and accurately amplified, because the RT-PCR was not run under optimal conditions. This step is dependent on several factors, of which the most critical is the diversity of primer sets covering the whole VL and VH gene repertoires. In contrast, we have constructed original primer sets that encompass theoretical whole repertoires using The Kabat Database [19] and previous report [20] as reference (Tables 1 and 2). In fact, in control comparative experiments, we were unable to amplify scFv genes even from the OKT9, W6/32, and TES23 hybridomas using previous primer sets [20] (data not shown). On the other hand, using our modified primer sets (Tables 1 and 2), the VL and VH genes were clearly amplified at 340 and 400 bp from these hybridomas. Moreover, in this step, the use of high fidelity *Taq* polymerase as well as the Expand High Fidelity PCR system, and specific primer sets, resulted in better coverage of the mouse antibody gene repertoire. In each case, PCR amplification using our improved primer sets yielded sufficient amounts of products of the predicted size, as shown by a sharp band (Fig. 2A). This result suggested that our primer sets possess sufficient diversity for constructing comprehensive non-immune scFv libraries.

Table 3
Selection of scFv displaying phage binding to Luciferase

Panning rounds	1st	2nd	3rd
Total input phage (CFU)	3.1×10^{11}	6.6×10^{11}	1.5×10^{12}
Total output phage (CFU)	2.7×10^{3}	2.1×10^{4}	1.4×10^{7}
Ratio (output phage/input phage)	8.7×10^{-9}	3.2×10^{-8}	9.3×10^{-6}

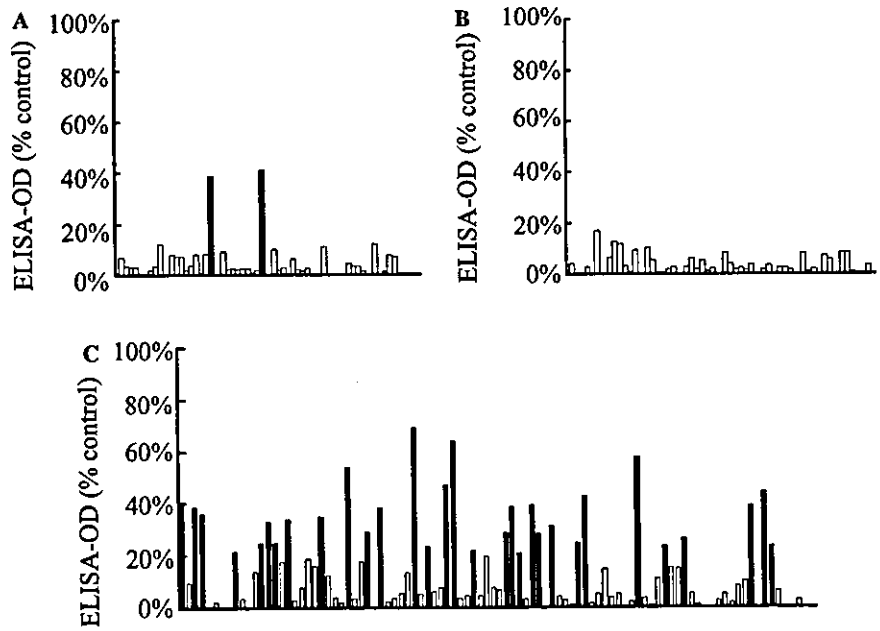

Fig. 4. Binding activity to Luciferase of output phage clones. After panning on Luciferase, the binding properties of selected clones were measured by ELISA. (A) Forty-eight clones from the first panning output phages, (B) 42 clones from the second panning output phages, and (C) 90 clones from the third panning output phages. Percentage control was (sample OD/positive control OD) × 100%. The positive control was the wild-type phage captured by anti-M13 Mab and detected by anti-M13 Mab HRP conjugate.

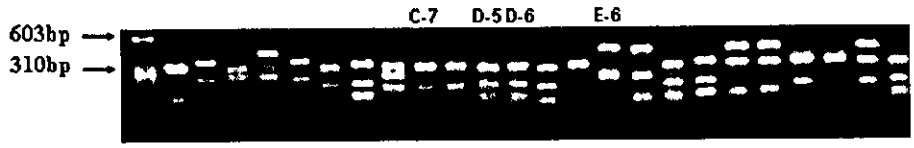

Fig. 5. Fingerprint analysis of anti-Luciferase scFvs. Anti-Luciferase scFv-encoding inserts of 21 phage clones were amplified by PCR using pCANTAB5-S1 and pCANTAB5-S6 primers. The amplified products were digested with the frequent-cutting enzyme *Bst*NI at 60 °C for 2 h. The restriction patterns of samples were analyzed on agarose gels.

In order to create scFv fragment genes (Fig. 1), 3′ ends of VL genes were fused to 5′ ends of VH genes through a $(G4S)_3$ linker peptide, and the VL and VH genes were assembled and amplified. We constructed VL-linker-VH-type scFv genes such that the 3′ end of the light chain was complementary to the 5′ end of the linker and the 3′ end of this linker DNA was in turn complementary to the 5′ end of the heavy chain. The scFv genes of the third PCR products were digested by *Sfi*I and *Not*I, and the bands obtained were trimmed of elongation sequences (Fig. 2B). By traditional methods, only a restricted repertoire of mouse antibody genes can be amplified and ligated using the pCANTAB5E vector. However, this method is not efficient, because scFv genes are commonly too short to be cut with some enzymes, i.e., in general, the sequences at the 5′ and 3′ ends of the PCR products are too short. To overcome this problem, we extended the 5′ end of VL and 3′ end of VH by approximately 100 bp at the third PCR, and improved the efficiency of cutting following the ligation step. The VH-linker-VL-type scFv formed by the inversion of VL and VH genes had lower affinity and bioactivity than the VL-linker-VH-type scFvs, which were created from an anti-TNF-α MAb-secreting hybridoma.

A combinatorial non-immune mouse scFv library of 5×10^8 clones was established by cloning the scFv fragments into a vector, which allowed the fusion of the heavy chain variable fragment to gene III protein of phage minor coat protein. To ascertain the scFv repertoire and the quality of the non-immune antibody library, DNA segments encoding the scFv genes from 21 randomly picked clones were identified from the primary library. These clones were amplified and digested with *Bst*NI, and their fingerprint patterns were compared. The patterns of these clones were found to vary, indicating an excellent diversity in the non-immune mouse scFv library (Fig. 2C). Using this method, we succeeded in establishing a non-immune mouse scFv library, which demonstrated a large diversity of scFv, and we also confirmed that our non-immune scFv phage library was composed of billions of independent clones.

In general, in order to select useful specific scFv antibodies for target antigens, it is important that the primary phage libraries demonstrate high quality. The term "high quality" implies several key features. Importantly, the library must have a large functional size to match diverse antibody sequences to facilitate the selection of a variety of high affinity scFvs. It is essential that the scFv genes be well expressed to allow the panning process. In this study, we established a large non-immune scFv phage display library and attempted to isolate specific scFvs for firefly Luciferase as a model antigen. Phage clones binding to immobilized Luciferase were selected on the basis of their affinity as described in Materials and methods. Fig. 3 shows the enrichment and screening of the phage scFv antibody library. The vertical axis indicates the ratio

Table 4
Amino acids sequence of anti-Luciferase scFv

Clone name	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4	(G4S)3
	VL							
C-7	DIQMMQSTSSLSAS LGDRVTISC	RTSQDIN YLN	WYQQKPDG TVKLLIY	YTSRL HS	GVPSRFSGSGSGTDYSLTISNL EQEDIATYFC	QQGNTL PLT	FGAGTK LEIKR	GGGGSGGGGS GGGS
D-5	DIVITQSPAILSVSP GERVSFSC	RASQSIG TSIH	WYQQRTNG SPRLLIE	YASESIS	GIPSRFSGSGSGTDFTLSINSVE SEDIADYYC	QQSNSW PTT	FGAGTK LTVL	GGGGSGGGGS GGGS
D-6	DIQMTQSPVILSVSP EGERVSFSC	RASQSIG TSIH	WYQQRING PPRPLIK	YASES IS	RIPSRFSGSGSGTDFTLSINSVE SEDIADYYC	QQSNSW PALT	FGAGTK LEIKR	GGGGSGGGGS GGGS
E-6	DILLTASPVILSVSP GERVSFSC	RASQNI GTSIH	WYQQRING SPRLLIK	YASESIS	RIPSRFSGSGSGTDFTLTINSVE SEDIADYYC	QQSNSW PALT	FGAGTK LEIKR	GGGGSGGGGS GGGS
	VH							
C-7	EVMLVESGPELVKPGASVKISC KASGYTFS	SYWMN	WMKQRPG KGLEWIG	QIYPGDGET NYNGKFKG	KATLTADKSSTAYMQLSS LTSEDSAVYFCAS	FDGYYVD Y	WGQGT TLQSS	
D-5	QVQLQQSGPELARPWASVKISC QAFYTFS	RRVHFAIR DTNYWMQ	WVKQRPG QGLEWIG	AIYPGNGDT SYNQKFKG	KALTLTADKSSSTAYMQLS SLTSEDSAVYFCAR	DPLVY	WGQGT TLTVSS	
D-6	QVHVKQSGAELVKPGAAVKVS CKASGYTFT	SYWMH	WVKQRPG HGLEWIG	QIYPGDGDT NYNGEFKG	KALTLTVDKSSSTAYMQLTS SLTSEDSAVYFCAS	QSSYVFDY	WGQGT TLTVSS	
E-6	EVQLQQSGPELVKPGASVKISC KASGYSFT	DYNMN	WVKQSNG KSLEWIG	VINPNYGTTS YNQKFKG	KALTLTVDQSSSTAYMQLS SLTSEDSAVYYCTR	ENYYGSSY LYYAMKDY	WGQGT TVTVSS	

of the number of recovered phages from the immunotube to the number of phages added. At the second round of panning, there was a 3-fold increase in enrichment, which was again increased approximately 300-fold by the third round. Thus, the overall enrichment was approximately 1000-fold (Table 3). These results suggest that high affinity scFv-displayed phage clones could be selected from the scFv library by affinity panning.

A total of three rounds of selection was performed on immobilized Luciferase. After each round, a total of 180 clones was randomly picked and their binding to Luciferase was tested by phage ELISA (Fig. 4). A few clones after the second selection and 31 clones after the third were positive for Luciferase binding. And then, we also confirmed that soluble formed scFvs of them could bind to Luciferase as well (data not shown). These results suggest high efficiency of the selection process. Additionally, crossreactivity on ovalbumin-immobilized phage ELISA demonstrated that 29 of the 31 scFvs were specific Luciferase binders (data not shown). DNA segments encoding the scFvs from 21 binders, identified in the third selection, were amplified, digested with *Bst*NI, and their fingerprint patterns were compared. A total of 18 different patterns was identified among the 21 clones, indicating an excellent diversity of the isolated anti-Luciferase scFvs (Fig. 5). Partial DNA sequencing data further confirmed that four clones were unique, with different nucleotide and amino acid sequences (Table 4). The fingerprints of clones D-5 and D-6 clones were apparently identical, although their amino acid sequences were different. Therefore, we have accomplished the generation of at least 18 different anti-Luciferase scFv antibody clones.

The large mouse scFv library was established in order to isolate multiple scFv binding to any antigen. To determine the general diversity of the scFv library, it was subjected to three rounds of selection on four different antigens, namely, immobilized VEGF, VEGF-receptor, TNF-α, and PE. After three rounds of selection, the ratio of the number of recovered selected phages from the immunoadsorption tubes to the number of phages added increased from 5- to 1000-fold (Fig. 6). In all cases, the scFv genes from randomly picked clones were analyzed by fingerprinting, and a unique pattern of scFv genes was identified from the non-immune mouse scFv library. Hence, we have succeed in establishing a non-immune mouse library, with a large functional size and a high antibody sequence diversity in order to facilitate the selection of a variety of high affinity scFvs. Further, it was essential that the scFv genes be well-expressed to allow for efficient panning.

In the last decade, recombinant antibody engineering has emerged as one of the most promising approaches for the design, selection, and production of reagents for basic research, medicine, and the pharmaceutical industry [21–23]. The in vitro selection and evolution of phage libraries now provide us with very powerful tools for producing antibodies without using animals and the immune system. Significant advances in the past decade with phage and other display methodologies,

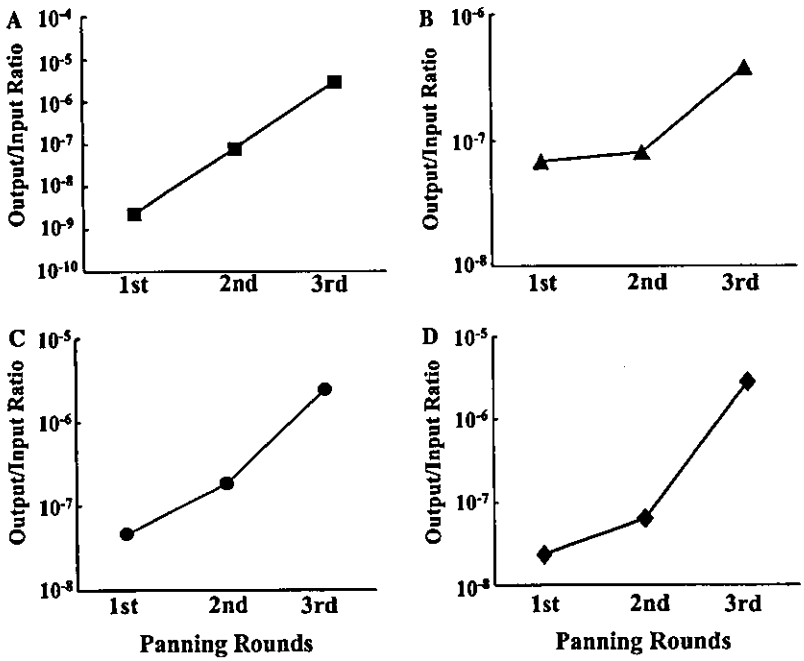

Fig. 6. Selection of phage-scFv libraries by panning. Selection on various antigens was performed by panning in antigen-coated immunotubes with (A) VEGF-receptor2, (B) VEGF, (C) TNF-α, and (D) PE.

library design, refined selection procedures, and instrumentation for automation have made display technologies increasingly popular for creating antibodies to be used in all areas of research, and also for medical and industrial applications. In the present study, we have explored the generation of useful antibodies such as cell type- and tissue-specific binders for application as cell markers and drug delivery carriers, by biopanning with cell and tissue in vivo. Moreover, to create more useful antibodies, we developed a novel modified methodology using polyethylene glycol to improve the stability and antigenicity of the antibodies. Previously, we reported that the site-specific modification of polyethylene glycol to protein improves the stability and other characteristics without deactivation of bioactivity [24]. Recently, we have used a number of such antibodies in clinical applications, and given their availability, these methodologies were used to develop antibody therapy to explore novel antibodies from the library and to improve their characteristics to make them more suitable as clinical tools. We believe that antibody therapy will be more widely applied in future and that this will be facilitated by the use of improved techniques for their production, such as those described here.

Acknowledgments

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Effective accumulation of poly(vinylpyrrolidone-*co*-vinyl laurate) into the spleen

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Abstract: To optimize polymer-conjugated drugs as a polymeric drug delivery system, it is essential to design polymeric carriers with tissue-specific targeting capacity. Previously, we showed that polyvinylpyrrolidone (PVP) was the most suitable polymeric carrier for prolonging the blood-residency of drugs, and was one of the best parent polymers to design the polymeric carriers with targeting capacity. In this study, we synthesized some hydrophobic PVP derivatives, poly(vinylpyrrolidone-*co*-styrene) [poly(VP-*co*-S)] and poly(vinylpyrrolidone-*co*-vinyl laurate) [poly(VP-*co*-VL)], and assessed their biopharmaceutical properties after intravenous administration in mice. The elimination of hydrophobic PVP derivatives from blood was the same as PVP, and the plasma half-lives of poly(VP-*co*-S) were almost similar to that of poly(VP-*co*-VL). Poly(VP-*co*-VL) efficiently accumulated in the spleen, whereas poly(VP-*co*-S) effectively accumulated in the liver. The level of poly(VP-*co*-VL) in the spleen was about 20 times higher than PVP and poly(VP-*co*-S). These hydrophobic PVP derivatives did not show any cytotoxicity against endothelial cells *in vitro*. Thus, poly(VP-*co*-VL) may be a useful polymeric carrier for drug delivery to the spleen. This study will provide useful information to design optimal polymeric carriers with targeting capacity to the spleen and liver. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 70A: 219–223, 2004

Key words: polymeric carrier; drug delivery system; targeting; polyvinylpyrrolidone; hydrophobic

INTRODUCTION

Because of recent advances in structural genomics and pharmacoproteomics, the functions of numerous proteins can be clarified. The therapeutic application of bioactive proteins, such as newly identified proteins and cytokines, is also expected.[1–4] Because these proteins are generally quite unstable *in vivo*, their clinical application requires frequent administration at high dosages. This often results in impaired homeostasis *in vivo* and may lead to severe adverse effects. Because cytokines such as interleukin (IL)-2 and tumor necrosis factor (TNF)-α have diverse effects on various tissues, it is not easy to selectively obtain a favorable function (therapeutic effects) of such proteins among their diverse functions to minimize their side effects.[5] The same problems exist in antitumor chemotherapeutic agents and immunosuppressive drugs.[6] To overcome these problems, we attempted to conjugate bioactive proteins with nonionic polymeric carriers such as polyethylene glycol (PEG).[7–11] Chemical conjugation of proteins with PEG, as a polymeric drug delivery system (DDS), increases their molecular size and enhances steric hindrance, both of which are dependent on the PEG attached to the protein. This results in improving the plasma half-lives of proteins and their stability against proteolytic cleavage, as well as a decrease in their immunogenicity. We also reported that PEGylation of proteins such as TNF-α, IL-6, and immunotoxins could enhance therapeutic potency and reduce undesirable effects.

For further improvement of this polymeric DDS, it is essential to design polymer-conjugated proteins with targeting capacity to the optimal tissue such as spleen and kidney. It is well known that the fate and

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distribution of the polymer-conjugated proteins can be attributed to the physicochemical properties of polymeric carriers such as molecular weight, electric charge, and hydrophilic–lipophilic balance. Therefore, to control the *in vivo* behavior of conjugated proteins with polymeric carriers, it is necessary to assess the pharmacokinetic characteristics of the polymeric carriers themselves. Previously, we showed that polyvinylpyrrolidone (PVP) had much longer circulation time than PEG, which has been used for bioconjugation (PEGylation) frequently, and tissue distribution of PVP was markedly restricted.[12] Therefore, we found that PVP was the most feasible polymeric modifier for increasing the half-life of cytokines and retention in the circulation. For example, bioconjugation of TNF-α with PVP showed 200 times higher antitumor effects than native TNF-α with decreased side effects and the antitumor effects of PVP-modified TNF-α was two times higher than that of PEG-modified TNF-α.[9] Our other studies on IL-6 yielded similar results.[13] In addition, it is easy to introduce various comonomers on radical polymerization to PVP for giving new function such as targeting capacity. For instance, we found that anionized PVP derivatives selectively accumulated into the urinary organ.[14] Briefly, carboxylated PVP efficiently accumulated in the kidney, whereas sulfonated PVP was rapidly excreted in the urine. Thus, these carboxylated and sulfonated PVPs may be useful polymeric carriers for drug delivery to kidney and bladder, respectively. Recently, we synthesized a novel polymeric drug carrier, polyvinylpyrrolidone-*co*-dimethyl maleic anhydride [poly(VP-*co*-DMMAn)], for its application in a renal DDS. About 80% of poly(VP-*co*-DMMAn) selectively accumulated in the kidneys 24 h after iv administration.[10] Poly(VP-*co*-DMMAn)-conjugated superoxide dismutase (SOD) accumulated in the kidneys after iv administration, and accelerated recovery from acute renal failure in a murine model. In contrast, PVP-modified SOD and native SOD were not as effective. These results suggested that PVP was the most feasible parent polymer for design of a novel polymeric carrier with targeting capacity.

In this study, we focused on assessing the *in vivo* behavior of hydrophobic PVP derivatives. To evaluate the relationship between pharmacokinetics and their hydrophobic functional groups, we synthesized poly(vinylpyrrolidone-*co*-styrene) [poly(VP-*co*-S)] and poly(vinylpyrrolidone-*co*-vinyl laurate) [poly(VP-*co*-VL)] by radical copolymerization. We found that poly(VP-*co*-VL) was useful for the targeting carrier to the spleen and poly(VP-*co*-S) was useful for the targeting carrier to the liver. This study is the first report of creation of the targeting carrier to the spleen. This study may provide useful information, which will facilitate the optimal molecular design of polymeric drug carriers applicable to therapeutic use for DDS to the spleen and liver.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). TSKgel G4000PW and TSKgel alpha-3000 columns were obtained from Tosoh Corporation (Tokyo, Japan). $Na^{125}I$ (3.7 GBq/mL) solution was obtained from NEN Research Products (Boston, MA).

Animals and cells

All experimental protocols for animal studies were in accordance with the Guide for Laboratory Animal Facilities and Care (National Institutes of Health publication 85-23, rev. 1985). These protocols have been approved by the committee at the school of Pharmaceutical Science, Osaka University. Male ddY mice were obtained from SLC (Hamamatsu, Japan). Sarcoma-180 cells (S-180; Cancer Cell Repository Institute of Development, Aging and Cancer, Tohoku University) were maintained intraperitoneally through serial passages in ddY mice.

Synthesis of PVP and its hydrophobic derivatives

PVP was synthesized by the radical polymerization method using 4,4′-azobis-4-cyanovaleric acid and β-mercaptopropionic acid as radical initiator and chain transfer agent, respectively. The hydrophobic PVP derivatives of both poly(VP-*co*-S) and poly(VP-*co*-VL) were prepared by radical copolymerization of VP and hydrophobic comonomers (styrene or vinyl laurate) in dimethylformamide with the aid of 4,4′-azobis-4-cyanovaleric acid and mercaptopropionic acid as for PVP. These polymers were separated into several fractions by gel filtration chromatography (GFC) to obtain polymers with a narrow molecular weight distribution. The number-average molecular weight of PVP and both hydrophobic PVP derivatives was about 10 kDa (polydispersity $[M_w/M_n]$ <1.39; PEG standards). The comonomer content determined by ^1H NMR was in good agreement with the feed molar ratio of the hydrophobic comonomer relative to the VP monomer.

In vivo behavior of polymers

^{125}I-radiolabeled polymers were prepared by the chloramine-T method and purified by GFC. The specific activities of ^{125}I-labeled polymers were about 4.44 μCi/mg/polymer. S-180 cells were implanted intradermally (5×10^5 cells/200 μL/site) in mice that were injected iv with ^{125}I-labeled poly-

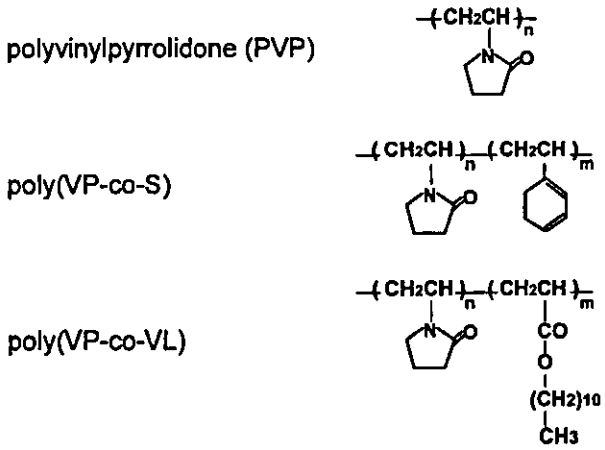

Figure 1. Chemical structures of PVP and hydrophobic PVP derivatives.

mer (1×10^6 cpm/200 μL) 7 days later when the diameter of the tumors exceeded 7 mm. Blood was collected from the tail vein at intervals and radioactivity was measured. GFC analysis confirmed that there was more than 95% of radioactivity in the circulating blood, 3 h after iv injection was derived from the intact ^{125}I-labeled polymers.

Pyrene fluorescence assay

Various concentrations of polymer solutions were added to each vial containing $1.0 \times 10^{-7}M$ pyrene and heated for 1 h at 70°C to equilibrate the pyrene and the polymers. Emission spectra of pyrene were recorded on a spectrofluorometer (excitation at 336 nm) and the intensity of emission peaks at 382 and 392 nm was determined.

RESULTS

Plasma clearance

The hydrophobic PVP derivatives, poly(VP-*co*-S) and poly(VP-*co*-VL), were synthesized by radical polymerization methods (Fig. 1). These products were separated and purified by GFC to adjust the molecular size (10 kDa) and polydispersity of PVP. We confirmed the successful synthesis of these products by NMR. These hydrophobic PVP derivatives at a dose of 10 mg/mL did not show any cytotoxicity against endothelial cells *in vitro* (data not shown). The plasma clearance of PVP and the hydrophobic PVP derivatives were compared after iv injection in mice bearing S-180 solid tumors (Fig. 2). Pharmacokinetics of ^{125}I-labeled polymers was not influenced by the ^{125}I-labeling method and the preparative method for activated polymers (data not shown). Additionally, almost all radioactivity in the blood was derived from the ^{125}I-labeled polymers by GFC analysis, 3 h after iv injection (data not shown). Therefore, it was considered that the pharmacokinetics of ^{125}I-labeled polymers was exactly correlated with that of the polymers. All polymers showed biphasic elimination patterns. Both poly(VP-*co*-S) and poly(VP-*co*-VL) as well as PVP, showed long retention in circulation and 25% of the injected dose remained in circulation after 180 min.

Tissue distribution

Next, we studied the tissue distribution of the polymers 3 h after iv injection (Fig. 3). Each polymer, with the same molecular weight, showed a characteristic distribution. PVP showed little tissue-specific localization. Poly(VP-*co*-S) (S: 1%) did not demonstrate specific tissue accumulation as well as PVP. However, poly(VP-*co*-S) (S: 3%) tended to accumulate in the liver, whereas the accumulation of poly(VP-*co*-S) (S: 3%) in other organs was similar to poly(VP-*co*-S) (S: 1%). In contrast, poly(VP-*co*-VL) effectively accumulated in spleen and accumulated more than 20 times higher than PVP or poly(VP-*co*-S) (S: 1%).

Detection of conformational shift of the polymers by pyrene fluorescence assay

In general, hydrophobic polymers tend to assemble each other in water. Therefore, the difference of phar-

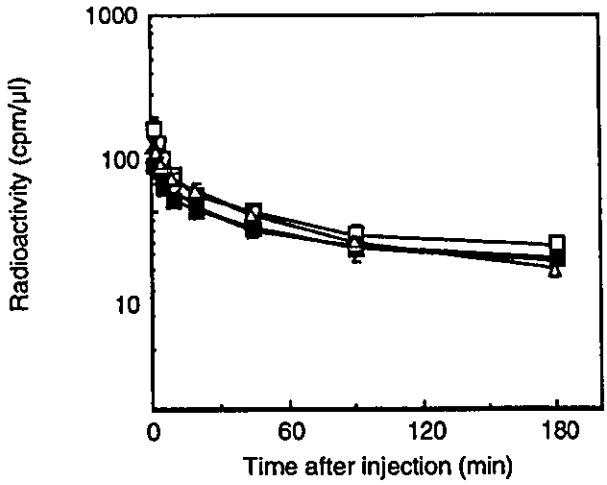

Figure 2. Plasma clearance of PVP and hydrophobic PVP derivatives after iv injection in mice. Mice were intravenously injected with ^{125}I-labeled polymers. After administration, blood was collected at indicated times and radioactivity was measured using a γ-counter. Mice were used in groups of five. Each value is the mean ± standard error. (○) PVP; (□) poly(VP-*co*-S) (S: 1%); (■) poly(VP-*co*-S) (S: 3%); (△) poly(VP-*co*-VL) (VL: 1%).

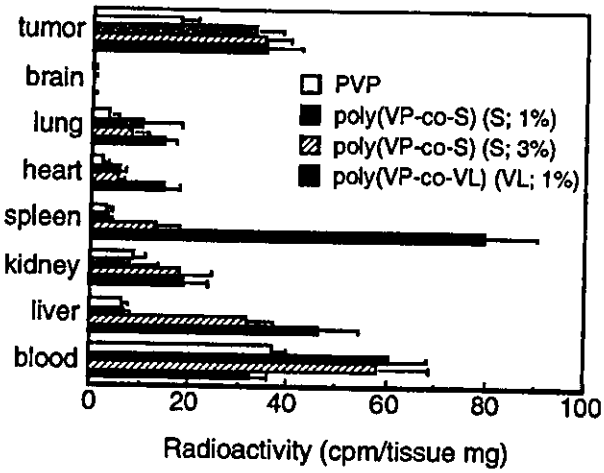

Figure 3. Tissue distribution of PVP and hydrophobic PVP derivatives at 3 h after iv injection in mice. At 3 h after iv injection, mice were killed and each organ was collected. The radioactivity was measured using a γ-counter. Mice were used in groups of five. Each value is the mean ± standard error.

macokinetics of the hydrophobic PVP derivatives would result from the difference of the state of the polymer in blood. Fluorescence probe technique was used to investigate the self-assembly of the polymers in water using pyrene as a hydrophobic probe.[15] The transition from an expanded, hydrophilic coiled polymer to a compact, globular structure can be detected in the presence of pyrene as the change of the ratio of the emission intensity at 372 nm to that at 382 nm. Phosphatidylcholine, as a positive control, assembled with increase of the concentration. In contrast, no changes were detected in all hydrophobic PVP derivatives (Fig. 4).

DISCUSSION

Systemic administration of potential activators of immune cells for tumor or virus therapy such as IL-2, IL-12, and IFN-γ, or potential inhibitors of immune cells for autoimmune disease such as IL-10 and immunosuppressive drugs, has attracted much attention.[16] However, systemic administration of these cytokines is often associated with severe toxic side effects before the curative dose. To overcome these problems, it is important to develop an effective spleen DDS that selectively carries bioactive proteins to the spleen with a high degree of safety. In this regard, we previously reported that PVP was a more suitable polymeric carrier for enhancing the blood residency of drugs than PEG, which has been used frequently.[9,13] Using this PVP as a backbone polymer, we have evaluated the *in vivo* pharmacokinetics of synthesized PVP derivatives with various electrically charge or hydrophilic/hydrophobic balance.[10,14] For instance, the copolymer between VP and dimethyl maleic anhydride showed a marked increase in accumulation in the kidney.[10] In this study, we focused on the assessment of the *in vivo* behavior of hydrophobic PVP derivatives for optimizing the spleen DDS.

In vivo behavior of hydrophobic PVP derivatives was changed by type and content (molar ratio) of hydrophobic groups. Poly(VP-*co*-VL) and poly(VP-*co*-S), as well as PVP, showed high retention in circulation (Fig. 2). Furthermore, poly(VP-*co*-VL) accumulated in the spleen, as compared with other tissues such as the liver, kidney, and lung at 3 h after their iv injection (Fig. 3). The level of poly(VP-*co*-VL) in spleen was about 20-fold higher than PVP and poly(VP-*co*-S). However, poly(VP-*co*-S) showed little accumulation in the spleen. The reason for this difference is not clear, but it is partially due to the difference in structure of hydrophobic polymers. These results suggested that optimal hydrophobic groups may produce the highest accumulation in the spleen. The spleen is a lymphoid organ composed of red pulp and white pulp, containing T and B cell areas. The spleen has a unique vascular network and its framework is formed by a reticular meshwork consisting of fiber-forming reticular cells. The accumulation site of poly(VP-*co*-VL), and the mechanism of accumulation are currently under investigation. To evaluate the relationship between accumulation efficiency in the spleen and the number of vinyl laurate groups, we are synthesizing various

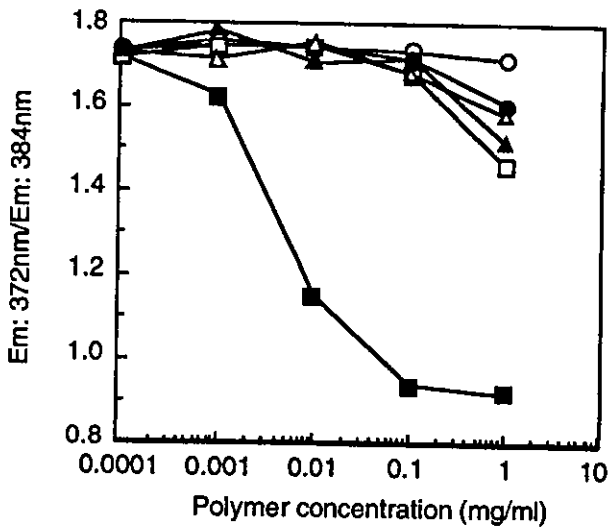

Figure 4. The ratio of pyrene emission intensity at 372 nm to that at 384 nm in the presence of poly(VP-*co*-S) and poly(VP)-*co*-VL. Pyrene and each polymer solution were mixed and incubated for 1 h at 70°C. After incubation, fluorescence activity (Ex: 336 nm, Em1: 372 nm, and Em3: 384 nm) was measured. (○) PEG; (•) PVP; (■) phosphatidylcholine; (▲) poly(VP-*co*-S) (S: 1%); (△) poly(VP-*co*-S) (S: 3%); (□) poly(VP-*co*-VL) (VL: 1%).

kinds of poly(VP-*co*-VL)s and examining the accumulation in the spleen. In addition, we are examining the therapeutic effects of poly(VP-*co*-VL) with immunosuppressive cytokine, IL-10, for the treatment of autoimmune diseases such as rheumatoid arthritis, and the usefulness of poly(VP-*co*-S) for the targeting carrier to liver is also under investigation.

This study may provide useful information that will facilitate the optimal molecular design of polymeric drug carriers applicable to therapeutic use for DDS to the spleen.

CONCLUSIONS

Both poly(VP-*co*-S) and poly(VP-*co*-VL), as well as PVP, showed long retention in circulation. Poly(VP-*co*-S) (S: 3%) tended to accumulate in the liver, whereas PVP showed little tissue-specific localization. Furthermore, poly(VP-*co*-VL) effectively accumulated in spleen and accumulated more than 20 times higher than PVP or poly(VP-*co*-S) (S: 1%).

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The optimal molecular design of polymeric drug carriers and its application for renal drug targeting

Review Article

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Abbreviations: Dimethyl maleic anhydride (DMMAn); immunotoxin (IT); interleukin-10 (IL-10); interleukin-6 (IL-6); poly(vinylpyrrolidone-co-dimethyl maleic acid), (PVD); polyacrylamide (PAAm); polydimethylacrylamide (PDAAm); polyethylene glycol (PEG); polyvinyl alcohol (PVA); polyvinylpyrrolidone (PVP); superoxide dismutase (SOD); tumor necrosis factor-a (TNF-a); vinylpyrrolidone (VP)

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Summary

Renal disease is a serious health problem which is on the increase in the world. Over time such conditions necessitate dialysis and may require a kidney transplant. However these therapies are expensive and do not restore normal health. Therefore, new therapeutic strategies must be developed for treating patients with renal disease. Drugs such as steroids have been used to prevent the progression of renal disease, but they produce toxicity because of their wide distribution in the body. The development of a renal delivery system that selectively carriers drugs to the kidneys is a promising approach for limiting tissue distribution and controlling toxicity. To overcome the problems associated with conventional therapies, bioactive proteins have been conjugated with water-soluble polymeric carriers. Conjugated bioactive protein with polymeric carriers regulate the tissue distribution of bioactive proteins, resulting in a selective increase in its desirable therapeutic effects, and a decrease in undesirable side effects. However, for further enhancement of the therapeutic potency and safety of conjugated bioactive proteins, more precise control of the *in vivo* behavior of each protein is necessary for selective expression of their therapeutic effect. Recently, we reported that the poly(vinylpyrrolidone-co-dimethyl maleic acid) [PVD] was selectively distributed into the kidneys after intravenous injection and it was conjugated with the amino groups of drugs. The conjugates demonstrated high accumulation and retention in the kidneys without any adverse toxicity. In this review, with reference to our recent studies, we propose that bioconjugation with the appropriate polymeric modifier of PVD can be a potential therapeutic agent for various renal diseases.

I. Introduction

In recent years, the clinical applications of bioactive proteins such as cytokines and growth factors have been studied. However, the clinical applications of most of these proteins are limited because of their various side effects (Blick et al, 1987; Rosenberg 1987). Generally, the plasma half- lives of bioactive proteins *in vivo* are very short (Donohue et al, 1983; Bollon et al, 1988; Tanaka and Tokiwa 1990). This necessitates their frequent administration at high dosage in order to obtain sufficient therapeutic effects. Such administration markedly destroys homeostasis, resulting in unexpected side effects. In addition, since bioactive proteins exhibit diverse pharmacological actions in various tissues, it is difficult to selectively obtain only the favorable actions (therapeutic effects). To overcome these problems, bioactive proteins have been conjugated with water-soluble polymeric carriers. We have already reported that polymer conjugation of cytokines typified with tumor necrosis factor-α (TNF-α) interleukin-6 (IL-6), and immunotoxin (IT), with polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP), improved their resistance to proteinase, enhanced their plasma half-lives, and resulted in greater therapeutic potency (Tsutsumi et al, 1997, 2000; Kaneda et al, 1998; Kamada et al, 2000; Yamamoto et al, 2003). We have also shown that conjugation with polymeric carriers regulates the tissue distribution of bioactive proteins, resulted in a selective increase in desirable therapeutic effects, and a decrease in undesirable side effects. However, for further enhancement of the therapeutic potency and safety of conjugated bioactive proteins, more precise control of the *in vivo* behavior of each protein is necessary for selective expression of their therapeutic effect. Thus, there is a need to develop novel

polymeric carriers capable of targeting specific tissue, while PEG and PVP are useful and powerful polymeric carriers for improving the plasma half-lives of proteins.

Renal disease is a serious problem that is on the rise all over the world. According to the Third National Health and Nutrition Examination Survey, about 10.9 million people in the United States have renal disease. (Jones et al, 1998). There is no cure for renal disease, and few strategies are available for prevention. Bioactive proteins such as superoxide dismutase (SOD) and interleukin-10 (IL-10) were believed to prevent the progression of renal disease; however, their therapeutic potency was too low as they were poorly distributed to the kidneys. The development of a renal delivery system that selectively targets the kidneys is a promising approach for limiting tissue distribution and controlling toxicity. Several renal drug delivery systems have been described. One approach involves prodrugs that are cleaved by kidney-associated enzymes to release the drugs in the kidney (Elfarra et al, 1995). However, these prodrugs tend not to accumulate in the kidneys because of plasma protein binding and limited transport to the kidney. Alternatively, low-molecular-weight proteins such as lysozyme have been used as carriers because they are easily reabsorbed by the kidneys. Unfortunately, they also result in considerable renal toxicity and cardiovascular side effects (Haverding et al, 2001). A third strategy has been based on the binding capacity of streptavidin carriers to biotin in the kidney. However, streptavidin is immunogenic and also results in limited renal accumulation because of its large molecular size (Schechter et al, 1995). The fate and distribution of conjugates between polymeric carriers and drugs is determined by their physicochemical properties such as electric charge and hydrophilic-lipophilic balance (Inoue et al, 1989). In this review, at first, we show that PVD is accumulated and retained in the kidney without any adverse toxicity. Additionally to assess the usefulness of PVD as a renal targeting polymeric carrier of drugs, we evaluated the relationship between PVD molecular weight and renal accumulation. We then prepared a conjugated SOD with PVD and evaluated its pharmacokinetic characteristics and therapeutic effects on $HgCl_2$-induced acute renal failure (ARF). This review will provide fundamental information enabling us to design of polymeric drug carriers and its application for renal drug targeting.

II. Pharmacokinetics of PVD

The *in vivo* pharmacokinetics of polymer-conjugated drugs such as bioactive proteins may be markedly influenced by the properties such as electric charge and hydrophilic-hydrophobic balance of polymeric carriers attached to the surface of the drugs. Therefore, in order to optimize drug therapy by polymer conjugation typified by PEGylation, we must initially design a polymeric carrier with useful functions such as targeting and controlled release capability, which can precisely regulate their behavioral characteristics *in vivo*. We reported that PVP was a more suitable polymeric carrier for enhancing the blood residency of drugs than PEG, polyacrylamide (PAAm), polydimethylacrylamide (PDAAm), and polyvinyl alcohol (PVA) (**Figure 1**). PVP, PAAm, and PDAAm could be functionalized by introduction of various comonomers on radical polymerization. PVA has several primary OH groups that can be used for bioconjugation on the side chain. Most appropriately, using this PVP as a backbone polymer, we have evaluated the in *vivo* pharmacokinetics of synthesized PVP derivatives with various electric charges or hydrophilic-hydrophobic balance. We assessed the pharmacokinetic properties of various PVP derivatives. We demonstrated PEGylated TNF-α was improved its anti-tumor effect than native TNF-α in mice bearing tumor, because their blood residency not tumor distribution (Tsutsumi et al, 1995). Among these, carboxylated PVP accumulated in the kidney 24h after intravenous injection (**Figures 2 and 3**). The *in vitro* cytotoxicity of carboxylated PVP against renal tubular cells was low, and its renal targeting capacity

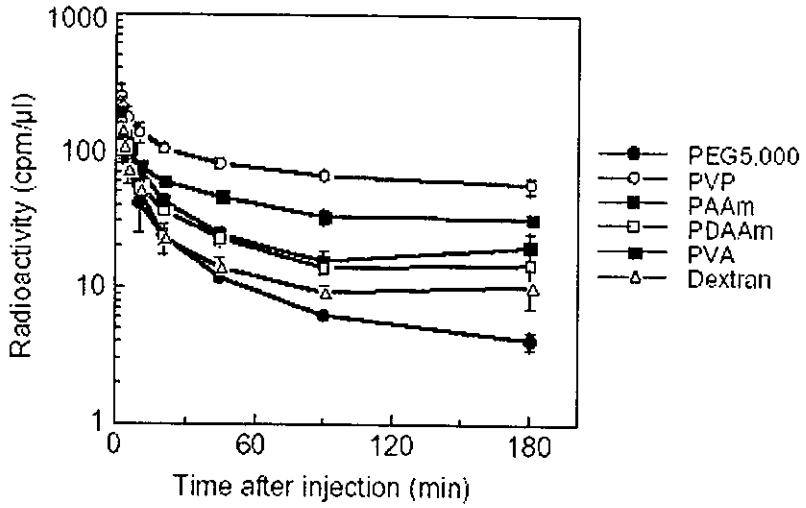

Figure 1. Plasma clearance of various water-soluble polymers in mice after intravenous injection.
From Biomaterials. 2004 Aug;25(18):4309-15

was better than that of other carriers (**Figure 5**). Anionic polyaspartamides are transiently distributed in the kidney and are rapidly excreted in the urine (Rypacek et al, 1982). However, we found that these anionic polymers were not suitable as renal targeting carriers, because the conjugates composed of these anionic polymers and the drug did not accumulate in sufficient quantities to produce therapeutic effects.

We synthesized PVD by radical copolymerization and mixed the reactive comonomers [Dimethyl maleic anhydride (DMMAn) and vinylpyrrolidone (VP)] to evaluate its use as a polymeric drug carrier for renal drug delivery systems. We found that about 80% of the dose of PVD selectively accumulated in the kidneys 24 h after intravenous injection (**Figure 4**). Although PVD accumulated in the kidneys was gradually excreted in the urine, about 40% was retained 96 h after beginning the treatment. The high renal accumulation and retention of PVD makes it a more useful targeting carrier than other agents. Although most anionized polymers are safer than cationized polymers, they exhibit cytotoxicity at high doses. Indeed poly(VP-co-MAn), PVD, which has the same molecular size, polydispersity, and carboxyl group content as PVD, produced cytotoxicity in LLC-MK2 cells at higher concentrations (**Figure 5**). In contrast, PVD produced no evidence of pathological effects in mice at a dose of 10mg/d for 28 d. A subcutaneous dose of 50mg PVD, which had a jelly-like consistency, was well tolerated by mice. The safety of PVD seems similar to that of PEG and PVP, which are used clinically. Thus, PVD seems to be a safe polymeric carrier with much higher renal targeting and retention capacity than any other renal targeting carrier. PVD was hydrolyzed at the maleic anhydride position to form carboxyl group, which produced polyanionic characteristics. Endothelial cells and the glomerular capillary wall are coated with highly polyanionic sialprotein (Simionescu, 1983). Therefore, anionic polymers such as anionized dextran are generally cleared more slowly from the circulation than are nonionic and cationic polymers (Chang et al, 1975). The reason for this discrepancy *in vivo* activity is not clear. In a preliminary study, the uptake of PVD by renal cells was inhibited by the energy inhibitor NaN_3 and was not affected by cytochalasin B.

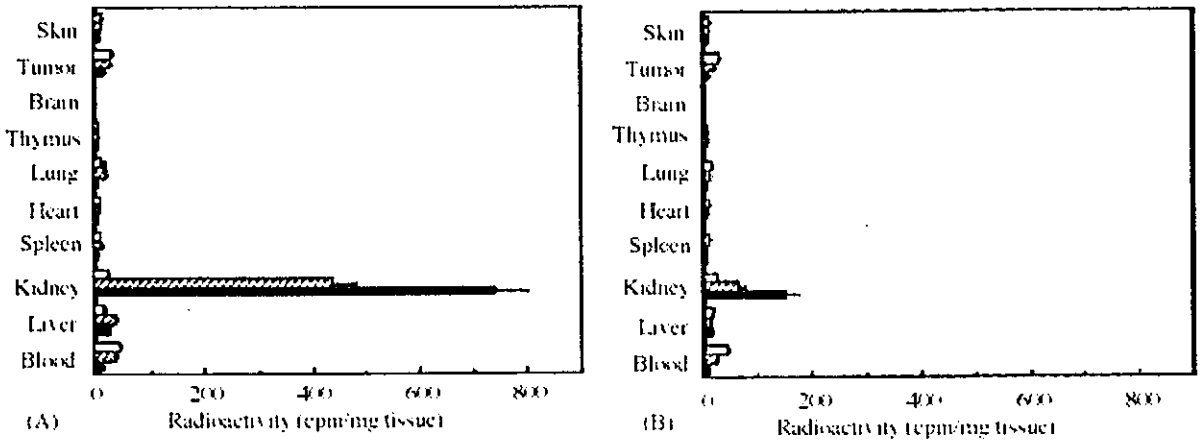

Figure 2. Tissue distribution of PVP and anionized PVP derivatives at 3h after intravenous injection in mice.
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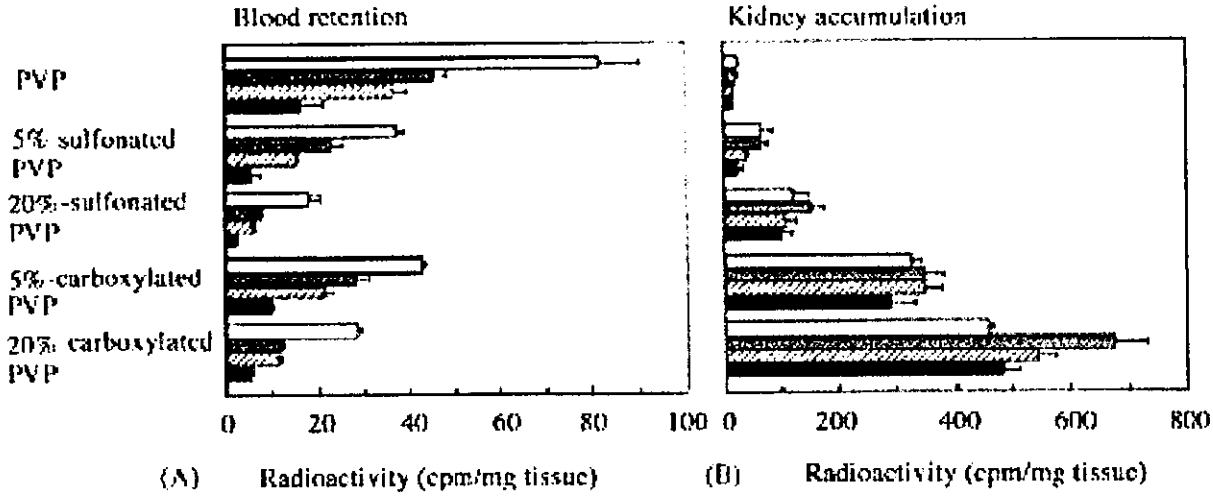

Figure 3. Blood retention and kidney accumulation of PVP and anionized PVP derivatives after intravenous injection in mice.
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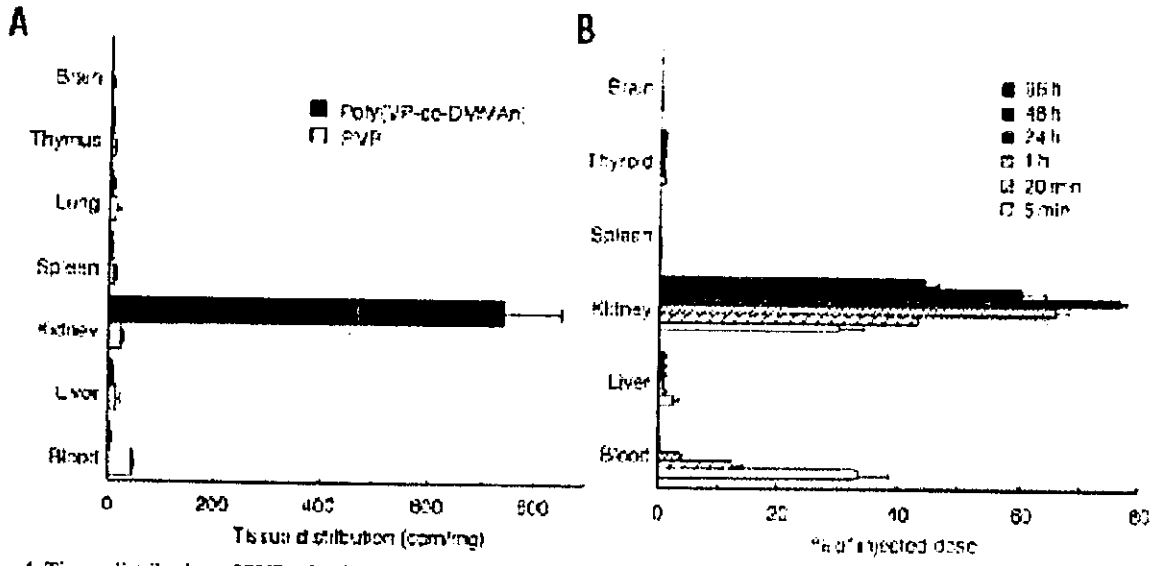

Figure 4. Tissue distribution of PVD after intravenous injection.
From Nat Biotechnol. 2003 Apr;21(4):399-404

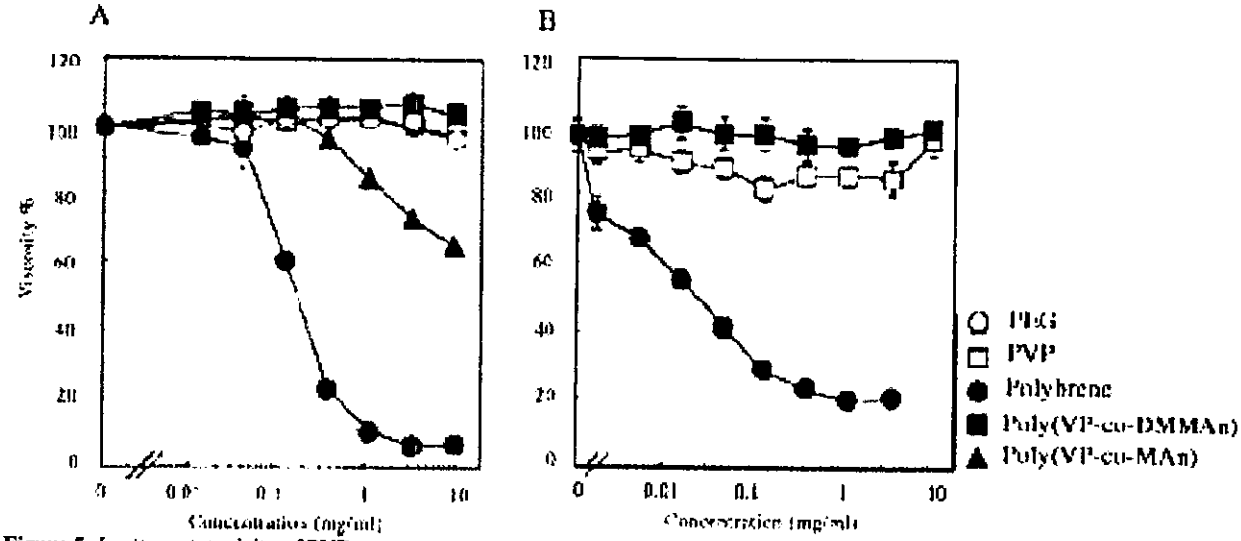

Figure 5. *In vitro* cytotoxicity of PVD.
From Nat Biotechnol. 2003 Apr;21(4):399-404

Thus, PVD may be taken in by an energy-dependent process other than endocytosis. Several specific molecules are involved in renal transport, and various organic anion transporters exist in the kidney (Moestrup et al, 1996; Sweet et al, 1997; Hosoyamada et al, 1999; Nakajima et al, 2000). However, these transporters generally carry low molecular weight drugs. Therefore, a new transport pathway may exist. It is important to consider the *in vivo* uptake pathway (reabsorption pathway or direct pathway) of PVD into the proximal tubule. We found that the *in vivo* behavior of PVD was similar in both normal and ARF mice. Since reabsorption did not occur in ARF mice, we believe that PVD was delivered directly to the proximal tubule.

We injected mice intravenously with fluorescenly labeled PVD and collected their kidneys after 3 h. We prepared sections and evaluated them by fluorescence microscopy (**Figure 3**). Most of the PVD accumulated in the cortex (data not shown). PVD was also present in renal tubules, especially proximal tubular epithelial cells, but not in glomeruli. In contrast, fluorescence-labeled PVP did not accumulate in the renal tubules. Neither amino-aceto-fluorescein nor the mixture with hydrolyzed PVD was detected in renal tubules.

Further we used ^{125}I-tyramine and amino-aceto-fluorescein as model drugs with low molecular weight, and showed that they specifically accumulated in the kidney after conjugation with PVD (data not shown). PVD may serve as a carrier for site-specific delivery of drugs with relatively low molecular weight to the kidney. These drugs may include radionucleotides or anti-inflammatory drugs, antibiotics, and other effector molecules. Furthermore, DMMAn is an amino-protective agent that binds to or separates from amino groups when the pH changes (Nieto and Palacian, 1983; de la Escalera and Palacian, 1989; Kaneda et al, 1998). PVD also has maleic anhydride groups that react with amino groups in drugs. In inflammatory tissue and tumor tissue, the pH is lower than

normal (Nakajima et al, 2000). Therefore, if PVD is used in nephritis and renal cancer, it is expected to accumulate in the kidneys and gradually release the drugs. In addition, the modification of proteins with polymeric modifiers has several advantages. TNF-α, IL-6, and functional single-chain Fv fragment bioconjugated with PEG or PVP are more effective than the native proteins (Tsutsumi et al, 1995, 1997, 2000; Kamada et al, 1999, 2000; Mu et al, 1999; Tsunoda et al, 2000, 2001). We have also shown that the fate and distribution of proteins with polymeric modifiers are strongly influenced by the polymeric modifiers. Therefore, PVD may be a useful modifier of bioactive proteins for targeting the kidney.

III. Therapeutic effect of PVD-SOD

We synthesized PVD as a new renal targeting carrier. About 80% of the dose of PVD was selectively distributed to the kidneys after intravenous injection and then gradually excreted through urine. Approximately 40% remained in the kidneys 4 days after the intravenous injection (**Figure 4**). No side effect occurred in the kidney and other tissues by administration of excessively high dose of PVD. Next, we assessed the usefulness of PVD as a renal targeting carrier. The relationship between the M_n of PVD and its renal accumulation after intravenous injection was investigated. To evaluate the influence of molecular weight on renal accumulation of PVD, we estimated the plasma clearance and tissue distribution of PVD with various M_n after intravenous injection (**Figure 7**). The radioactivity in the supernatant of homogenized kidneys was measured after acid precipitation to distinguish between bound polymer and free tyramine, it was confirmed that the PVD did not release the free tyramine and it was not degraded in the kidneys (data not shown). The blood retention increased as the molecular weight increased (**Figure 7A**). On the other hand, PVD with an average molecular weight of 6–8kDa (PVD_{6k} and PVD_{8k}) showed the highest renal accumulation and about 80% of the administered dose accumulated in the kidneys at 3 h after injection (**Figure 7B**). Accumulation rates decreased to 60% for PVD_{14k} and 30% for PVD_{3k}. We examined the clearance, which was calculated on the basis of radioactivity at 3 h after intravenous injection of various PVDs in mice (Nishikawa et al, 1996; Nishikawa et al, 2003). The uptake clearance of PVD_{6k} was the highest among various PVDs. PVD_{6k} and PVD_{8k} were rapidly eliminated from the blood and specifically accumulated in the kidneys only 1 h after intravenous injection without being distributed to other tissues.

In addition, PVD_{6k} and PVD_{8k} showed high retention in the kidneys and about 60% of the injected dose was retained in the kidneys 24 h after intravenous administration. By the measurement of the urinary radioactivity excretion, it became clear that the PVD which accumulated in the kidney was gradually excreted through the urine. Furthermore, measurement of urinary radioactivity excretion revealed a significantly higher value for PVD_{3k} with the lowest molecular size (**Figure 8**).

We further evaluated the usefulness of PVD as a renal targeting carrier by polymer conjugation to SOD, which is viewd as a potential drug for renal disease. Several recent studies have reported an association between activated oxygen species such as superoxide

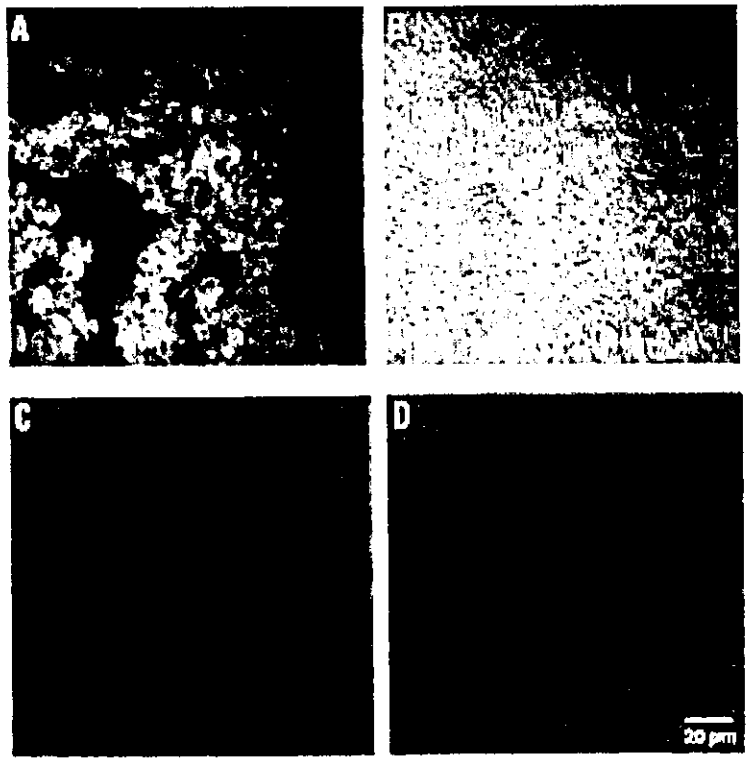

Figure 6. Histological sections of renal tissues in mice receiving an injection of fluorescein-labeld PVD.
From Nat Biotechnol. 2003 Apr;21(4):399-404

radical, hydrogen peroxide hydroxyl radical, and NO with various pathologic diseases processes such as cancer, inflammation, septicemia, and necrosis associated with ischemic reperfusion. Several studies have investigated the use of activated oxygen metabolic enzymes and antioxidants as therapeutic agents in diseases where stress oxidation plays a prominent role. SOD has shown promise as a therapeutic agent capable of eliminating superoxide radical in the early stages of formation of highly reactive oxygen species such as hydroxyl radical. Developments in genetic engineering have now enabled the production of large quantities of human Cu/Zn-SOD, which has attracted attention as a therapeutic agent. Hashida et al. reported that cationized SOD and PEGylated SOD exhibited significant therapeutic effects on ischemic acute renal failture (Fujita et al, 1992; Mihara et al, 1994). However, there is no report as to delivery of drug to the kidney specifically. With respect to kidney disease, activated oxygen is known to play an indispensable role in the mechanisms of ARF, complications associated with long-term maintenance dialysis, drug toxicity, and various inflammatory conditions. The PVD-SOD was prepared via formation of amide bound between the SOD lysine residues and carboxyl groups of PVD_{6k}. The resultant PVD-SOD was separated into three fractions of different molecular sizes (high = H, middle = M, low = L) by gel filtration HPLC, and then, specific activities were measured. The separated PVD-SODs, with molecular sizes of 73, 120, and 220 kDa, were termed L-PVD-SOD, M-PVD-SOD, and H-PVD-SOD, respectively. Although specific activity decreased with an increase in the molecular size, even H-PVD-SOD with the largest molecular size still had 60% activity compared with native SOD.

We then evaluated the pharmacokinetics of the three kinds of PVD-SODs after intravenous administration. Native SOD was rapidly cleared from the blood circulation (**Figure 9A**) 3 h after injection, accumulation of native SOD into the kidneys was observed in small quantities (**Figure 9B**), and almost all native SOD was found to be eliminated in the urine (data not shown). On the other hand, the blood residency and renal distribution

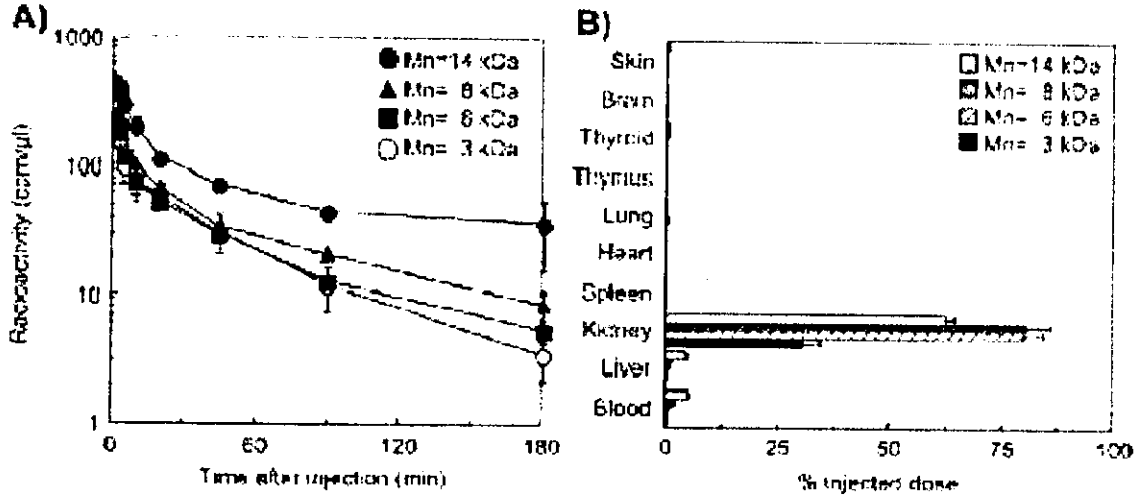

Figure 7. Plasma clearance and tissue distribution of PVDs with various molecular weight (M_n) after intravenous injection. From J Control Release. 2004 Mar 5;95(2):229-37.

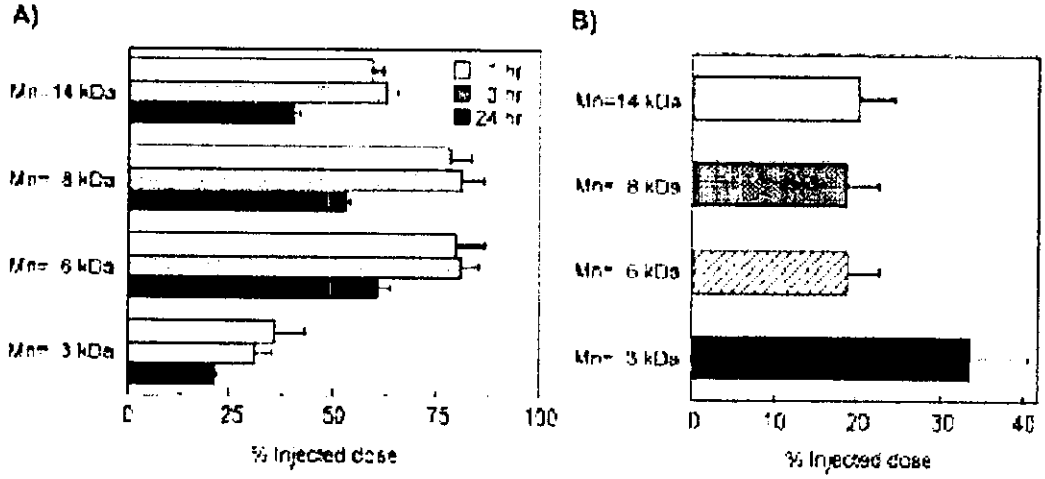

Figure 8. Renal accumulation and urinary excretion of PVD with various molecular weight after intravenous injection. From J Control Release. 2004 Mar 5;95(2):229-37

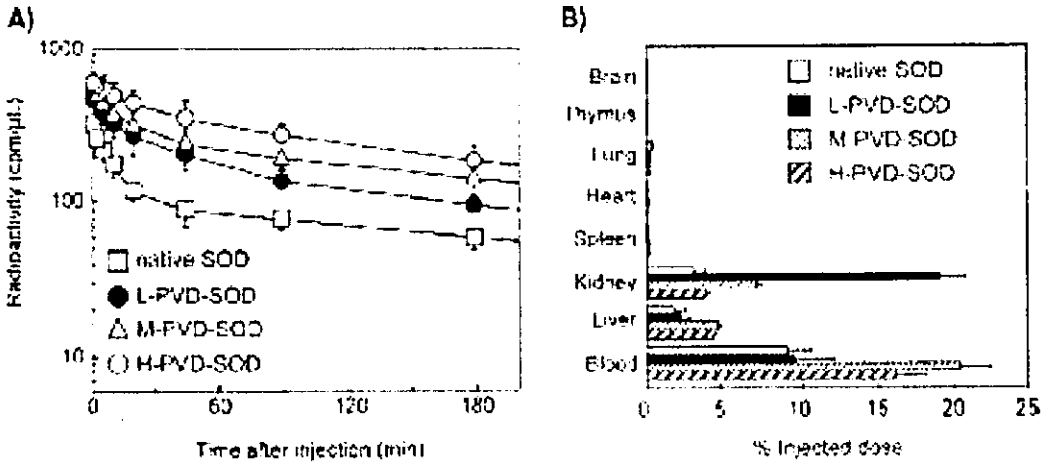

Figure 9. Plasma clearance and tissue distribution of native SOD and PVD-SODs 3 h after intravenous injection. From J Control Release. 2004 Mar 5;95(2):229-37

of PVD-SOD increased with a decrease in their molecular size. For L-PVD-SOD with an activity almost equivalent to native SOD, the renal accumulation was about six times higher than that of native SOD. Moreover L-PVD-SOD did not show with selective distribution to other major organs such as the liver or spleen. M-PVD-SOD and H-PVD-SOD showed higher distribution to the liver than native SOD and L-PVD-SPD, probably due to their high blood concentration.

We also assessed the therapeutic effect of L-PVD-SOD on ARF (**Table 1**). ARF was induced by subcutaneous injection of $HgCl_2$ at a dose of 8 mg/kg. SOD may be a defective agent to protect against the damaging effect of reactive oxygen species involved in inflammatory joint disease, such as ARF and rheumatoid arthritis (Corvo et al, 1997). The clinical application of SOD is limited because of its poor stability and pharmacokinetic properties (Veronese et al, 2002). Not only the levels of urinary ALP, Á-GTP, NAG, and serum creatinine, but also the urinary content of hemoglobin, ketone glucose, and protein rapidly increased after 12 h later. Native SOD and L-PVD-SOD were injected intravenously at a dose of 4 mg protein/kg12 h after injection of $HgCl_2$. The therapeutic efficacy was assessed 48 h after the administration of $HgCl_2$ (**Table 1**), because ARF markers reached the highest levels in untreated ARF mice. Native SOD showed weak therapeutic effects, because of its poor renal accumulation. PVP-SOD accumulated poorly in the kidney and did not produce substantial effects (data not shown). However L-PVD-SOD effectively accelerated the recovery from ARF. L-PVD-SOD showed great potential as a renal antioxidant agent against ARF. Drugs that prevent ARF pathopoiesis were given before the induction of ARF in almost all previous studies. In our study, L-PVD-SOD effectively accelerated recovery from ARF. These results suggest that L-PVD-SOD may be a candidate for a novel therapeutic agent with high renal targeting capability.

IV. Conclusion

Recently, the focus of life science research has shifted from genome analysis to genetic and protein function analysis resulting in drastic advances in pharmaco-proteomics. Recent advances in structural genomics will help clarify the function of numerous proteins. Therefore, it is highly probable that bioactive proteins such as newly identified proteins and cytokines will find therapeutic applications. (Furman et al, 1993; Glue et al, 2000; Barnard, 2001; Kreitman et al, 2001). However, most of these proteins are limited in their clinical application because of unexpectedly low therapeutic effects. The reason for this limitation is that these proteins are immediately decomposed by various proteases *in vivo*, and are rapidly excreted from the blood circulation. Therefore, frequent administration at an excessively high dose is required to obtain their therapeutic effects *in vivo*. As a result, homeostasis is destroyed, and unexpected side effects occur. Many cancer chemotherapies utilizing anticancer antibiotics are also limited by such problems. Therefore, in order to overcome the limitations peculiar to many proteins, we attempted to perform chemical modification (bioconjugation) with water-soluble polymers. Bioconjugation with polymeric modifiers improves plasma clearance and body distribution, resulting in an increase in therapeutic effects and decrease in side effects. We suggest that the investigation of the relationship between the degree of modification by the polymer, molecular size, and specific activity on bioactive protein bioconjugation may accomplish an increase in therapeutic effect and a decrease in side effects. In addition, our previous study indicates that optimally bioconjugated drugs can achieve well-balanced tissue transport, receptor binding, and plasma clearance, resulting in a selective increase in therapeutic effects.

On the other hand, in order to deliver a bioconjugated drug to the targeted tissue, the conjugate must be designed to possess desirable pharmacokinetic characteristics such as plasma clearance and tissue distribution. It is well known that the fate and the distribution of the conjugates are governed by the physicochemical properties of polymeric modifiers, such as molecular weight, electric charge, and hydrophilic-lipophilic balance. The increase in the therapeutic effect of a drug bioconjugated with

Table 1 Therapeutic effects of L-PVD-SOD to $HgCl_2$-induced ARF

		Intact mice	ARF mice	Native SOD-treated ARF mice	L-PVD-SOD-treated ARF mice
Urinary levels	Hemoglobin	- (<0.06 mg/dl)	++(>0.75 mg/dl)	+	-
	Ketone	-(<5 mg/dl)	++(>20 mg/dl)	±	-
	Glucose	-(<100 mg/dl)	++(>2000 mg/dl)	+	±
	protein	-(<10 mg/dl)	++(>1000 mg/dl)	+	±
	γ-GTP	-(<0.7 IU/LOG)	++(>550 IU/l)	++	+
	ALP	-(<14 IU/l)	++(>400 IU/l)	+	±
	NAG	-(<12 IU/l)	++(>17 IU/l)	++	-
Serum levels	creatinine	-(<0.5 mg/dl)	++(>1.5 mg/dl)	+	±

From J Control Release. 2004 Mar 5;95(2):229-37

polymeric modifier is attributed to the pharmacokinetics of the bioconjugated drug. Therefore, selecting the polymeric modifier by considering the influence of physicochemical characteristics on its pharmacokinetics is markedly important. As mentioned above, sequential and multiple strategies are needed for the optimization of drug therapy based on bioconjugation: (I) optimum selection of the polymeric modifier considering the disposition of the drugs and objectives such as targeting or controlled release; (ii) bioconjugation based on estimation of characteristics such as molecular size, modification site, degree of modification, and specific activity; and (iii) assessment of therapeutic effect and pharmacokinetics of bioconjugated drug. Further this methodology may be applied to not only bioactive protein but also gene therapy, we demonstrated bioconjugated adenovirus vectors enhanced transduction efficiency longer than naked virus vector *in vitro* and *in vivo* (data not shown). These results, in concert with the pharmacokinetic profiles, indicate that bioconjugation does protect the virus from inactivation in the serum and, as a result, improves the transduction efficiency of in susceptible organs *in vivo* (O'Riordan et al, 1999; Croyle et al, 2004). Bioconjugation prolonged transgene expression and allowed partial readministration with native virus or with a virus bioconjugated with a heterologous chemical moiety. Apparently, modification of the capsid leads to a shift in antigenic epitopes because vector readministration was not possible when the immunizing vector had been modified by the same bioconjugation chemistry used to modify the second vector. This concept of improving the performance of virus vectors through modification of the capsid with the optimum molecular design of a polymeric modifier shows promise. Our fundamental approach will enable the establishment of such a methodology ofbioconjugation. This approach may facilitate the optimum molecular design of a polymeric modifier in a drug delivery system.

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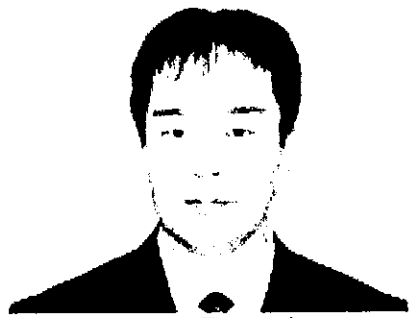

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