ACCOUNTS
of chemical research

# Hyperbranched Polymeric "Star Vectors" for Effective DNA or siRNA Delivery

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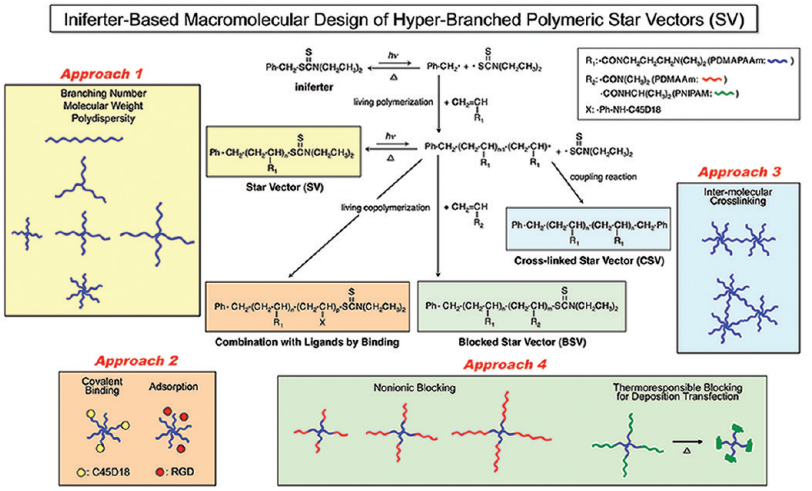
RECEIVED ON AUGUST 24, 2011

## CONSPECTUS




Although gene therapy offers an attractive strategy for treating inherited disorders, current techniques using viral and nonviral delivery systems have not yielded many successful results in clinical trials. Viral vectors such as retroviruses, lentiviruses, and adenoviruses deliver genes efficiently; however, the possibility of negative outcomes from viral transformation cannot be completely ruled out. In contrast, various types of nonviral vectors are attracting considerable attention because they are easier to handle and induce weak immune responses.

Cationic polymers, such as polyethylenimine (PEI) and poly(*N*,*N*-dimethylaminopropyl acrylamide) (PDMAPAAm), can generate nanoparticles through the formation of polyion complexes, "polyplexes" with DNA. These nonviral systems offer many advantages over viral systems. The primary obstacle to implementing these cationic polymers in an effective gene therapy remains their comparatively inefficient gene transfection in vivo.

We describe four strategies for the development of hyperbranched star vectors (SVs) for enhancing DNA or siRNA delivery. The molecular design was performed by living radical polymerization in which the chain length can be controlled by photoirradiation and solution conditions, including concentrations of the monomer or iniferter (a molecule that serves as a combination of initiator, transfer agent, and terminator). The branch composition is controlled by the types of monomers that are added stepwise. In our first strategy, we prepared a series of only cationic PDMAPAAm-based SVs with no branches or 3, 4, or 6 branching numbers. These SVs could form polyion complexes (polyplexes) by mixing with DNA only in aqueous solution. The relative gene expression activity of the delivered DNA increased according to the degree of branching. In addition, increasing the molecular weight of SVs and narrowing their polydispersity index (PDI) improved their activity. For targeting DNA delivery to the specific cells, we modified the SV with ligands. Interestingly, the SV could adsorb the RGD peptide, making gene transfer possible in endothelial cells which are usually refractory to such treatments. The peptide was added to the polyplex solution without covalent derivatization to the SV. The introduction of additional branching by cross-linking using iniferter-induced coupling reactions further improved gene transfection activity. After block copolymerization of PDMAPAAm-based SVs with a nonionic monomer (DMAAm), the blocked SVs (BSVs) produced polyplexes with DNA that had excellent colloidal stability for 1 month, leading to efficient in vitro and in vivo gene delivery. Moreover, BSVs served as carriers for siRNA delivery. BSVs enhanced siRNA-mediated gene silencing in mouse liver and lung. As an alternative approach, we developed a novel gene transfection method in which the polyplexes were kept in contact with their deposition surface by thermoresponsive blocking of the SV. This strategy was more effective than reverse transfection and the conventional transfection methods in solution.



Published on the Web 02/21/2012 www.pubs.acs.org/accounts
10.1021/ar200220t 

## Introduction

Gene therapy is an attractive approach for treating inherited disorders. However, current techniques have not yielded many successful results in clinical trials. There are two primary gene delivery systems, viral and nonviral. Viral vectors such as retroviruses, lentiviruses, and adenoviruses have been proven to deliver genes efficiently; however, the possibility of negative outcomes resulting from viral transformation cannot be completely ruled out. In contrast, various types of nonviral vectors have been recently reported and are attracting considerable attention because they are easier to handle and induce weak immune responses.

Cationic polymers such as polyethylenimine (PEI) and poly(*N*,*N*-dimethylaminopropyl acrylamide) (PDMAPAAm), which can generate nanoparticles by forming polyion complexes, that is, "polyplexes" with DNA, are highly anticipated to be major carriers in nonviral gene delivery systems due to their many advantages compared to virus systems. However, the primary obstacle to implementing an effective gene therapy using cationic polymers remains their relatively inefficient gene transfection in vivo when compared with those of viral vectors.

To enhance gene transfection using cationic polymers, numerous studies have been performed by various approaches as follows: (1) chemical synthetic engineering, in which the kind and composition of the polymers are modified,[1,2] (2) biochemical, in which targeting ligands such as galactose, mannose, transferring, or antibodies are incorporated into the polymers,[3,4] and (3) functional molecular engineering, in which stimulus-response polymers with light- and thermal reactivity are designed as high performance vectors.[5−7] However, few studies have examined the molecular structure of cationic polymers, which are usually synthesized by conventional radical polymerization. Some exceptions include analysis of the effects of polymer chain length changes, polymer composition, and complex multibranching polymers. In these cases, structural analysis is impossible. Because achieving precise molecular design, including the molecular weight and three-dimensional structure, by conventional radical polymerization is quite difficult in general, the systematic structure-dependence of cationic polymers in gene transfection has not been established.

Iniferter-based living radical polymerization as a base for macromolecular architecture design of hyperbranched SVs will be considered here. Furthermore, we will describe four strategies for designing SV families using different approaches, which include (1) number of branches and precise chain lengths of branches; (2) biochemical modification of SVs by combination with targeting ligands; (3) cross-linking of SVs for the formation of further complex structures, namely, cross-linked SVs (CSVs); and (4) block copolymerization to form blocked SVs (BSVs). Finally, (5) a novel transfection method using thermoresponsive BSVs will be described.

## Iniferter-Based Living Radical Polymerization

Photoliving polymerization can control polymer properties without catalysis and can be conducted under mild or extreme conditions using inexpensive instruments. This is in contrast to other precision polymerizations such as metal-catalyzed controlled radical polymerization, nitroxide-mediated controlled radical polymerization, and reversible addition−fragmentation chain transfer (RAFT) polymerization. One of the most popular photoliving polymerizations is iniferter (an iniferter acts as an initiator, transfer agent, and terminator)-based radical polymerization, which was pioneered by Otsu et al. in 1982.[8] *N*,*N*-Diethyldithiocarbamylmethylbenzene is a frequently used iniferter. The unique feature of this iniferter polymerization is that it proceeds in a controlled manner, in which "active" and "dormant" propagating chain ends are reversibly equilibrated under ultraviolet (UV) light irradiation. Using this polymerization, we successfully synthesized block copolymers having the ability to control the block chain lengths with minimal transfer or termination reactions.[9,10] Photoreactions are a suitable technology in the field of biomaterials because they can proceed without toxic catalysts. Therefore, iniferter-based polymerization was applied to modify naturally occurring polymers.[11] Furthermore, homo- or block-graft polymerized surfaces with regional precision were designed in order to create biocompatible surfaces.[12−15]

However, the standard iniferter polymerization method is not perfect with respect to conversion and termination. Most studies over the years have employed UV light sources, which frequently induce irreversible termination because of the exposure to excessive high-energy wavelengths. Therefore, we determined the optimal irradiation wavelength for precise polymer design using monochromatic light in the range from 330 to 400 nm at approximately 10 nm intervals using the tetrafunctional iniferter 1,2,4,5-tetrakis(*N*,*N*-diethyldithiocarbamylmethyl)benzene in the presence of *N*,*N*-dimethylaminopropyl acrylamide (DMAPAAm).[16] Polymerization was maximum at 370 nm and proceeded in a controlled manner up to approximately 30% conversion and up to a molecular weight of approximately 40 000 with a relatively limited polydispersity index (PDI: approximately 1.6).

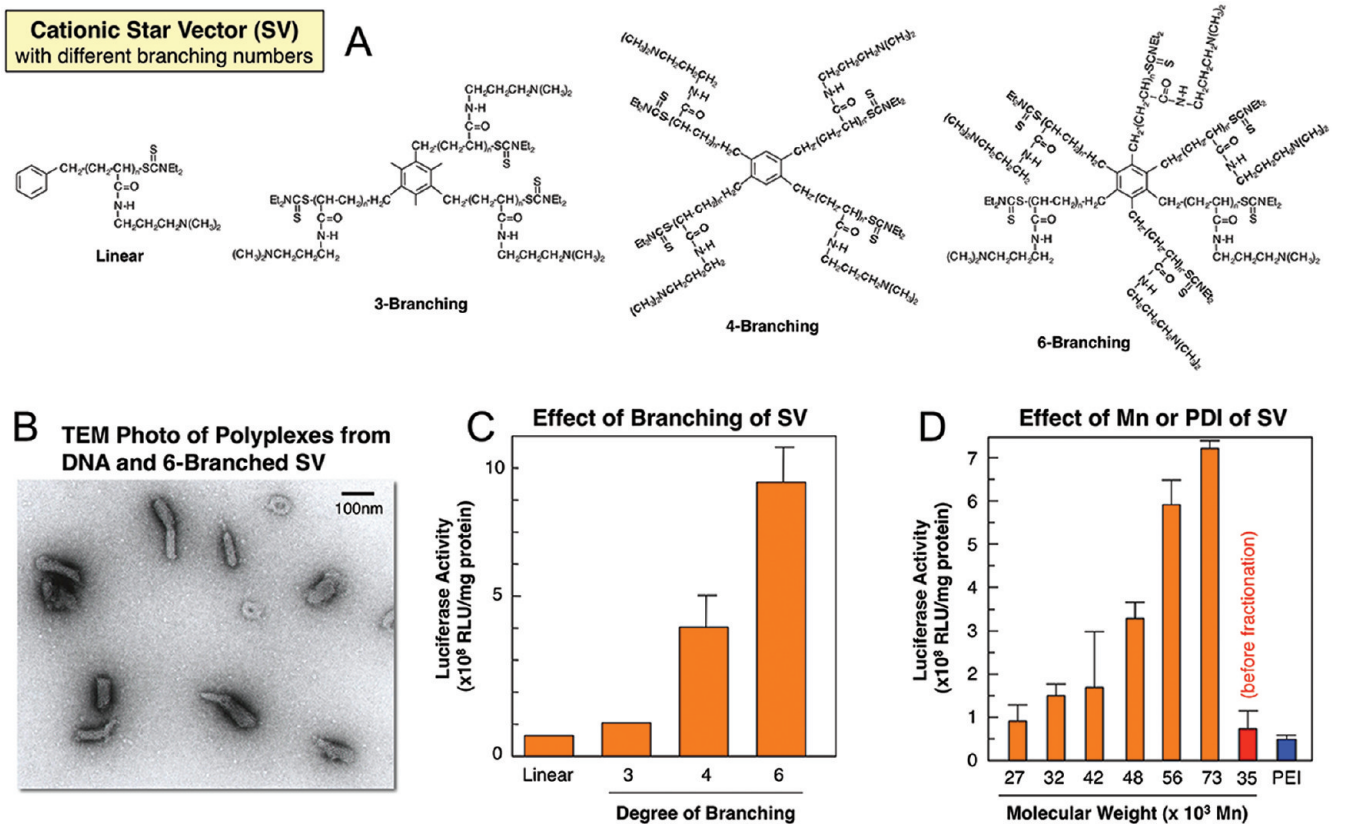



**FIGURE 1.** (A) Chemical structures of SVs with different branching numbers, which were synthesized by iniferter-based photoliving radical polymerization of 3-(*N*,*N*-dimethylamino)propylacrylamide (PDMAPAAm) from the respective multifunctional iniferters, *N*,*N*-diethyldithiocarbamate-derivatized benzenes. (B) Transmission electron microscopy (TEM) image of a polyplex from DNA (pGL3-control) and 6-branched SV of PDMAPAAm ($M_n$: 18 000, PDI: 1.4). (C) The transfection activity of the polyplexes prepared from DNA (pGL3-control) and 6-branched SVs ($M_n$: 18 000, PDI: 1.4) with varying degrees of branching in COS-1 cells. (D) The transfection activity of the polyplexes prepared from DNA and the 6 fractionated 4-branched SVs of PDMAPAAm ($M_n$: 27 000−73 000) with narrow PDI (1.1−1.2) or linear PEI in COS-1 cells.

The theoretical number of *N*,*N*-diethyldithiocarbamate functional groups after polymerization was obtained. Thus, this simple optimization procedure improved the reliability of the iniferter method.

## Approach 1: Effects of Branching Number, Molecular Weight, and Polydispersity of SVs

We attempted to improve the utility of star-shaped, hyperbranched cationic polymers as a base chemical structure for a novel high-performance gene carrier. SVs were prepared by iniferter-based living radical polymerization of DMAPAAm using the respective multidithiocarbamate-derivatized benzenes (multifunctional iniferters). As the first approach, we synthesized a series of linear and branched (3, 4, or 6) cationic polymers, with the same approximate molecular weight ($M_n$: 18 000) and PDI (1.4) (Figure 1A).[17] The PDI was determined by GPC using a high-performance liquid chromatography system, calibrated for molecular weight with narrow weight distribution poly(ethylene glycol) standards. All polymers produced polyion complex (polyplexes) when mixed with a luciferase-encoding plasmid DNA (pGL3-control plasmid). The size of the polyplexes, determined by dynamic light scattering (DLS), ranged from approximately 150 nm in diameter. Similar value was observed by TEM (Figure 1B). Higher gene expression was obtained with increased branching accompanied by little cytotoxicity. Gene expression relative to the linear polymer was about 2, 5, and 10 times higher for the 3-, 4-, and 6-branched polymers, respectively (Figure 1C). The precise change in polymer branching permitted the control of transfection activity. As the degree of branching increases, the density of cationic charges in the branched polymers increases, which may result in high compaction of DNA polyplexes to achieve high gene expression levels.

The gene transfection efficiency of cationic polymers strongly depends on their structures as demonstrated by the branching number. Similarly, molecular weight, which is one of the main structural parameters, exerts the greatest influence on gene transfection efficiency and has been evaluated by many researchers.[18−20] Synthetic polymers

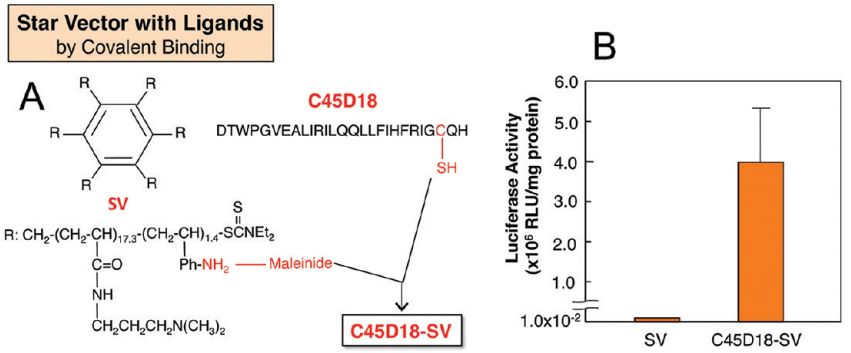



**FIGURE 2.** (A) Chemical structure of 6-branched SVs of PDMAPAAm (Mn: 18300, PDI: 1.4) derivatized with C45D18 peptide (C45D18-SV). (B) Gene transduction into human macrophages. Monocyte-derived macrophages were prepared from healthy humans by culturing peripheral blood mononuclear cells for 6 days in the presence of 100 ng/mL macrophage colony-stimulating factor (M-CSF). Then, the cells were deprived of M-CSF for 4−5 days and subjected to experiments.

invariably have heterogeneous molecular weights even if they are synthesized by living radical polymerization. Unfortunately, molecular weight distribution, including PDI, has not been necessarily described in reports in this field. Using uniform polymers with monodispersity is ideal for precisely evaluating transfection efficiency.

To examine precisely the effect of molecular weight of the SV in terms of gene transfection efficiency, we separated 4-branched SVs of PDMAPAAm ($M_n$: 35 000, PDI: 1.6) into several different molecular weight fractions with low PDI ranging from 1.1 to 1.2 by using size-exclusion chromatography.[21] The highest luciferase transfection activity was obtained for the SVs with the highest molecular weight (73 000) and was more than 7 times greater than that for the SVs with the lowest molecular weight (27 000), the nonfractionated SVs or PEI (ExGen500), which is one of the major commercially available typical polymeric vectors used as a positive control (Figure 1D). Transfection activity correlated more with SV amphiphilicity or hydrophobicity, surface potential, and condensate density of the polyplexes than with particle size. It was very difficult to clearly explain the relationship between the transfection activity and the physicochemical properties of SVs or the polyplexes, such as critical micellar concentration of the SV, mean diameter, $\zeta$-potential of the polyplexes, and pDNA condensation potential of the polyplexes. However, SVs with the highest molecular weight and activity could be easily isolated by using size-exclusion chromatography. In comparison with fractionated SVs with narrow molecular weight distribution, slight differences in molecular weight resulted in considerable differences in the physicochemical properties and transfection efficiencies. We consider that the optimal molecular weight is different for each polymeric vector species because the vectors exhibit different physicochemical properties. Therefore, precise and reliable evaluation of transfection activity must be performed using polymeric vectors with narrow molecular weight distributions. Size fractionation will also be useful for enhancing the gene transfection activity of other cationic polymers.

## Approach 2: Combination with Ligands

Vectors that express ligands are effective in delivering a target to specific cells. Vpr, a human immunodeficiency virus type-1 nonstructural gene product,[22] is thought to transport viral DNA from the cytoplasm to the nucleus in resting macrophages.[23] A peptide encompassing amino acids 52−78 of Vpr (C45D18) promotes nuclear trafficking when conjugated to recombinant proteins.[24] Gene expression in resting macrophages is facilitated when C45D18 is conjugated to 6-branched SVs of PDMAPAAm ($M_n$: 18 300, PDI: 1.4) terminated with oligo(4-aminostyrene) (1.4 of 4-aminostyrene units in average were terminated for each branch) (Figure 2A).[25] Although there was no difference between SV alonse and C45D18-SV with respect to gene transfer into growing cells, C45D18-SV resulted in more than 40-fold greater expression of the exogenous gene when transfected into chemically differentiated macrophages and quiescent monocyte-derived human macrophages (Figure 2B). These data suggest that C45D18 contributes to improving the ability of a nonviral vector to transduce macrophages with exogenous genes. We postulate that the C45D18-SV system will be effective for transfecting other types of resting cells.

In contrast, the tripeptide sequence arginine (Arg)-glycine (Gly)-aspartic acid (Asp) (RGD) found in the active site of vitronectin binds to integrin $\alpha_V\beta_3$ and almost half of the other 22 known integrins.[26] RGD peptides are exploited by pathogenic microorganisms, such as the foot-and-mouth disease virus, for cell entry.[27] A great advantage of integrin targeting is that internalization occurs by a "zippering"

mechanism that allows the uptake of relatively large structures such as bacteria. Therefore, several cationic polymer vectors combined with RGD peptides were developed for tumor-targeted gene delivery.[28] For example, RGD-containing peptides were coupled with PEI with or without a PEG spacer. The RGD-modified PEI showed a significant increase in transfection efficiency as compared with only PEI in endothelial cells. Almost all RGD-mediated gene delivery systems have been described with the chemical derivatization of RGD peptides to vector compounds.

We investigated the feasibility of using SV in endothelial cells for enhancing gene transfection by adding the RGD peptide (Figure 3A).[29] We strongly believed that if the coating of the polyplex surface with the RGD peptides occurs only by the addition of these peptides, then the gene transfection efficiency for endothelial cells would be enhanced, similar to that in the case of chemical derivatization of the RGD peptides. The addition of the RGD-containing peptide (GRGDNP) to the solution of polyplex from 4-branched SVs of PDMAPAAm ($M_n$: 18 000, PDI: 1.4) and the luciferase-encoding plasmid DNA led to a marked inhibition of polyplex aggregation, indicating the coating of the polyplex surface with RGD peptides (Figure 3B). A transfection study on endothelial cells showed that luciferase activity increased with the amount of RGD peptides added to the polyplexes (Figure 3C). The activity further increased 8-fold compared to that without RGD addition when cyclic RGD peptides (RGDFV) were used. In both cases, the surplus RGD peptides might bind to the integrin receptor and prevent binding with RGD peptide, thereby coating the polyplexes. Gene delivery to endothelial cells was significantly enhanced by the addition of only RGD peptides to SV-based polyplexes. The enhancement of gene transfer by the addition of the RGD peptide may be applied to other cationic polymer vectors as well because the RGD peptide could coat the cationic surface of the polyplexes. The surface-coating ability may be enhanced by the introduction of anionic amino acids such as aspartic acid or glutamic acid into the RGD peptide.

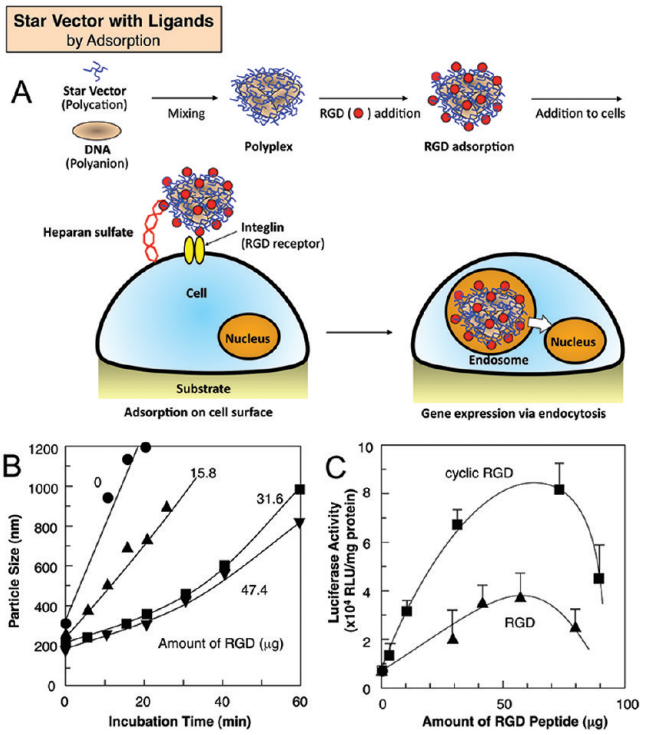



**FIGURE 3.** (A) Illustration of the method of gene transfection to endothelial cells by using 4-branched SVs of PDMAPAAm with the addition of the RGD peptide. (B) Incubation time-dependent changes in the cumulant diameter of the polyplexes from DNA (pGL3-control) and 4-branched SVs ($M_n$: 18 000, PDI: 1.4) in aqueous solutions in the presence of RGD peptides (GRGDNP). (C) Transfection of the same polyplexes in the presence of RGD peptides (linear GRGDNP or cyclic RGDFV) to endothelial cells.

## Approach 3: Intermolecular Cross-Linking

In the first approach, the greater the number of branches, the higher is the efficiency of gene expression. Because additional complex branching by cross-linking SVs was expected to further improve gene transfection efficiency, we attempted a photocross-linking strategy using macroiniferters.[30] As demonstrated in the iniferter-based living radical polymerization section, polymerization due to an iniferter occurs in the presence of a monomer. However, in the absence of monomers, chain transfer of the generated benzyl radicals and dithiocarbamyl radicals to the solvent or polymer matrix results in simultaneous coupling between the radicals. Coupling between benzyl radicals and dithiocarbamyl radicals is reversible, and these radicals are regenerated by UV reirradiation. Coupling between dithiocarbamyl radicals generates *N*,*N*,*N*′,*N*′-tetraethylthiuram disulfide, which is dissociated to dithiocarbamyl radicals by UV irradiation because the coupling is also reversible. In contrast, coupling between benzyl radicals generates bibenzyl, which is not dissociated by UV irradiation. Therefore, benzyl radicals are selectively consumed by UV irradiation to generate bibenzyl. As expected, a dimer was produced from a PEG derivative with dithiocarbamate at one terminus, and a polymer was produced from the PEG derivative with dithiocarbamate at both termini.[31] This study indicated that the terminal ends of the dithiocarbamate-derivatized polymers could be cross-linked by photoirradiation alone, without using a chemical cross-linking agent such as glutaraldehyde or diisocyanate. The cross-linking may be applied to molecular architecture to prepare more complex-shaped polymers, such as mesh or hyper-branch structures.

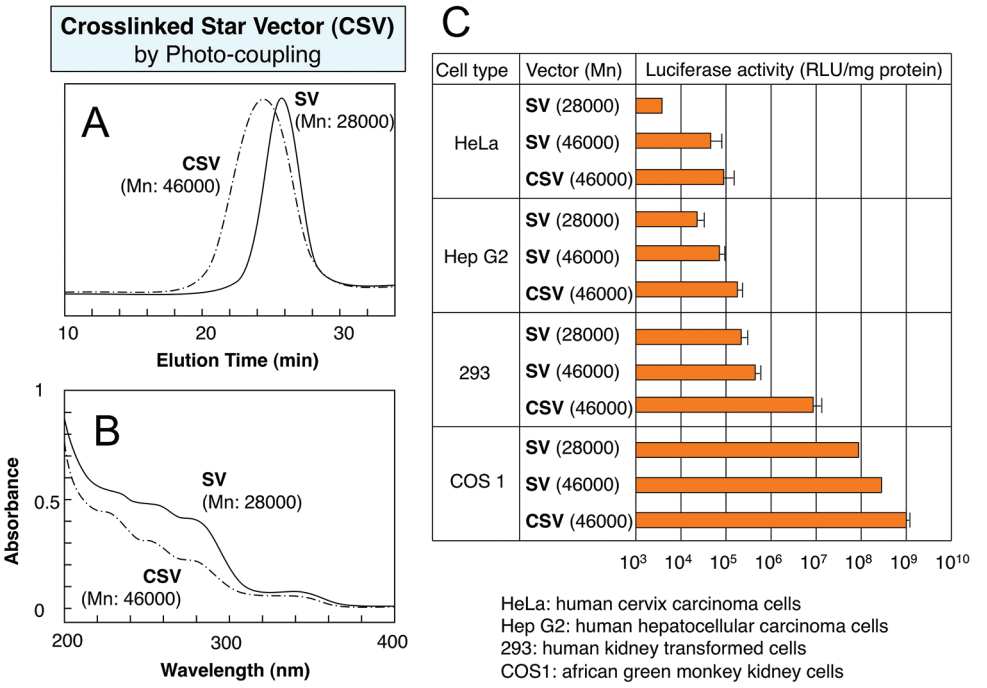



**FIGURE 4.** (A) GPC curves and (B) UV adsorption spectral changes after photoirradiation of 4-branched SVs of PDMAPAAm ($M_n$: 28 000, PDI: 1.4) to obtain CSVs ($M_n$: 46 000, PDI: 2.0) (irradiation time, 240 min). (C) Transfection of four different cell lines using polyplexes prepared by mixing the DNA (pGL3-control) and the SV or CSV.

We used 6-branched SVs of PDMAPAAm as a precursor for photocross-linking. With increasing irradiation time, the molecular weight of the CSVs increased through terminal coupling of the SVs (Figure 4A); this effect was confirmed by a decrease in the absorbance of dithiocarbamate (Figure 4B).[30] As expected, cross-linking the SVs the gene transfection efficiency increased dramatically in several types of cells (Figure 4C). The luciferase activity with CSVs ($M_n$: 46 000, PDI: 2.0) was at least 1 order of magnitude in the original SV ($M_n$: 28 000, PDI: 1.4). The activity of CSVs was at least 2-fold higher than that of non-CSVs of the same molecular weight ($M_n$: 46 000, PDI: 1.4). Designing nanoarchitectural macromolecular polymeric DNA carriers by hyper-branching or cross-linking or other methods has been considered an important strategy for improving the transfection efficiency. Therefore, we consider that SV cross-linking, which can induce an increase in the cationic charge density, could be a key strategy for enhancing the transfection efficiency of cationic polymers.

## Approach 4: Blocking of SVs

**Nonionic Blocking.** The conjugation of cationic polymers with a hydrophilic and biocompatible polymer such as polyethylene glycol (PEG)[32,33] provides a major vector-modification strategy to improve transfection efficiency.[34,35] Surface modification of these PEG conjugates increases the DNA delivery system's half-life in blood circulation. Therefore, linking the nonionic hydrophilic chains with SV DNA condensates was expected to improve gene transfection efficiency by shielding the particles from nonspecific interactions and conferring greater stability upon them by a process similar to PEGylation of polyplexes.

As the fourth approach, we designed polymers consisting of cationic and nonionic chains as inner and outer domains, respectively, by iniferter-based block radial polymerization (Figure 5A).[36] We synthesized 4-branched PDMAPAAm-poly(*N*,*N*-dimethylacrylamide) (PDMAAm)-blocked copolymers with copolymer compositions (unit ratio of DMAAm to DMAPAAm) ranging from 0.18 to 1.0 for 4-branched PDMAPAAm with molecular weights ranging from 20 000 to 50 000, and a PDI of 1.3−1.4. The polyplexes formed by block copolymers with molecular weights of 50 000 in the case of PDMAPAAm branches and 33 000 in the case of PDMAAm branches supported the highest luciferase activity, which was approximately 5-fold higher than that achieved with nonblocked SVs (Figure 5B). PDMAPAAm-PDMAAm-blocked star-shaped polymers (blocked star vectors [BSVs]) are an attractive novel class of nonviral gene delivery systems.

Interestingly, because the polyplexes were very stable in aqueous media even at 1 month after their formation, they

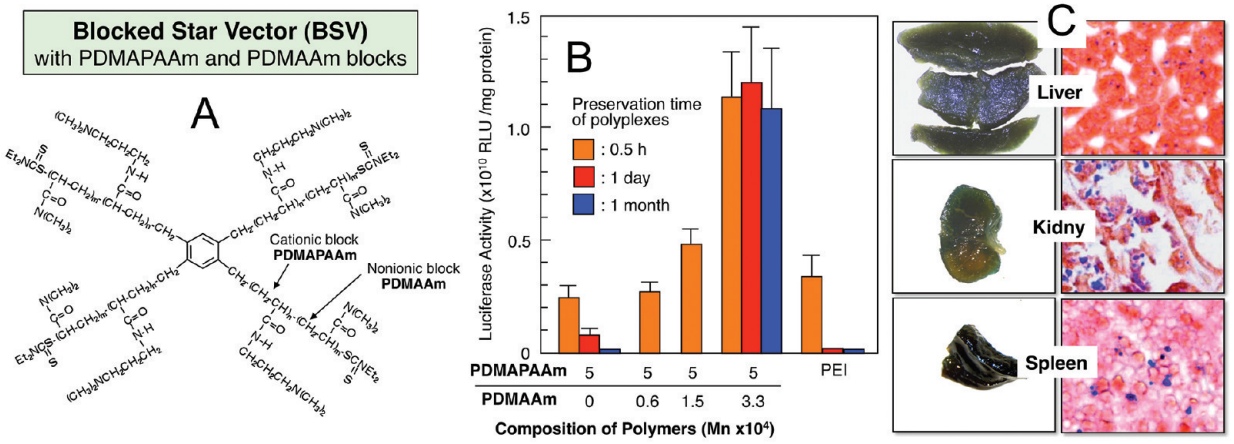



**FIGURE 5.** (A) Chemical structure of 4-branched BSVs containing an inner domain of cationic PDMAPAAm chains and an outer domain of nonionic PDMAAm chains. (B) Transfection efficiency of polyplexes prepared from DNA (pGL3-control) and the 4-branched BSV at different preservation times after preparation. For comparison, transfection efficiency data from PEI ($M_n$: 18 000) is also provided. (C) Expression of $\beta$-galactosidase in mouse organs (liver, kidney, and spleen) 48 h after injection of the polyplexes from DNA (*lac Z* gene, *pcDNA3.1/His B/lac Z)* and the 4-branched BSV with PDMAPAAm block ($M_n$: 50 000) and PDMAAm block ($M_n$: 33 000) from jugular vein. (left side) Macroscopic photos after staining with X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). (right side) Histological sections further stained with eosin.

continued to show high activity, whereas nonblocked SVs showed approximately 60% loss of the activity after 1 day (Figure 5B). In the case of PEI, more than 90% of the activity was lost within 1 day after polyplex formation.

Furthermore, in preliminary in vivo studies conducted over 48 h, mice injected with the polyplexes containing the $\beta$-galactosidase-expression gene showed high levels of gene expression in the liver, kidneys, or spleen, without any detectable tissue damage (Figure 5C).[37] Upon staining with X-gal, these resected tissues showed complete green coloration because of the enzymatic decomposition of X-gal by the produced $\beta$-galactosidase. In addition, many blue spots of 5,5′-dibromo-4,4′-dichloro-indigo, produced by the decomposition, were clearly observed. These results suggest that BSV has potential for clinical use as a nonviral vector.

**siRNA Delivery by BSV.** RNA interference (RNAi) is the process by which double-stranded RNA (dsRNA) directs the sequence-specific degradation of complementary mRNA.[38,39] It is therefore an effective method for gene-function analysis as well as a potentially powerful therapeutic modality for silencing pathogenic gene products associated with cancer, viral infections, and autoimmune disorders. Small interfering RNAs (siRNAs) can be directly introduced into cells as synthetic linear siRNAs or short hairpin RNAs (shRNAs).[40] RNA polymerase III driven expression cassettes can be used to constitutively express shRNA molecules. Although both viral and nonviral vectors can be used to deliver siRNA into cells, viral vectors are not sufficiently capable of delivering siRNA-expressing constructs such as shRNAs. Commercially available cationic lipids such as Oligofectamine can efficiently deliver siRNAs into cells;[41] however, such cationic lipids are highly toxic and hence cannot be effectively used for systemic delivery of siRNAs in vivo.

We developed a novel nonviral gene silencing system using siRNA or shRNA complexes and BSVs with 4-branched PDMAPAAm ($M_n$: 50 000) and PDMAAm ($M_n$: 33 000) blocks (PDI: 1.4).[42] BSV is shown to condense and interact with siRNAs to yield stable BSV/siRNA polyion complexes, approximately 90 $\mu$m in diameter. Using these complexes, siRNA was successfully delivered to almost all human hepatocellular carcinoma cells, as confirmed by fluorescence microscopic examination, which showed Cy3 labeling in almost cells (Figure 6A). siRNAs could induce significant gene silencing in these cells without affecting cell viability (Figure 6B). We selected the lamin gene as the target for siRNA-mediated gene silencing. Lamin siRNA was used to assess the correlation of fluorescence with transfection efficiency and target gene silencing. Moreover, lamin A/C is abundantly expressed in most human, mouse, and rat cells, and knockdown of lamin mRNA does not affect cell viability. The gene-silencing efficacy of the BSV/siRNA polyion complexes was similar to that of a commercially available high-efficiency siRNA transfection reagent (Dharmafect 4; TR).

After injecting BSV/siRNA complexes into mice, effective gene silencing was observed in the liver and lungs (Figure 6C), indicating that these complexes were stable in vivo and retained their transfection efficiency after

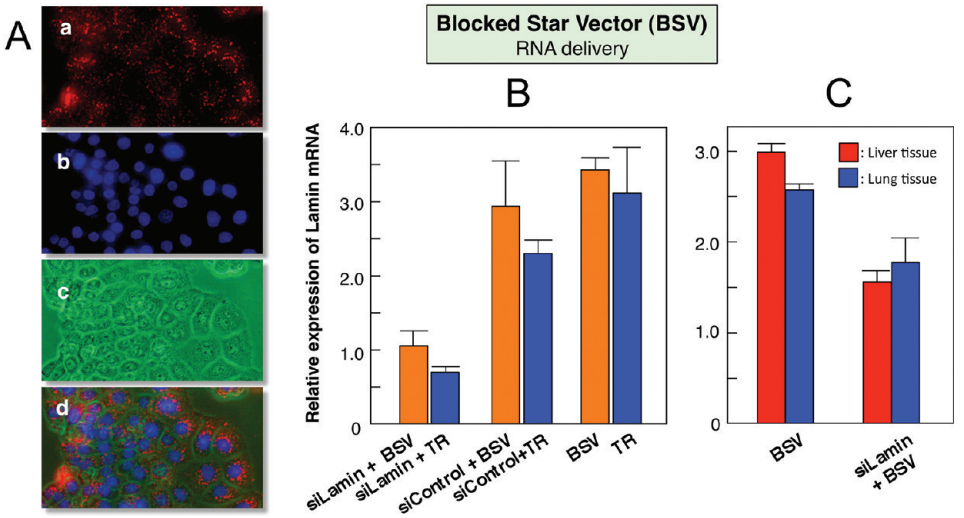



**FIGURE 6.** (A) Fluorescence microscopic image of Cy3-labeled siLamin (red color, siGL2 Lamin A/C siRNA, sense: 5′-GGUGGUGACGACGAUCUGGGCUdTdT-3′; antisense: 5′-AGCCCAGAUCGUCACCACCdTdT-3′) in human hepatocellular carcinoma cells delivered by polyplexes from the siRNA and 4-branched BSV with PDMAPAAm block ($M_n$: 50 000) and PDMAAm block ($M_n$: 33 000) (a), after staining the nucleus with diaminophenyl indole (DAPI; blue) (b), phase-contrast microscopic image for the observation of cell structure (c), and all three images merged into one (d). (B) In vitro lamin mRNA silencing efficiency of the polyplexes in human hepatocellular carcinoma cells, as analyzed by quantitative RT-PCR. siControl is designed to minimize the potential for targeting any known human or mouse genes (sense, 5′-UAGCGACUAAACACAUCAAUU-3′; antisense, 5′-UUGAUGUGUUUAGUCGCUAUU-3′). (C) In vivo lamin mRNA silencing efficiency of the polyplexes in mice liver or lung tissues, as analyzed by quantitative RT-PCR. The polyplexes were transfected by tail vein injection.

intravenous administration. Thus, BSVs can serve as carriers for siRNA and shRNA delivery both in vitro and in vivo and may thus offer a new approach to gene therapy.

**Deposition Transfection by BSV.** A reverse transfection method was developed as another practical approach for gene delivery.[43] This method is performed by culturing cells on a plasmid DNA-loaded substrate. DNA-loaded substrates are generally prepared by mixing DNA with cationic polymers that have been physically adsorbed or chemically bonded to the substrate. The cells were in direct contact with the DNA-loaded surface during the culture period; this differs from conventional transfection culture in which the contact time is limited. Furthermore, transfectional microarrays that permit parallel transfer of multiple genes into cultured cells were developed for high-throughput reverse genetics research because the reverse transfection method can be performed with spatial and temporal control.[44] However, it cannot be said that the transfection efficiency of this method is as good as that of the conventional transfection method. Therefore, an additional physical stimulus, such as an electric pulse, was used to enhance the transfection efficiency.

We designed a unique gene-adsorbent material possessing thermoresponsive properties in order to improve the reverse transfection method.[45] The SVs had 4-branched PDMAPAAm ($M_n$: 3000, PDI: 1.3) for binding to a plasmid DNA to form polyplexes and were blocked with poly(*N*-isopropylacrylamide) (PNIPAM) chains ($M_n$: 6000, PDI: 1.4) for surface deposition on a hydrophobic substrate (Figure 7A). PNIPAM is one of the most well-known thermoresponsive polymers.[46] Therefore, the SV was precipitated at approximately 35 °C owing to hydrophilic to hydrophobic conversion of the thermoresponsive polymer chains (Figure 7C). In ordinary reverse transfection, it is necessary to coat DNA on the culture substrate with cationic matrix materials to ensure that the DNA is firmly impregnated in the matrix that is adhered or bonded to the substrate. In contrast, in our method, the DNA was precipitated onto a culture substrate using a thermoresponsive polymeric adsorbent material immediately before the cells were seeded (Figure 7B). Further, in contrast to the conventional transfection and reverse transfection methods, our method allowed transfection experiments to be performed without the need for preculturing cells or precoating genes, respectively, at least 1 day in advance. Furthermore, serum-containing culture medium could be used.

When COS-1 cells were cultured on the polyplex-coated substrate in a culture medium, the luciferase activity was higher than that observed when (1) a DNA-coated substrate was used with or without SVs, before and after complete adhesion, or (2) a conventional transfection solution containing the polyplexes was used. With the proposed method, the luciferase activity was enhanced with an increase in the charge ratio and with

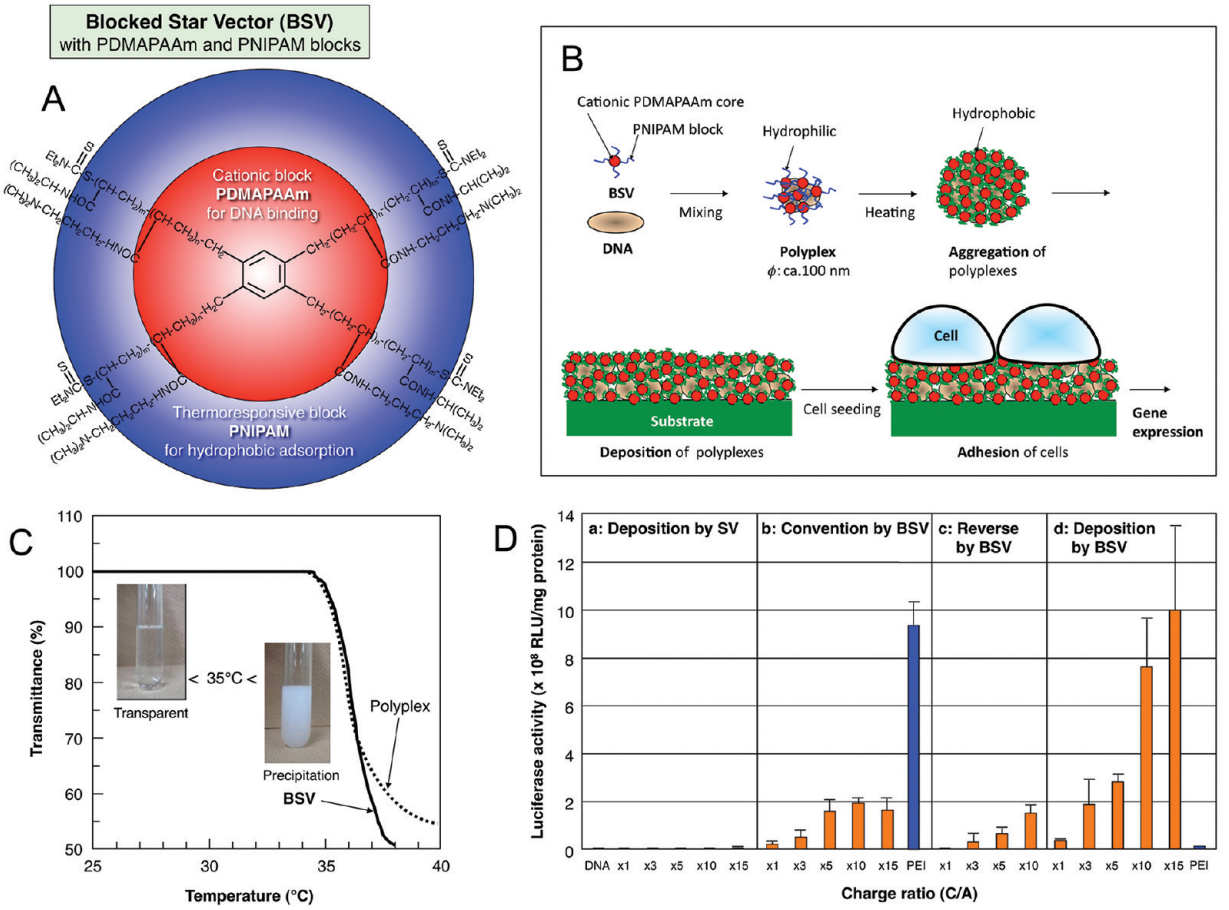



**FIGURE 7.** (A) Chemical structure of the 4-branched cationic thermoresponsive BSVs with PDMAPAAm ($M_n$: 3000, PDI: 1.4) and PNIPAM ($M_n$: 6000, PDI: 1.4) blocks as an adsorbent material for the deposition transfection method. (B) Illustration of the deposition transfection method. (C) Thermoresponsive changes in the transmittance of the BSV and its polyplex with DNA (pGL3-control). BSV concentration: 10 mg·mL$^{-1}$, DNA concentration: 5 mg·mL$^{-1}$. Heating rate: 0.5 °C·min$^{-1}$. (D) Transfection activity using the 4-branched SV of PDMAPAAm ($M_n$: 3000) (a) and the BSV in (b) the conventional solution method, (b) the reverse transfection method, and (d) the deposition method under different CA ratio.

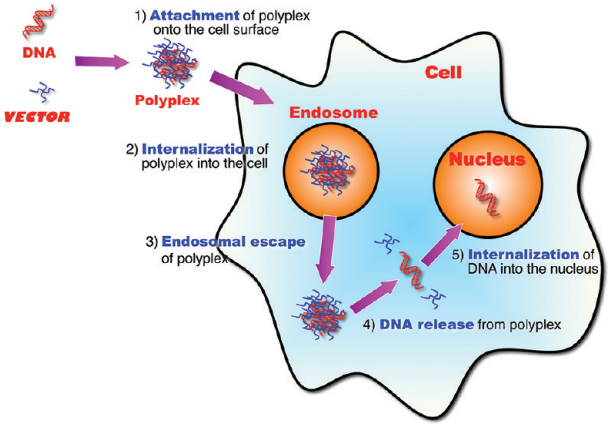



**FIGURE 8.** Estimated DNA delivery mechanism by cationic polymers including SV.

permissible levels of cellular cytotoxicity (Figure 7D). This novel transfection method, which is a modified reverse transfection method, is termed the deposition transfection method.

Interestingly, thermoresponsive SVs can bind to other anionic compounds.[47] When heparin was mixed with the thermoresponsive SVs, the resultant hydrophobic aggregates could be stably adsorbed onto the surfaces of several polymeric materials for antithrombogenic coating. Recently, by taking advantage of the cationic and thermoresponsive characteristics of PDMAEMA, a novel heparin bioconjugate with SVs was developed for highly effective heparin surface immobilization, based on a strategy similar to that described above.[48] In addition, PDMAEMA-based SVs could be applied as a novel agent for deposition transfection, where high and sustaine transgene expression was achieved by using small amounts of PDMAEMA.[49]

## Conclusion

Four approaches for effective DNA or siRNA delivery based on optimizing the macromolecular design by using the

photochemistry of iniferters were described. All strategies improved the transfection activity. However, it was very difficult to clearly explain the relationship between transfection activity and physicochemical properties, such as amphiphilicity or hydrophobicity, particle size, surface potential, and condensate density of the polyplexes. DNA expression through the polyplexes probably occurs via five sequential steps as shown in Figure 8: (1) attachment of the polyplex onto the cell surface, (2) internalization of the polyplex into the cell, (3) endosomal escape of the polyplex, (4) DNA release from the polyplex, and (5) internalization of DNA into the nucleus. Virus are naturally equipped with unique mechanisms which they overcome obstacles at each step. Therefore, virus vectors such as retroviruses, lentiviruses, and adenoviruses have proven to be efficient means for gene delivery; however, the possibility of negative outcomes resulting from viral transformations cannot be completely ruled out. On the other hand, cationic polymers used for nonviral vectors have little physiological properties for overcoming the above-mentioned obstacles. Therefore, DNA transfection is induced by the progression of the obstacles at the sequential steps depending the physiochemical properties of the polymers or the polyplexes. However, despite our improved understanding of nonviral gene delivery systems, many questions related the underlying mechanisms remain unanswered. Any single physicochemical property of the system can have a positive or negative influence at each step. Therefore, an optimum balance in the properties involved is very important. Once a system for evaluating each stage of the transfection process has been established, the combination of properties required will become apparent, and this in turn will lead to the development of an ideal polymeric vector. At present, we believe that at least the following macromolecular properties are essential for structural designing useful polymeric vectors: (1) high molecular weight, (2) high degree of branching or complexity, and (3) narrow polydispersity.

*The author thanks Dr. Yasushi Nemoto of the Chemical Products Development Department, Bridgestone Company; Dr. Taisuke Mori of the Division of Molecular Pathology, National Cancer Center Research Institute; Dr. Yukihito Ishizaka of the Research Institute, National Center for Global Health and Medicine; and all members of the Division of Medical Engineering and Materials, National Cerebral and Cardiovascular Center Research Institute.*

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**Yasuhide Nakayama** received his Ph.D. degree from Osaka University in 1991. He has been a group leader of the Division of Medical Engineering and Materials at the National Cerebral and Cardiovascular Center Research Institute and a Visiting Professor at the Graduate School of Chemical Science and Engineering, Hokkaido University, from 2005, and at the Graduate School of Engineering Science, Osaka University, from 2007. He has written over 150 articles and has over 150 issued or pending patents. He pioneered the development of "star vectors" for gene therapy, "microporous covered stents" for endovascular therapy, and "biovalves" or "biotubes" for regenerative medicine.

## FOOTNOTES

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**Note Added after ASAP Publication.** This paper was published on the Web on February 21, 2012, with minor errors in reference 49. The corrected version was reposted on February 24, 2012.