of nonviral gene vectors. In the present study, the mouse earlobe was noninvasively fixed beneath the coverslip, and the vein was imaged at the dermis layer. Confocal imaging eliminated light from out-of-focus sections in the ear lobe such as the epidermis and hypodermis. Furthermore, we kept the confocal slice thinner (5.11 μm) than the diameter of the vein, so that the signal was detected only from inside the vasculature. High-speed scanning was essential to obtain unambiguous images to quantify the aggregates and colocalization between nonviral gene vectors and platelets because conventional galvano scanners are too slow to distinguish the individual aggregates and platelets rapidly flowing in the bloodstream, providing insufficient and blurred images (Supplementary Videos 7 and 8).

We investigated the polycations BPEI and PLys. They are widely used to construct polyplexes and PAsp(DET) has reduced cytotoxicity and high transfection efficiency [13]. To evaluate the improvement of biocompatibility via PEGylation, PEG-PLys/pDNA and PEG-PAsp (DET)/pDNA micelles were examined. A simple and effective way to PEGylate polyplexes is, as we reported [10–12], to use PEG-based cationic block copolymers as counterpart polycations to pDNA. The block copolymers are characterized by tandem alignment of a hydrophilic PEG segment and a cationic segment, leading to the formation of stable and biocompatible micelles with a core of polycation/pDNA complex surrounded by a dense PEG palisade and size of approximately 100 nm. Indeed, the micelle composed of PEG-PLys and pDNA achieved higher stability than that of unmodified PLys/pDNA polyplex in a medium containing serum and showed prolonged blood circulation [18,24]. The block copolymer possessing a cationic polyaspartamide segment carrying an ethylenediamine unit at the side chain, PEG-PAsp(DET), also formed the micelle with pDNA, which prevented nonspecific interaction with biological components such as erythrocytes and platelets under *in vitro* conditions [8].

IVRTCLSM was used to directly investigate the interaction between these gene vectors and platelets in the bloodstream. IVRTCLSM could be used to evaluate the dynamic states of nonviral gene vectors rapidly flowing in the bloodstream over time *in situ* (Fig. 1 and Supplementary Videos 1–6). This is the first report to visualize the formation of aggregates and the prevention by PEGlyation of polyplexes *in situ* in the bloodstream.

To quantify the aggregates, we adopted the CV. CV values reflected the nonuniform fluorescence distribution of polyplexes and uniform fluorescence distribution of micelles (Fig. 2). It is noteworthy that our IVRTCLSM started video acquisition 10 s before administration, allowing us to follow aggregate formation immediately after injection. CV values of the polyplexes rapidly increased approximately 20–30 s after injection, and corresponded well with the entry of polyplexes, indicating instantaneous formation of aggregates (Fig. 2). CV values also fluctuated over time, depending on the amount of aggregates at those time points. Furthermore, CV values of polyplexes decreased with time due to their disappearance from the bloodstream. In contrast, CV values of micelles were moderately elevated when micelles passed the ROI first. This moderate elevation was because of the admixture of micelles and blood without aggregate formation. Moreover, CV values were retained at a plateau after this moderate elevation, suggesting persistent circulation and uniform distribution of micelles in the bloodstream.

IVRTCLSM was also useful for the investigation of the dynamic interaction between nonviral gene vectors and platelets. Indeed, we succeeded in visualizing the interaction between polyplexes and platelets *in situ*. This dynamic information could not be revealed without IVRTCLSM.

To quantify the platelet interaction, we adopted PCC between polyplexes/micelles and platelets (Fig. 3). PCC values of polyplexes did not increase at the time point when CV values started to increase. PCC values began to increase after approximately 1 min after injection, and indicated strong correlation between polyplexes and platelets 2 min after injection. This temporal gap between aggregate formation and platelet interaction strongly indicated that aggregate formation was not triggered by platelets. To confirm this, we conducted the study in mice that were administered aspirin (Fig. 4). Aspirin induces a long-lasting functional defect in platelets [25], and thus may inhibit platelet interaction with polyplexes. The CV and PCC quantitatively demonstrated that oral administration of aspirin successfully inhibited platelet interaction with aggregates (Fig. 4b), but did not inhibit aggregate formation itself (Fig. 4a). This result indicates that the aggregate formation of polyplexes does not involve platelets (at least in the initial stage). Presumably, some protein components in plasma may have a role in aggregate formation, but further investigation is needed to clarify the mechanism.

Aggregate formation in the range of several micrometers immediately after intravenous injection should crucially affect the efficiency of systemically injected polyplexes. The aggregated polyplexes cannot extravasate into the targeted tissues or cells. Moreover, they might lead to thrombosis through the interaction with platelets to obstruct microvessels in normal tissue, including the lungs and liver, resulting in nonspecific accumulation of polyplexes in these tissues. This accumulation caused by aggregate formation will lead to unfavorable effects such as pulmonary embolism. The micelles, in contrast, did not form aggregates, and also showed no interaction with platelets. Thus, they are expected to prevent adverse effects caused by polyplex agglomeration, which cannot be inhibited even by oral administration of aspirin. This result confirms that PEGylation is a rational strategy to improve the biocompatibility of nonviral gene vectors based on polyplex formation [3,10–12].

In the present study, IVRTCLSM was used to visualize and quantify the dynamic states of polyplexes flowing in the bloodstream. Moreover, with respect to ethics, IVRTCLSM excels conventional *ex vivo* methods that involve the sacrifices of numerous animals to acquire pharmacokinetic information. IVRTCLSM provides temporal and spatial information at 30 time points in 1 s with a single mouse, which is desirable for high-throughput screening of newly developed DDSs.

In conclusion, IVRTCLSM was developed and applied to directly investigate the dynamic state of gene vectors in the bloodstream. Aggregate formation of the polyplexes and its prevention by PEGylation was observed *in situ* for the first time under the flow in the capillary. Thus, IVRTCLSM could provide the requisite information that has not been obtained by conventional methods, thereby giving a new facet in the research on systemic gene delivery.

Supplementary materials related to this article can be found online at doi:10.1016/j.jconrel.2011.02.011.

Acknowledgment

This work was supported in part by Core Research Program for Evolutional Science and Technology (CREST) from the Japan Science and Technology Corporation (JST) and Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program) from Japan Society for the Promotion of Science (JSPS).

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