

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 (DMMAAn); 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- α (TNF- α); 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 carries 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, resulting 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 HgCl₂-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

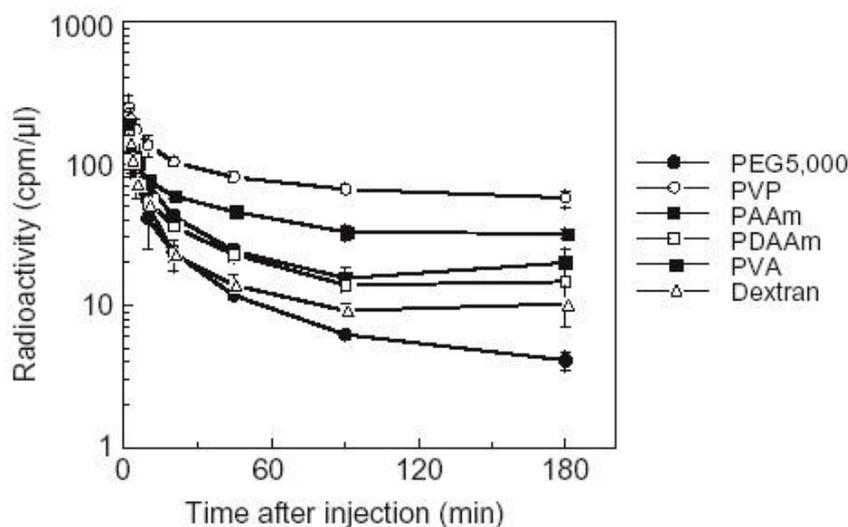


Figure 1. Plasma clearance of various water-soluble polymers in mice after intravenous injection. Reproduced from Kodaira et al, 2004 with kind permission from Biomaterials

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 (DMMA) 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 sialoprotein (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.

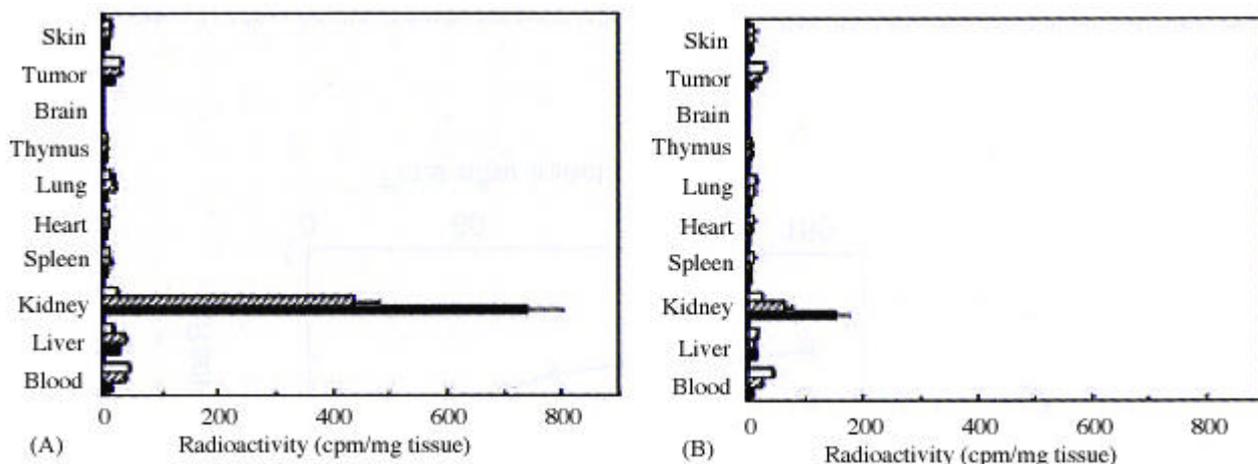


Figure 2. Tissue distribution of PVP and anionized PVP derivatives at 3h after intravenous injection in mice. Reproduced from Kodaira et al, 2004 with kind permission from Biomaterials

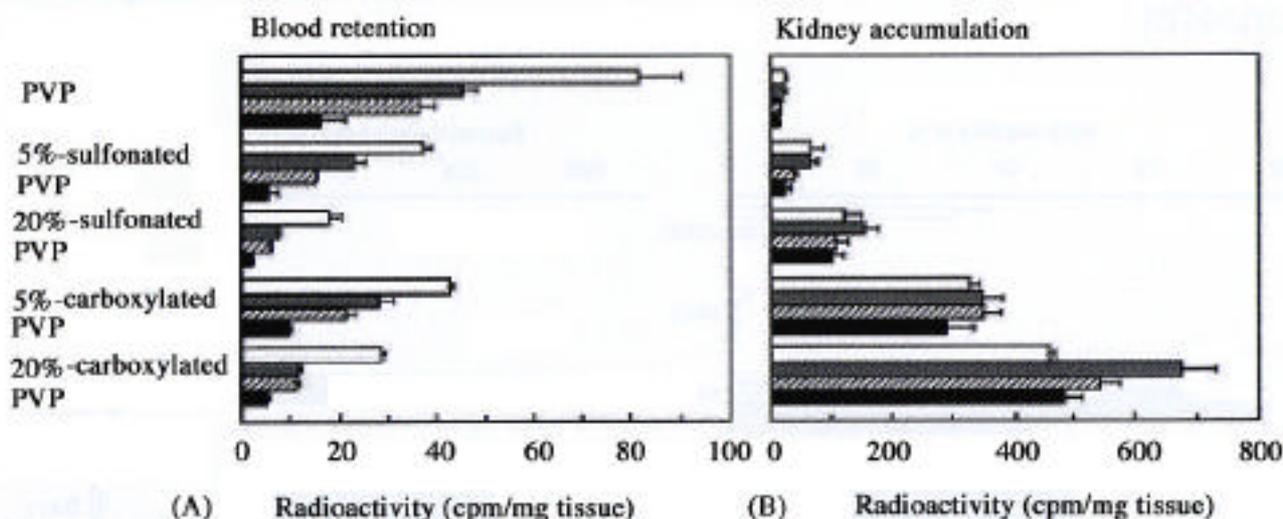


Figure 3. Blood retention and kidney accumulation of PVP and anionized PVP derivatives after intravenous injection in mice. Reproduced from Kodaira et al, 2004 with kind permission from Biomaterials

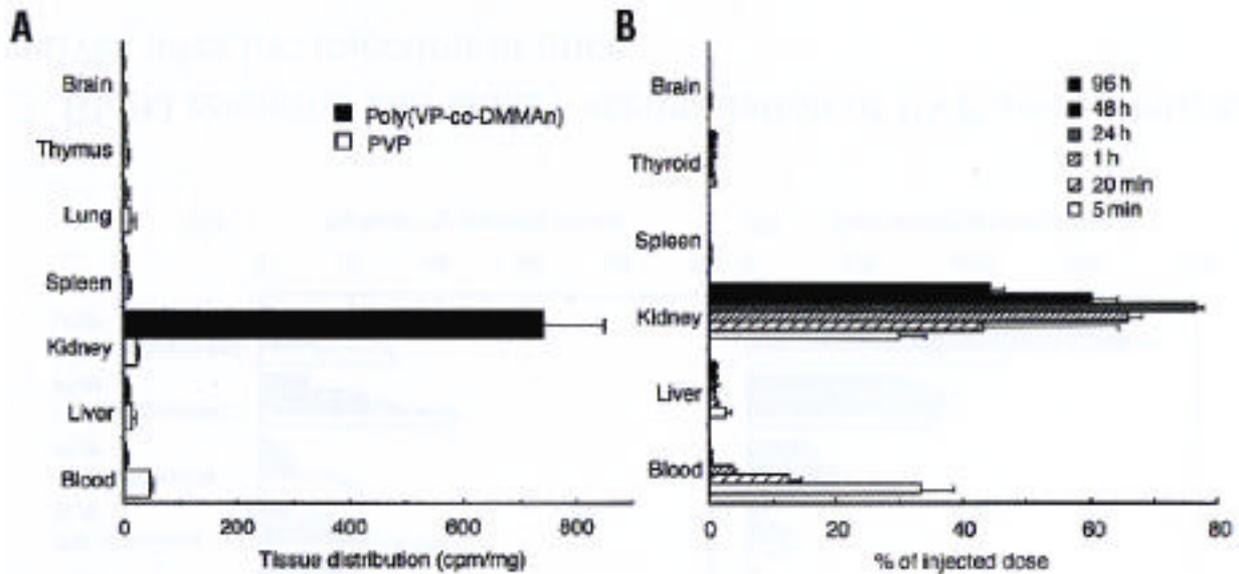


Figure 4. Tissue distribution of PVD after intravenous injection. Reproduced from Kamada et al, 2003 with kind permission from Nature Biotechnology

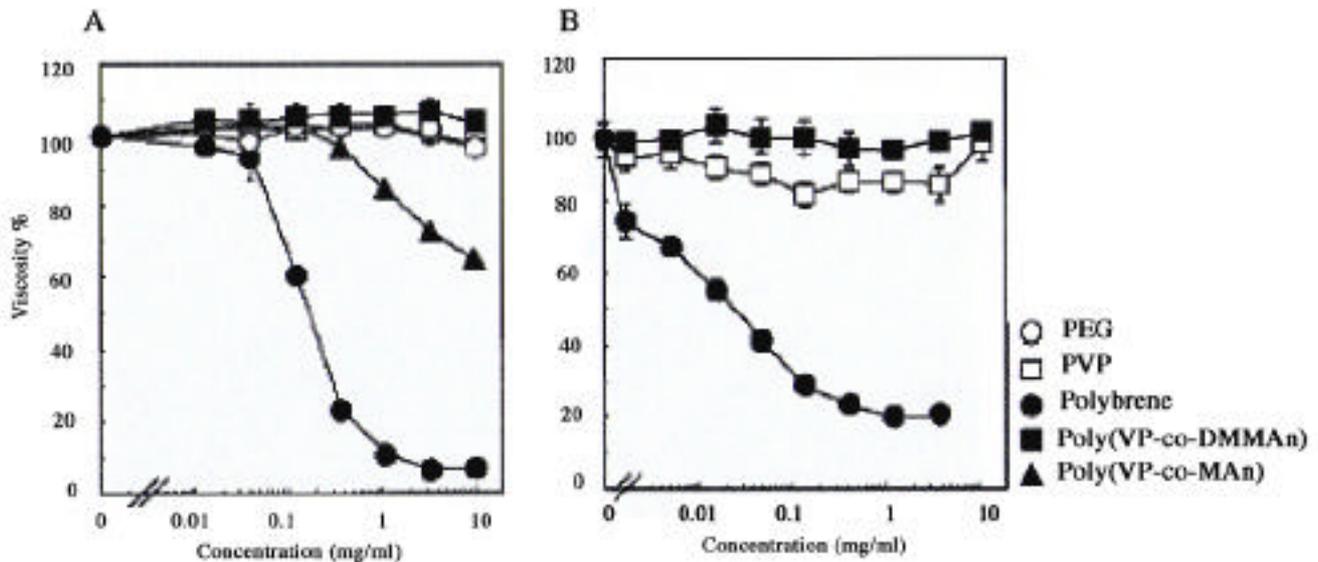


Figure 5. *In vitro* cytotoxicity of PVD. Reproduced from Kamada et al, 2003 with kind permission from Nature Biotechnology

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 fluorescently 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,

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

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.

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 viewed as a potential drug for renal disease. Several recent studies have reported an association between activated oxygen species such as superoxide

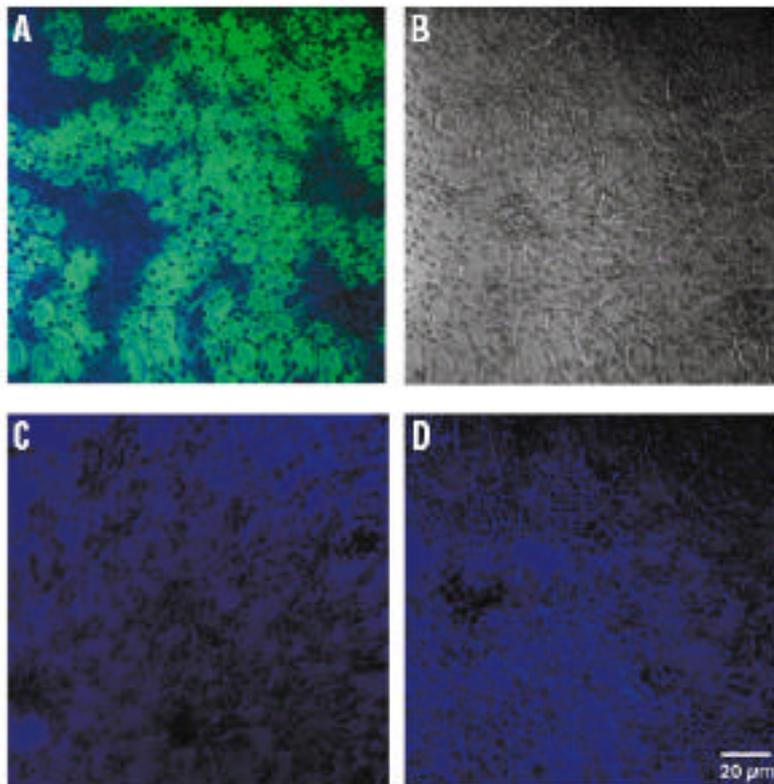


Figure 6. Histological sections of renal tissues in mice receiving an injection of fluorescein-labeled PVD.

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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 failure (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

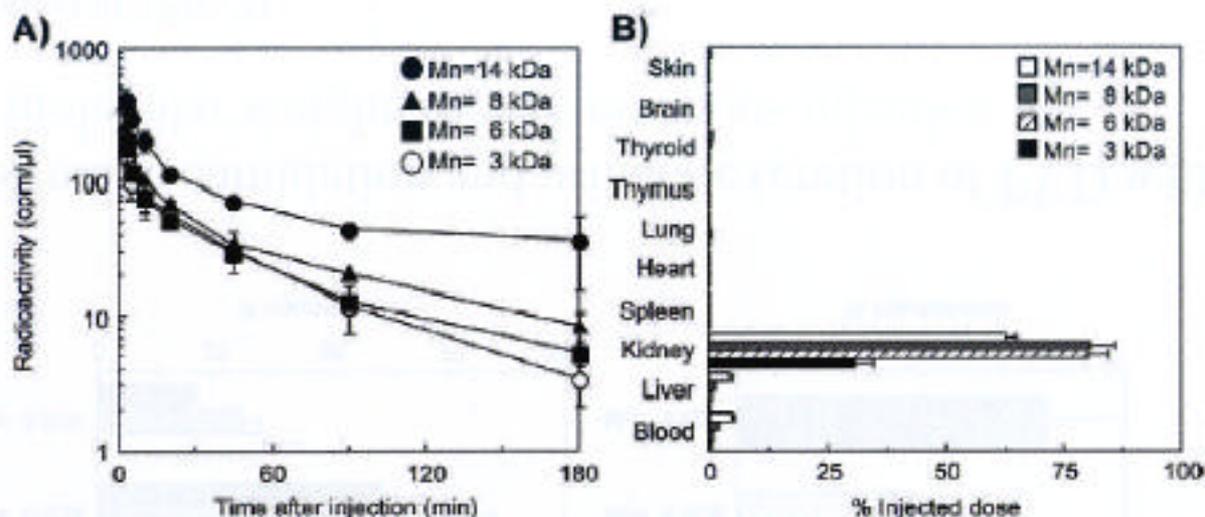


Figure 7. Plasma clearance and tissue distribution of PVDs with various molecular weight (M_n) after intravenous injection. Reproduced from Yamamoto et al, 2004 with kind permission from Journal of Controlled Release

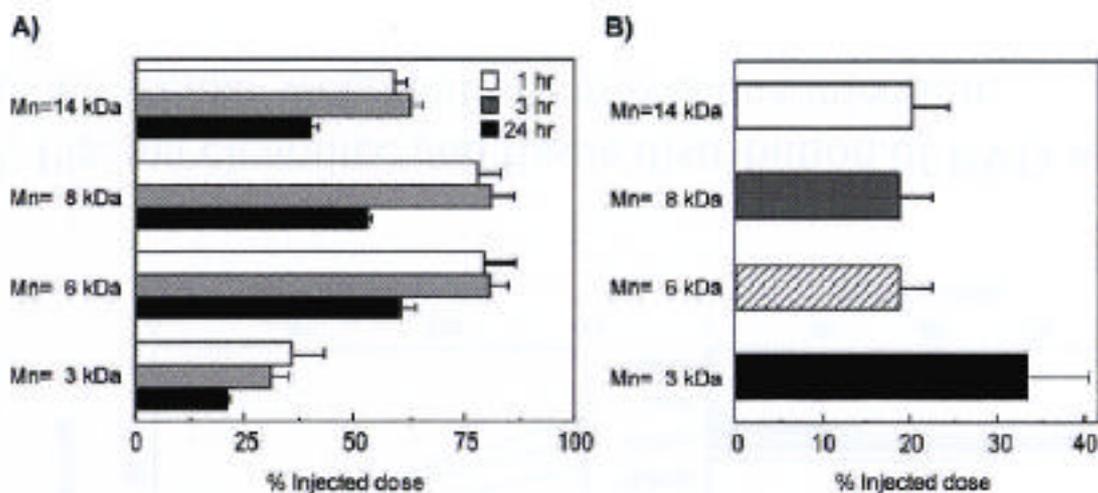


Figure 8. Renal accumulation and urinary excretion of PVD with various molecular weight after intravenous injection. Reproduced from Yamamoto et al, 2004 with kind permission from Journal of Controlled Release

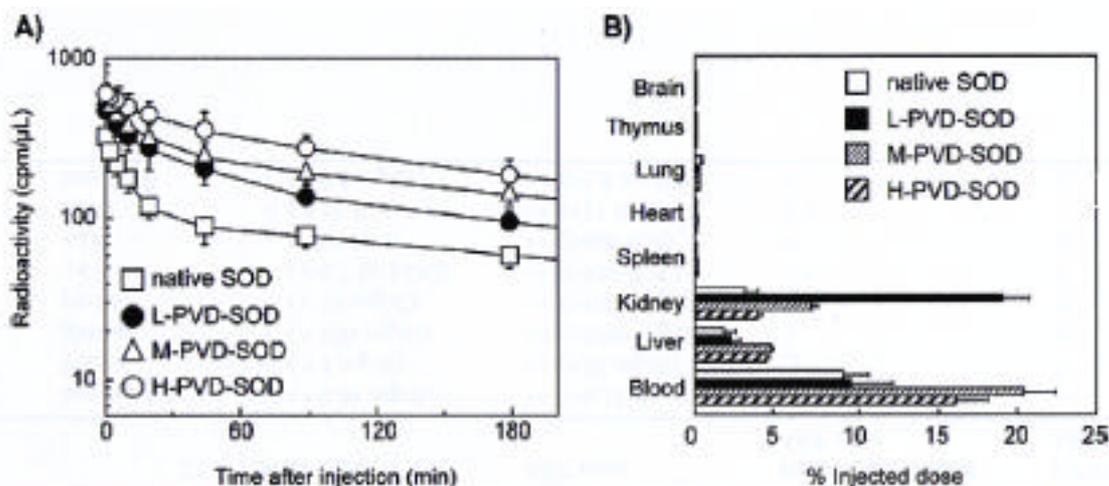


Figure 9. Plasma clearance and tissue distribution of native SOD and PVD-SODs 3 h after intravenous injection. Reproduced from Yamamoto et al, 2004 with kind permission from Journal of Controlled Release

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-SOD, 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, \AA -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/kg 12 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 pathogenesis 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 HgCl₂-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)	+	±
	-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)	+	±

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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 of bioconjugation. This approach may facilitate the optimum molecular design of a polymeric modifier in a drug delivery system.

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