for this system through our previous study, namely single pDNA molecule was packaged into rod structure through highly regulated folding fashion, with rod length l_n at folding number n being quantized to 1/(2(n+1)) multiples of pDNA contour length,



Scheme 1. Polyplex micelles formation through polyion complexation of PEG-PLys and pDNA

offering necessary geometry to calculate surface area. Moreover, PEG tethering number can be determined using ultracentrifuge analysis, thereby fulfilling requirements to estimate σ. Taking consideration that there is a distribution of *n* in each sample, number-average σ (<σ>) of polyplex micelles is presented (Table 1). From the results, shorter rods formed by longer PLys DP were found to be associated with lower σ, while longer rods formed by shorter PLys DP were found to be associated with higher σ. It is also convenient to express σ in reduced tethering density $\pi R_g^2\sigma$ (Table 1), defined as number of tethered chains that occupies a projected area of isolated polymer chain (πR_g^2), because $\pi R_g^2\sigma$ provides a clearer picture of crowdedness, and can predict conformation (see footnote of Table 1): PEG was squeezed for PLys 19 and 39, while mushroom for PLys 70. This prediction was confirmed by measuring PEG height obtained from careful analysis of Zernike phase contrast (ZPC) cryo-TEM images of polyplex micelles at locally concentrated region. It is thus evident that longer polyplex micelles (short PLys DP) had PEG height pertaining to squeezed conformation (proving higher $\pi R_g^2\sigma$), while shorter polyplex micelles (long PLys DP) had PEG height pertaining to mushroom conformation (proving lower $\pi R_g^2\sigma$) (Table 1) conclusively supporting that crowdedness is associated to rod length.

Table 1. Physical description of polyplex micelles.

	PLys 19	PLys 39	PLys 70
DNA/PLys complex core:			
Average rod length (nm)	162 (longest)	123	91 (shortest)
Number-averaged rod surface area (nm²)	5844	5045	4388
PEG shell:			
Tethering number of PEG[#]	436 ± 31.2	258 ± 10.4	168 ± 2.5
<σ> (chains/nm²)	0.075	0.051	0.038
$<\pi R_g^2\sigma>$	5.2 (Highest)	3.5	2.6 (Lowest)
Monomer volume fraction Φ_f (%)	3.1	3.0	3.1
Osmotic pressure Π (10^5 dyne/cm²)	1.1	1.1	1.1
PEG height <*H*> (nm)[*]	12.4 ± 1.6	10.9 ± 1.8	9.6 ± 2.0
PEG conformation prediction from $\pi R_g^2\sigma$*	Squeezed	Squeezed	OvLap.Mushroom
PEG conformation from <*H*> **	Squeezed	Squeezed	Mushroom

*Based on $\pi R_g^2\sigma$ value, polymer conformation on a surface can be tethered random coil (mushroom) ($\pi R_g^2\sigma \leq 1$), overlapping mushroom ($1 < \pi R_g^2\sigma \leq \sim 3$), squeezed ($\sim 3 \leq \pi R_g^2\sigma \leq \sim 6$), and ultimately, brush conformation ($\pi R_g^2\sigma >> 6$).

**Inferred from PEG height from ZPC Cryo-TEM. PEG with <*H*> of $2R_g$ (=9.4 nm for PEG 12k) is classified as mushroom, while PEG with <*H*> higher than $2R_g$ is classified as squeezed.

3. The role of PEG crowdedness on biological performance of polyplex micelle gene carriers

In order to clarify role of PEG crowdedness on gene delivering performance, retention in blood compartment after injection was examined for polyplex micelles of known PEG <σ> using intravital real-time confocal scanning microscopy (IVRTCLSM), which allows *in situ* monitoring of fluorescence-labeled carriers. The results showed that polyplex micelles with higher <σ>, associated with squeezed PEG conformation (PLys 19 and 39), had better retention in blood circulation than that with lower <σ>, associated with overlapping mushroom PEG (PLys 70) (Figure 1). Since it was previously suggested in a study that inhibition of adsorption of serum proteins (including opsonins) started when PEG chains become overlapping mushrooms, and maximized when they become squeezed PEG, it is possible that the higher retention for polyplex micelles with higher <σ> suggests that they were less easily marked by opsonins, and thus less susceptible to rapid RES uptake. Inversely, lower retention for polyplex micelles with lower <σ> may suggest that they were more marked by opsonins, and thus more susceptible to rapid RES uptake. After all above considerations, it is important to note that all polyplex micelles circulated

with similar decay curve and were eliminated at 40 minutes, even for squeezed PEG, implying that there might be factors in blood circulation that gradually eliminated the polyplex micelles. In this regard, it is interesting to note a recent report that electrostatically-assembled nanoparticles may be disassembled in glomerulus basement membrane of kidney by its abundant heparan sulfate, and to use this information to speculate it as an account for gradual elimination of polyplex micelles. If this is the case, PEG might actually still be useful because it can act as a barrier to prevent core contact with heparan sulfate, but obviously squeezed PEG was not yet enough. Therefore, it might be useful to further increase PEG crowdedness, and also reinforce polyplex micelles with core crosslinking.

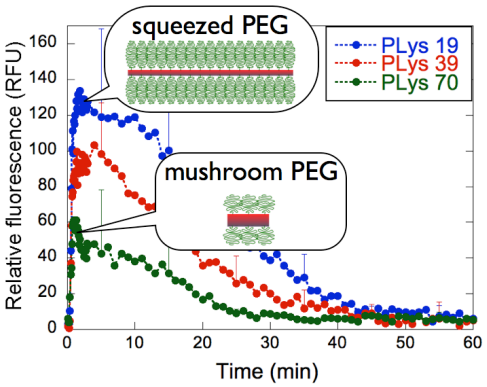


Figure 1. Blood circulation profile of polyplex micelles prepared by fluorescence-labelled pDNA, observed by IVRTCLSM on the ear of mice

4. Governing principles in engineering tethered PEG density

The implication that squeezed PEG may not be enough to completely prevent elimination of polyplex micelles from blood compartment brought up the necessity to further increase PEG crowdedness (Section 3). Thus, it is useful to explore governing principles to increase PEG crowdedness through analyzing the energetic balance of polyplex micelles. In this regard, the correlation between PEG crowdedness and rod length of polyplex micelles (Section 2) may be indicative of an intricate intercorrelation of the two determining factors for σ on the polyplex micelle structure, namely tethering number and surface area (Table 1). Principally, DNA packaging into core of polyplex micelles is driven by the need to minimize surface energy of polyion-complexed pDNA in contact with water molecules, through minimization of surface area. In this regard, smaller surface area, associated with shorter rods, is basically more favorable for the core because it minimizes surface energy, although it should be recognized that packaging process of semi-flexible polymer such as DNA is not as easy as the case of flexible polymer because of rigidity barrier to packaging. On the contrary, smaller surface area may not be favorable for PEG because it reduces available space, onto which PEG tether. This induces PEG crowding, which coincides with energetically unfavorable situation for PEG. In this circumstance, larger surface area, associated with longer rods, is more favorable for crowded PEG. Based on these arguments, it is thus reasonable that low σ is associated with shorter rods, and high σ is associated with longer rods. In addition, this observation also revealed that σ could be achieved by increasing tethering number, but not completely, because the achieved increase in σ was relaxed to a certain extent by PEG resisting minimization of surface area. To further understand how PEG crowdedness prevents surface area minimization, PEG osmotic pressure Π should be principally considered because it may produce repulsive force of PEG. However, calculated Π, based on PEG monomer volume content were the same in all polyplex micelles (Table 1). This suggests that other energetic factor, namely conformational entropy may be more involved, because minimization of surface area for tethered PEG may cause conformational entropic loss. Summing up, factors governing PEG crowdedness could be accordingly analyzed by considering the balance between free energy for PEG repulsion (from conformational entropy contribution) and free energy for DNA compaction.

If the above description is correct and rod structure is under thermodynamic equilibrium, then it follows that rod length should continuously shorten as PEG gradually detach off from core surface. To directly prove PEG crowdedness sustaining the core surface energy, a polyplex micelles with acidic-pH detachable PEG was constructed from PEG-[acetal]-PLys. Indeed, the design was successful because while PEG-[acetal]-PLys based polyplex micelle was stable at pH 7.4, PEG detachment was proven to occur at pH 4.0 by following GPC profile change, where

detachment rate was estimated to be 0% (0h), 20% (3h), 40% (6h), 65% (12h), 85.5% (16h) and 92.5% (at 24h). The structure of polyplex micelles upon detachment was followed by TEM, and was measured for rod length. From this study, (1) there was no significant change of rod length distribution at 0-12h, and (2) transition to globule had been observed at 16h and 24h. Note that structural change occurred upon PEG detachment is consistent with the above-mentioned thermodynamic consideration, but unexpectedly, rod length was not continuously shorter upon gradual PEG detachment. In this case, it is reasonable to speculate that trapping might be involved. The parameter that may be most responsible for retaining DNA in rod-shape might be DNA rigidity. Overcoming this barrier may set the design of a new polyplex micelle system, as will be described in the following section.

5. Engineering high PEG crowdedness by modulating packaged DNA core

Examination of polyplex micelle gene carrier system showed that σ could not be very effectively increased simply by increasing tethering number onto the core because the achieved increase in σ may be relaxed to a certain extent by PEG resisting minimization of core surface area (Section 4). Thus, it is imperative to find ideal ways to increase σ through minimization of surface area. Taking the lessons from Section 4, it may be possible to achieve this by reducing rigidity barrier to DNA packaging. Indeed, in this study, it is possible to prepare very small polyplex micelle with spherical-shaped core (diameter<50 nm), and thus producing polyplex micelles with the highest σ.

6. Conclusion

In the effort to clarify the relationship between tethered PEG crowdedness and performance of polyplex micelles in systemic application, PEG crowdedness value of polyplex micelles was successfully estimated for the first time in this study. This results showed that σ increased with decreasing PLys DP, driving PEG conformation from overlapping mushroom for PLys DP 70 to upward squeezed conformation for PLys DP 39 and 19. This opened a comprehensive understanding on the effect of PEG crowdedness and gene delivering performance of polyplex micelles, where blood circulation profile successfully revealed that σ played a critical role in determining retention amount of polyplex micelles, with squeezed PEG showing better retention than mushroom PEG. Nevertheless, further examination of blood circulation profile indicated that polyplex micelles of all PLys DP were finally eliminated at 40 min, suggesting that it might be necessary to further increase PEG crowdedness. To accommodate the demand for higher PEG crowdedness, governing principles to increase PEG crowdedness was analyzed from energetic balance on polyplex micelles. By means of these governing principles, further increased σ was accommodated by minimizing surface area through overcoming rigidity barrier to DNA condensation, producing polyplex micelles with highest PEG crowdedness. Thus, by providing the principles to engineer tethered σ to optimize polyplex micelles' structure for systemic application, this study gives its strong impact to polymer-based biomedical field.