Table 1
Preparation and characterization of PEG-introduced cationized Pronectin F^+ with different extents of PEGylation

Cationized Pronectin F^+ concentration (mg/ml)	PEG concentration (mg/ml)	The molar percentage of PEG introduced
1.0	0.25	0.02 ± 0.01[a]
1.0	0.15	1.1 ± 0.15
1.0	3.0	5.1 ± 0.9
1.0	7.0	10.1 ± 0.8
1.0	14.0	20.1 ± 1.2
1.0	19.0	30.1 ± 0.8
1.0	25.0	50.1 ± 2.2

[a] Mean ± S.D.

DP50 digital camera (KS Olympus, Tokyo, Japan). As controls, RITC-labeled plasmid DNA alone was used to evaluate the tissue localization similarly.

2.10. Statistical analysis

All the data were statistically analyzed to express the mean ± the standard deviation (S.D.) of the mean. Student's t test was performed and $p < 0.05$ was accepted to be significance.

3. Results

3.1. Preparation and characterization of PEG-introduced cationized Pronectin F^+ and the complexation with plasmid DNA

Table 1 shows the preparation condition and characterization result of cationized Pronectin F^+ with or without PEGylation prepared under different reaction

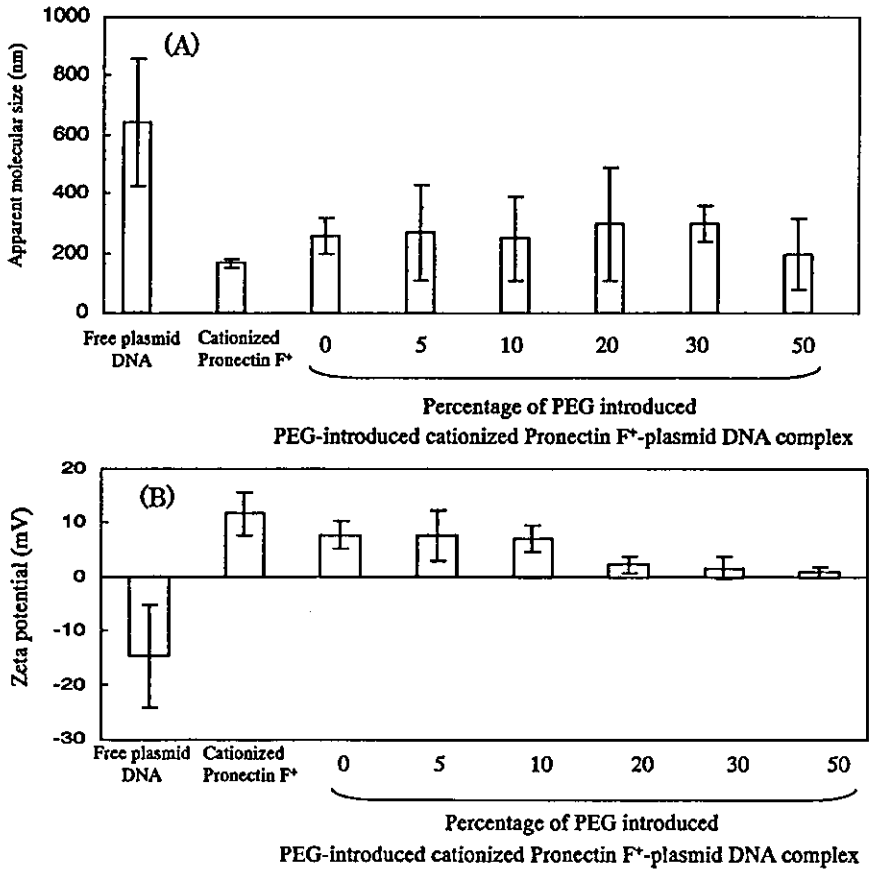

Fig. 2. Apparent molecular size (A) and zeta potentials (B) of free plasmid DNA, cationized Pronectin F^+, PEG-introduced cationized Pronectin F^+ with different extents of PEGylation, and their plasmid DNA complexes. The concentration of plasmid DNA used is 0.25 mg/ml. The N/P charge ratio is 2.3.

conditions. It is apparent that the percentage of PEG introduced increased with the amount of PEG added.

Fig. 1 shows the electrophoretic patterns of cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation prepared at different N/P molar ratios. Irrespective of the cationized type of Pronectin F^+, the migration of plasmid DNA was retarded with an increase in the N/P molar ratio, but not observed any more at the ratio higher than a certain value. Neither the original Pronectin F^+ without any cationization nor cationized Pronectin F^+ with the smallest extent of aminization induced the electrophoretic migration of plasmid DNA. PEGylation of cationized Pronectin F^+ at the lower introduction percentage of PEG did not affect the electrophoresis migration of plasmid DNA at the higher N/P ratios, although higher introduction percentage of PEG greatly affected the electrophoresis migration of plasmid DNA. PEGylation tended to allow the cationized Pronectin F^+ to reduce the complexation with plasmid DNA.

Fig. 2 shows the molecular size and surface charge of PEG-introduced cationized Pronectin F^+–plasmid DNA complexes prepared by use of the cationized Pronectin F^+ at different PEGylation extents. It is clear that lower introduction percentage of PEG would not be enough to reduce the surface charge of complexes. When the introduction percentage of PEGylation reached into 20 mol%, the zeta potential of complexes became nearly zero.

Our previously results showed that complexation of cationized Pronectin F^+ enabled the apparent molecular size of plasmid DNA to reduce to about 250 nm, the size decreasing with the increased cationized Pronectin F^+/plasmid DNA charge ratio. However, PEGylation did not change the apparent molecular size of cationized Pronectin F^+–plasmid DNA complexes and the size was similar to that of complexes with cationized Pronectin F^+.

3.2. Gene expression of tumor and organs of mice following intravenous injection of cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation

Fig. 3 shows the in vivo gene expression of tumor 2 days after intravenous injection of PBS, free plasmid DNA, cationized Pronectin F^+, PEG-introduced cationized Pronectin F^+, cationized Pronectin F^+–plasmid DNA complex, and PEG-introduced cationized Pronectin F^+–plasmid DNA complex. Significantly enhanced level of gene expression was observed in the tumor for the PEG-introduced cationized Pronectin F^+–plasmid DNA complex when in-

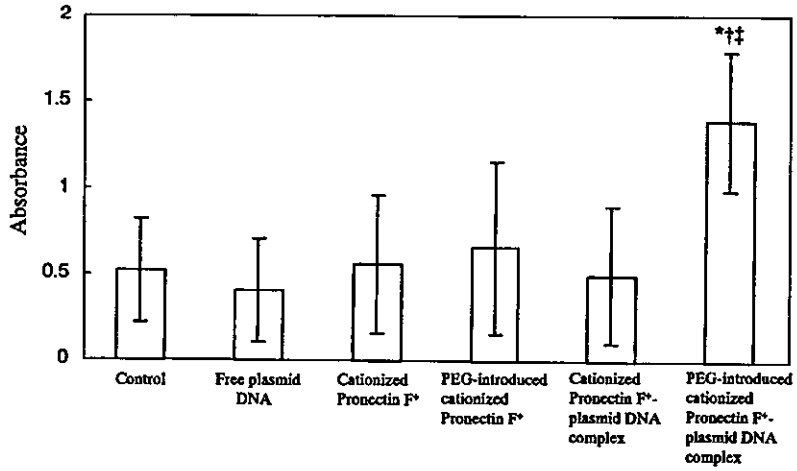

Fig. 3. In vivo gene expression of tumor 2 days after intravenous injection of free plasmid DNA, cationized Pronectin F^+ with or without PEGylation, and cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation. The dose of plasmid DNA used is 0.25 mg/ml and the molar percentage of PEG introduced to the cationized Pronectin F^+ is 20.1 mol%. The N/P ratio of Pronectin F^+ to plasmid DNA is 2.3. $^*p<0.05$, significant against the absorbance of mice tumor after injection of PBS. $^\dagger p<0.05$, significant against the absorbance of mice tumor after injection of free plasmid DNA. $^\ddagger p<0.05$, significant against the absorbance of mice tumor after injection of cationized Pronectin F^+–plasmid DNA without PEGylation.

Table 2
Gene expression at different organs 2 days after intravenous injection of cationized Pronectin F^+–plasmid DNA complexes with and without PEGylation or other control agents[a]

Organ	Absorbance			
	PBS	Free plasmid DNA	Cationized Pronectin F^+–plasmid DNA complex	PEG-introduced cationized Pronectin F^+–plasmid DNA complex
Blood	0.30 ± 0.1[b]	0.40 ± 0.2	0.08 ± 0.2	0.17 ± 0.08
Heart	0.24 ± 0.30	0.33 ± 0.12	0.24 ± 0.07	0.22 ± 0.020
Lung	0.30 ± 0.20	0.34 ± 0.1	0.20 ± 0.02	0.25 ± 0.16
Liver	0.48 ± 0.19	0.56 ± 0.30	0.50 ± 0.40	0.73 ± 0.31
Spleen	0.14 ± 0.13	0.17 ± 0.12	0.36 ± 0.21	0.42 ± 0.14
Kidney	0.39 ± 0.08	0.38 ± 0.24	0.40 ± 0.20	0.62 ± 0.23
Tumor	0.41 ± 0.12	0.36 ± 0.18	0.41 ± 0.02	1.33 ± 0.22[c,d,e]

[a] The dose of plasmid DNA injected is 0.25 mg/ml and the PEG introduction percentage is 20.1 mol%. The charge ratio of Pronectin F^+ to plasmid DNS is 2.3.
[b] Mean ± S.D.
[c] $p < 0.05$, significant against the absorbance of mice tumor 2 days after injection of PBS at the corresponding organ.
[d] $p < 0.05$, significant against the absorbance of mice tumor 2 days after injection of free plasmid DNA at the corresponding organ.
[e] $p < 0.05$, significant against the absorbance of mice tumor 2 days after injection of cationized Pronectin F^+–plasmid DNA complex at the corresponding organ.

travenously injected, in contrast to that of the cationized Pronectin F^+–plasmid DNA complex.

Table 2 shows the in vivo gene expression of tumor and different organs 2 days after intravenous injection of PBS, free plasmid DNA, the cationized Pronectin F^+–plasmid DNA complex, and the PEG-introduced cationized Pronectin F^+–plasmid DNA complex. The level of gene expression induced by the PEG-introduced cationized Pronectin F^+–plasmid DNA complex was significantly high in the tumor compared with in other organs. On the contrary, neither free plasmid DNA nor cationized Pronectin F^+–plasmid

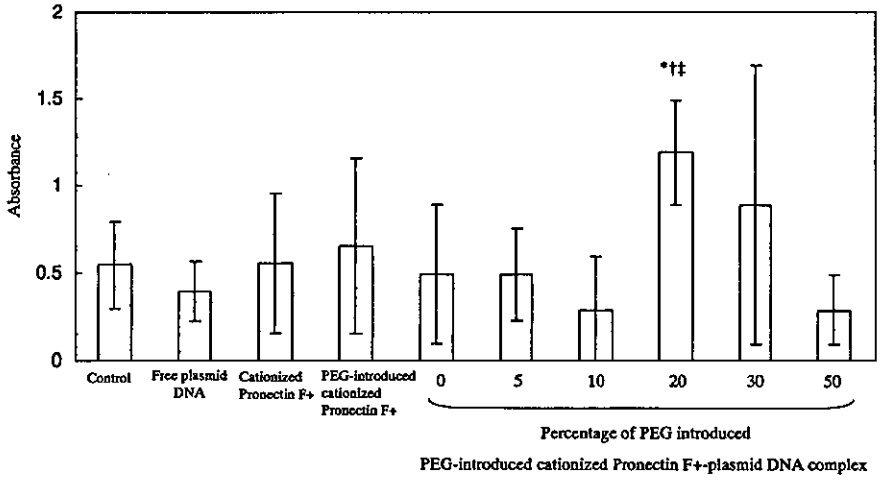

Fig. 4. Effect of percent PEG introduced on the in vivo gene expression of tumor 2 days after intravenous injection of free plasmid DNA, cationized Pronectin F^+ with or without PEGylation, and complexes of plasmid DNA and cationized Pronectin F^+ with or without PEGylation. The dose of plasmid DNA used is 0.25 mg/ml. The N/P ratio of Pronectin F^+ to plasmid DNA is 2.3. $^*p < 0.05$, significant against the absorbance of mice tumor after injection of PBS. $^\dagger p < 0.05$, significant against the absorbance of mice tumor after intravenous injection of free plasmid DNA. $^\ddagger p < 0.05$, significant against the absorbance of mice tumor after injection of cationized Pronectin F^+–plasmid DNA complex with 0 and 5.1 mol% PEGylation.

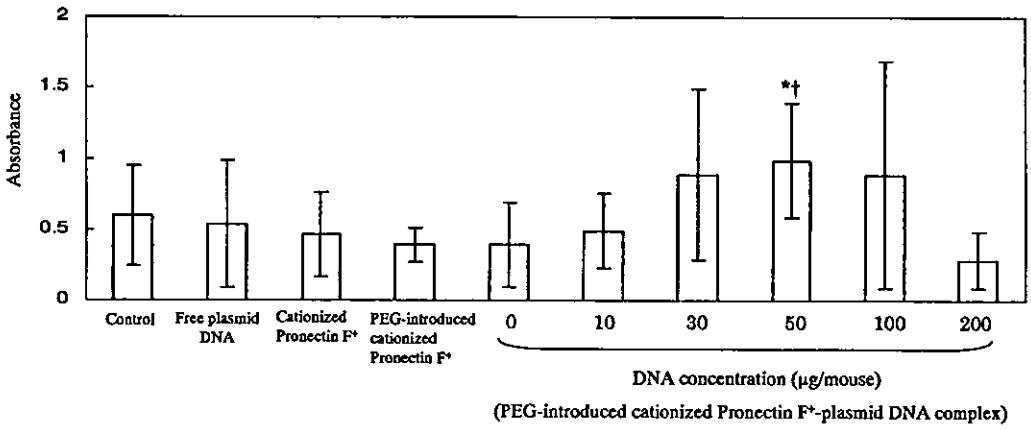

Fig. 5. Effect of plasmid DNA dose on the in vivo gene expression of tumor 2 days after intravenous injection of free plasmid DNA, cationized Pronectin F^+ with or without PEGylation, and complexes of plasmid DNA and cationized Pronectin F^+ with or without PEGylation. The molar percentage of PEG introduced to the cationized Pronectin F^+ is 20.1 mol%. The N/P ratio of Pronectin F^+ to plasmid DNA is 2.3. $^*p<0.05$, significant against the absorbance of mice tumor after injection of PBS. $^\dagger p<0.05$, significant against the absorbance of mice tumor after injection of PEG-introduced cationized Pronectin F^+.

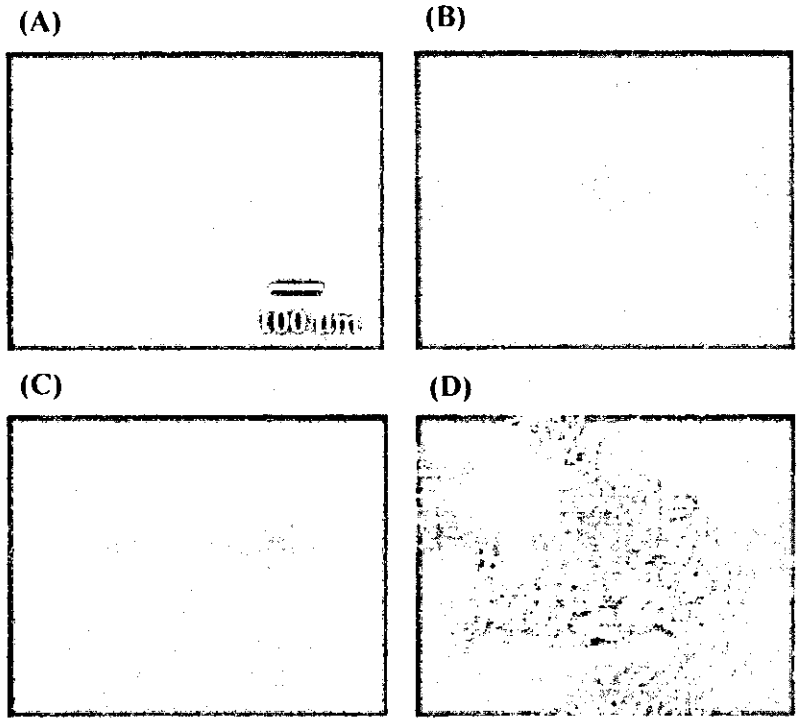

Fig. 6. Fluorescent microphotographs of tumor 2 days after intravenous injection of cationized Pronectin F^+–plasmid DNA complex with or without PEGylation or other control agents: (A) control, (B) free plasmid DNA, (C) cationized Pronectin F^+–plasmid DNA complex, and (D) PEG-introduced cationized Pronectin F^+–plasmid DNA complex. The dose of plasmid DNA used is 0.25 mg/ml and the molar percentage of PEG introduced to the cationized Pronectin F^+ is 20.1 mol%. The N/P ratio of Pronectin F^+ to plasmid DNA is 2.3.

DNA complex enhanced the gene expression in every organ, and the level of gene expression was similar to those of PBS-injected control.

Fig. 4 shows the effect of percentage of PEG introduced on the in vivo gene expression of tumor 2 days after intravenous injection of the PEG-introduced cationized Pronectin F^+–plasmid DNA complexes or other control agents. The tumor level of gene expression increased with an increase in the amount of PEG introduced to attain maximum at the percentage of 20 mol%, and thereafter decreased.

Fig. 5 shows the effect of plasmid DNA dose on the in vivo gene expression of tumor 2 days after intravenous injection of the PEG-introduced cationized Pronectin F^+–plasmid DNA complexes or other control agents. The gene expression increased with an increase in the dose of plasmid DNA used and showed a maximum level at dose of 50 μg/mouse, although lower level was observed at the higher DNA doses.

3.3. Tissue localization of gene expression after intravenous injection of cationized Pronectin F^+–plasmid DNA complexes with or without PEGylation

Fig. 6 shows the fluorescent microscopic photographs of tumor section following the intravenous injection of cationized Pronectin F^+-RITC-labeled plasmid DNA complexes with or without PEGylation or free RITC-labeled plasmid DNA. Only for the tumor section of mice injected with the PEG-introduced cationized Pronectin F^+–plasmid DNA complex, the fluorescent location of plasmid DNA was observed. After injection of free plasmid DNA, cationized Pronectin F^+–plasmid DNA complex without PEGylation, no fluorescence was detected in the tumor.

4. Discussion

This study demonstrates that PEG introduction enabled cationized Pronectin F^+ with repeated RGD sequences to significantly enhance the in vivo expression of plasmid DNA in the tumor. Liposomes or polymer particles intravenously applied are often unspecifically interacted with blood cells and the components. These undesired interactions must be avoided in order to deliver pharmaceutically active substances to certain target tissues. Hydrophilic polymers such as poly(ethylene glycol) (PEG) have been used for this purpose. Modification of the liposome surface by PEG chains strongly prolonged their circulation time in blood [9–12].

Introduction of PEG to cationized Pronectin F^+ was controllable by changing the amount of PEG used for introduction reaction. The physicochemical data of plasmid DNA before and after complexation with cationized Pronectin F^+ with or without PEGylation can be explained in terms of the electrostatic interaction. The cationized Pronectin F^+ was electrostatically interacted with the plasmid DNA (Fig. 1). Retarded or no electrophoretic migration of plasmid DNA complexed with the cationized Pronectin F^+ without PEGylation is ascribed to the neutralization of DNA negative charge and an increase in the molecular size. However, lower introduction percentage of PEG into cationized Pronectin F^+ did not affect on the electrophoretic migration of plasmid DNA at higher charge ratios. Higher introduction percentage of PEG into cationized Pronectin F^+ greatly affected the electrophoretic migration of plasmid DNA at higher charge ratios. Our previous results demonstrated that the surface charge and molecular size were not different between the plasmid DNA complexes with cationized Pronectin F^+ [75]. However, no difference was observed in molecular size of plasmid DNA complexes between the cationized Pronectin F^+ and PEG-introduced cationized Pronectin F^+. PEGylation greatly affected the zeta potential of the plasmid DNA complexes with cationized Pronectin F^+ (Fig. 2). The zeta potential of cationized Pronectin F^+–plasmid DNA complexes decreased by increasing the percentage of PEG introduced and became almost zero at the percent introduction of higher than 10%.

The physical and colloidal parameters of transfection complexes, such as particle size, charge, and stability, are critical factors which determine the biodistribution and expression profiles of plasmid DNA complexes. The parameters should be considered to design gene delivery vectors for the in vivo application. It is well known that the gene carriers of positively charged are prone to nonspecifically interact with a variety of components in the blood, extracellular matrix, and non-target cells because they are negatively charged and can electrostatically inter-

act with the carriers [26]. The nonspecific interactions result in degradation of the carriers and the nonspecific cell uptake which cause their in vivo instabilization of plasmid DNA–carrier complexes. Mixing of plasmid DNA with the cationized Pronectin F^+ without PEGylation did not show any gene expression in the tumor. This may be due to the electrostatic interaction with negatively charged blood cells and components that prevents the plasmid DNA from reaching to the tumor tissue (Fig. 3). From the viewpoint of tumor targeting, the complex charge should be neutral or negative because of less or no interaction with the blood cells and components. In addition, the condensed size of complex is also advantageous in terms of passive targeting of complex to the tumor. Mixing with cationized Pronectin F^+ with PEGylation neutralized the surface charge of plasmid DNA and condensed the size. The change of zeta potential was caused by molecular covering of Sm residues in cationized Pronectin F^+ with PEG chains. It has been reported that the size of substances injected intravenously is one of the key factors contributing to the passive accumulation in the tumor [76]. Enhanced tumor level of gene expression by the PEG-introduced cationized Pronectin F^+–plasmid DNA complex is due to the enhanced tumor accumulation. It is conceivable that the Sm residue can condense the plasmid DNA and PEGylation is effective enough to carry the plasmid DNA to the tumor without enzymatic degradation in the blood circulation. The enhancement of tumor gene expression depended on the PEGylation extent into cationized Pronectin F^+ and the maximum gene expression level was found at the molar percentage of 20% (Fig. 4). This will be explained by the in vivo stability of complex. At the low introduction percentages, it is possible that the amount of PEG molecule is too small to electrostatically cover the complex of plasmid DNA and cationized Pronectin F^+, resulting in insufficiently prolonged circulation, because the surface positive charge may induce the interaction with the blood components of negatively charged. Oppositely, too large amount of PEG molecules may not allow the cationized Pronectin F^+–plasmid DNA complex to suppress the interaction with the blood components, resulting in reduced tumor targetability. It has been demonstrated that an excessive surface modification by PEG chains enhanced the protein adsorption onto the surface of polymers [77,78]. As a result, there would be an optimal extent of PEG introduction to form complexes for a maximum gene transfection in the tumor. When the introduction percentage was low, the complexation would not be strong enough to carry the plasmid DNA to the tumor. It is conceivable that the features of a PEG-introduced cationized Pronectin F^+–plasmid DNA complex result in the superior gene expression.

PEGylation and complexation enabled the plasmid DNA to significantly enhance the gene expression (Fig. 3). PEG-introduced cationized Pronectin F^+ enhanced the level of gene expression compared with PEG-introduced cationized gelatin (data not shown). This can be explained from the viewpoint of an enhanced cell attachment through the RGD sequence. Both cationized Pronectin F^+ and gelatin showed ability to form a complex with negatively charged plasmid DNA and PEGylation strongly reduced their positive charge for tumor targeting. Higher gene transfection may be due to the higher cell attachment. This is because the Pronectin F^+ has larger number of RGD sequences than the gelatin. It has been shown that the RGD sequence can bind to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on angiogenic endothelial cells [66]. Besides the angiogenic endothelial cells, $\alpha_v\beta_3$ integrins also become expressed on some tumor cells, and on a small percentage of activated leukocytes, macrophages, and osteoclasts [79]. We have to bear in mind that our complex with its increased binding capacity may also bind to and become internalized into these cells. Yet, several studies with α_v-integrin-directed therapies have shown promising anti-tumor results, without serious side effects on other $\alpha_v\beta_3$-expressing cells [80,81].

In conclusion, the Pronectin F^+ with repeated RGD sequences was a promising candidate of plasmid DNA non-viral vector for gene transfection in the tumor if it is cationized for the complexation with plasmid DNA and PEG-introduced to form the complex with a prolonged life-period in the blood circulation. The RGD sequence enabled the plasmid DNA to effectively internalize into tumor cells, resulting in enhanced gene expression. This is the first report to use the Pronectin F^+ with the repeated RGD sequence for gene delivery into tumor. This feature makes the Pronectin F^+ a carrier suitable for the intracellular delivery of potent anti-angiogenic drugs into endothe-

lial cells for the treatment of cancer and chronic inflammatory diseases. We conclude that PEG-introduced cationized Pronectin F^+ is promising to target the plasmid DNA to the tumor for therapeutically promoted gene expression.

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Gene Therapy (2004) 11, 194–203

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RESEARCH ARTICLE

Dextran–spermine polycation: an efficient nonviral vector for in vitro and in vivo gene transfection

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Dextran–spermine cationic polysaccharide was prepared by means of reductive amination between oxidized dextran and the natural oligoamine spermine. The formed Schiff-base imine-based conjugate was reduced with borohydride to obtain the stable amine-based conjugate. The transfection efficiency of the synthetic dextran–spermine was assessed in vitro on HEK293 and NIH3T3 cell lines and found to be as high as the DOTAP/Chol 1/1 lipid-based transfection reagent. Modification of the dextran–spermine polycation with polyethylene glycol resulted in high transfection yield in serum-rich medium. Intramuscular injection in mice of dextran–spermine–pSV-LacZ complex induced high local gene expression compared to low expression of the naked DNA. Intravenous injection of a dispersion of the dextran–spermine–pSV-LacZ complex resulted with no expression in all examined organs. When the partially PEGylated dextran–spermine–pSV-LacZ complex was intravenously applied, a high gene expression was detected mainly in the liver. Preliminary targeting studies indicated that the PEGylated dextran–spermine–pSV-LacZ complex bound to galactose receptor of liver parenchymal cells rather than the mannose receptor of liver nonparenchymal cells. This work offers a new biodegradable polycation based on natural components, which is capable of transfecting cells and tissues in vitro and in vivo.

Gene Therapy (2004) **11**, 194–203. doi:10.1038/sj.gt.3302159

Keywords: *dextran–spermine; polycation; PEG; gene delivery; transfection; in vivo*

Introduction

DNA can be delivered into the cell nucleus using physical means or using specific carriers that carry the genes into the cells for gene expression.[1] Gene carriers are divided into two main groups: viral carriers where the DNA to be delivered is inserted into a virus,[2] and cationic molecular carriers that form an electrostatic interaction with the DNA.[3] From the various methods developed for delivering genes, gene carriers have been extensively investigated as transfecting agents for therapeutic genes in *Gene Therapy*.[4] Although at present, the *in vivo* expression levels of the synthetic molecular gene vectors are lower compared to viral vectors and gene expression is transient, these vehicles are likely to present several advantages including safety, low immunogenicity, capacity to deliver large genes, and large-scale production at low cost.[5] The two leading classes of synthetic gene delivery systems that have been mostly investigated involve the use of either cationic lipids or cationic polymers.[1]

Polycations used for gene delivery are considered to be promising candidates for nonviral gene delivery in part because of their molecular diversity that can be modified to fine-tune their physicochemical properties.[6,7] These polycations are able to condense large genes into compact structures and to mask the negative DNA charges, necessities for transfecting most types of cells.[8,9] Polycations used for gene complexation are polyamines that become cationic at physiologic conditions. The most studied polycations used for gene complexation and delivery are the branched/linear polyethylene imine (PEI),[10,11] poly(L-lysine),[12] poly (dimethyl aminoethyl methacrylate, pDMAEMA),[13] poly(trimethyl aminoethyl methacrylate, pTMAEMA),[14] poly(vinylpyridine),[15] chitosan,[16] and diethylaminoethyl dextran (DEAE-dextran).[17] Most polycations are toxic to cells and nonbiodegradable, while the polymers based on amino acids such as poly(lysine) are immunogenic.[12] More advanced polymeric gene delivery systems employ macromolecules with a very high cationic charge density that act as endosomal buffering systems, thus suppressing the endosomal enzyme activities and protecting the DNA from degradation. The high cationic charge mediates both DNA condensing and buffering capacities that diminish the requirement for the addition of endosomolytic agents.[18,19]

Success of nonviral gene delivery depends on the type of carrier materials to bind plasmid DNA and facilitate the cell-specific uptake of carrier–DNA complex.[20–22] In addition, the *in vivo* gene expression is greatly influenced by the body distribution of the carrier–DNA complex intravenously injected. The gene transfection with naked plasmid DNA always

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Received 22 May 2003; accepted 05 August 2003

shows low efficiency *in vivo* although it is simple and safe.[23,24] On the other hand, when a plasmid DNA is complexed with a vector, the molecular size and surface charge of the complex affect the body fate of the plasmid DNA.[25] For example, the complex of plasmid DNA with cationic liposomes mainly accumulates in the lung, liver, and spleen.[26,27] In an attempt to alter the organ distribution of lipid complexes, their surface was modified by polyethylene glycol (PEG) and other hydrophilic polymers.[28,29] This modification enabled the plasmid DNA to prolong the half-life in the blood circulation by suppressing their interaction with plasma components and erythrocytes, which strongly modifies the *in vivo* characteristics of DNA. The particle size, charge, and the surface characteristics of the complex have a strong influence on the plasmid DNA body distribution and transfection efficiency.[24,26,30–33]

In recent publications,[34,35] we reported on a new class of biodegradable polycations capable of complexing and transfecting various genes to many cell lines in relatively high yields. More than 300 different polycations were prepared starting from various natural polysaccharides and oligoamines having two to four amino groups. These cationic polysaccharides were prepared by reductive amination of the oligoamine and periodate-oxidized polysaccharides. Although most of these cationic conjugates formed stable complexes with plasmid DNAs as determined by ethidium-bromide quenching assay,[36] only the dextran–spermine-based polycations were found to be highly effective in transfecting cells *in vitro*.

The main objective of this study was to evaluate the efficiencies of dextran–spermine in delivering genes *in vivo* after intramuscular (i.m.) and intravenous (i.v.) injection of its complex with plasmid DNA, and also to test the transfection efficiencies and biodistribution of PEGylated dextran–spermine–DNA complex relative to the corresponding non-PEGylated carrier after i.v. administration.

Results

Chemistry

Dextran–spermine-based conjugate (Figure 1) was prepared by reductive amination between oxidized dextran and spermine. Dextran was initially oxidized with potassium periodate and the obtained dialdehyde derivative was allowed to react under basic conditions with spermine. Three dextran–spermine-based conjugates (G7TA103, G7TA107, and G7TA141) were prepared under similar conditions and characterized as illustrated in Table 1. The content of substituted spermine moieties was determined from nitrogen content (% N) and found to be nearly 50% (50 spermine moieties in each 100 repeating sugar units).

The corresponding G7TA141 dextran–spermine polycation was further modified with increasing amount of PEG to obtain a partially shielded polycation. PEG monomethyl ether (mPEG, M_w=2000 Da) was initially treated with *p*-nitrophenyl chloroformate to obtain the active *p*-nitrophenyl carbonate derivative (mPEG-OPNC) in relatively good yields (Figure 2a). Substitution of the PEG moieties to dextran–spermine was simply achieved by mixing an aqueous solution of the mPEG-OPNC with a concentrate aqueous solution of the polycation at pH 9. The amount of PEG was fixed at 1, 3, and 5% (mol/mol) of PEG moieties to primary amines (ε-NH_2), respectively. The mixture was allowed to stir at room temperature overnight, purified by column chromatography and lyophilized to dryness. The PEGylated dextran–spermine (Figure 2b) derivatives show nearly similar molecular weights to the starting dextran–spermine (G8TA34A to G8TA34C, Table 1).

In vitro transfection

The *in vitro* transfection efficiency of the cationic dextran–spermine-based conjugates was tested in NIH3T3 and HEK293 cell lines applying *p*-luc and β-Gal encoding plasmid DNAs. Figure 3a shows the *in vitro* transfection efficiencies in serum-free medium

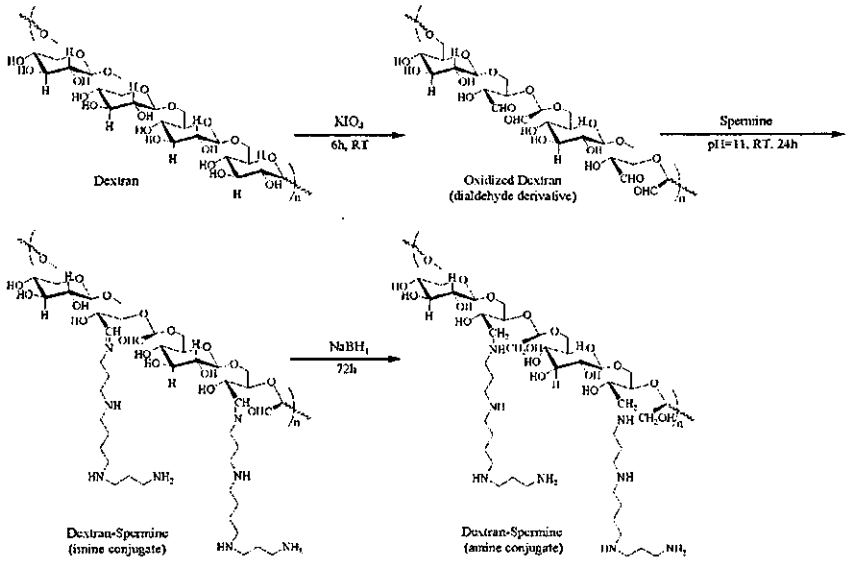

Figure 1 Synthesis of dextran–spermine-based conjugate.

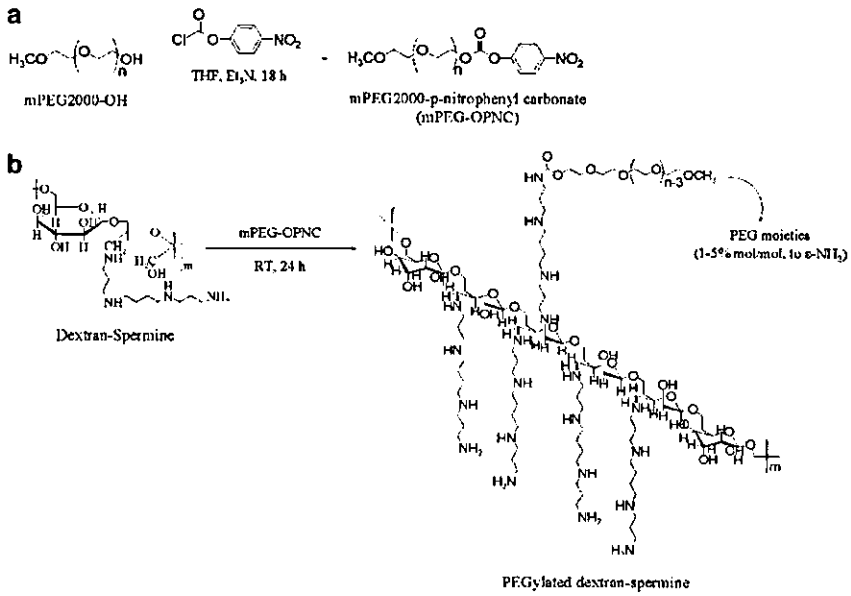

Figure 2 (a) Synthesis of mPEG2000-p-nitrophenyl carbonate (mPEG-OPNC) and (b) modification of dextran–spermine-based conjugate with PEG.

Table 1 Chemical characterization of dextran–spermine-based conjugates

Polymer code	*%N*[a]	*% binding*[b]	*TNBS*[c]	*GPC*[d]		
				M_w	M_n	P
G7TA103	10.99	52	1.45±0.05	10826	4466	2.42
G7TA107	10.82	50	1.19±0.04	11674	5136	2.27
G7TA141	11.20	53	1.18±0.08	10071	4504	2.23
G8TA34A[e]	10.47	ND[f]	1.15±0.05	10854	4820	2.25
G8TA34B[g]	10.96	ND[f]	1.10±0.02	11790	4792	2.46
G8TA34C[h]	10.21	ND[f]	1.02±0.06	11264	5120	2.20

[a]Found nitrogen content (elemental microanalysis).
[b]Percent of calculated spermine moieties substituted to 100 repeating sugar units.
[c]Amount of primary amine (μmol/mg) determined by the TNBS method (n=3).
[d]M_w, M_n and polydispersity (P=M_w/M_n) were determined by GPC (Materials and methods).
[e]PEGylated dextran–spermine derivatives of G7TA141 starting polycation. The degree of PEGylation was fixed at 1% mol/mol (PEG/ε-NH_2).
[f]Degree of spermine substitution could not calculated from % N due to PEG modification.
[g]PEGylated dextran–spermine derivatives of G7TA141 starting polycation. The degree of PEGylation was fixed at 3% mol/mol (PEG/ε-NH_2).
[h]PEGylated dextran–spermine derivatives of G7TA141 starting polycation. The degree of PEGylation was fixed at 5% mol/mol (PEG/ε-NH_2).

(SFM) of the representative dextran–spermine-based conjugates (G7TA141, G7TA107, and G7TA103, Table 1) in NIH3T3 cells applying *p*-luc as the marker gene. The purpose of this experiment was to evaluate the reproducibility in the transfection efficiency of the synthetic polycations. Transfection yields were recorded as a function of polycation/DNA weight-mixing ratios, and expressed as relative light units (RLU) normalized to the total protein contents in cell wells. At weight-mixing ratio of 2 (polymer/DNA), negligible degree of transfection was observed in all conjugates. Maximum transfection was obtained in the range of 4–8 weight-mixing ratios (polymer/DNA) and luciferase readings were similar to the positive control (ie DOTAP/Chol 1/1). At this range, nearly 7500 RLU/mg protein was obtained. Higher weight-mixing ratios (10–15, polymer/DNA) resulted with a remarkable decrease in luciferase expression.

PEGylated dextran–spermine polycations were tested for their transfection activities both in SFM and 10% fetal calf serum (FCS). The active dextran–spermine polycation (G7TA141, Table 1) was modified with increasing amount of PEG (up to 5% mol/mol, Table 1), and the *in vitro* transfection efficiency in HEK293/β-Gal system was tested as functions of %PEGylation and medium type (Figure 3b). The transfection efficiency of each PEGylated derivatives was recorded at the best weight-mixing ratio and expressed as OD at 420 nm (see Materials and methods) without normalization to total protein contents in cell wells. The non-PEGylated dextran–spermine polycation (ie G7TA141, 0% PEGylation) was used as positive reference in this experiment. As expected,

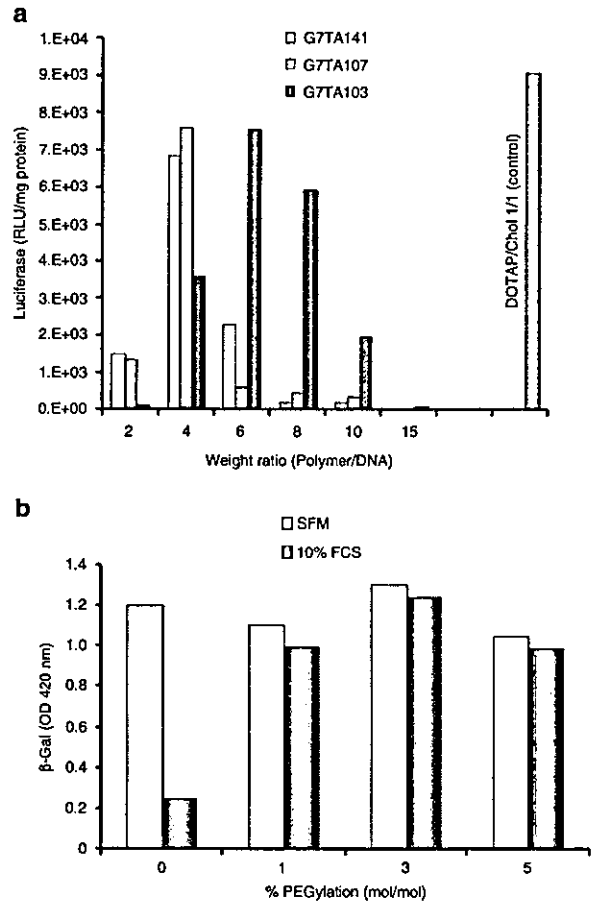

Figure 3 (a) p-luc transfected NIH3T3 cells applying dextran–spermine-based conjugates: G7TA141 (□), G7TA107 (□), and G7TA103 (■). Transfection was performed in SFM and luciferase content was expressed as RLU/mg protein. DOTAP/Chol 1/1 commercial transfecting reagent was used as positive control. (b) β-Gal plasmid DNA transfected HEK293 cells applying dextran–spermine polycation (G7TA141) and its PEGylated derivatives (1, 3 and 5% mol/mol of PEG to ε-NH_2). Transfection was performed both in serum-free (□) and 10% FCS (■).

non-PEGylated dextran–spermine (ie 0% PEGylation) showed high OD value (~1.2) in SFM but low OD value (~0.2) in 10% FCS (Figure 3b). PEGylated derivatives (1, 3, and 5% mol/mol) showed high transfection yields both in SFM and 10% FCS cultures. Also, it was noticed that minor differences in β-Gal expression in full-medium cultures were monitored at all degrees of PEGylation.

In vivo studies

Intramuscular injection of dextran–spermine–pSV-LacZ complex

G7TA141 polycation (Table 1) was randomly selected for the *in vivo* studies and all *in vivo* injections were made in triplicate and average values and SD were recorded. Figure 4 shows the average specific β-Gal activities (mU/mg protein, see Materials and methods) of mice muscles as a function of complex weight-mixing ratio (polycation/pSV-LacZ) 2 days post treatment. Intramuscular

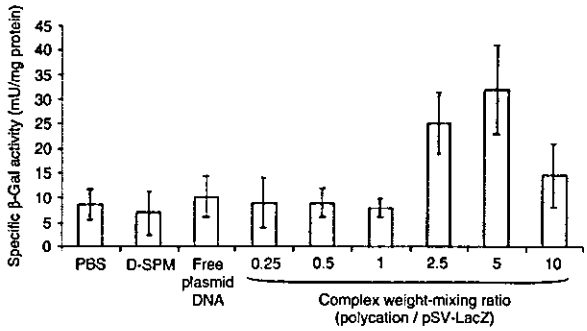

Figure 4 In vivo β-Gal activities (mU/mg protein) of mice muscles as a function of weight-mixing ratio (polycation/DNA). Mice were intramuscularly injected with dextran–spermine–pSV-LacZ complex at weight-mixing ratios of 0.25–10 (polycation/plasmid DNA) and the gene level was evaluated 2 days post injection. PBS, dextran–spermine (D-SPM) and free plasmid DNA solutions were injected as control references.

injection with the control buffer (ie phosphate-buffered saline solution (PBS)) resulted with background β-Gal reading (~7±2 mU/mg protein). Similar background β-Gal activities were obtained with naked pSV-LacZ DNA and the dextran–spermine polycation (coded as D-SPM) control solutions. At complex weight-mixing ratios of 0.25:1 to 1:1 (polycation/pSV-LacZ), low to negligible β-Gal activities were obtained. At 2.5 weight-mixing ratio, high gene expression was detected (25±7 mU/mg protein). The highest β-Gal activity was obtained at 5 weight-mixing ratio where nearly 34±8 mU/mg protein was obtained. Further increase in the polycation ratio (ie 10 polycation/pSV-LacZ) resulted with a decrease in the gene expression.

Figure 5a shows the average specific β-Gal activities of mice muscles, 2 days after receiving the i.m. injection complexes containing varying amounts of pSV-LacZ amounts. The weight-mixing ratio was fixed to 5 and the amount of injected pSV-LacZ was varied from 10 to 200 μg per mouse. PBS, naked pSV-LacZ, and dextran–spermine solutions were applied as controls and resulted with low and background β-Gal activities. At pSV-LacZ injected amounts of 10 and 25 μg/mouse, low β-Gal activities were obtained similar to the controls. The level of gene expression gradually increased with the increase in pSV-LacZ amount up to 50 μg/mouse. At this injected amount, nearly 35±8 mU/mg protein of β-Gal activity was monitored. Higher injected amounts of pSV-LacZ (ie 75–200 μg/mouse) resulted with a gradual decrease in the gene expression. Figure 5b shows the level of gene expression as a function of the time after treatment. The amounts of injected pSV-LacZ and weight-mixing ratio were fixed at 50 μg/mouse and 5 (polycation/DNA), respectively. The level of β-Gal activity after 1 day was low, similar to the controls. Maximum gene expression was obtained 2 days post injection and the level of β-Gal activity was calculated to be 33±8 mU/mg protein. At days 3–5 post injection, gradual decrease in β-Gal readings was observed.

Intravenous injection of PEGylated/non-PEGylated polycations complexes with pSV-LacZ

Preliminary i.v. injection to mice of free plasmid DNA and its complex formulation with non-PEGylated

Table 2 Gene expression at different organs 2 days after i.v. injection of 5% PEGylated–dextran–spermine-pSV-LacZ complex and other agents

Organ	*Specific β-Gal activity (mU/mg protein)*			
	PBS[a]	*PEGylated-D–SPM*[b]	*Free plasmid DNA*[c]	*PEGylated-D–SPM–pSV-LacZ complex*[d]
Blood	6.22±0.82[e]	6.42±3.18	6.24±0.73	7.12±1.56
Heart	7.23±2.23	4.45±2.40	5.54±3.23	8.23±1.10
Lung	6.94±3.12	8.25±0.75	9.02±0.52	7.29±0.14
Liver	7.45±1.03	8.25±2.55	8.17±1.23	**28.45±3.38**
Spleen	5.87±1.14	4.45±3.46	7.30±1.05	9.13±1.45
Kidney	6.98±1.08	6.45±2.34	7.34±1.34	**17.30±1.15**
Gastrointestinal tract	7.14±4.20	5.32±0.34	6.23±5.32	9.10±1.02
Carcass	8.72±2.14	9.04±1.01	8.06±1.17	9.26±1.08
Excretion	7.94±0.15	8.16±0.23	6.24±3.90	5.45±3.34

[a]Phosphate-buffered saline control (200 μl per single injection).
[b]PEGylated–dextran—spermine-based conjugate (250 μg in 200 μl PBS per mouse).
[c]Free plasmid DNA (50 μg in 200 μl PBS per mouse).
[d]5% PEGylated-dextran–spermine–pSV-LacZ at weight-mixing ratio of 5 (polycation/DNA) and 50 μg/mouse of the plasmid in 200 μl PBS. β-Gal activities were evaluated as described in Materials and methods.
[e]Mean±s.d. (n=3).

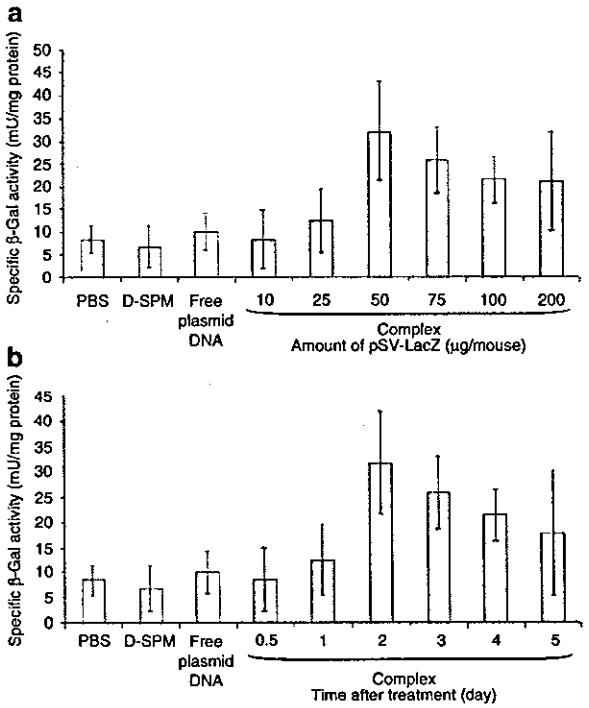

Figure 5 In vivo β-Gal activities in mice muscles as functions of amount of injected plasmid DNA (a) and time after treatment (b). The weight-mixing ratio of complex mixtures was fixed at 5 (polycation/plasmid DNA). PBS, dextran-spermine and free plasmid DNA were injected as control references.

dextran–spermine polycation did not induce gene expression in all tested organs (data not shown). Modification of the dextran–spermine polycations with the nonionic hydrophilic polymer PEG remarkably improved the transfection efficiency of the polycations in serum-rich media.

Table 2 summarizes the *in vivo* gene expression of different organs 2 days after i.v. injection of PBS, free plasmid DNA, 5% PEGylated dextran–spermine polycation (without DNA), and the 5% PEGylated dextran–spermine–pSV-LacZ complex. PBS, PEGylated polycation, as well as free-plasmid DNA did not induce any β-Gal activities in all tested organs. On the other hand, when 5% PEGylated dextran–spermine–pSV-LacZ complex was applied, a high gene level was observed in the liver. The β-Gal activity in the liver was calculated to be 28.45±3.38 mU/mg protein in contrast to the free plasmid DNA and other controls (ie PBS and polycations), which resulted with negligible activities.

In another *in vivo* experiment, the level of gene expression in the liver after i.v. injection of PEGylated dextran–spermine–pSV-LacZ complex was monitored as a function of %PEGylation and time after treatment. Figure 6a shows the calculated β-Gal activities in the liver, 2 days after i.v. injection of PEGylated-dextran–spermine–pSV-LacZ complex solutions. The percentages of PEGylated dextran–spermine were 1, 3 and 5% mol/mol (PEG to ε-NH_2). PBS, free plasmid DNA, and the PEGylated polycations (without DNA) solutions were similarly injected as controls and resulted as expected with no gene expression. In all, 1% PEGylated polymers showed minor transfection activity, while the 3 and 5% PEGylated dextran–spermine showed high β-Gal transfection yields of 23 and 30 mU/mg protein, respectively. Figure 6b shows the level of gene expression in the liver after i.v. injection of 5% PEGylated dextran–spermine–pSV-LacZ complex solution as a function of time after treatment. The highest level of gene expression was obtained 2 days post injection.

To assess the reason for this liver targeting obtained with the PEGylated dextran–spermine gene carrier, the animals were pretreated with galactosylated and mannosylated agents that bind to liver galactose and mannose receptors, respectively. Figure 7 shows the gene expression of β-Gal in the liver 2 days after i.v. injection of 5% PEGylated-dextran–spermine–pSV-LacZ complex with and without preinjection of galactose and mannose agents. Without preinjection of the blocking agents, high β-Gal activity was observed in the liver (~30 mU/mg protein) similar to the results reported in

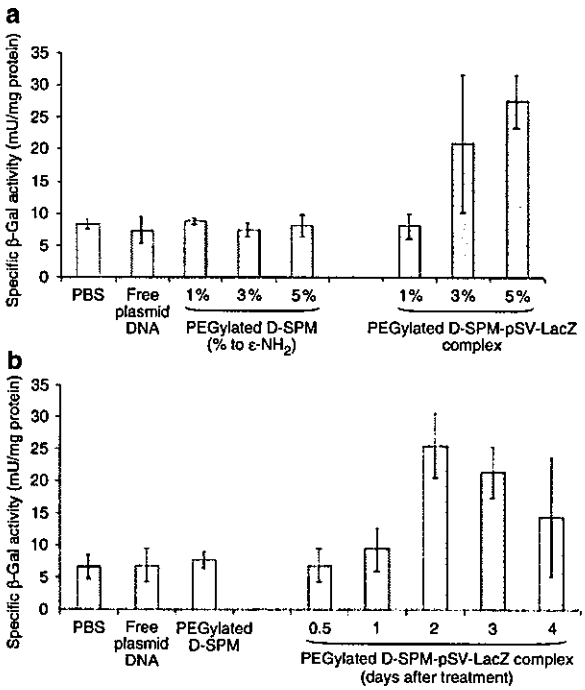

Figure 6 In vivo β-Gal activities in mice livers as functions of %PEGylation of dextran–spermine (a) and time after treatment (b). The weight-mixing ratio of injected complexes was fixed to 5 (polycation/plasmid DNA) at a total plasmid dose of 50 (μg/mouse). PBS, free plasmid DNA and PEGylated-dextran–spermine derivatives (without DNA) solutions were injected as control references.

Figure 6. Preinjection with both arabinogalactan and D-galactosylated bovine albumin resulted in a remarkable decrease of an average of 10 mU/mg protein in the β-Gal activity in the liver. In addition, when D-mannosylated bovine albumin that does not bind to liver galactose receptor was preinjected, high transfection yield was obtained.

Discussion

Dextran–spermine-based polycation was prepared by reductive amination synthesis between oxidized dextran (dialdehyde derivatives) and the naturally occurring tetramine spermine. The synthetic route of dextran–spermine-based conjugates was found to be reproducible in terms of degree of conjugation and grafted spermine moieties (Table 1).[34] The *in vitro* transfection efficiencies of the synthetic conjugates, on the other hand, were also found to be reproducible in terms of gene level, but the weight-mixing ratio needed for optimal transfection varied from polymer to polymer. The optimal weight-mixing ratio needed for transfection in all tested dextran–spermine conjugates was found to be in the range of 4–8 (polymer/DNA). This variation could be explained by the slight changes in spermine content and degree of grafting, which in part could result with major changes in DNA-condensation properties.

When 10% FCS culture medium was applied instead of SFM in the *in vitro* transfection experiments, 80% reduction in the gene expression was observed (Figure

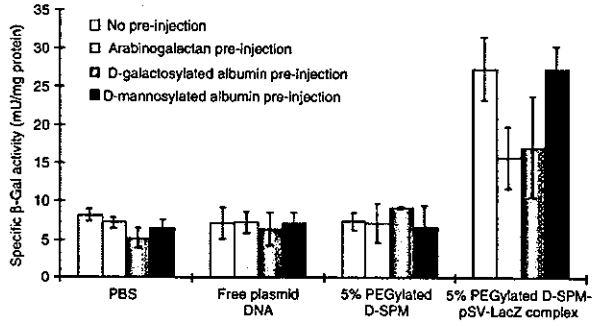

Figure 7 Suppression effect of preinjection of blocking agents on the in vivo gene expression of liver 2 days after i.v. injection of 5% PEGylated-dextran–spermine–prSV-LacZ complex with or without blocking agents. At 1 h prior to complex injections, mice were preinjected with 20 mg/kg dose of arabinogalactan (▥), D-galactosylated bovine albumin (▥) and D-mannosylated bovine albumin (■). A direct injection of the complex without preinjection of blocking agents (□) was applied as positive control. PBS, free plasmid DNA and 5% PEGylated dextran–spermine polycation (without DNA) solutions were injected as negative controls.

3b). This phenomenon is well known and is attributed to proteins (serum components) adsorption on the surface of the complex (ie polycation/DNA), which in part could induce the aggregation/deactivation of the complex and finally to the reduction in the transfection efficiencies.[37] In analogy to literatures, it was decided to modify our developed polycation with hydrophilic polymer such as PEG in order to increase their stability and transfection efficiencies in serum-rich media.[29,37] PEG molecules are considered to be the most attractive materials for this purpose. They are safe, nontoxic, cheap, and do not interact with plasma components.[38] Transfection efficiencies in 10% FCS medium applying the PEGylated-dextran–spermine polycations as the gene carriers resulted with the high gene level (Figure 3b), indicating a remarkable increase in the complex stability. No remarkable differences in the gene level were observed with the all PEGylated derivatives (ie 1, 3, and 5% PEGylation).

Intramuscular injection of the dextran–spermine–pSV-LacZ complex solution to mice resulted with a high local gene level in comparison to the naked DNA, which resulted with no gene expression. The optimal weight-mixing ratio for proper expression was found to be 5 (polycation/DNA) applying 50 μg/mouse plasmid injected dose. The non-PEGylated dextran–spermine–pSV-LacZ complex when applied by i.v. injection to mice did not show gene expression. Typical polycation–DNA complexes are believed to be rapidly removed from the bloodstream by the reticuloendothelial system, which decreases their potential for specific targeting *in vivo*. In analogy with STEALTH liposomes, it was suggested that incorporation of hydrophilic, nonionic polymer onto the surface of the complexes can help to solve this problem.[39] Therefore, PEGylated-dextran–spermine derivatives were synthesized as gene carriers for i.v. administration. Intravenous administration of the PEGylated dextran–spermine–pSV-LacZ complex resulted with a high gene expression in the liver. The 5% PEGylated-dextran–spermine was found to be the most active PEGylated derivative for systemic administration.

The gene expression in the liver by the PEGylated dextran–spermine–pSV-LacZ complex was markedly

reduced by preadministration of both arabinogalactan and D-galactosylated bovine albumin, whereas the mannosylated albumin had no influence on the liver level of gene expression. It is well recognized that both arabinogalactan and D-galactosylated bovine albumin interact with asialoglycoprotein receptor located in the surface of liver parenchymal cells *in vitro* and *in vivo*.[40,41] On the other hand, albumin bovine-α-D-mannopyranosyl phenyl isothiocyanate is bound to the mannose receptor, which is located on the surface of the nonparenchymal cells and responsible for this uptake.[42] Taken together, the present results strongly indicate that the targeting of PEGylated dextran–spermine–plasmid DNA complex to the liver is probably mediated by the galactose receptor of the liver parenchymal cells rather than the mannose receptor of liver nonparenchymal cells.

Materials and Methods

Chemistry

All solvent and reagents were of analytical grade and were used as received. Dextran with an average molecular weight of 40 kDa was obtained from Sigma Chemical Co. (St Louis, MO, USA). Potassium periodate (KIO_4), sodium borohydride ($NaBH_4$), mPEG (mPEG2000), *p*-nitrophenyl chloroformate and spermine were obtained from Aldrich (Milwaukee, WI, USA). Water-free mPEG2000 was obtained by azeotropic distillation from toluene and vacuum-dried at 60°C over P_2O_5. A sage-metering pump model-365 (Orion, NJ, USA) was used for slow and reproducible addition of reactants. Oxidized dextran was obtained by reacting dextran with 1 equimolar of potassium periodate (to saccharide unit) in water followed by purification with DOWEX-1 anion-exchange chromatography (acetate form), dialysis against double-distilled water (DDW), and finally by lyophilization. The aldehyde content of the oxidized dextran was determined by the hydroxylamine hydrochloride method and found to be 6.9 ± 0.6 mmol/g (aldehyde/1 g polymer, $n=5$).[43] ^{1}H-NMR spectra were recorded on Varian 300 MHz instrument using D_2O or d_6-DMSO as solvents and TMS as internal standard. Average molecular weights of polycations were estimated by GPC-Spectra Physics instrument (Darmstadt, Germany) consisting of a pump, column (Shodex KB-803) and refractive index (RI) detector. Average molecular weights were determined according to Pullulan standards (PSS, Mainz, Germany) with molecular weights between 5800 and 212 000 Da. A volume of 5% (w/v) Na_2HPO_4 in 3% (v/v) acetonirile (pH 4) was used as an eluent for the cationic conjugates.[44] Degree of conjugation was estimated by elemental microanalysis of nitrogen (% N) using a Perkin-Elmer 2400/II CHN analyzer. Primary amine content (ε-NH_2) in the polymer was determined by the TNBS method applying spermine tetrahydrochloride (Aldrich) as a standard.[45]

Synthesis of dextran–spermine conjugate

Dextran–spermine-based conjugate was prepared as described elsewhere.[34,35] Briefly, a solution of oxidized dextran (1 g) in 100 ml DDW (6.9 mmol aldehyde groups) was slowly added during 5 h to a basic solution containing 1.25 equimolar amount of oligoamine (to aldehyde) dissolved in 50 ml borate buffer (0.1 M, pH 11). The mixture was stirred at room temperature for 24 h and excess of sodium borohydride (1 g) was added and stirring was continued for 48 h at room temperature. The reduction was repeated with additional portion of $NaBH_4$ (1 g) and stirring for 24 h under the same conditions. The resulting light-yellow solution was dialysed against DDW (6×6 l) applying 3500 cutoff cellulose tubing (Membrane Filtration Products Inc., San Antonio, TX, USA) followed by lyophilization to obtain a yellowish reduced amine-based conjugate in 50% overall yield (to polysaccharide).

^{1}H-NMR (D_2O): 1.645 (m, 4H, dextran-$CH_2NH(CH_2)_3$ $NHCH_2CH_2CH_2CH_2NH(CH_2)_3NH_2$), 1.804 (m, 4H, dextran-$CH_2NHCH_2CH_2CH_2NH(CH2)_4NHCH_2CH_2CH_2NH_2$), 2.815 (m, 14H, dextran-$CH_2NHCH_2CH_2CH_2NHCH_2$ $(CH2)_2CH_2NHCH_2CH2CH_2NH_2$), 3.52–4.19 (m, sugar hydrogens) and 5.02 p.p.m. (m, 1H, anomeric hydrogens of sugars). % N=10.90 ± 0.5% (equal to ~50% substitutions). TNB=1.33 ± 0.15 μmol/mg (primary amino content). M_w=$10\,000 \pm 1500$ Da (n=10).

Synthesis of methoxy PEG2000 terminated p-nitrophenyl carbonate (mPEG-OPNC)

Methoxy PEG2000 (5 g, 2.5 mmol of hydroxyl groups) was dissolved in 100 ml of anhydrous THF and 1.4 ml of anhydrous triethylamine (10 mmol) was added under nitrogen atmosphere. The mixture was cooled to 0°C (ice bath) and 1.5 g of *p*-nitrophenyl chloroformate (7.5 mmol) was added and the mixture was stirred for 2 h at 0°C and 16 h at room temperature. Triethylammonium hydrochloride salt was discarded by filtration and the filtrate was evaporated to dryness under reduced pressure. The crude was redissolved in dichloromethane (150 ml) and washed with 5% citric acid (2×50 ml) and DDW (2×50 ml). The organic phase was dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure to a final volume of 15 ml. Precipitating of the product was achieved by dropwise addition of the concentrate solution into anhydrous diethylether (400 ml) and incubation at –20°C for 24 h, followed by filtration and vacuum-dried over P_2O_5 to yield 3.15 g (~60%) of the product. The degree of modification was calculated by UV spectrophotometer (λ=410 nm) after hydrolysis of the *p*-nitrophenyl carbonate group with 0.1 M NaOH (standardized *p*-nitrophenol solution in 0.1 M NaOH was used for the calibration curve). The degree of conversion was found to be approximately 97% relative to starting terminal hydroxyl functionalities.

^{1}H-NMR (d_6-DMSO): 3.227 (m, 3H, $CH_3(OCH_2CH_2)$-$nOCH_2CH_2OCOOC_6H_4NO_2$), 3.4–3.6 (m, ~90H, CH_3 $(OCH_2CH_2)nOCH_2CH_2OCOOC_6H_4NO_2$), 3.701 (t, 2H, $CH_3(OCH_2CH_2)nOCH_2CH_2OCOOC_6H_4NO_2$), 4.36 (2, 2H, $CH_3(OCH_2CH_2)nOCH_2CH_2OCOOC_6H_4NO_2$), 7.559 (d, 2H) and 8.312 p.p.m. (d, 2H) are the aromatic hydrogens bearing to carbonate and nitro groups, respectively.

Synthesis of PEGylated dextran–spermine conjugates

Dextran–spermine conjugate (100 mg, ~118 μmol of ε-NH_2) was dissolved in 2.5 ml. DDW and the pH was adjusted to 9 by 1 M HCl aqueous solution. To this solution, 118 μl (1% mol/mol to ε-NH_2) of mPEG-OPNC aqueous solution (21.76 mg/ml in DDW, equal to 10 μmol/ml) was added, and the mixture was stirred at room temperature for 16 h. The PEGylated derivative

was purified from the released *p*-nitrophenol and unbound PEG by Sephadex G-25 column chromatography using DDW as the eluent. Fractions containing the PEGylated derivative (positive Ninhydrin test) were collected and lyophilized to dryness. This method was similarly adapted for other percents of PEG substitution (ie 3 and 5% mol/mol). The average yield of the synthesis was nearly 90% (w/w).

The degree of PEGylation was found to be ~95% (to starting feed) as determined by spectrophotometric measurement of the released *p*-nitrophenol (UV, λ=410 nm).

Preparation of plasmid DNA

The plasmid DNA used in this study was the expression vector consisting of the coding sequence of LacZ and an SV40 promoter inserted at the upstream (pSV-LacZ, 7931 bp). The plasmid DNA was amplified in a transformant of *E. coli* bacteria and isolated from the bacteria by Qiagen Maxi kit-25 (Qiagen KK, Tokyo, Japan). Briefly, the grown bacteria were harvested and lysed in the aqueous solution of 1 wt% sodium dodecyl sulfate (SDS) and 0.1 wt% RNase A solution in NaOH (pH 8.0). The lysate was neutralized by the addition of 3.0 M potassium acetate (pH 5.5). After separation of the insoluble portion by the use of the QIA filter cartridge, the lysate was applied to the Qiagen-tip (anion-exchange resin), followed by washing with a buffer containing 1 M NaCl to remove the trace of RNA and protein. The plasmid DNA was eluted with an elution buffer containing 1.25 M NaCl at pH 8.5, de-salted, and precipitated by 2-propanol. The precipitated plasmid DNA was centrifuged at 14 000 r.p.m. for 10 min at 4°C and washed twice with 70% ethanol aqueous solution to substitute 2-propanol for ethanol. After centrifugation (14 000 r.p.m., 2 min, 4°C), the resulting plasmid DNA was air-dried and dissolved in a small volume of 10 mM Tris-HCl and 1 mM EDTA buffer solution. When measured to assess the purity of plasmid DNA obtained, the absorbance ratio at the wavelength of 260 to 280 nm ranged from 1.8 to 2.0.

In vitro transfection

Transfection with dextran–spermine polycation was performed and analyzed using expression plasmid pCMV-luc containing luciferase gene under the control of cytomegalovirus long terminal repeat enhancer/promoters. A measure of 0.5 μg of purified plasmid (Qiagen kit) per well of transfected cells was mixed with dextran–spermine (or PEGylated derivatives) at various weight-mixing ratios ranging from 1 to 20 w/w (polycation/DNA, respectively). The polycation/DNA complexes at particular weight ratios were diluted to a final volume of 200 μl with SFM and allowed to stand at room temperature for 30 min. The 24-well plates, seeded 24 h before the transfection with 1.5×10^5 cells per well (NIH3T3 or HEK293), were washed with SFM and the solution of the complexes (at various polymer/DNA ratios) were added to the corresponding wells and incubated for 4 h at 37°C under growth conditions. The medium containing the complexes was then replaced with 10% FCS and the cells were incubated in culture for 48 h. At this time, the growth medium was removed, and cell lysates were formed and analyzed for luciferase activity (Promega luciferase assay kit) and for total protein content (BCA assay, Pierce). pCMV-Gal encoding to β-galactosidase was also used in parallel experiments and the enzyme activity was measured using Invitrogen kit (Invitrogen Co., CA, USA). For the transfection experiments in serum-containing medium, SFM was replaced with 10% FCS at the complexation and uptake stages, followed by the same procedure described above.

DOTAP/Chol 1/1 transfecting reagent (Avanti Polar Lipids Inc., Alabaster, AL, USA) was used as positive control according to the manufacturer's protocol. Briefly, 2.32 μl of 1 mM DOTAP/Chol 1/1 solution in 10 mM HEPES buffer saline (pH 7.4) was added to 2 μg DNA solution and the mixture was diluted to 200 μl with the same buffer and allowed to stand at room temperature for 30 min. Then, it was added to cell well in SFM, incubated for 4 h and SFM was replaced with growth medium (10% FCS) and incubated for 48 h. Cells were lysed and the gene expression was evaluated as described above.

In vivo studies

Complexation of dextran–spermine with plasmid DNA for the *in vivo* studies was performed by mixing the two components at various weight-mixing ratios in the aqueous solution. Briefly, 150 μl of 0.1 M PBS (pH 7.4) containing 2.5, 5, 10, 25, 50, and 100 μg of dextran–spermine was added to the same volume of PBS containing 10 μg of plasmid DNA. The solution was gently agitated at 37°C for 30 min to form cationized dextran–plasmid–DNA complexes. Complexes having higher DNA content (up to 200 μg) were similarly formed as described above.

Intramuscular injection of dextran–spermine–DNA complexes

All the experimental procedures were performed in accordance with the specifications of Guideline for Animal Experiments of Kyoto University. DDy male mice (6 weeks old, body weight around 15 g, Shimizu Laboratory Supplies Co. Ltd, Kyoto, Japan) were anesthetized by intraperitoneal injection of pentobarbital (160 mg/kg body weight) and an incision was performed on the skin overlying the inguinal portion of the left leg. One thread was sutured on the left femoral muscle exposed to mark the site of DNA injection. The pSV-LacZ-cationized–dextran complexes containing different amounts of pSV-LacZ were intramuscularly injected at the sutured point. Mice were killed with cervical dislocation and the muscle samples (5 mm in diameter and 5 mm in depth) were taken around the marking points 0.5, 1, 2, 3, 4, and 5 days after DNA-complex injection.

Intravenous administration of PEGylated-dextran–spermine–DNA complexes

DDy male mice (6 weeks old, 20 g body weight) were used in this study. All mice received an i.v. injection of 0.1 M PBS (pH 7.4) or that containing free plasmid DNA, PEGylated-dextran–spermine and the PEGylated-dextran–spermine–plasmid DNA complex mixtures in a total volume of 200 μl. The complex was prepared at weight mixing ratio of 5 (polymer/plasmid) and the amount of plasmid DNA injected was fixed at 50 μg/mouse. At 0.5–5 days post injections, mice were killed with cervical dislocation and the blood sample was taken out directly from the heart by syringe aspiration, and the organs and carcass (residual body portions) were taken

and washed with PBS, frozen in liquid nitrogen, and stored at −85°C.

Preadministration of galactosylated and mannosylated reagents

Arabinogalactan, albumin bovine-galactosamide and albumin bovine-α-D-mannopyranosylphenyl isothiocyanate were purchased from Sigma-Aldrich Japan KK (Tokyo, Japan).

Mice were intravenously injected with 100 μl of 20 mg/kg of arabinogalactan, albumin bovine-galactosamide and bovine albumin-α-D-mannopyranosylphenyl isothiocyanate in PBS. After 1 h, mice were administered with PBS, free plasmid DNA, PEGylated dextran–spermine, and PEGylated dextran–spermine–plasmid DNA complex as described above. At 2 days post injection, mice were killed and the liver organs were taken out to evaluate the level of gene expression.

Evaluation of gene expression

For the evaluation of gene expression, β-galactosidase activity was determined using Invitrogen kit. Briefly, the muscle samples or other organs were immersed and homogenized in the lysis buffer (0.1 M Tris-HCl, 2 mM EDTA, 0.1% Triton X-100) at a lysis buffer volume (ml)/sample weight (mg) ratio of 4:1 in order to normalize the influence of weight variance on the β-galactosidase assay. The sample lysate (2 ml) was transferred to a centrifuge tube, followed by freeze-and-thaw three times and centrifugation at 15 000 *g* at 4°C for 5 min. The supernatant (30 μl) was mixed with 70 μl of 4 mg/ml *o*-nitrophenyl β-D-galactopyranoside (OPNG) aqueous solution and 200 μl of cleavage buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, and 1 mM $MgSO_4 \cdot 7H_2O$, pH 7) in a fresh microcentrifuge tube. After incubation at 37°C for 30 min, 500 μl of 1 M sodium carbonate solution was added to each sample and the absorbance at 420 nm was measured for the β-galactosidase activity. All *in vivo* experiments were conducted in triplicate and the average results were recorded. The protein concentration of each lysate sample was assayed by the Lowry kit (Nacalai tesque, Japan). Briefly, 50 μl of lysate was mixed with 1 ml of the copper solution and allowed to stand at 25°C for 10 min. Then, 0.1 ml of 1 N phenol aqueous solution was added and the mixture was incubated for 30 min at 25°C. The absorbance was recorded at a wavelength of 750 nm and the protein concentration was calculated based on the calibration curve prepared by use of standard albumin bovine solution.

Acknowledgements

Tony Azzam is grateful to the Ministry of Science, Israel, for its generous financial assistance. This work was supported in part by the AFIRST, French–Israeli Cooperation on Gene Therapy, and by the US–Israel Binational Fund (BSF).

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Synthesis and Physical Characterization of Poly(ethylene glycol)-Gelatin Conjugates

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Received August 21, 2003; Revised Manuscript Received November 13, 2003

Poly(ethylene glycol) (PEG) with the terminal group of active ester was coupled to the amino group of gelatin to prepare PEG-grafted gelatin (PEG-gelatin). The affinity chromatographic study revealed that the PEG-gelatin with high degrees of PEGylation did not adsorb onto the gelatin affinity column, in remarked contrast to gelatin alone and the PEG-gelatin with low PEGylation degrees. The former PEG-gelatin showed a critical micelle concentration while it had the apparent molecular size of about 100 nm and a surface charge of almost zero. These findings indicate that the PEG-gelatin formed a micelle structure of which the surface is covered with PEG molecules grafted. When the body distribution of ^{125}I-labeled gelatin and PEG-gelatin after intravenous injection was evaluated, the radioactivity of micellar PEG-gelatin was retained in the blood circulation compared with that of gelatin and the PEG-gelatin of no micelle formation. At the same PEGylation degree, the blood concentration was significantly higher for the PEG-gelatin prepared from PEG with a molecular weight of 12 000 than that of molecular weights of 2000 and 5000. It is concluded that the PEG-gelatin is a drug carrier with a micelle structure which retains in the blood circulation.

Introduction

It is one of the fast growing areas of drug delivery system (DDS) research to design and develop long-circulating pharmaceuticals and pharmaceutical carriers.[1−4] It has been recognized that they are gradually accumulated in the tumor and inflammation site because of the anatomical vascular characteristics, such as the leaky vasculature and the lack of lymph vessels, the so-called enhanced permeability and retention (EPR) effect.[5−7] Generally, the rapid uptake of colloidal drug carriers by the mononuclear phagocyte system (MPS) after intravenous administration is one of the major events, which often prevents a drug that is injected from delivering to the sites other than the MPS tissue and organ.[1,8−10] A large amount of work has been performed over the previous 10 years to optimize carriers that have a longer half-life in the blood stream and altered body distribution, allowing them to reach other cells and tissues, such as circulating cells themselves, solid tumors, and inflammatory sites.[11,12] As one practical way to minimize the MPS uptake, the surface coating of drug carriers with poly(ethylene glycol) (PEG) or PEG-like polymers is effective. In the different types of polymeric nanoparticles available, those currently receiving increased attention are the nanoparticles prepared from diblock poly(lactide)−monomethoxypoly(ethyleneglycol) (PLA−mPEG) and poly(lactide-*co*-glycolide)−monomethoxypoly(ethyleneglycol) (PLGA−mPEG) copolymers or from blends of these copolymers with PLA or PLGA.[13−17] PLA−mPEG and PLGA−mPEG nanoparticles combine a number of desirable, important characteristics for application in controlled drug delivery and in drug targeting, such as biocompatibility and biodegradability,[13,14] persistence in blood following intravenous administration,[13−16] good encapsulation properties for lipophilic drugs,[13,18] and neutral oligonucleotide complexes.[19] Interestingly, Kataoka et al. reported the polyion complex (PIC) micelles having cationic polymer, poly-L-lysine (PLL), poly(ethylenimine) (PEI), and poly(di-methylaminoethyl methacrylate) (PAMA), as the micelle core.[20] Moreover, intact DNA was observed in the blood circulation up to 3 h after intravenous administration of optimized PIC micelles. For in vivo transfection, injection of the PIC micellar vector with an optimized composition showed significant gene expression in the liver.[21] Such PIC micelles entrapping drug or DNA in the core are expected to be useful as functional materials including carrier systems in drug delivery applications.[22]

Gelatin has extensively been used for industrial, pharmaceutical, and medical applications, and the bio-safety has been proved through its long clinical usage as the surgical biomaterials and drug ingredients. Another unique advantage of gelatin is the electrical nature of gelatin, which can be readily changed by the processing method of collagen.[23] We have designed the controlled release system of drugs by taking advantage of their interaction with the carrier and the carrier degradation. Various growth factors, such as basic fibroblast growth factor (bFGF),[24] bone morphogenetic protein-2 (BMP-2),[25] transforming growth factor beta1 (TGF-beta1),[26] and hepatocyte growth factor (HGF),[27] are immobilized into the biodegradable hydrogel of gelatin on the

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10.1021/bm0343139 CCC: $27.50
Published on Web 12/18/2003