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BRIEF COMMUNICATION

Erythrocyte ghost-mediated gene delivery for prolonged and blood-targeted expression

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This study reports the use of erythrocyte ghosts (EG) as a biocompatible nonviral delivery system for extended circulation and prolonged expression of plasmid DNA in the blood. Murine interleukin-2-expressing plasmid DNA was efficiently loaded to EG by electroporation in hypotonic condition. The presence of plasmid DNA in EG was confirmed by fluorescence-labeled plasmid DNA. At 21 min after intravenous administration into mice, the level of plasmid DNA in the blood was 92 000-fold higher following EG-mediated delivery as compared to the injection of naked form. EG-mediated gene delivery revealed higher and more prolonged mRNA expression levels of plasmid DNA in the blood until 9 days after the single intravenous injection. Moreover, plasmid DNA-loaded EG showed gene expression targeted to the blood cells. At 3 days post-dose, substantial expression levels of plasmid DNA delivered in EG were observed only in the blood and not in the other organs. Of the blood cells, the subpopulation containing granulocytes showed higher expression of plasmid DNA than mononuclear cells. These results indicate the potential of EG as a safe, prolonged and blood-targeted delivery system of therapeutic genes.
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Introduction

For systemic gene therapy, there is a need for the development of safe delivery systems allowing prolonged circulation and expression of administered genes *in vivo*.[1] Given the short half-lives of intravenously administered plasmid DNA,[2] nonviral cationic vectors have been variously modified to extend the systemic circulation of DNA. Encouraging results on the longer circulation life times of DNA have been observed after the intravenous administration of poly(L-lysine)/DNA complexes,[3] polyethylene glycol-conjugated polyethylenimine/DNA complexes,[4] and stabilized plasmid/lipid particles.[5] However, the safety of these cationic vectors still remains unresolved. Aggregation of erythrocytes has been observed after the administration of polyethylene glycol-conjugated polyethylenimine/DNA complexes.[6] Acute inflammatory toxicity has been reported in cationic lipid-based systemic gene delivery.[7]

Meanwhile, erythrocyte ghost (EG) particles made of autologous red blood cells have been studied as safe and sustained delivery systems of therapeutic compounds,[8] oligonucleotides,[9] and proteins such as adenosine deaminase and aldehyde dehydrogenase.[10,11] Taking advantages of the nontoxic and long-circulating properties of erythrocytes,[12] we tested whether we could modulate the circulation time and expression of plasmid DNA by EG-mediated delivery. Here, we report that the EG-mediated gene delivery provided much higher and more prolonged levels of gene expression in the blood. Moreover, following EG-mediated gene delivery, we observed the expression of gene targeted to the blood for prolonged periods. Our results suggest the new application of EG for a prolonged and blood-targeted gene expression in systemic gene therapy.

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Results

Systemic circulation of plasmid DNA delivered by EG

EG-mediated gene delivery showed a significant increase in the systemic circulation time of plasmid DNA. For *in vivo* intravenous administration, plasmid DNA was loaded to EG by electroporation in the hypotonic medium using 1 mg of a loading dose. The same dose of plasmid DNA (50 μg) in naked form or loaded in EG was intravenously injected into mice. At 21 min after administration, the serum level of plasmid DNA was 91 200-fold higher in plasmid DNA-loaded EG relative to free plasmid DNA (Figure 1). The plasmid DNA levels decreased to lower than 0.1 ng/ml within 15 min in the naked DNA-treated group. In contrast, it took more than 120 min for the plasmid levels to decrease to the same concentration in EG-mediated delivery. The area under the curve value, calculated by WinNonlin 4.0.1 (Pharsight, USA), was $26\,091 \pm 4405$ ng/ml • min in the plasmid DNA-loaded EG, showing 26-fold higher values than the free plasmid DNA.

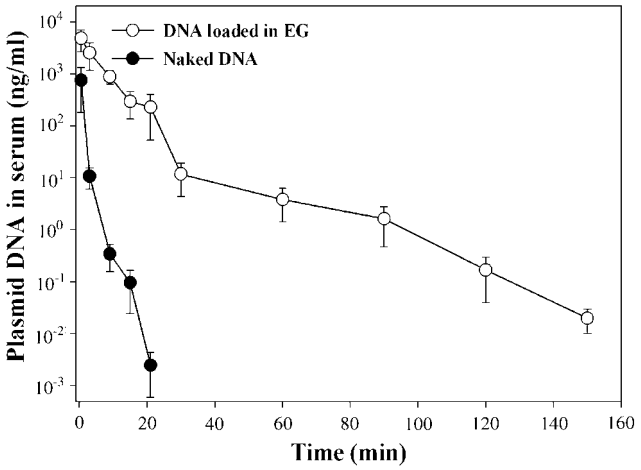

Figure 1 *Circulation levels of plasmid DNA. Mouse erythrocytes were loaded with murine interleukin-2 (mIL-2) expressing plasmid DNA, pVAXmIL-2 (3.5 kb), by electroporation. pVAXmIL-2 was constructed by inserting the 529-bp mIL-2 gene from pCIneoIL-2 into the pVAX1 expression vector (Invitrogen) using NheI and BamHI restriction enzymes.[13] Washed erythrocytes (3×10^9 cells) were suspended in 1 ml of hypotonic phosphate buffer (5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 5 mM $MgCl_2$, pH 7.4)[18] containing 1 mg of plasmid DNA, electroporated at 0.3 V and 0.25 capacity (Gene Pulser II, Biorad), and then NaCl was added to recover the isotonicity of the medium. After incubation at 37°C for 30 min, the resulting EG were washed free of extracellular plasmid DNA by centrifugation. Plasmid DNA (50 μg) in EG or in free form was intravenously administered into BALB/c mice. At various time points after the intravenous administration, approximately 30 μl of blood was collected from the tail vein. The total DNA of serum was extracted and the amount of administered plasmid DNA was measured by quantitative PCR according to the method described previously.[19] The results are expressed as the mean ± s.e. (n=5).*

To confirm the presence of plasmid DNA in EG, plasmid DNA was labeled using a fluorescence dye and electroporated into EG. After loading of fluorescence dye-labeled plasmid DNA into EG, the cells were observed under phase contrast (Figure 2a) and fluorescence (Figure 2b) microscopy. The fluorescence microscopy indicates the presence of plasmid DNA in EG. Moreover, EG containing plasmid DNA observed at the higher magnification view ($\times 1000$) showed spherical morphology with a relatively indistinct rim under phase-contrast microscopy (Figure 2c).

To further test whether the plasmid DNA is encapsulated inside EG, the stability of plasmid DNA against externally added nuclease was studied. Quantitative PCR results obtained using an internal standard deletion mutant[13] of pVAXmIL-2 showed that plasmid DNA in EG was stable in the presence or absence of DNase I (Figure 2d). The same amount of naked plasmid DNA used as a control was completely degraded after treatment with DNase I (data not shown).

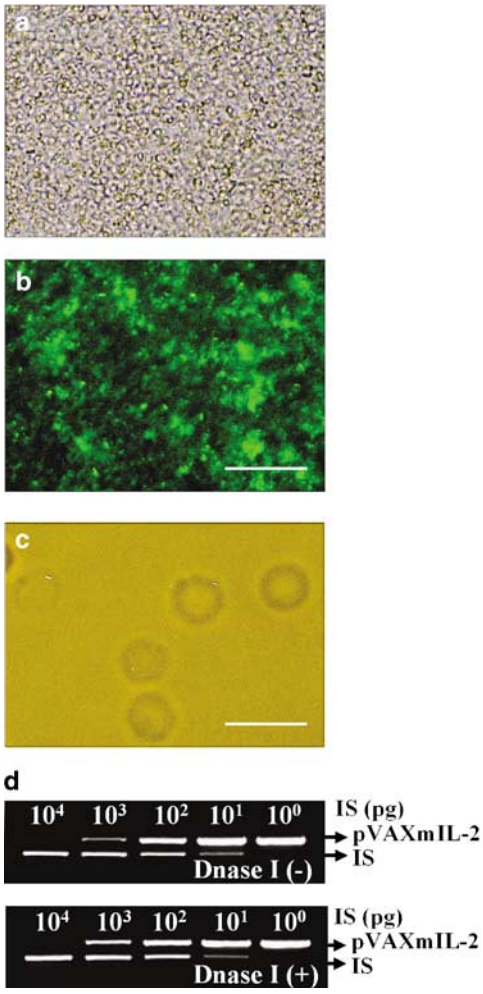

Figure 2 *Encapsulation of plasmid DNA in EG. For identification of plasmid DNA in EG, pVAXmIL-2 was labeled using an ARES Alexa Fluor® 488 DNA labeling kit (Molecular Probes, OR, USA), loaded into erythrocytes by electroporation in the hypotonic buffer medium, and observed by phase-contrast (a) and fluorescence microscopy (b). Scale bar: 100 μm. (c) Phase-contrast microscopy of EG containing plasmid DNA at a higher magnification view. Scale bar: 10 μm. (d) EG containing 1 μg of pVAXmIL-2 was incubated with or without 0.5 U of DNase I (Gibco, USA) at 37°C for 30 min. Following incubation, the plasmid DNA was extracted using DNAzol® (Invitrogen) and the amounts of plasmid DNA were analyzed by quantitative PCR using various amounts of an internal standard (IS) deletion mutant of pVAXmIL-2 (IS) as described previously.[13]*

Prolonged expression and biodistribution of plasmid DNA given in EG

EG loaded with plasmid DNA, pVAXmIL-2, showed higher and more prolonged expression of mIL-2 in the blood than did the naked plasmid DNA following single intravenous administration. At 3 days post-dose, the mRNA expression levels of mIL-2 were 10 times higher in the plasmid DNA-loaded EG compared to the naked DNA. Although the mRNA levels could be detected up to 3 days after administration of the naked plasmid DNA, the mRNA levels of mIL-2 were detected until 9 days following gene delivery in EG (Figure 3a). As a control, pVAX1 vector, the plasmid backbone of pVAX-mIL-2, was loaded into EG and administered into mice. EG loaded with pVAX1, however, did not show detectable levels of mRNA from 3 days post-dose. Similar to pVAX1-loaded EG, empty EG showed low levels of mIL-2 mRNA ($<10^2$ fmole/ml) at 1 day after administration, but no expression of mIL-2 from 3 days post-dose.

The organ distribution kinetics differed between the naked DNA and the DNA given in EG. As compared to the naked plasmid DNA, the plasmid DNA-loaded EG showed lower levels of plasmid DNA in all organs at 10 min post-administration (Figure 3b). Between the two

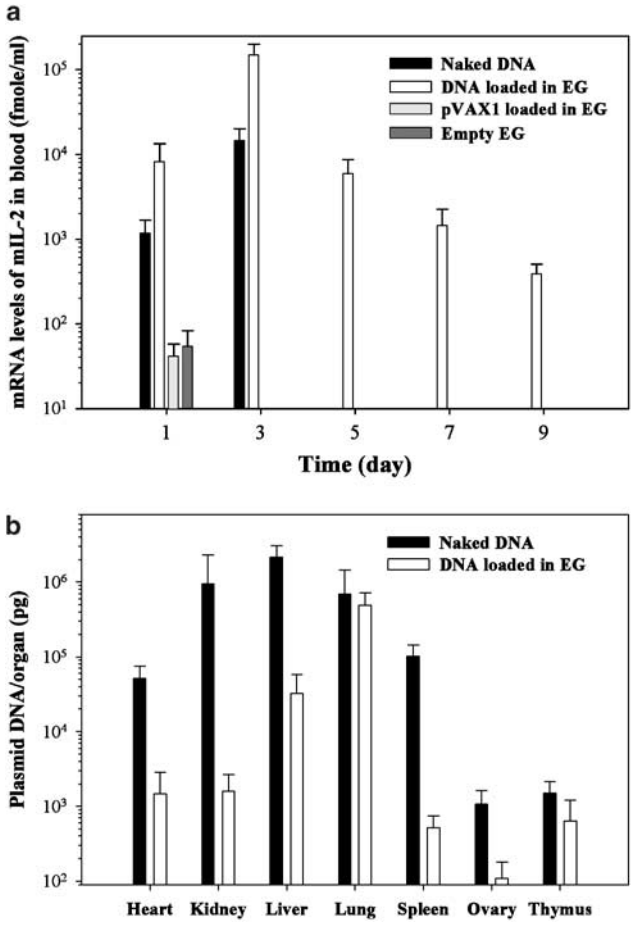

Figure 3 *Expression and biodistribution of plasmid DNA following EG-mediated delivery. (a) Mice were intravenously administered with naked pVAXmIL-2 plasmid DNA, pVAXmIL-2 in EG, pVAX1 expression vector in EG, or empty EG. To quantitate the mRNA expression levels of administered plasmid DNA in the blood, the mice were killed at various time points after intravenous administration of plasmid DNA (50 μg/mouse) in various forms. Total RNA was extracted from each sample using a TRIzol® reagent (Gibco BRL, NY, USA). The cDNA was prepared using a first-strand cDNA synthesis kit (Boehringer Mannheim, IN, USA). The mRNA levels of mIL-2 were determined by a quantitative RT-PCR using a synthetic standard RNA of mIL-2 kindly provided by Dr Kagnoff (University of California, San Diego) as described.[13,20] The results are expressed as the mean±s.e. (n=5). (b) After administration of plasmid DNA in free or EG-encapsulated form, mice were killed at 10 min post-dose. The tissue samples were then suspended into DNAzol® (Gibco BRL, NY, USA) at a concentration of 50 mg tissue per ml. The homogenates (500 μl) were then loaded onto a Wizard® DNA clean-up column (Promega, WI, USA). After the washing steps, the DNA was eluted with 50 μl of TE buffer. The levels of plasmid DNA in each organ were determined by quantitative PCR.*

groups, the highest difference in the organ levels was observed in the kidney (601-fold), followed by the spleen (198-fold) and the liver (66-fold).

Blood-targeted expression of plasmid DNA delivered using EG

In addition to the prolonged expression of plasmid DNA, EG provided blood-targeted gene expression. Given that the biodistribution of plasmid DNA administered in EG was the highest in the lung followed by the liver and other tissues, the expression levels of mIL-2 in the lung, liver, kidney, and spleen were further studied at 3 days

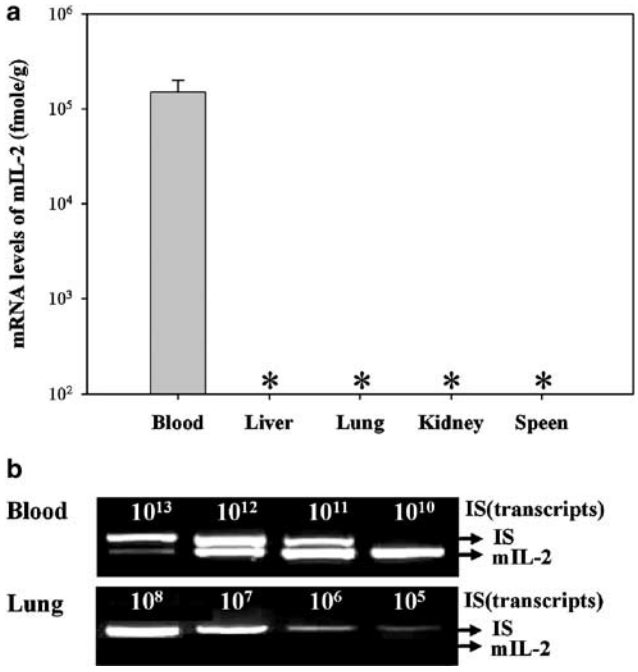

Figure 4 *Blood-targeted expression of plasmid DNA delivered in EG. (a) Mice were intravenously administered with pVAXmIL-2 loaded in EG. At 3 days post-dose, the mRNA levels of mIL-2 in the blood and various organs were determined by quantitative RT-PCR. *Under the detection limit. (b) The results are representative gel pictures of quantitative RT-PCR products. Following RT-PCR, the PCR products were electrophoresed on a 2% agarose gel. For detetion of mRNA in the blood, 10^{10}–10^{13} transcripts of synthetic internal standard (IS) RNA were used. For detetion of mRNA in the lung, 10^{5}–10^{8} transcripts of synthetic IS RNA were used.*

after administration of plasmid DNA in EG. Unlike the substantial expression of mIL-2 in the blood, the expression of mIL-2 in the lung, liver, kidney, and spleen was under the detection limit at 3 days post-dose (Figure 4a). The representative competitive RT-PCR results in the blood and the lung are shown in Figure 4b. Following EG-mediated pVAXmIL-2 delivery, the mRNA transcripts in the blood were detected using 10^{13} internal standard RNA in the competitive RT-PCR. In contrast, there was no signal even at 10^{5} internal standard RNA in the lung.

To further study the blood cell types involved in the expression of plasmid DNA administered in EG, the mRNA expression levels of mIL-2 were measured in the subpopulations of the blood cells such as erythrocytes/granulocytes, lymphocytes, and monocytes (Figure 5a). The murine housekeeping gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for testing the presence of total cDNA in each sample (Figure 5b). Although the mRNA expression of plasmid DNA was observed in every subpopulation at 1 and 5 days post-dose, erythrocyte/granulocyte subpopulation showed a higher mRNA expression than did monocytes and lymphocytes over 9 days post-dose.

Although EG might be assumed as a biocompatible delivery system, we tested the histology of the organs after repeated dosing of plasmid DNA-loaded EG. Given the distribution results, we examined organs such as the lung, liver, kidney, and spleen. Twice a week intravenous injections of plasmid DNA-loaded EG over 6 consecutive

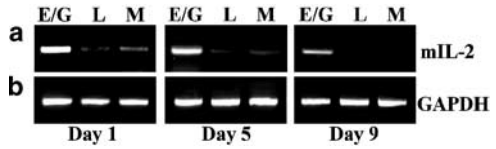

Figure 5 *Expression of plasmid DNA in the subpopulation of blood cells. At various time points after intravenous injection of plasmid DNA (50 μg) loaded in EG into mice, the blood was collected and separated using Histopaque®-1077 (Sigma). The mononuclear cells were further separated into monocytes and lymphocytes by their adherency on plastic cultureware. RNA was extracted from the subpopulations of the blood cells such as erythrocytes/granulocytes (E/G), lymphocytes (L), and monocytes (M) using Trizol® (Gibco). RT-PCR was performed for mIL-2 and a housekeeping gene, GAPDH, using primers described previously.[2,13] The representative mRNA expression patterns of mIL-2 (a) and GAPDH (b) at 1, 5, and 9 days post-dose are presented. Similar results were obtained in four separate experiments.*

weeks had no discernible effects on histopathology in all the organs tested (data not shown).

Discussion

In this study, we demonstrated the new usage of EG as a biocompatible nonviral gene delivery vector. The prolonged and targeted expression of plasmid DNA given in EG over several days in the blood indicates the application of EG as a potential nonviral vector for various systemic gene therapy.

In this study, we loaded plasmid DNA into EG via electroporation in hypotonic condition. The presence of plasmid DNA in EG (Figure 2b) and the stability of plasmid DNA in EG against DNase I (Figure 2d) indicates that plasmid DNA might be encapsulated inside EG rather than bound to the surface. The encapsulation of plasmid DNA in EG was affected by loading methods. The higher entrapment efficiency was observed after electroporation in the hypotonic medium rather than electroporation in the isotonic medium or incubation in the hypotonic medium alone (data not shown). Among the three methods, the hypotonic medium-based osmotic shock method showed the lowest entrapment. The increase of incubation time in the hypotonic medium had limits in increasing the entrapment of plasmid DNA. During the preparation of EG, the loading doses of DNA above 1 mg did not significantly increase the encapsulation. The enhanced entrapment efficiency following electroporation in the hypotonic medium might be of value in delivering therapeutically effective amounts of plasmid DNA into systemic circulation via EG. Previously, there was a great deal of work to load drugs and proteins into EG via electroporation or electrofusion. Electroporation technique was used to load erythrocytes with the low molecular drug methotrexate,[14] and the high molecular weight enzyme pronase.[15] Electrofusion between mammalian tumor cells and EG was studied to mediate drug and gene transfer to target tumor cells or to form hybrid cells.[16]

Our observation on the prolonged circulation of EG-mediated plasmid DNA delivery agrees with the previous reports on the extended circulation time of small molecules[8] and proteins[10,11] delivered in EG. Although consistent with these reports, our results first demonstrated the prolonged circulation of plasmid DNA by EG-mediated delivery. The circulation times of plasmid DNA were, however, shorter than the natural circulation times of red blood cells. One possibility is the leakage of plasmid DNA from EG *in vivo*, subject to degradation by nucleases. Moreover, the possibility exists that the membrane properties of EG might be slightly altered during the *ex vivo* encapsulation process.

We observed that EG loaded with plasmid DNA showed lower levels of plasmid DNA in all the organs as compared to the free plasmid DNA at 10 min administration. Such lower organ levels of plasmid DNA following EG-mediated delivery might be partially due to the prolonged circulation of plasmid DNA, limiting the chance to be taken up by other tissues of the body at the early distribution phase of post-dose.

It is unlikely that such prolonged expression could be due to stimulation of endogenous mIL-2 expression by EG since the empty EG did not produce detectable levels of mIL-2 from 3 days after administration. Moreover, the lack of mIL-2 expression following the administration of pVAX1 vector plasmid in EG eliminates the possibility that the detected production of mIL-2 could be an inflammatory response due to the CpG motifs in the plasmid DNA delivered in EG. Rather, the prolonged expression of mIL-2 might be attributed to the longer circulation of pVAXmIL-2 plasmid DNA given in EG. Meanwhile, given our observation that empty EG and pVAX1-loaded EG showed much lower IL-2 expression than pVAXmIL2-loaded EG at day 1, we cannot exclude the possibility that the levels of IL-2 seen with plasmid-loaded EG at early time points may still somehow relate to endogenous levels stimulated by the injection process of EG.

In gene therapy, the targeting of gene expression is one of the desired characteristics in gene delivery systems. We observed that pVAXmIL-2 loaded in EG provided the substantial expression of mIL-2 in the blood but not in the other organs such as the lung and liver (Figure 4a). Such a targeted expression of gene delivered in EG indicates the potential of EG as a blood-targetable gene delivery system. Recently, polyethylene glycol modification approach has been tried to prolong the circulation of plasmid DNA in the blood.[4] However, our results support that EG can provide prolonged circulation as well as sustained and targeted expression of delivered genes in the blood over several days.

Following administration of plasmid DNA loaded in EG, we observed higher expression of the plasmid in the subpopulation of erythrocytes and granulocytes as compared to other cell types such as monocytes and lymphocytes. Since mature erythrocytes lack a nucleus, it is unlikely that erythrocytes actively expressed the mRNA of the plasmid DNA in the subpopulation fraction containing both erythrocytes and granulocytes. Rather, it appears that granulocytes might be the major cell types involved in the expression of plasmid DNA given in EG. Among granulocytes including neutrophils, basophils, and eosinophils, neutrophils are the most numerous cells with phagocytic activity. Neutrophils have been considered as the emergency response unit within the body's circulatory system since they are the first to be recruited at sites of tissue trauma, infection, or inflammation.[17] Given the phagocytic function of neutrophils in the circulation and the highest expression of plasmid DNA in the subpopulation with neutrophils, we cannot exclude the possibility that EG containing

plasmid DNA could be mainly taken up by neutrophils and be expressed there for prolonged periods.

Safety is of paramount importance in designing or evaluating a system for gene therapy. Although nonviral vectors are generally considered to be safer than viral vectors, the toxicity of cationic lipids and polymers following repeated dosing still remains to be solved. We previously showed that polyethylenimine, one of the extensively studied nonviral gene delivery systems, might result in a sign of inflammation in the liver after six repeated dosings of polyethylenimine/DNA complexes over 3 consecutive weeks.[2] Neither inflammation nor tissue necrosis observed after 12 repeated administration of plasmid DNA-loaded EG over 6 consecutive weeks (data not shown) suggests that EG would be a proper candidate as a safer and biocompatible nonviral vector system.

In conclusion, the extended circulation time, and prolonged and blood-targeted expression of autologous EG-mediated plasmid DNA delivery suggest the potential of EG as a promising biocompatible nonviral vector for systemic gene therapy.

Acknowledgements

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References

1 Fenske DB, Maclachlan I, Cullis PR. Long-circulating vectors for the systemic delivery of genes. *Curr Opin Mol Ther* 2001; **3**: 153–158.
2 Oh YK *et al*. Prolonged organ retention and safety of plasmid DNA administered in polyethylenimine complexes. *Gene Therapy* 2001; **8**: 1587–1592.
3 Ward CM, Read ML, Seymour LW. Systemic circulation of poly(L-lysine)/DNA vectors is influenced by polycation molecular weight and type of DNA: differential circulation in mice and rats and the implications for human gene therapy. *Blood* 2001; **97**: 2221–2229.
4 Oupicky D, Ogris M, Howard KA, Dash PR. Importance of lateral and steric stabilization of polyelectrolyte gene delivery vectors for extended systemic circulation. *Mol Ther* 2002; **5**: 463–472.
5 Tam P *et al*. Stabilized plasmid-lipid particles for systemic gene therapy. *Gene Therapy* 2000; **7**: 1867–1874.
6 Ogris M *et al*. PEGylated DNA/transferrin–PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. *Gene Therapy* 1999; **6**: 595–605.
7 Tan Y *et al*. Sequential injection of cationic liposome and plasmid DNA effectively transfects the lung with minimal inflammatory toxicity. *Mol Ther* 2001; **3**: 673–682.
8 Rossi L *et al*. Erythrocyte-mediated delivery of a new homodinucleotide active against human immunodeficiency virus and herpes simplex virus. *J Antimicrob Chemother* 2001; **47**: 819–827.
9 Magnani M *et al*. Erythrocyte-mediated delivery of drugs, peptides and modified oligonucleotides. *Gene Therapy* 2002; **9**: 749–751.
10 Bax BE *et al*. *In vitro* and *in vivo* studies with human carrier erythrocytes loaded with polyethylene glycol-conjugated and native adenosine deaminase. *Br J Haematol* 2000; **109**: 549–554.
11 Lizano C, Perez MT, Pinilla M. Mouse erythrocytes as carriers for coencapsulated alcohol and aldehyde dehydrogenase obtained by electroporation *in vivo* survival rate in circulation, organ distribution and ethanol degradation. *Life Sci* 2001; **68**: 2001–2016.
12 Bax BE *et al*. Survival of human carrier erythrocytes *in vivo*. *Clin Sci* 1999; **96**: 171–178.
13 Oh YK *et al*. Polyethylenimine-mediated cellular uptake, nucleus trafficking and expression of cytokine plasmid DNA. *Gene Therapy* 2002; **9**: 1627–1632.
14 Kruse CA, Mierau GW, James GT. Methotrexate loading of red cell carriers by osmotic stress and electric-pulse methods: ultrastructural observations. *Biotechnol Appl Biochem* 1989; **11**: 571–580.
15 Haritou M, Yova D, Koutsouris D, Loukas S. Loading of intact rabbit erythrocytes with fluorophores and the enzyme pronase by means of electroporation. *Clin Hemorheol Microcirc* 1998; **19**: 205–217.
16 Li LH, Hensen ML, Zhao YL, Hui SW. Electrofusion between heterogeneous-sized mammalian cells in a pellet: potential applications in drug delivery and hybridoma formation. *Biophys J* 1996; **71**: 479–486.
17 Simon SI, Goldsmith HL. Leukocyte adhesion dynamics in shear flow. *Ann Biomed Eng* 2002; **30**: 315–332.
18 Oh YK, Swanson JA. Different fates of phagocytosed particles following delivery into macrophage lysosomes. *J Cell Biol* 1996; **132**: 585–593.
19 Oh YK *et al*. Nasal absorption and biodistribution of plasmid DNA: an alternative route of DNA vaccine delivery. *Vaccine* 2001; **19**: 4519–4525.
20 Jung HC, Kim JM, Song IS, Kim CY. *Helicobacter pylori* induces an array of pro-inflammatory cytokines in human gastric epithelial cells: quantification of mRNA for interleukin-8, -1α/β, granulocyte–macrophage colony-stimulating factor, monocyte chemoattractant protein-1 and tumour necrosis factor-α. *J Gastroenterol Hepatol* 1997; **12**: 473–480.