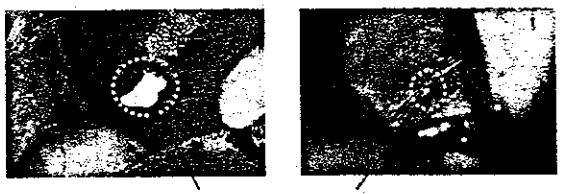

Fig. 4. Hemostases of bleeding from canine liver (left) and rat aorta (right) covered with PNIPAM–gelatin solution (20 w/v%). Spontaneous hydrogel formation and hemostases were observed. No re-bleeding was observed during 1-h after coating. Pulsatile-induced periodic dilation/contraction was observed on the dissected rat aorta.

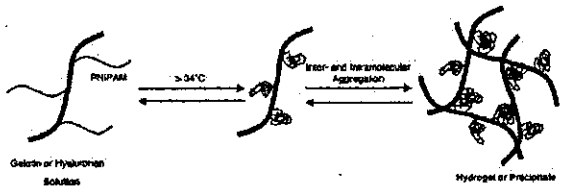

Fig. 5. Thermoresponsive gelation mechanism of PNIPAM–HA and PNIPAM–gelatin. Due to the dehydration of the hydrated amide group above lower critical solution temperature (LCST), PNIPAM graft chains were precipitated to form multimolecular aggregates. At high concentrations of PNIPAM-grafted biomacromolecules, the entire solution gelled to produce an opaque hydrogel and at low concentrations, a white precipitate was obtained.

completely cover complex-shaped tissue surfaces, (2) rapid formation of a swollen gel-like film or a precipitate when applied to injured tissues, (3) non-cell adhesiveness for tissue adhesion prevention and cell adhesiveness for hemostatic aids, and (4) appropriate biodegradability. That is, a wound-healing material should be biodegraded and sorpted with normal healing. Although various approaches and attempts have been carried out to meet the requirements listed above, in situ-applicable liquid-type materials, which include cyanoacrylate and fibrin glue, are limited (the major drawbacks associated with these hemostatic aids are described in Section 1) [7–9]. In this article, we applied thermoresponsive biomacromolecules (PNIPAM–HA and PNIPAM–gelatin) as wound-healing materials to meet the target requirements as listed above.

On a PNIPAM–HA film, cast from its aqueous solution at room temperature, the adhesion and spreading of fibroblasts were markedly inhibited (Fig. 1), which is due to the very highly swollen gel-like structure of HA (Fig. 5). When such a solution was applied to a rat cecum tissue, a slightly opaque precipitate was spontaneously formed. One week after application, markedly reduced adhesion of the PNIPAM–HA-treated cecum to adjacent tissues was observed while the non-treated cecum adhered to adjacent tissues, producing collagenous tissues (Fig. 3 and Table 1). Thus, in situ swollen precipitate of PNIPAM–HA effectively functioned in preventing tissue adhesion between the cecum and adjacent tissues.

As for thermoresponsive hemostatic aids, our previous study showed that PNIPAM–gelatin served as an artificial extracellular matrix material: the aqueous solution of PNIPAM–gelatin was immediately converted to a hydrogel, in which cells can adhere and proliferate. When PNIPAM–gelatin-containing PBS was coated on the bleeding sites of the canine liver and rat aorta, the solution was immediately converted to an opaque elastic hydrogel (Fig. 4). The hydrogel fully covered and weakly adhered to the bleeding sites, resulting in complete hemostasis on both tissues. The PNIPAM–gelatin-coated aorta pulsated well, indicating that the PNIPAM–gelatin formed quite elastic hydrogel and the hydrogel did not interfere with the periodic pulsation of a high-pressure circulatory system.

5. Conclusion

Although this article describes very limited experiments on tissue adhesion control using PNIPAM–HA and PNIPAM–gelatin, the bioconjugation of thermoresponsive synthetic materials and extracellular-matrix derived biomacromolecules, which thermally form precipitate or hydrogel, can provide a new prototype of wound-healing materials and promising procedures. Further studies to improve the properties required for both tissue adhesion and tissue adhesion prevention and to examine a longer term performances are needed. Such studies are ongoing in our laboratory.

Acknowledgements

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High Performance Gene Delivery Polymeric Vector: Nano-Structured Cationic Star Polymers (Star Vectors)

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Abstract: Nano-structured hyperbranched cationic star polymers, called star vectors, were molecularly designed for a novel gene delivery non-viral vector. The linear and 3, 4 or 6 branched water-soluble cationic polymers, which had same molecular weight of ca. 18,000, were synthesized by iniferter (initiator-transfer agent-terminator)-based photo-living-radical polymerization of 3-(*N,N*-dimethylamino)propyl acrylamide, initiated from respective multi-dithiocarbamate-derivatized benzenes as an iniferter. All polymers produced polyion complexes 'polyplexes' by mixing with pDNA (pGL3-control plasmid), in which the particle size was ca. 250 nm in diameter [the charge ratio < 2/1 (vevtor/pDNA)] and ca. 150 nm (the charge ratio > 2.5/1), and the ζ-potential was ca. +10 mV (the charge ratio > 1/1). When COS-1 cells were incubated with the polyplexes 12h after preparation under the charge ratio of 5/1, higher gene expression was obtained as an increase in branching, with a little cytotoxicity. The relative gene expression to the linear polymer was about 2, 5, and 10 times in 3-, 4-, and 6-branched polymers, respectively. The precise change in branching of polymers enabled the control of the gene transfer activity.

Keywords: Non-viral vector, star polymer, polyplex, branched polymer, gene transfection, molecular design.

INTRODUCTION

The cationic polymers, which can generate nano-particles by formation of polyion complexes 'polyplexes' with DNA irrespective of its size and kind, are highly expected as one of the major materials for non-viral vectors [1-4]. However, the primary obstacle toward implementing an effective gene therapy using the cationic polymers remains their relatively inefficient gene transfection *in vivo* than virus vectors.

To achieve an enhancement of gene transfection using cationic polymers, numerous studies have been performed by various approaches; e.g., the chemical synthetic engineering approach in which the kind and composition of the polymers are modified [5,6], biochemical approach in which targeting ligands such as galactose, mannose, transferring, or antibodies into the polymers [7-11], functional molecular engineering approach in which stimulus-response polymers with light and thermal reactivity are designed as high performance vectors [12-14], and physical engineering approach in which physical stimulation with electroporation, gene gun, ultrasound and hydrodynamic pressure are provided at the transfection [2,15,16]. However, few studies in the molecular structure of cationic polymers, which are usually synthesized by conventional radical polymerization, has been reported, except for the effects of changes in the polymer chain length and composition of polymers [17-20] and complex multi-branching polymers, of which structural analysis is impossible [21-24]. Since precise molecular design, including the molecular weight and three-dimensional structure, by conventional radical polymerization was quite difficult in general, the systematic structure-dependency of cationic polymers in gene transfection has not been established.

In this study, for examination of the effects of the molecular structure on gene expression we designed novel cationic polymers with star-shaped and symmetric structure, which is determined by 2-parameters, the degree of branching and chain length. Molecular design was performed by the iniferter (acts as *ini*tiator-trans*fer* agent-*ter*minator)-based photo-living-radical polymerization method pioneered by Otsu *et al.* [25-30]. An iniferter, benzyl *N,N*-diethyldithiocarbamate (DC) is dissociated into a benzyl radical and a dithiocarbamyl radical by ultraviolet light (UV) irradiation. The reaction involving an *N,N*-diethyldithiocarbamyl radical favors chain termination with a growing polymer chain radical end rather than a reaction with a vinyl monomer, whereas a benzyl radical reacts with a vinyl monomer to produce a polymer. These reactions proceed only during irradiation. Therefore, the chain length of the growing polymer is controlled by irradiation condition such as irradiation time or light intensity and the composition of the solution. We previously used the living radical polymerization for designing of various surface graft architectures [31-34] controlling the chain length, block graft chain, gradient chain length and regionally graft polymerized pattern surface. As the first step of the study, star polymers of the same molecular weight at a precise degree of branching of 0, 3, 4, and 6 were synthesized. The effects of the degree of branching on gene expression by measuring the luciferase activity were examined.

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MATERIALS AND METHODS

Materials

Benzyl chloride, 2,4,6-tris(bromomethyl)mesitylene, 1,2,4,5-tetrakis(bromomethyl)benzene, and hexakis(bromomethyl)benzene were obtained from Sigma-Aldrich (Milwaukee, WI). Sodium *N,N*-diethyldithiocarbamate and *N,N*-dimethyaminopropyl acrylamide were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Solvents and other reagents, all of which were of special reagent grade, were obtained from Wako and used after conventional purification. Plasmid DNA (pGL3-control), which contains the firefly luciferase gene, was obtained from Promega Inc., (Tokyo, Japan). ExGen 500 [poly(ethylene imine)] was obtained from Euromedex Inc., (Cedex, France).

Synthesis of Cationic Star Polymers

Cationic polymers including linear and three types of star polymers with 3, 4, or 6 branches per molecule were prepared by iniferter-based photo-living-radical polymerization of 3-(*N,N*-dimethylamino)propyl acrylamide as a monomer from respective inifeters such as benzyl *N,N*-diethyldithiocarbamate, 2,4,6-tris (*N,N*-diethyldithiocarbamylmethyl)mesitylene, 1,2,4,5- tetrakis(*N,N*-diethyldithiocarbamylmethyl)benzene, and hexakis (*N,N*-diethyldithiocarbamylmethyl)benzene, which were obtained by *N,N*-diethyldithiocarbamylation from respective benzyl halogenate derivatives such as benzyl chloride, 2,4,6-tris (bromomethyl)mesitylene, 1,2,4,5-tetrakis(bromomethyl) benzene, and hexakis(bromomethyl)benzene.

The general preparation method of iniferter is followed. An ethanol solution (10 ml) of chloromethyl benzene (4.8 g, 38 mmol) was added to an ethanol solution (50 ml) of sodium *N,N*-diethyldithiocarbamate (10.3 g, 46 mmol) at 0°C. After the mixture was stirred at room temperature for 24 h, the resulting sodium chloride was separated by filtration. The filtrate was concentrated under reduced pressure. The residue was added into 150 ml of water and extracted with ether (200 ml x 2) and washed successively with deionized water (100 ml x 3), followed the separation of the organic layer, drying over $MgSO_4$, condensation to give benzyl *N,N*-diethyldithiocarbamate: yield, 17.6g (93%); ^{1}H NMR (DMSO-d_6 with Me_4Si) δ 7.34 (m, 5H, C_6H_5), 4.54 (s, 2H, CH_2-S), 4.05 (q, 2H, N-CH_2), 3.73 (q, 2H, N-CH_2), 1.28 (m, 6H, CH_2CH_3).

The general procedure of iniferter-induced photo-living-radical polymerization is followed. A methanol solution (20 ml) of benzyl *N,N*-diethyldithiocarbamate (24 mg, 0.1 mmol) and 3-(*N,N*-dimethylamino)propyl acrylamide (3.9 g, 25 mmol) was placed into 50 ml quartz crystal tube. A stream of dry nitrogen was introduced through a gas inlet to sweep the tube for 5 min or more. The solution was then irradiated for 30 min with a 200 W Hg lamp (SPOT CURE, USHIO, Tokyo, Japan) in nitrogen atmosphere at 20~25 °C. Light intensity was set to 1 mW/cm^2 at the wavelength of 250 nm (UVR-1, TOPCON, Tokyo, Japan). The reaction mixture was concentrated under reduced pressure. The residue was dissolved in a small amount of methanol. The precipitate, obtained by the addition of a large amount of ether, was separated by filtration. Reprecipitation was performed in the methanol-ether system. The last precipitate was dried in a vacuum to yield poly[3-(*N,N*-dimethylamino)propyl acrylamide] as a white powder. The molecular weight, determined by GPC analysis, was 18,000 g mol^{-1}. ^{1}H NMR (DMSO-d_6 with Me_4Si) δ 7.60 (br, 1H, N-*H*), 3.22 (br, 2H, NH-CH_2), 2.30 ((br, 2H, $N(CH_3)_2$-CH_2), 2.15 (br, 6H, N-CH_3), 1.65 (br, 2H, CH_2-CH_2).

General Methods

GPC analysis was carried out on a RI-8012 (TSK_{gel} α-3000 and α-5000; Tosoh, Tokyo, Japan) after calibration with standard polyethylene glycol samples. The eluent was *N,N*-dimethylformamide. ^{1}H-NMR spectra were obtained on a Valian Gemini-300 (300 MHz) spectrometer (Tokyo, Japan). All ^{1}H-NMR spectra were recorded in DMSO-d_6 solutions using tetramethylsilane as the internal standard. Dynamic light scattering (DLS) measurements were carried out using a DLS-8000 instrument (Otsuka Electrinics, Tokyo, Japan). An Ar ion laser (λ_0 = 488 nm) was used as the incident beam. The sample was prepared by direct mixing of pDNA solution and the polymer in Tris-HCl buffer (pH 7.4). The DNA concentration of the mixture was then adjusted to 23 μg cm^{-3}.

Cell Culture and Transfection

COS-1 cells (ca. 3 x 10^4 cells per well) were seeded prior to treatment in 24-well plates and grown for 24 h in DMEM (Gibco, Invitrogen Corp., Carlsbad, CA) containing 10% fetal calf serum (Hyclone Laboratories Inc., Logan, UT), penicillin (200 units/ml, ICN Biomedicals Inc., Aurora, OH), and streptomycin (200 mg/ml, ICN) in an atmosphere of 5% CO_2 at 37 °C. Transfections were performed with 0.5 μg of plasmid DNA (pGL3-control) in 24-multi well dishes in 0.2 ml of OPTI-MEM I (Gibco). After 3 h of incubation, the cells were washed once with PBS, and cultured in 1 ml of DMEM containing 10% fetal calf serum for an additional 48 h. The medium was removed and the cells were washed twice with PBS. The cells were lysed with 0.2 ml of cell lysis buffer (Promega, Madison, WI) and mixed by vortexing. The lysate was centrifuged at 15,000 rpm for 1 min at 4 °C and 5 μl of the supernatant was analyzed for luciferase activity using a Luminous CT-9000D (Dia-Iatron, Tokyo, Japan) luminometer. The relative light unit/s (RLU) were converted into the amount of luciferase (pg) using a luciferase standard curve, which was obtained by diluting recombinant luciferase (Promega) in lysis buffer. The protein concentrations of cells lysates were measured by Bio-Rad protein assay (BIO-RAD, Hercules, CA) using bovine serum albumin as a standard. The expressed luciferase represented the amount (mole quantity), which is standardized for total protein content of cell lysate. The data are presented as means±S.D. (n=5).

Cytotoxicity

Cytotoxicity was assessed by cell viability assay using WST-8 method (Dojindo, Kumamoto, Japan). COS-1 cells were seeded 24 h prior to treatment in 96-well plates at 5,000 cells per well. Cells were treated with the same conditions used for luciferase assays, with a volume of 6.2 μl of the transfection mixture including 0.124 μg of pDNA added to

each well. Cells were treated with the appropriate conditions for 3 h, washed once with PBS, and cultures in 50 μl of DMEM (Gibco) containing 10% fetal calf serum for an additional 24 h. Each well was added with 10 μl of WST-8 reagent (5 mmol/l). After 2 h of incubation at 37 °C, absorbance at 450 nm was read in a BIO-RAD microplate reader (Model 680). The data are presented as means±S.D. (n=5).

RESULTS AND DISCUSSION

Preparation of Cationic Star Polymers

Four kinds of cationic polymers, consisting of one linear polymer and three star polymers precisely controlled the degree of branching to 3, 4, and 6, were molecularly designed (Fig. 1). The polymers were synthesized by the iniferter living radical polymerization using respective initiators, multi-dithiocarbamate-derivatized benzenes, which were prepared corresponding to the degree of branching. As the monomer, a cationic vinyl monomer with tertiary amino residues, 3-(*N,N*-dimethylamino)propyl acrylamide was used. Since polymerization could proceed only during irradiation, the chain length of the polymers could be easily controlled by the irradiation condition and the composition of the solution. One linear and three kinds of star polymers with a molecular weight of about 18,000 with low polydispersity of about 1.5, irrespective of the degree of branching, were obtained. Therefore, the chain length in the polymers was set to about 6,000 with a degree of branching of 3, about 4,500 with a degree of branching of 4, and about 3,000 with a degree of branching of 6.

Polyplex Formation

When aqueous solutions of all obtained branched cationic polymers with same molecular weight were mixed with a Tris-HCl buffered saline of pDNA, marked high scattering intensity in quasi-elastic (dynamic) light scattering (DLS) measurements was immediately observed regardless of the degree of branching, indicating polyplexes formation from all cationic polymers. It was considered that the polyplexes formed by electrostatic interactions are same as other cationic polymeric vectors. The particle sizes of the polyplexes were measured using DLS. The DLS measurements showed that the cumulant diameter of the polyplexes was about 250 nm at a charge ratio less than 2/1 (vector/pDNA) and decreased to about 150 nm at a charge ratio more than 2.5/1(vector/pDNA). However, the particle

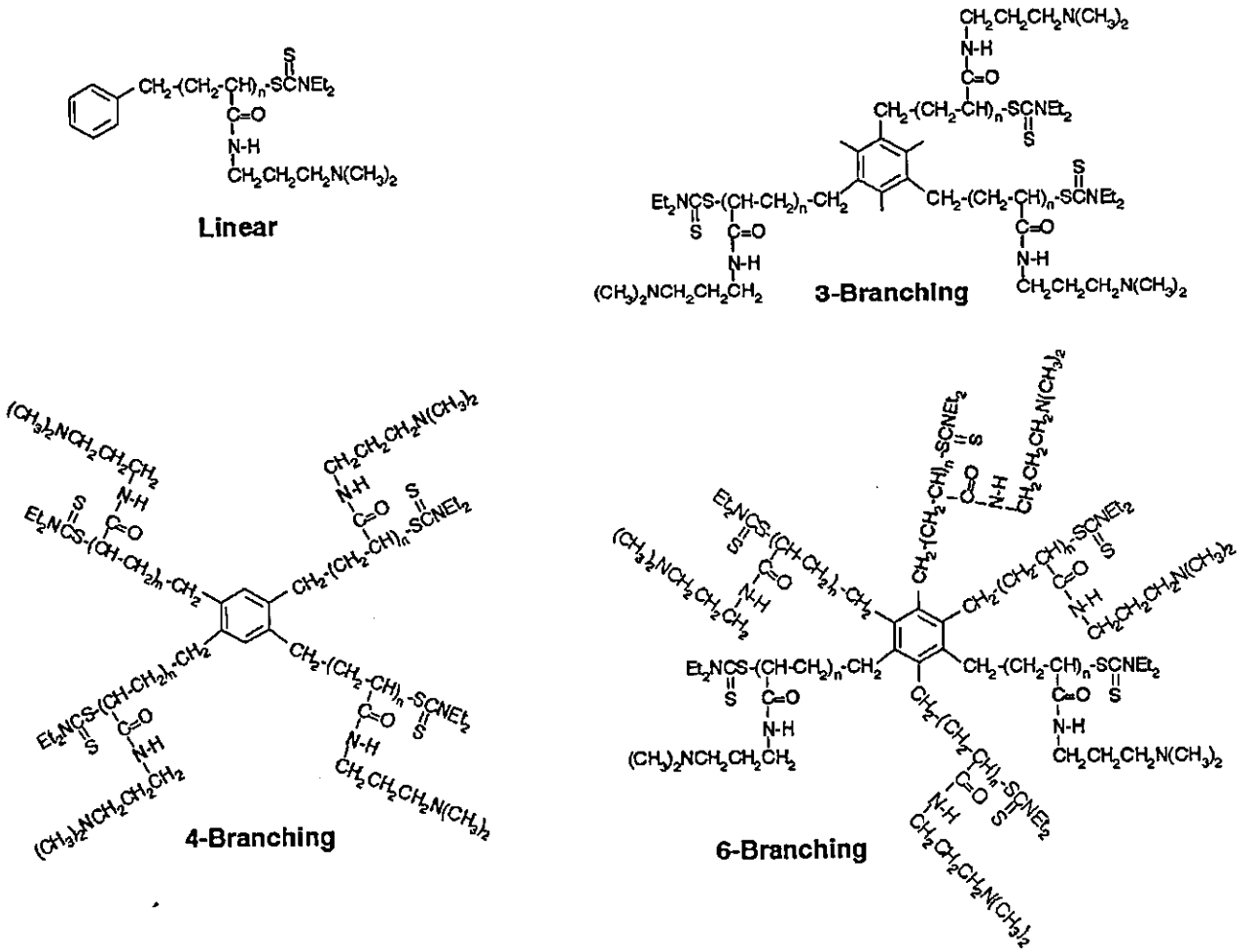

Fig. (1). Structural formulas of the star polymers, which were synthesized by iniferter-induced photo living radical polymerization of 3-(*N,N*-dimethylamino)propyl acrylamide from the respective multi-iniferters, *N,N*-diethyldithiocarbamate-derivatized benzenes.

sizes of the polyplexes were not significantly affected by the branching. In addition, ζ-potentials of pDNA polyplexes with the cationic polymers were measured to examine their electric property. The ζ-potential of the pDNA polyplexes was about +10 mV at a charge ratio more than 1/1(vector/pDNA). The difference in ζ-potential value between the polymers was little in each branching. Therefore, it can be said that there is little difference in physiochemical properties of the polyplexes produced from cationic polymers with different branching.

Cytotoxicity

Cytotoxicity of the pDNA polyplexes with the 6-branching polymer to COS-1 cells was studied by the cell survival rate using the WST-8 method. As shown in (Fig. 2) the cytotoxicity of the polyplexes was negligible up to a charge ratio of 5/1 (vector/pDNA). At charge ratios more than 5/1, the cytotoxicity was gradually reduced, and it was about 60% at a charge ratio of 20/1 (vector/pDNA).

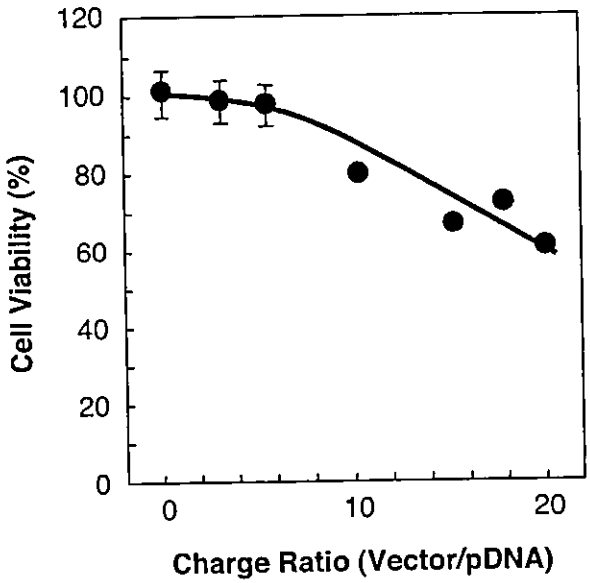

Fig. (2). Cytotoxicity of the polyplexes obtained immediately after mixing of DNA (pGL3-control) and 6-branching star polymer under the changing of a charge ratio (vector/pDNA), which was determined by cell viability assay of COS-1 cells using a WST-8 method. The data are presented as means±S.D. (n=5).

Gene Expression and Cell Viability

Gene transfer activity of the cationic polymers with same molecular weight of about 18,000 was examined and compared with that of ExGen 500 [35,36], which was one of major commercially available typical cationic polymeric vectors as a positive control. Figure 3 shows gene transfer activity of the cationic polymers at the charge ratio of 5/1 (vector/pDNA) in COS-1 cells. When pDNA alone was transfected, little luciferase activity was observed (data not shown). On the other hand, the luciferase was produced in all pDNA polyplexes. The enhancement of gene transfer activity in the use of the polyplexes may be due to acceleration of cellular uptake of pDNA polyplexes by

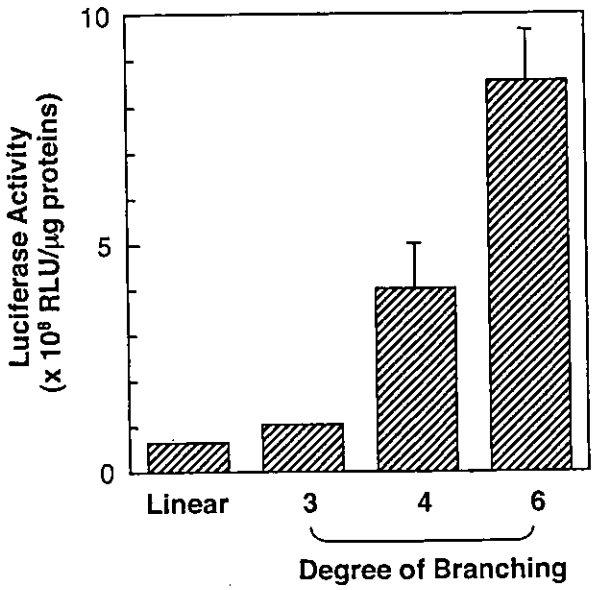

Fig. (3). Effect of branching of the star polymers on the level of luciferase gene transfer activity in COS-1 cells. COS-1 cells were treated with the polyplexes prepared by mixing of the star polymers and DNA (pGL3-control) under a charge ratio of 5/1 (vector/pDNA) 12 h after those preparation. The expression level was increased with increases in the degree of branching. The data are presented as means±S.D. (n=5).

endocytosis and endosomal release of the polyplexes by the proton sponge effect [37,38] in endosomes, similar to the other cationic polymers. The gene transfer activity of the pDNA polyplexes with the non-branched, linear cationic polymer was lowest, which was comparable with that of ExGen 500. However, the activity was increased by stage, corresponding to the degree of branching. The relative transfer activity to the linear polymer was about 2, 5 and 10 times in 3-, 4- and 6-branched polymers, respectively. As an increase in the degree of branching the transfer activity was almost exponentially increased. It can be said that the highly branched polymer called star vectors is useful for a gene delivery vector.

Cationic polymer-mediated transfection should overcome three major barriers for transfection, which includes binding of pDNA polyplexes to cell surface, endosormal release, and entry of pDNA into the nucleus. These barriers are strongly depended on the physicochemical properties of polyplexes such as net charge and particle size. Therefore, such properties markedly determine transfection efficiency. However, in the present study, transfection efficiency was strongly affected with the branching degree regardless of almost same physcochemical properties in pDNA polyplexes formed from the all branched polymers. The branching degree-dependent transfer activity changing may be estimated below. As an increase in the degree of branching the density of cationic charges in the branched polymers is increased. Higher charge density may affect the formation of higher compaction of pDNA polyplexes. The condensed pDNA polyplexes thus obtained may be stable in endosomes and also in aqueous media, which may prevent degradation

and aggregation of the polyplexes, respectively. Therefore, higher branching resulted in higher gene transfer activity.

The other star polymers as a gene delivery vector are easily designed by iniferter-based photo-living-radical polymerization. The composition of polymer chains can be determined by the kind of monomers, and the molecular weight by the irradiation time. Therefore, in addition to allowing design of the basic skeletal structure, the composition and length of polymer chains can be optimized as schematically shown in (Fig. 4). Changing the kind of monomers can control the composition of the polymer chains continuously or stepwise. To further increase the degree of branching, we will design the core molecules from benzene ring to naphthalene ring or combinations of benzene rings as multi-iniferters. Furthermore, formation of hyper branching structure by diverging of branching chains will be possible [34]. In the near future, the correlation between the three-dimensional structure in a star vector and the efficiency of gene expression will be clarified in detail.

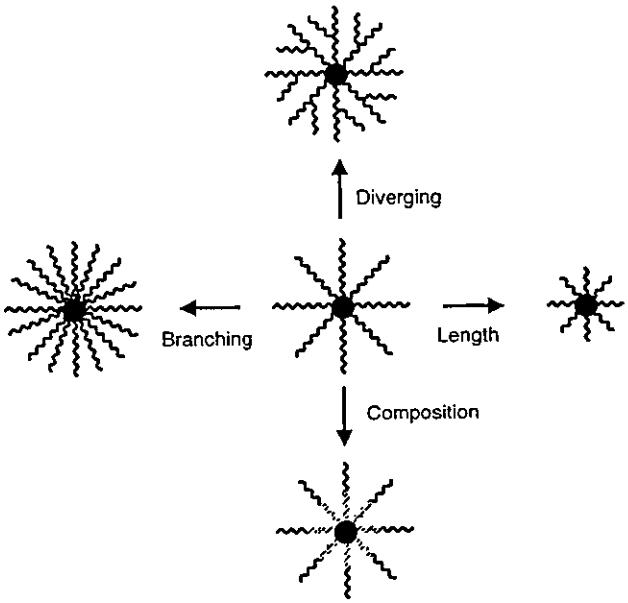

Fig. (4). Possibility in molecular design of various star polymers having different branching, diverging, chain length, or composition, which are based on iniferter-induced living radical polymerization.

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Photo-Control of the Polyplexes Formation between DNA and Photo-Cation Generatable Water-Soluble Polymers

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Abstract: Photo-cation generatable water-soluble polymers (Mw: approximately 1 x 10^5) were prepared by radical copolymerization of *N,N*-dimethylacrylamide and vinyl monomer of triphenylmethane leucohydroxide (malachite green), which generate a cation upon ultraviolet light (UV) irradiation at wavelengths of between 290 and 410 nm. The malachite green contents of the copolymers were 3.6 (0.4 mol %), 7.9 (0.7 mol %), and 15.0 (2.7 mol %) per molecule. When an aqueous solution of the photo-cationized copolymers generated by UV irradiation was mixed with a Tris-HCl buffer (pH 7.4) of DNA (pGL3-control plasmid), dynamic light scattering (DLS) measurements showed the formation of polyplexes between the cationic copolymers and anionic DNA by non-specific electrostatic interaction, which was visualized with a confocal laser scanning microscopy (CLMS). Their mean cumulant diameter was about 150 nm with low polydispersity irrespective of the malachite green content in the copolymers. In the copolymer with the lowest malachite green content, almost all of the amount of the polyplexes was maintained by repeated UV irradiation, but the amount gradually decreased in the dark at 37 °C due to dissociation of the polyplexes, synchronized with the neutralization of the cation form of malachite green. The photo-cation generatable copolymers designed here can undergo photo-induced formation of the polyplexes with DNA and thermal polyplex dissociation, which may be used as a model for a novel photo-induced gene delivery system into cells.

Keywords: Photo-induced polyplex formation, Photo-control, Photo-cation generatable polymer, Malachite green, Polyplex dissociation.

1. INTRODUCTION

Viral vectors with high gene transfection efficiency both *in vitro* and *in vivo*, such as adenovirus and retrovirus, have been widely used for gene therapy [1-8] since its first use for the treatment of adenosine deaminase (ADA) deficiency in 1990 [8]. However, since a fatal accident with an adenovirus vector in the USA in 1999, high safety standards have been required in the use of such vectors. Therefore, in recent years the development of non-viral vectors, which can be expected to have high biological safety, has been further accelerated. One major approach in the development of non-viral vectors is based on synthetic cationic polymers [9-20], including poly(ethylene imine) [13,14], poly-L-lysine [15,16], poly (amidoamine) dendrimer [17,18], and poly[(2-dimethylamino)ethyl methacrylate] [19,20], which can spontaneously form 'polyplexes' as a result of electrostatic interactions between the positively charged groups of the cationic polymers and the negatively charged phosphate groups of DNA. The cationic polymer-induced gene delivery systems have several advantages in addition to 1) high biological safety, and these are: 2) low costs, 3) ease of preparation and manipulation, and 4) no limitation of the size of transduced genes. However, the primary obstacle toward implementing a clinically available gene therapy using cationic polymers as vectors remains their extremely low efficiency in gene transfection *in vivo* compared with virus vectors.

It is strongly indicated that polyplexes are usually taken up by cells in endosormal compartments via endocytosis induced by non-specific electrostatic interactions between positively charged groups on the polyplex surface and negatively charged residues on the cell surface. When the polyplexes are released from the highly acidic endosormal compartment, and if the polyplexes can be easily dissociated, DNA released from the polyplexes will be more efficiently taken in by nuclei, resulting in higher transcription and gene expression. Therefore, the promotion of gene transfection may be improved by increasing: 1) the introduction of polyplexes into cells, 2) release of DNA from introduced polyplexes into the cytoplasm, and 3) delivery of DNA to nuclei. To enhance the introduction of polyplexes into cells, many research groups have attempted the introduction of targeting ligands such as galactose, mannose, transferrin, or antibodies into cationic polymers [19,21-25]. Such biochemical approaches have provided high gene expression. On the other hand, even using existing vectors, the promotion of gene transfection has been obtained by physical stimulation of transfection using electroporation, gene gun, ultrasound and hydrodynamic pressure [9,26,27]. These physicochemical approaches are very simple, and can be widely used.

Recently, a new gene delivery system, which can control the release of DNA, has been developed by the molecular design of polymeric vectors. For example, Yokoyama *et al.* constructed a temperature-controlled gene expression system,

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in which a thermosensitive random copolymer, based on cationized poly(*N*-isopropylacrylamide) (PNIPAM), was synthesized as a thermoreactive vector [28]. Increased cellular uptake of the polyplexes, consisting of the cationized PNIPAM and DNA, at 37 °C and its disintegration by the reduction of temperature to 20 °C were demonstrated. A photo-controlled gene transfection system was proposed by Nagasaki *et al.* [29]. A photoreactive vector was designed, consisting of cationic lipids with an o-nitrobenzyl moiety as a photocleavable spacer. The DNA complex that had been taken up by the lipids was disintegrated by UV irradiation. In both systems external physical stimulation with temperature or light could promote enhancement of gene expression.

Malachite green is a well-known photochromic molecule that reversibly dissociates into ion pairs under ultraviolet light (UV) irradiation, producing intensely deep-green-colored triphenylmethyl cations and counter hydroxide ions within a few minutes (Fig. **2**) [30,31]. The generated cations are stable under acidic condition. In our previous study, we synthesized water-soluble polyacrylamide-based copolymers with malachite green side chains, which could be immediately taken up into cells by non-specific electrostatic interactions due to conversion from the nonionic copolymers to polycations by UV irradiation [32]. The photo-generated cations slowly reverted to the original leucohydroxide species on cessation of the UV irradiation even under physiological temperature within approximately 1 has shown in Fig. **2**. Therefore, if using the malachite green-derivatized polymers as a vector, it is expected that upon UV irradiation polyplexes can be formed from the photo-generated cationic polymers and DNA, and these can be dissociated by the time-dependent decrease in cation concentration in the dark (Fig. **1**). In this study, water-soluble poly(*N,N*-dimethy-

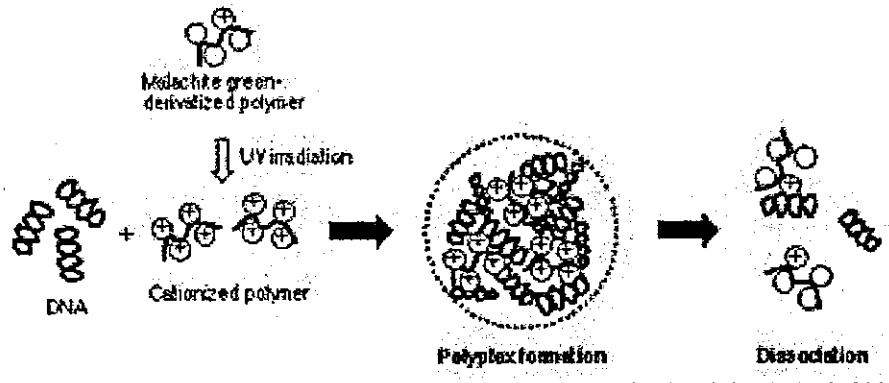

Fig. (1). Schematic illustration of the principle of photo-induced polyplex formation and its dissociation, both of which were based on the specific photochemical/thermal reactivity of the malachite green-derivatized water-soluble polymer.

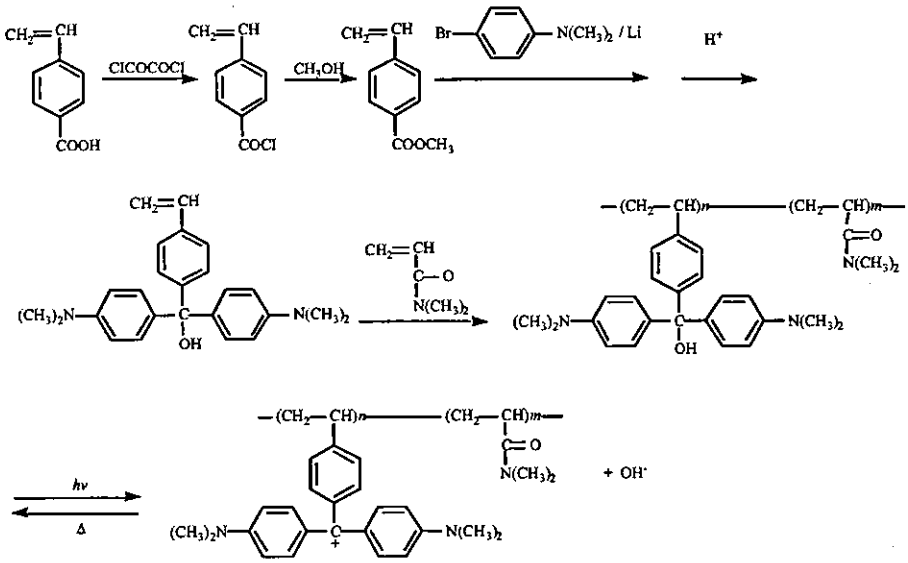

Fig. (2). Reaction scheme of the preparation of photo-cation generatable water-soluble copolymer, which is a radical copolymer of diphenyl(4-vinylphenyl)methane leucohydroxide and *N,N*-dimethylacrylamide. Chemical reaction in photoinduced dissociation of triphenylmethane leucohydroxide (malachite green) derovatized in the copolymer to triphenylmethyl cation and counter hydroxide ion and thermal recombination between them.

acrylamide)-based copolymers with malachite green groups as side chains were synthesized (Fig. 2). Their photo-induced polyplex formation with DNA and thermal dissociation reactions were evaluated by measuring the light scattering intensity using a dynamic light scattering (DLS) measurement apparatus. The possibility of using the malachite green-derivatized polymers for a vector material in a novel photo-induced gene delivery system is discussed.

2. MATERIALS AND METHODS

2.1. Materials

4-Vinylbenzoic acid and 4-bromo-*N,N*-dimethylaniline were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Oxalyl chloride, *N,N*-dimethylacrylamide, 2,2'-azobis(isobutyronitrile) (AIBN), and *n*-butyl lithium hexane solution were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other regents and solvents were obtained commercially and were purified by distillation.

2.2. Synthesis of Diphenyl(4-Vinylphenyl)methane Leuco-hydroxide [33]

Oxalyl chloride (21.4 g, 170 mmol) was added to 4-vinylbenzoic acid (5 g, 34 mmol) at less than 0 °C and stirred at room temperature for 8 h. After the oxalyl chloride was evaporated off under vacuum, dry methanol (50 mL) was added to the residue. Solvent evaporation from the reaction mixture afforded methyl 4-vinylbenzoate: 5.1 g (93%); ^{1}H NMR ($CDCl_3$): δ 7.99 (d, *J*=8.1 Hz, 2H, m-H of PhC=C), 7.46 (d, *J*=8.7 Hz, 2H, o-H of PhC=C), 6.75 (q, *J*=10.8 Hz, 1H, PhCH=C), 5.86 (d, *J*=17.4 Hz, 1H, cis-H of PhCH=CH_2), 5.38 (d, *J*=10.2 Hz, trans-H of PhCH=CH_2), 3.91 (s, 3H, $COOCH_3$).

4-Bromo-*N,N*-dimethylaniline (12.3 g, 61.7 mmol) was dissolved in anhydrous tetrahydrofuran (THF) (100 cm^3) and the solution was kept at –78 °C in a liquid nitrogen bath under an argon atmosphere. A hexane solution of butyllithium (BuLi) (48 mL, 77 mmol) was injected gradually into the THF solution with stirring. To the mixture was added dropwise a THF (70 cm^3) solution of methyl 4-vinylbenzoate (5 g, 30.8 mmol). The reaction mixture was allowed to warm slowly to room temperature and then stirred for an additional hour. After the reaction, the THF was evaporated off under vacuum and water (150 cm^3) was added to the residue. The aqueous phase was then neutralized by the addition of 0.1 mol dm^{-3} hydrochloric acid. Extraction with dichloromethane, followed by vacuum evaporation of the solvent, afforded a dark-green oily product of diphenyl(4-vinylphenyl)methane leucohydroxide **1**. Recrystallization of the crude product from methanol yielded a pale-green solid of **1** (42%). ^{1}H NMR (DMSO-d_6): δ 7.34 (d, *J* =9.0 Hz, 2H, *m*-H of PhC=C), 7.28 (d, *J* =9.0 Hz, 2H, *o*-H of PhC=C), 7.12 (d, 4H, *J* =9.0 Hz *o*-H of NPh), 6.74 (dd, *J* =10.8, 18.0 Hz, 1H, PhC*H*=C), 6.65 (d, *J* =8.1 Hz, 4H, *m*-H of NPh), 5.72 (d, *J* =16.2 Hz, 1H, *cis*-H of PhCH=CH_2), 5.21 (d, *J* =10.8 Hz, 1H, *trans*-H of PhCH=CH_2), 2.94 (s, 12H, -NCH_3).

2.3. Synthesis of Photo-cation Generatable Water-soluble Polymer [32]

The photo-cation generatable water-soluble polymers were prepared by radical copolymerization of diphenyl(4-vinylphenyl)methane leucohydroxide with *N,N*-dimethylacrylamide in benzene at 60 °C for 24 h, in a glass tube sealed after several freeze-pump-thaw cycles under vacuum. The total monomer concentration was set at 0.7 mol dm^{-3} (feed of diphenyl(4-vinylphenyl)methane leucohydroxide was changed from 0.1 to 0.5 mol % in the text), and α,α'-azobis(isobutyronitrile) (AIBN) was used as the initiator (0.5 mol % relative to the total monomer). The polymer, precipitated by addition of a large amount of ether, was separated from the solution by filtration. Reprecipitation was carried out sufficiently from the methanol solution to ether three times to exclude non-reacted monomer and initiator completely. The last precipitate was dried under a vacuum and stored in a dark desiccator. The molecular weight was determined by gel permeation chromatograph (GPC) analysis. The triphenylmethane leucohydroxide group contents were 3.6 (0.4 mol %), 7.9 (0.7 mol %), and 15.0 (2.7 mol %) per molecule, which was determined from the absorption spectrum using the absorption coefficient of malachite green carbinol base, which had a maximum absorption at a wavelength of 620 nm in an aqueous solution (ε = 6.7 x 10^4 $dm^3mol^{-1}cm^{-1}$). Polymerization yield was adjusted less than about 20 % to obtain copolymers with homogeneous composition. ^{1}H NMR ($CDCl_3$): δ 7.2-7.4 (Ph-C), 6.9-7.15 (*o*-H of NPh), 6.45-6.70 (*m*-H of NPh), 2.7-2.9 (PhNCH_3, CONCH_3), 1.1-1.6 (-CH_2-).

2.4. General Methods

Irradiation was carried out using a mercury-xenon arc lamp (L2859-01; Hamamatsu Photonics Inc., Shizuoka, Japan). The illumination wavelength (290 < λ < 410 nm) was selected with the aid of cutoff filters (UV-D33S, Toshiba, Tokyo, Japan). The light intensity, measured with a photometer (UVR-1, TOPCON, Tokyo, Japan), was fixed at 1.0 mW/cm^2. The absorption spectra were measured using a UV/visible light (VIS) spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan). GPC analysis was carried out on a RI-8012 (TSK_{gel} α-3000 and α-5000, Toso, Tokyo, Japan) after calibration with standard poly(ethylene glycol) samples. The eluent was *N,N*-dimethylformamide. ^{1}H-NMR spectra were obtained on a Varian Gemini-300 (300 MHz) spectrometer (Tokyo, Japan). ^{1}H-NMR spectra were recorded in deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6) solutions using tetramethylsilane (TMS) as the internal standard. Dynamic light scattering (DLS) measurements were carried out using a DLS-8000 instrument (Otsuka Electrinics, Tokyo, Japan). An argon (Ar) ion laser (λ_0 = 488 nm) was used as the incident beam. The sample was prepared by direct mixing of the DNA solution and polymer in Tris-HCl buffer (pH 7.4). The DNA concentration of the mixture was then adjusted to 23 μg cm^{-3}. The fluorescence image of the polyplexes was obtained using a confocal laser scanning microscope (CLSM, 543 nm, Radiance2100, Bio-Rad Lab., Hercules, CA).

3. RESULTS

3.1. Preparation and Physical Properties of Photo-cation Generatable Water-soluble Polymers

Photo-cation generatable water-soluble polymers were prepared by free radical copolymerization of *N,N*-dimethylacrylamide with the photo-dissociable monomer,

Table 1. Preparation of Photo-Cation Generatable Water-Soluble Copolymers and their Composition

Feed of MG[a] monomer (mol %)	Yield (%)	MG[a] content (mol %)	Mn^b (x10^4)	polydispersity	number of MG[a] / molecule
0.1	22	0.4	9.1	2.6	3.6
0.2	20	0.7	11.4	2.1	7.9
0.5	17	2.7	5.9	2.3	15.0

Polymerization conditions: initiator, AIBN; [monomer]/[initiator] = 200; Solvent; benzene; [monomer] = 0.7 mol/l; temp., 60°C; Polym. Time, 17 h. [a]MG means malachite green. [b] Number-average molecular mass determined by gel-permeation chromatography (PEO standard). [c] Determined by absorption spectra at 620 nm.

diphenyl(4-vinylphenyl)methane leucohydroxide according to the previously reported method [32] (Fig. 2). The content of the photodissociable group, triphenylmethane leucohydroxide, in the copolymers was determined by absorption spectroscopy using the absorption coefficient at an absorption maximum (620 nm) of a malachite green carbinol base. Table 1 summarizes the preparation conditions and compositions of the copolymers. ^{1}H NMR spectra indicated that by repetition of sufficient reprecipitation no non-reactive monomer and initiator was detected completely in the all polymers obtained. Polymer yield was adjusted under about 20 % to obtain homogeneous copolymers with narrow distribution of copolymerization ratio. The malachite green contents in the copolymers of molecular weight about 0.6-1x10^5, were 3.6 (copolymerization ratio; 0.4 mol %), 7.9 (0.7 mol %) and 15.0 (2.7 mol %) units per molecule.

Aqueous solutions of the three copolymers at pH 7.4 and 37 °C were light green before UV irradiation. Upon UV irradiation at a wavelength of between 290 and 410 nm, the aqueous solutions spontaneously turned to deep green with an increase in absorption at 620 nm (Fig. 3) and exhibited a considerably elevated pH to about 8.5. These were attributed to the generation of a triphenylmethyl cation according to Fig. 2. Within 1 min of UV irradiation almost all malachite green groups were converted to the cationic form. On cessation of the UV irradiation the absorption at 620 nm reverted to the initial level (one quarter of the maximum level) within about 1 h regardless of the malachite green contents in the copolymers, indicating that three quarters of all cationic forms slowly reverted to the original leucohydroxide, nonionic form. Therefore, one quarter of the malachite green groups were present as cations at equilibrium state at pH 7.4 and 37 °C.

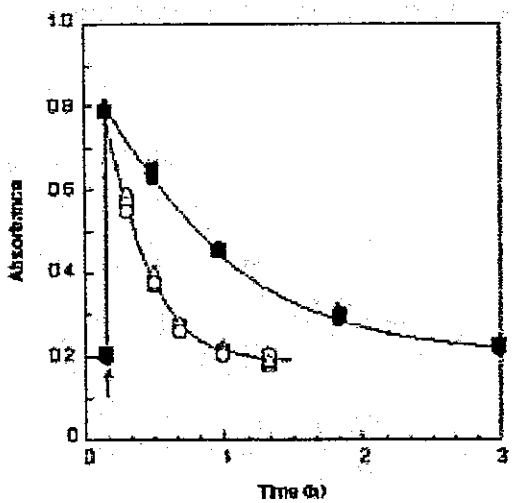

Fig. (3). Absorbance changes at 620 nm in aqueous solutions of photo-cation generatable water-soluble copolymers (malachite green content per molecule, 3.6: _; 7.9: _; 15.0: _), and the polyplexes from the photo-cation generatable water-soluble copolymer (malachite green content, 3.6: _; 7.9: _; 15.0: _) and DNA (pGL3-control plasmid, 0.5 μg). Arrow indicates the time of UV irradiation for 2 min.

3.2. Polyplex Formation and Degradation

When DNA was added to Tris-HCl buffered solution of the cationic copolymers produced from the malachite green-derivatized copolymers by UV irradiation, a marked high scattering intensity in DLS measurements was immediately observed regardless of the malachite green contents of the copolymers (Table 2). In contrast, low scattering intensities were detected in saline solutions of DNA or the copolymers with or without UV irradiation, or even after mixing of DNA with the copolymers without UV irradiation (Table 2). For all copolymers, the scattering intensity was highest at C/A ratios between 0.5 and 1. An example in the use of the copolymer, with a malachite green content of 15.0, is shown in Fig. 4. The larger number and size of the particles caused a larger scattering intensity. Therefore, it can be said that the photo-cation-generated copolymers produced polyplexes upon mixing with DNA at the appropriate mixing ratio. The cumulant analysis of the DLS measurements showed that the diameter of the polyplexes produced at C/A ratios ranging from 0.25 to 5 was about 150 nm with low polydispersity (about 0.4) (Fig. 4). In addition, the polyplexes formation was visualized by fluorescent microscopic image because the malachite green is a fluorescent dye, where almost all polyplexes observed by exposure to light of wavelength 543 nm were in spherical shape (Fig. 5).

Upon incubation at 37 °C of the polyplex solutions, prepared from the copolymer with the lowest malachite green-content, the scattering intensities gradually decreased up to 3 h (Fig. 6), which was well synchronized with the change in absorbance of the malachite green groups (Fig. 3). After 3 h of incubation the scattering intensity had declined to one half that obtained immediately after mixing of the photo-cationized copolymer and DNA. In addition, in this time the cumulant diameter of the polyplexes decreased from approximately 150 to 100 nm. However, little change in the

Table 2. Scattering Intensity of Tris-HCl Buffered (pH 7.4) DNA and/or Malachite Green-Derivatized Copolymer (Malachite Green Content; 15.0) with or without UV Irradiation

Run	Solution condition			Scattering intensity
	DNA[a]	copolymer[a]	*hν*[a]	
1	-	-	-	49.1 ± 21.8
2	O	-	-	158.2 ± 27.3
3	-	O	-	169.1 ± 12.4
4	-	O	*O*	174.5 ± 38.2
5	O	O	-	916.3 ± 299.9
6	O	O	*O*	2345.2 ± 97.3

[a]Key: o, presence; -, absence.

scattering intensity and the cumulant diameter were observed in the high malachite green content (7.9 and 15.0) copolymers (Fig. 6) despite a decrease in the amount of cations in the copolymer (Fig. 3). These results indicated that the polyplexes were formed immediately upon mixing of the photocationized copolymers and DNA for all the copolymers studied, and gradually dissociated only in the lowest malachite green-content copolymer to release of DNA without any damages, which was confirmed by the observation of the shift of DNA electrophoretic migration in agarose gel (data not shown).

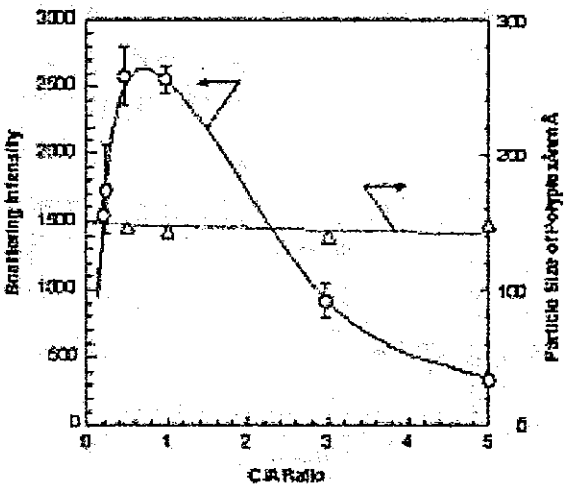

Fig. (4). Scattering intensity (○) and cumulant diameter (○) of an aqueous solution of the polyplexes prepared by mixing of photocation generatable water-soluble copolymer (malachite green content per molecule, 15.0) and DNA (pGL3-control plasmid, 0.5 μg) in different ratios.

When 1-min UV irradiation of the polyplex solutions was repeated every 10 min the absorbance at 620 nm was maintained to some extent for the lowest malachite green content copolymer for up to 2 h (Fig. 7), indicating that almost all cationized malachite green groups existed without conversion

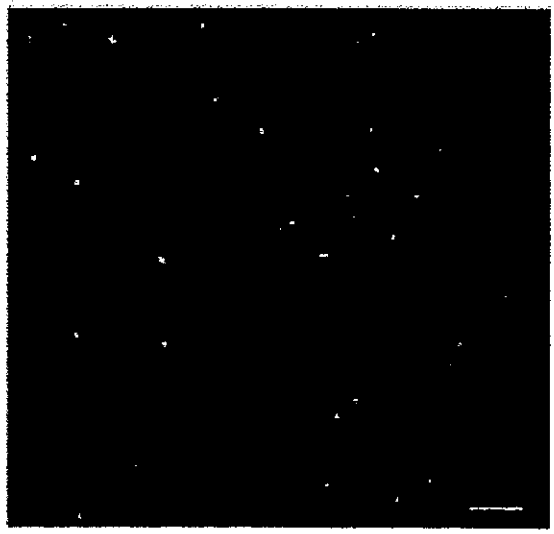

Fig. (5). Fluorescence microscopic image of the polyplexes from the photo-cation generatable water-soluble copolymer (malachite green content per molecule, 15.0) and DNA at C/A ratio of 1. Bar=10 μm.

to the nonionic form due to the action of the intermittent UV irradiation. Simultaneously, under the same UV irradiation conditions, the scattering intensity derived from the polyplexes was also maintained at the initial value to some extent (Fig. 6), indicating the dissociation of the polyplexes was prevented under UV irradiation. However, upon cessation of UV irradiation the scattering intensities started to spontaneously decrease, due to disappearance of the cations (Fig. 6). Therefore, it can be said that the formation and dissociation of the polyplexes were photochemically controlled.

4. DISCUSSION

In this study, as a model compound as a vector for a proposed new gene delivery system a photoreactive water-soluble polymer derivatized with a photochromic compound, malachite green was molecularly designed [30,31]. Since malachite green can reversibly generate a cation as shown in (Fig. 2), the malachite green-derivatized water-soluble poly

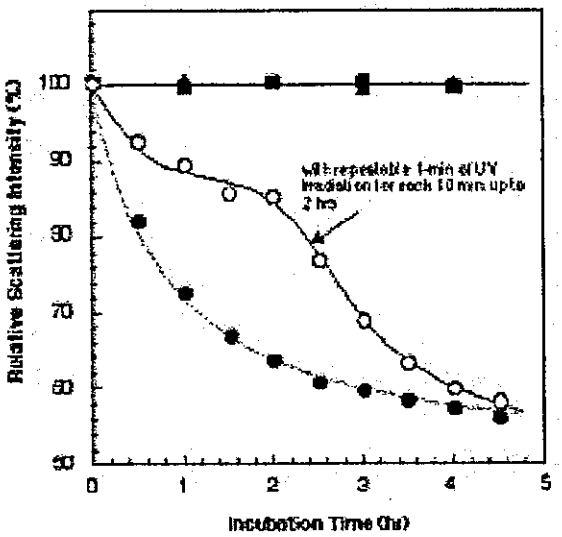

Fig. (6). Changes in scattering intensity of an aqueous solution of the polyplexes prepared by mixing photo-cation generatable water-soluble copolymer (malachite green content per molecule, 3.6: _ and _; 7.9: _; 15.0: _) with 2-min of UV-irradiation, and DNA (0.5 μg) at C/A ratio of 1. (_): 1-min of UV irradiation was performed repeatedly every 10 min up to 2h.

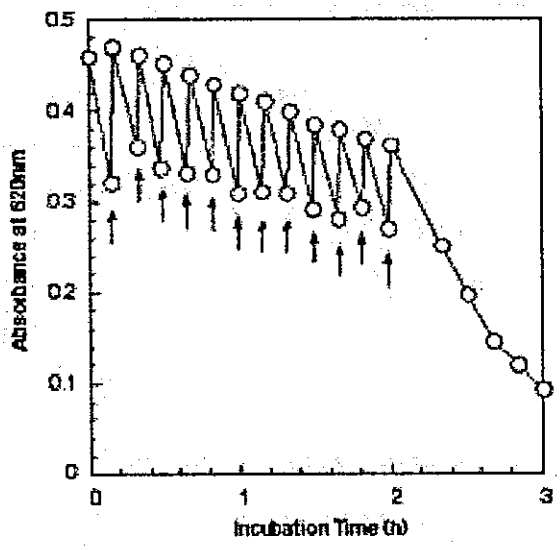

Fig. (7). Change in absorbance at 620 nm of an aqueous solution of the polyplexes prepared by mixing of photo-cation generated water-soluble copolymer (malachite green content; 3.6) and DNA at C/A ratio of 1. Arrows indicate the time of 1-min UV irradiation.

mers developed here can be reversibly converted from a nonionic to cationic form by UV irradiation. Therefore, the following three functions will be expected when using the polymers as a vector. Namely, cationization upon UV irradiation induces 1) acceleration of the formation of polyplexes with DNA and their subsequent cellular uptake due to enhancement of electrostatic interactions. Since malachite green exists as a stable cation under highly acidic conditions, the polyplexes are stably maintained in endosomal compartments. Therefore, 2) DNA can be protected from enzymatic degradation and hydrolysis in the endosome. If the polyplexes are released into the pH-neutral cytoplasm, thermal deionization of cations will occur, which causes: 3) dissociation of the polyplexes, resulting in the release of DNA. The last function, 3), may provide the most effective enhancement of the gene delivery to nuclei in addition to the others 1) and 2), both of which are usually present in other cationic copolymers designed as vectors.

The malachite green-derivatized water-soluble polymers synthesized in this study were radical copolymers of malachite green-derivatized vinyl monomer and a water-soluble monomer, *N,N*-dimethylacrylamide. Poly(*N,N*-dimethylacrylamide), is a widely used medical material, for biocompatible surface coatings for medical devices [34,35] and for the modification of drugs [36]. Malachite green has been studied as a functional material in drug delivery systems [32]. In addition, an affinity chromatography of DNA was developed by using A.T-base-pair-specific affinity of the chloride derivative of malachite green [37]. There have been few reports showing toxicity in these materials. In our previous study, there was little significant difference between the control and endothelial cells (ECs) after treatment with the copolymers synthesized here, regardless of the presence of UV irradiation in the exclusion test of trypan blue [32]. In addition, the long-range viability and integrity of the ECs were not altered from the control upon incubation with the copolymers at the concentration less than 1 mg/mL, which is about 10 times larger than that used in standard *in vitro* transfection experiments [32]. On the other hand, about 70 % of cell viability was reported in Exgen500, which is polyethylenimine, one of the commercially avairable cationic polymer vectors. Therefore, it can be said that the copolymers were biocompatible with little cytotoxicity. The copolymers will be excluded from the cells without any cellular damages because they can not degraded by hydrolysis *et al.*

In our previous study, cationized malachite green-derivatized water-soluble copolymers generated by UV irradiation were taken up in cells by electrostatic interactions, whereas the non-ionic form of the copolymers before irradiation displayed no detectable interaction with cell membranes [32]. The strength of interaction was increased by the amount of malachite green residues introduced into the copolymers and the irradiation time, i.e., the amount of generated cations. To obtain high interaction strength with cells, copolymers with many malachite green residues are required. However, to maintain the water-solubility of copolymers, the content of malachite green residues must be less than several mol % due to their hydrophobicity.

In this study, 3 kinds of photoreactive copolymers with 3.6 (content of malachite green residues; 0.4 mol %), 7.9 (0.7 mol %), and 15 (2.7 mol %) of malachite green groups per molecule were prepared. In all copolymers, upon mixing with DNA aqueous solutions and after irradiation there was a marked increase in scattering intensity (Table 2), indicating the generation of polyplexes by non-specific electrostatic interaction. The formation of the polyplexes was also con-

firmed by observation of CLSM. The diameter of the obtained polyplexes was approximately 150 nm, irrespective of the malachite green content (Fig. 4). The amount of malachite green cations in the polyplexes returned to the initial level, i.e., the level prior to irradiation, (about 25% of the entire malachite green groups) in about 3 h (Fig. 3). Since the amount of cations in an aqueous solution of the copolymers alone returned to the initial level in about 1 h (Fig. 3), binding of malachite green cations with hydroxyl anions may have been inhibited in the polyplexes. The scattering intensity of the polyplex solution prepared from the lowest malachite green content copolymer 3 h after irradiation was reduced to approximately 50% that immediately after irradiation (Fig. 6), indicating dissociation of the polyplexes. If the dissociation of the polyplexes occurs in the cytoplasm, gene delivery to nuclei will be enhanced.

Only the lowest malachite green content copolymer polyplexes were dissociated (Fig. 6). About 4 cations remained in the highest malachite green content copolymer approximately 3 h after irradiation and were sufficient to form polyplexes. Deionization of malachite green cations was prevented to some extent by repeated irradiation at intervals (Fig. 7). Therefore, maintaining the amount of cations through continuous irradiation could control the condition of the complex. These results suggested that generation and dissociation of the complex could be controlled by irradiation. However, amount of cations was gradually decreased even intermittent irradiation, which may be due to decreasing of malachite green molecules by photochemical side reaction, resulted in prevention of maintenance of stable polyplexes.

In the copolymer with 3.6 malachite green residues per molecule, in principle, less than 1 cation exists in a molecule in the dark. Therefore, it is expected that the copolymer cannot bind to more than 1 DNA molecule, indicating that no polyplex can be generated. However, complete dissociation of the polyplexes could not be achieved even in the use of the copolymer. This may be due to the relatively high polydispersity of the copolymer. To completely dissociate the polyplexes in neutral solution, it is necessary to design a new malachite green-derivatized polymer, in which the number of malachite green units is strictly controlled to 2-4 per molecule, or a functional molecule that is completely deionized in neutral solution. With regard to the former material, we have been investigating synthesis by controlled polymerization methods, such as living radical polymerization. With regard to the latter, since malachite green is converted into cations by dissociation of hydroxyl anions, it tends to be in the leuco form at equilibrium under alkaline conditions because of suppression of dissociation (Fig. 2). Therefore, even in the polyplex with the highest malachite green content, the scattering intensity was reduced to about 25% at pH 9 24 h after irradiation, and to about 10% at pH 10 within about 1 h after irradiation, whereas little change in the scattering intensity was observed at pH 6 (Fig. 8). At pH 10, the change in scattering intensity was well correlated with that in absorbance (Fig. 8). In other words, complete deionization of malachite green led to almost complete dissociation of the complex. The other approach to complete dissociation of the polyplexes is the synthesis of leuconitrile derivates of malachite green as model compounds, which could exist in a complete non-ionic form before irradiation, and would not be affected by the pH of the solution.

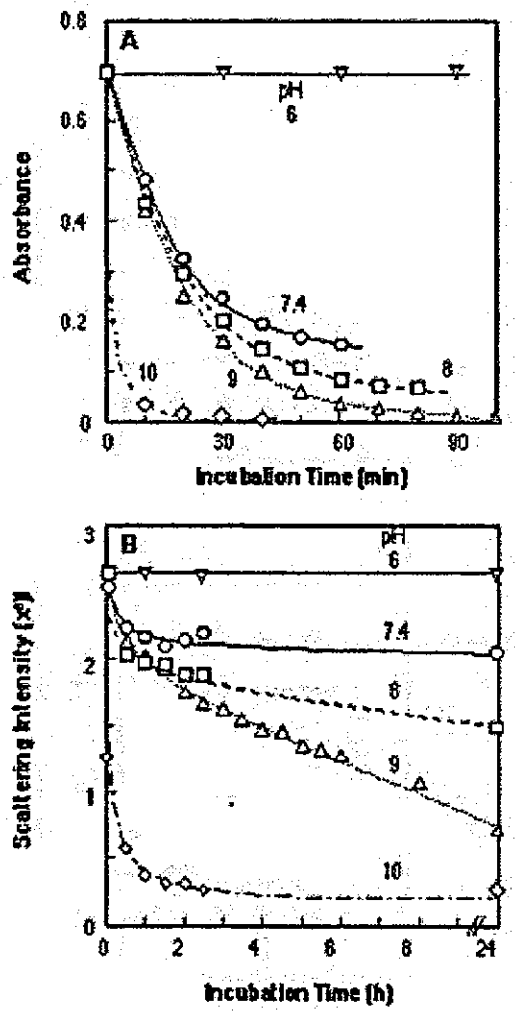

Fig. (8). Changes in absorbance (A) and scattering intensity (B) of an aqueous solution of the polyplexes of photo-cation generatable water-soluble copolymer (malachite green content per molecule, 15.0) and DNA at C/A ratio of 1 at different pHs.

This study indicated that generation and dissociation of polyplexes of water-soluble polymer and DNA was controlled to some extent using the specific photo/thermoreactivity of malachite green (Fig. 1). It can be said that a new model for gene delivery system into cells has been demonstrated. For the next stage of this study, we are planning to examine *in vitro* transfection efficiency. It is highly expected that only by mixing with the malachite green-derivatized copolymers after irradiation DNA will deliver effectively to inside of cells by endocytosis. The degree of delivery will be controlled easily by irradiation time and malachite green content. In addition, thermal dissociation ability of the photo-generated polyplexes after escaping from

endosomes will promote DNA delivery to nuclei, which will cause the enhancement of transfection efficiency. Such study will be reported in the near future.

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光反応によるバイオマテリアル界面の精密創製

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第2章　光反応によるバイオマテリアル界面の精密創製

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1　はじめに

光反応は，微細加工に適した以下に示す優れた特徴を有している。すなわち，①レンズ，シャッター，電圧等によってエネルギー量を変化できるので，反応量を容易に調節することができる（量制御能）　②照射時のみに反応を起こさせることができるので，時間を限定して反応させることができる（時間制御能）　③照射波長を調節することで，種を特定して反応させることができる（選択能）　④室温下，大気中での穏和な条件下で反応させることができる（低障害能）。さらに，⑤フォトマスクや光ファイバーを用いて照射領域を制限することで，領域を限定して反応させることができる（空間制御能），などがあげられる。

これらの特徴を生かして，光反応は，これまで半導体や記録媒体の開発など，主に電子情報分野の発展を支える超精密加工技術として大きく貢献してきた。また最近では，DNAチップやタンパクチップに端を発して，さらに複雑な化学システムやバイオシステムをマイクロチップ上に集積化する精密基盤の作製技術としても利用され，ナノーマイクロ化学実験空間が現実のものとなりつつある。これらのマイクロチップ化技術は，既にゲノム解析，遺伝子診断，プロテオーム解析などに応用されている。また，人体の構造情報や機能情報を視覚的に把握するために，光透視や光CTなど，光の性質を利用したより低侵襲な画像化機器の開発も進められている。さらに，molecular imagingに代表される分子遺伝子細胞レベルを対象とした可視化技術は，光分子プローブの開発によって細胞内情報を解析する手法として絶大な威力を発揮して分子生物学の発展に貢献し，病態の解明などに応用が期待されている。いずれにおいても光は，次世代医療の診断領域における要素技術として欠かすことができないであろう。

このように，光の利用は医療分野においてすでに身近なものとなりつつあり，治療領域においても，レーザーアブレーションによる狭窄血管病変の血管形成術や，光感受性薬剤と組み合わせたレーザー癌治療など光そのものの性質を利用した治療法が開発されている。また，光反応の利用は，1980年代より治療デバイスの表面に機能性や生体適合性を付与するための表面修飾・改質法として，使用する光反応性化合物の開発に基づいて検討されてきている。本稿では治療デバイスの素材として主に用いられている高分子バイオマテリアルを対象として，その生

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体成分との接触界面における，光反応を用いた精密創製に焦点をあて，循環器領域での治療デバイスへの応用例を交えて紹介する。

2　界面設計に使用されている光反応性化合物

医療分野において界面設計に利用されている光反応性化合物の代表例を図1に示す。いずれも医療目的に特別に開発されたものではないが，毒性を含めた生体適合性が使用する第一の選択理由となっている。それらを光反応機構別に分類すると，二量化するタイプとラジカルを発生するタイプに大きく分けられる。前者にはシナモンの構成成分であるケイ皮酸やクマリンなどα,β-不飽和カルボニル類が含まれる。これらは紫外光照射によって不飽和結合部が分子間で付加環化反応を起こし二量化する。また核酸の構成成分の一つであるチミンも同様な環化二量化反応を起こすことが知られている。一方，後者には紫外光照射によって解裂反応を起こしてラジカルを生成するフェニルアジド類，可逆的にラジカルを生成するジチオカルバメート類，紫外光照射によって水素引き抜き反応を起こしてラジカルを発生するベンゾフェノンやアセトフェノンなどの芳香族ケトン類，さらには可視光照射でラジカルを発生するカンファキノン類，ならびにフルオレセインやエオシン，ローズベンガルなどの食用色素を含むキサンテン系色素が含まれる。その他，ビタミンB_{12}であるリボフラビンも可視光でラジカルを発生することが報告されている。

3　従来の界面設計方法

良好な成型加工性を有する高分子バイオマテリアルは，人工臓器を含む医療デバイスの素材として広く用いられている。例えば，高分子エラストマーであるセグメント化ポリウレタンは，伸縮耐久性に優れているため，拍動による大きな形状変化を持続的に伴う人工心臓の基材として極めて有用である。しかし，このウレタン素材を含めて医療デバイスに使用されている高分子マテリアルは，そのほとんどが汎用の工業用マテリアルを医療用に純度を高めたものとして提供されている。つまり，素材として選択する際には生化学的性質より機械的性質が重視されているため，医療デバイス化して使用する場合には，炎症や拒絶反応を防ぐために薬物投与が必要となる。

そこで，高分子マテリアルの機械的性質を大きく損なうことなく生体に適合化させるために，さまざまな表面修飾や改質が検討されてきた。これまでに，電子線照射やコロナ放電，プラズマ処理，薬品処理など物理的あるいは化学的手法によって，ぬれ性や接着性など特定の機能の向上が図られている。しかし，これらの方法はいずれも，表面層の高分子を化学的に反応させて極性基など特定種の官能基を無作為に導入しているため，制御された界面設計は極めて困難である。

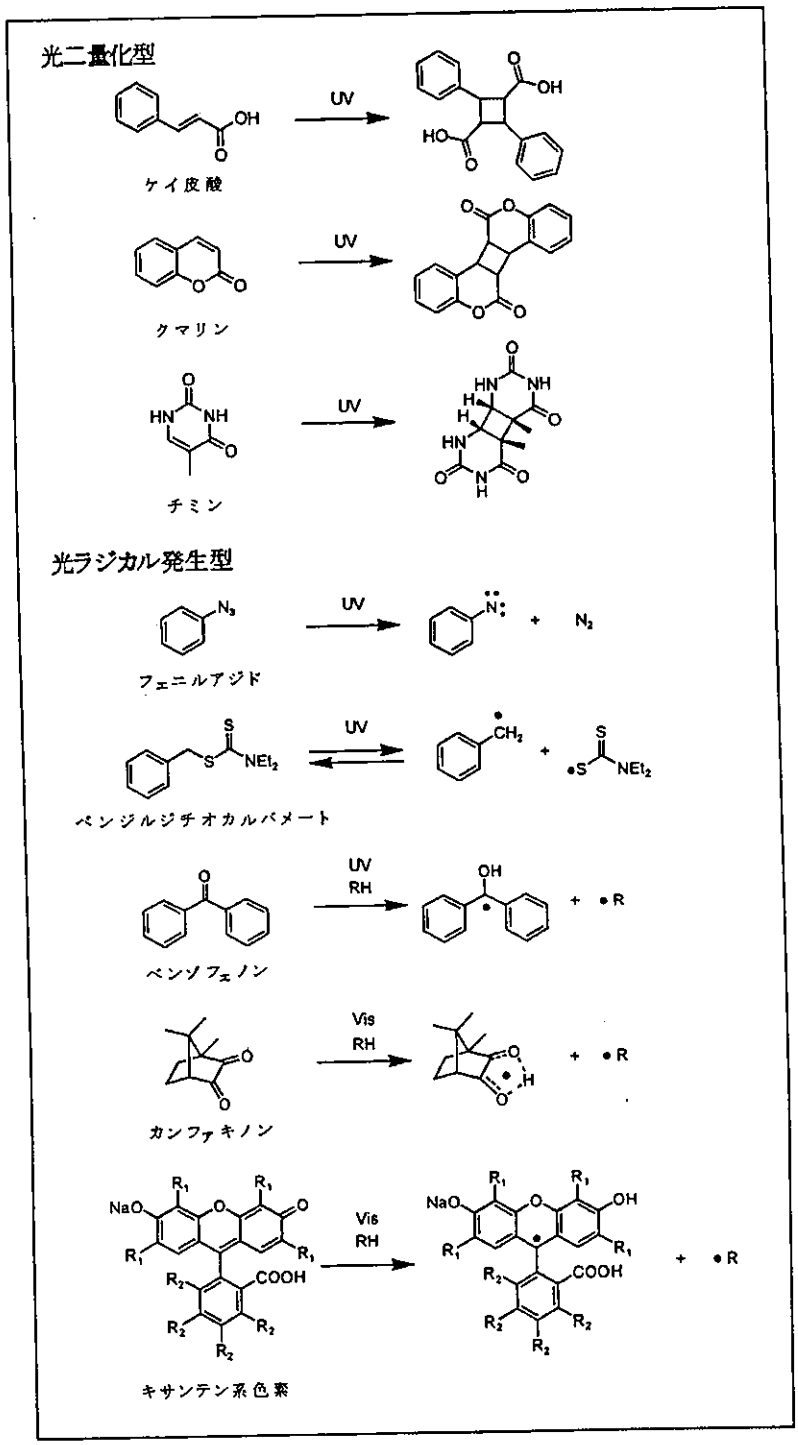

図1　界面設計に利用されている光反応性化合物

4　光グラフト化による界面設計

光反応は照射時に照射領域のみに限定して起こさせることができるので，任意の微細領域のみの界面を設計することができる。また，単純な官能基の導入に加えて，生理活性物質や，タンパク質，糖などの生体高分子，さらには合成高分子を任意の微細領域に固定化させて，機能性表面や生体適合性表面を創製することが可能である。この目的のために，フェニルアジドの光反応が多用されている。先ず，固定化させたい生理活性物質や高分子にフェニルアジド基を導入することによって光反応性化合物が合成されている。これら光反応性化合物を高分子マテリアル表面にコーティングし紫外光照射することによって，フェニルアジド基に生成した高反応性のビラジカル（ナイトレン）が，高分子マテリアルの表面から水素を引き抜くと同時に共有結合を形成して基材と架橋反応を起こし，目的化合物がマテリアル表面に固定化される。

例えば，細胞接着性タンパク質（フィブロネクチン，ラミニンなど）の細胞接着活性部位であるarginine-glycine-aspartic acid (RGD) を含むオリゴペプチドの末端にフェニルアジド基を導入した光反応性ペプチドは，コーティングした後に紫外光を照射するだけで，容易に基材表面に固定化されている[1]。この表面では積極的な細胞接着性が獲得されている。また，Chungらは，生体高分子であるキトサンの表面にRGDペプチド鎖の光固定を行い，キトサン表面を細胞接着性に改変している[2]。この方法は，他の生理活性物質の固定化にも利用されており，例えば，伊藤らは，細胞成長因子 (EGF) を微細領域に固定化させると，限定した固定化領域内でのみで細胞の増殖を促進できることを報告した[3]。この際，細胞成長因子の機能発現は，表面へ固定された状態においても十分に発揮することが示された。従って，発現にはそれらが必ずしも細胞内に取り込まれる過程を必要としないことが明らかとなり，さらには触媒反応的に繰り返し機能させられることも明らかにされた。その他，神経成長因子 (NGF) や腫瘍壊死因子 (TNF-α) を固定化させることによって各種細胞の分化やアポトーシスの誘導が観測されている。一方，Kooleらは，高度な生体適合性を付与することを目的として細胞膜の主構成成分であるリン脂質の極性基の一種であるフォスファチジルコリン(PC) 基の固定化を行っている[4]。

紫外光照射は，基材に対して固定化層となる最外面部を除いてバルクの性質にほとんど影響を与えない。一方，固定化物に対しては，直接照射を受けるため一部に障害を受ける可能性が指摘されている。そこで，固定化物への障害を回避する工夫が考案されている。つまり，Cassらは，ビオチン化フェニルアジドを合成し，先ずこれを基材表面に光固定した[5]。次いで，表面をアビジン化させた後に，ビオチン化した酵素やタンパク質を作用させると，ビオチン-アビジン-ビオチン架橋を介してほぼ非侵襲で目的物を固定化させることを可能とした。

生理活性物質以外にも合成高分子材料の固定化による基材表面の改質が検討されている。例えば，Kooleらは，フェニルアジド基を側鎖に多数有するアジドスチレン共重合体を接着層と