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

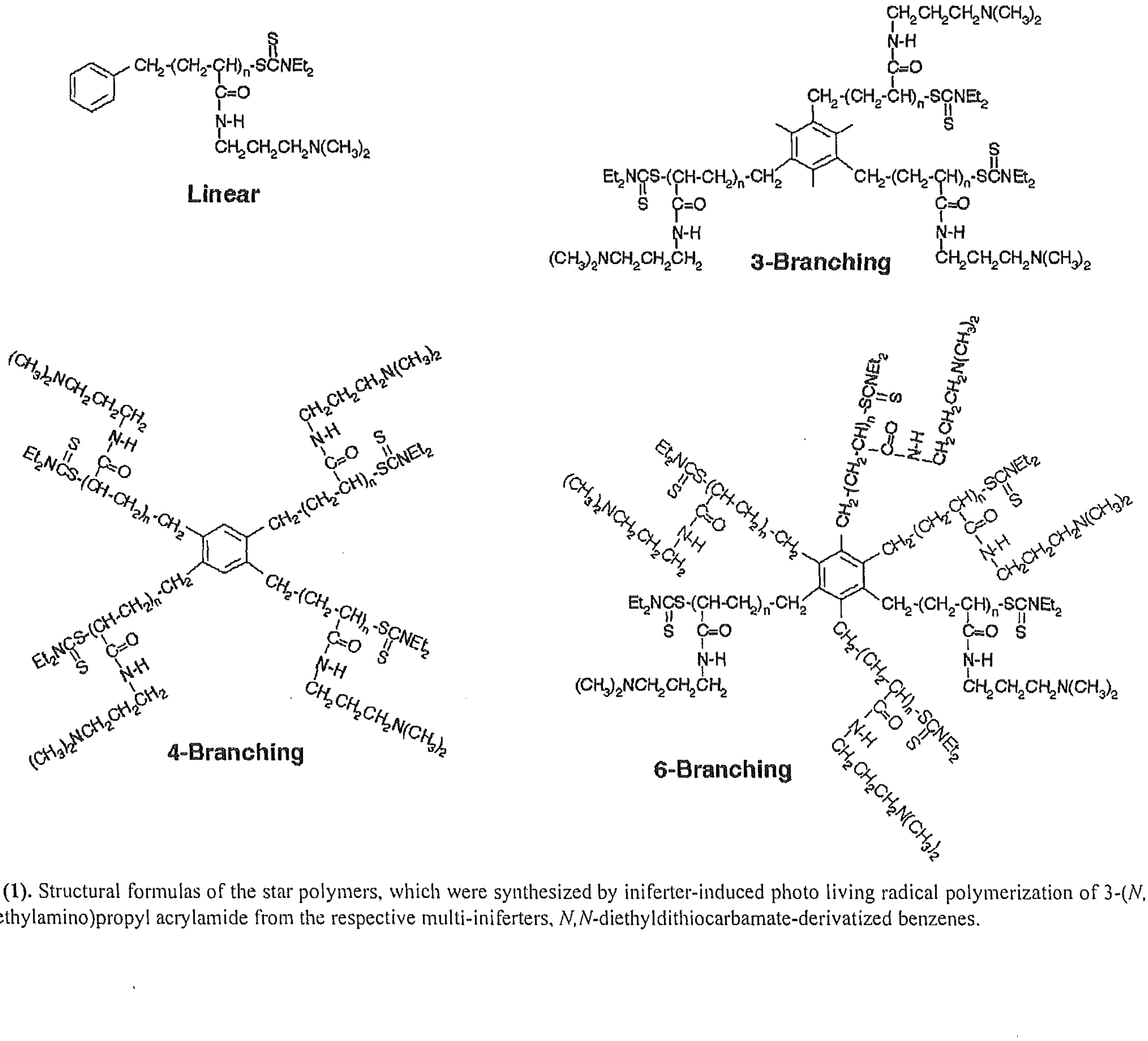

Fig. (1). Structural formulas of the star polymers, which were synthesized by iniferter-induced photo living radical polymerization of 3-(*N,N*-dimethylamino)propyl acrylamide from the respective multi-iniferters, *N,N*-diethyldithiocarbamate-derivatized benzenes.

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).

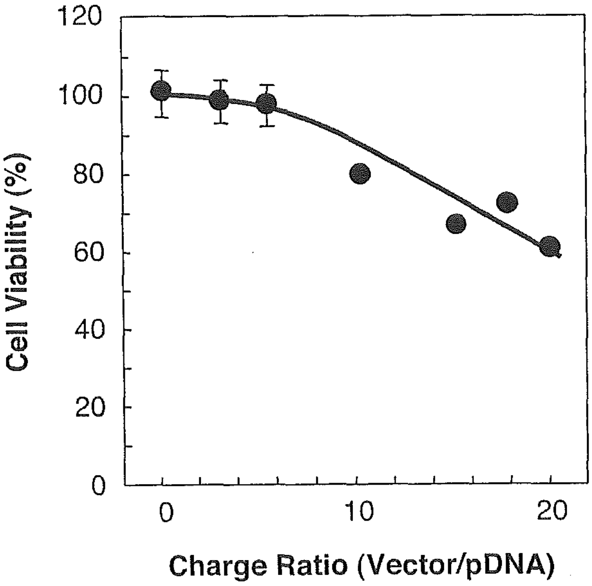

Fig. (2). Cytotoxicity of the polyplexes obtained immediately after mixing of DNA (pGL3-control) and 6-branching star polymer under the changing of a charge ratio (vector/pDNA), which was determined by cell viability assay of COS-1 cells using a WST-8 method. The data are presented as means±S.D. (n=5).

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 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.

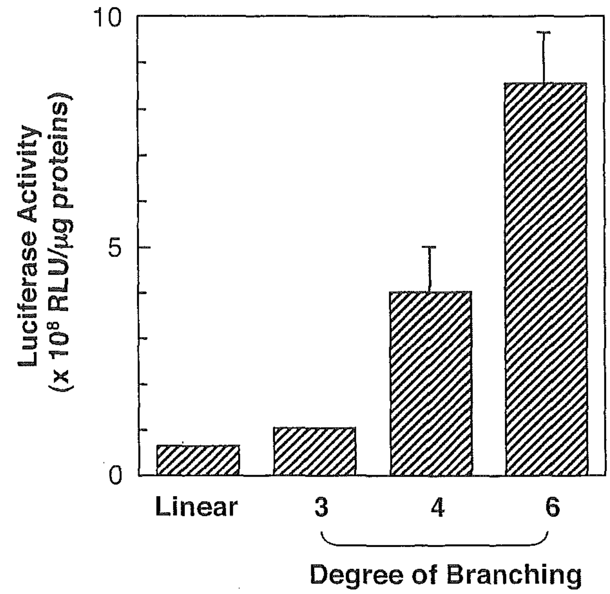

Fig. (3). Effect of branching of the star polymers on the level of luciferase gene transfer activity in COS-1 cells. COS-1 cells were treated with the polyplexes prepared by mixing of the star polymers and DNA (pGL3-control) under a charge ratio of 5/1 (vector/pDNA) 12 h after those preparation. The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

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.

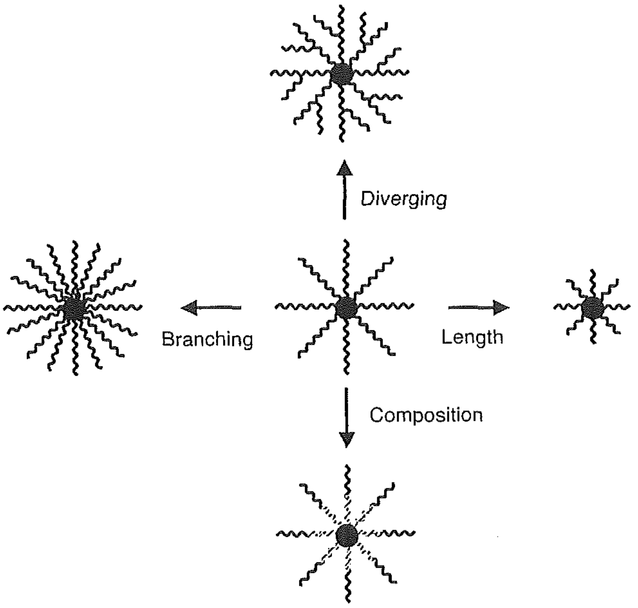

Fig. (4). Possibility in molecular design of various star polymers having different branching, diverging, chain length, or composition, which are based on iniferter-induced living radical polymerization.

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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-

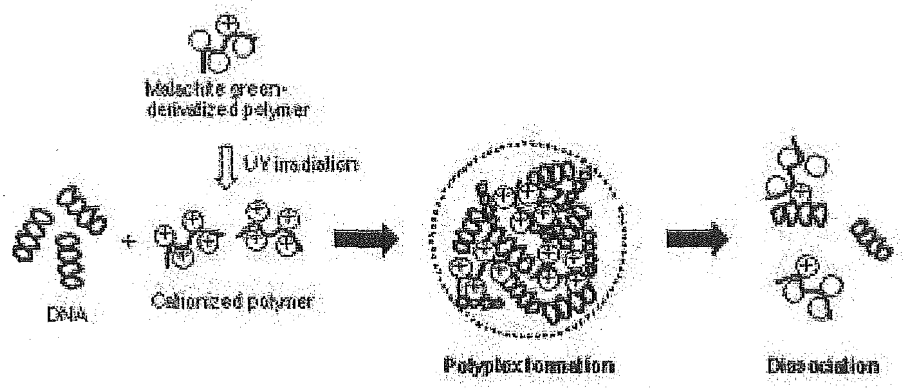

Fig. (1). Schematic illustration of the principle of photo-induced polyplex formation and its dissociation, both of which were based on the specific photochemical/thermal reactivity of the malachite green-derivatized water-soluble polymer.

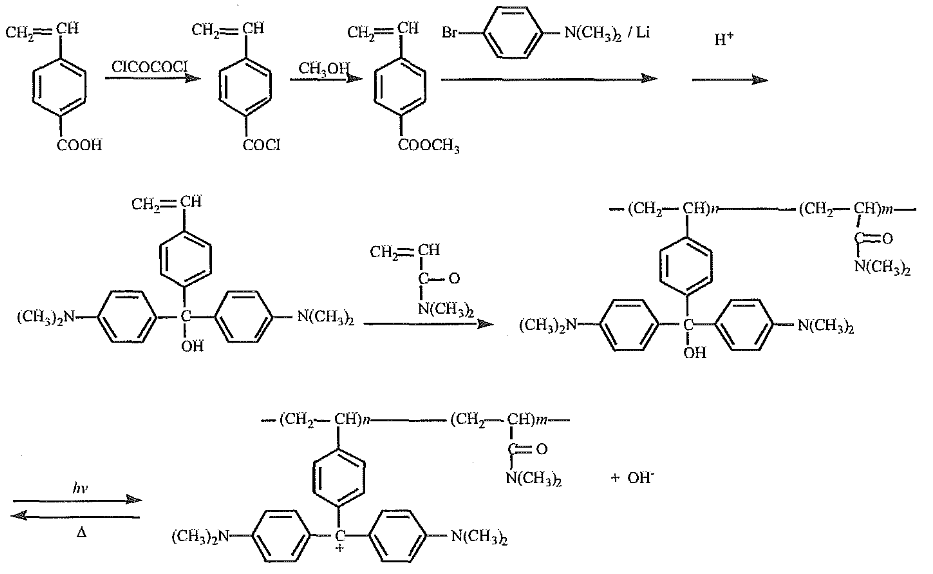

Fig. (2). Reaction scheme of the preparation of photo-cation generatable water-soluble copolymer, which is a radical copolymer of diphenyl(4-vinylphenyl)methane leucohydroxide and *N,N*-dimethylacrylamide. Chemical reaction in photoinduced dissociation of triphenylmethane leucohydroxide (malachite green) 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 Leucohydroxide [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=C*H*$_2$), 5.38 (d, *J*=10.2 Hz, trans-H of PhCH=C*H*$_2$), 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=C*H*$_2$), 5.21 (d, *J* =10.8 Hz, 1H, *trans*-H of PhCH=C*H*$_2$), 2.94 (s, 12H, -NC*H*$_3$).

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 ($\varepsilon = 6.7 \times 10^4$ dm^{3}mol^{-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 (PhNC*H*$_3$, CONC*H*$_3$), 1.1-1.6 (-C*H*$_2$-).

2.4. General Methods

Irradiation was carried out using a mercury-xenon arc lamp (L2859-01; Hamamatsu Photonics Inc., Shizuoka, Japan). The illumination wavelength ($290 < \lambda < 410$ 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] ($\times10^4$)	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.

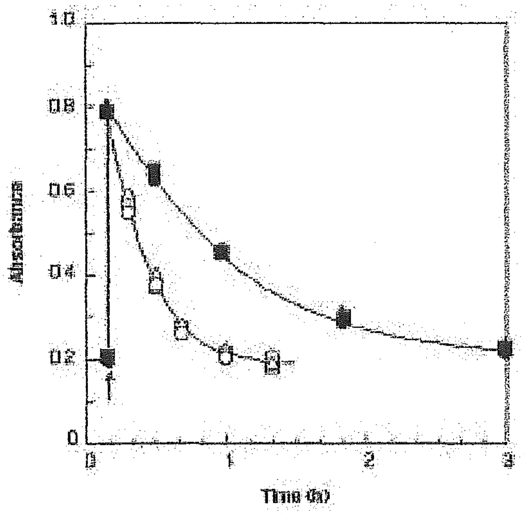

Fig. (3). Absorbance changes at 620 nm in aqueous solutions of photo-cation generatable water-soluble copolymers (malachite green content per molecule, 3.6: _; 7.9: _; 15.0: _), and the polyplexes from the photo-cation generatable water-soluble copolymer (malachite green content, 3.6: _; 7.9: _; 15.0: _) 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]	*hv*[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 photo-cationized 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).

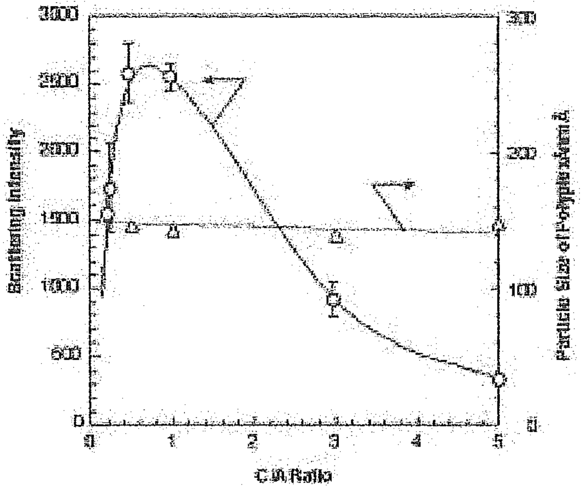

Fig. (4). Scattering intensity (_) and cumulant diameter (_) of an aqueous solution of the polyplexes prepared by mixing of photo-cation generatable water-soluble copolymer (malachite green content per molecule, 15.0) and DNA (pGL3-control plasmid, 0.5 μg) in different ratios.

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 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.

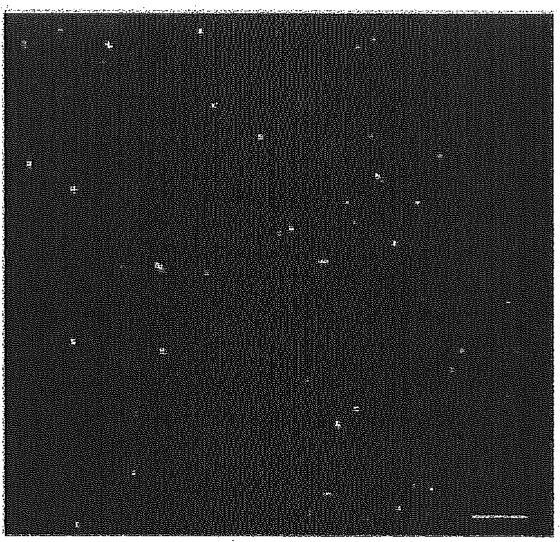

Fig. (5). Fluorescence microscopic image of the polyplexes from the photo-cation generatable water-soluble copolymer (malachite green content per molecule, 15.0) and DNA at C/A ratio of 1. Bar=10 μm.

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

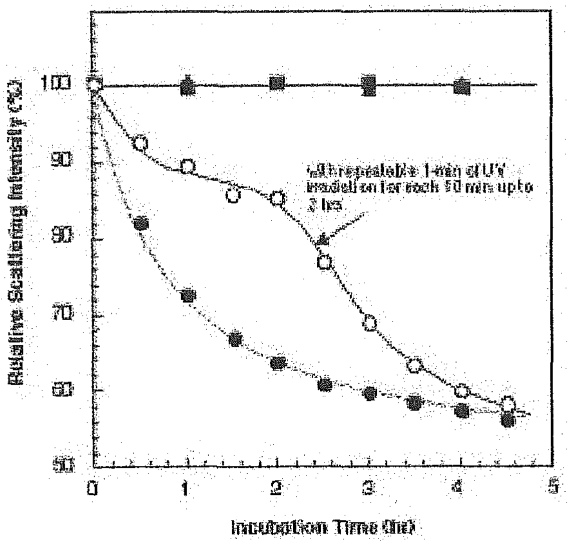

Fig. (6). Changes in scattering intensity of an aqueous solution of the polyplexes prepared by mixing photo-cation generatable water-soluble copolymer (malachite green content per molecule, 3.6: _ and _; 7.9: _; 15.0: _) with 2-min of UV-irradiation, and DNA (0.5 µg) at C/A ratio of 1. (_): 1-min of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

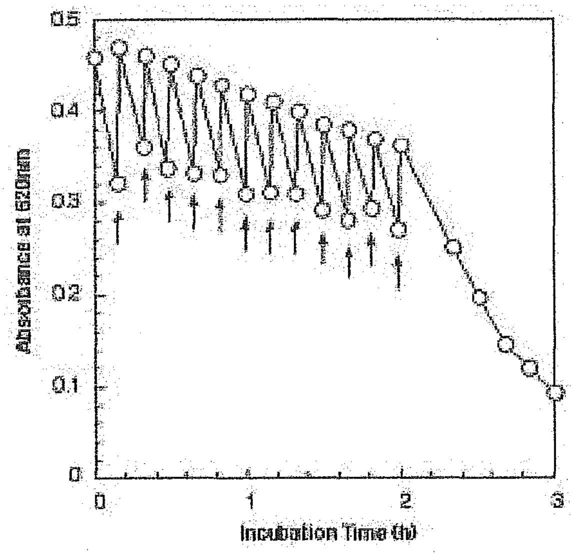

Fig. (7). Change in absorbance at 620 nm of an aqueous solution of the polyplexes prepared by mixing of photo-cation generated water-soluble copolymer (malachite green content; 3.6) and DNA at C/A ratio of 1. Arrows indicate the time of 1-min UV irradiation.

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 confirmed 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.

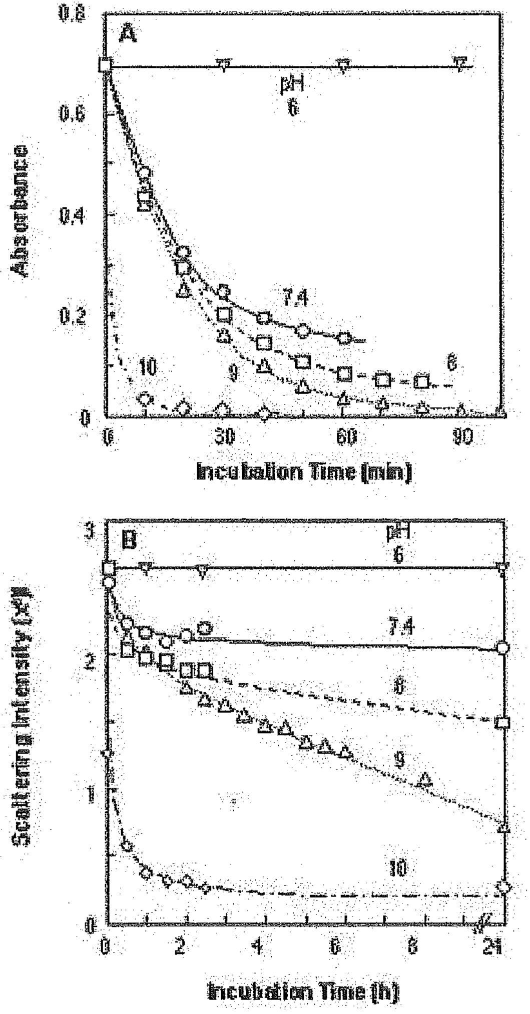

Fig. (8). Changes in absorbance (**A**) and scattering intensity (**B**) of an aqueous solution of the polyplexes of photo-cation generatable water-soluble copolymer (malachite green content per molecule, 15.0) and DNA at C/A ratio of 1 at different pHs.

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。

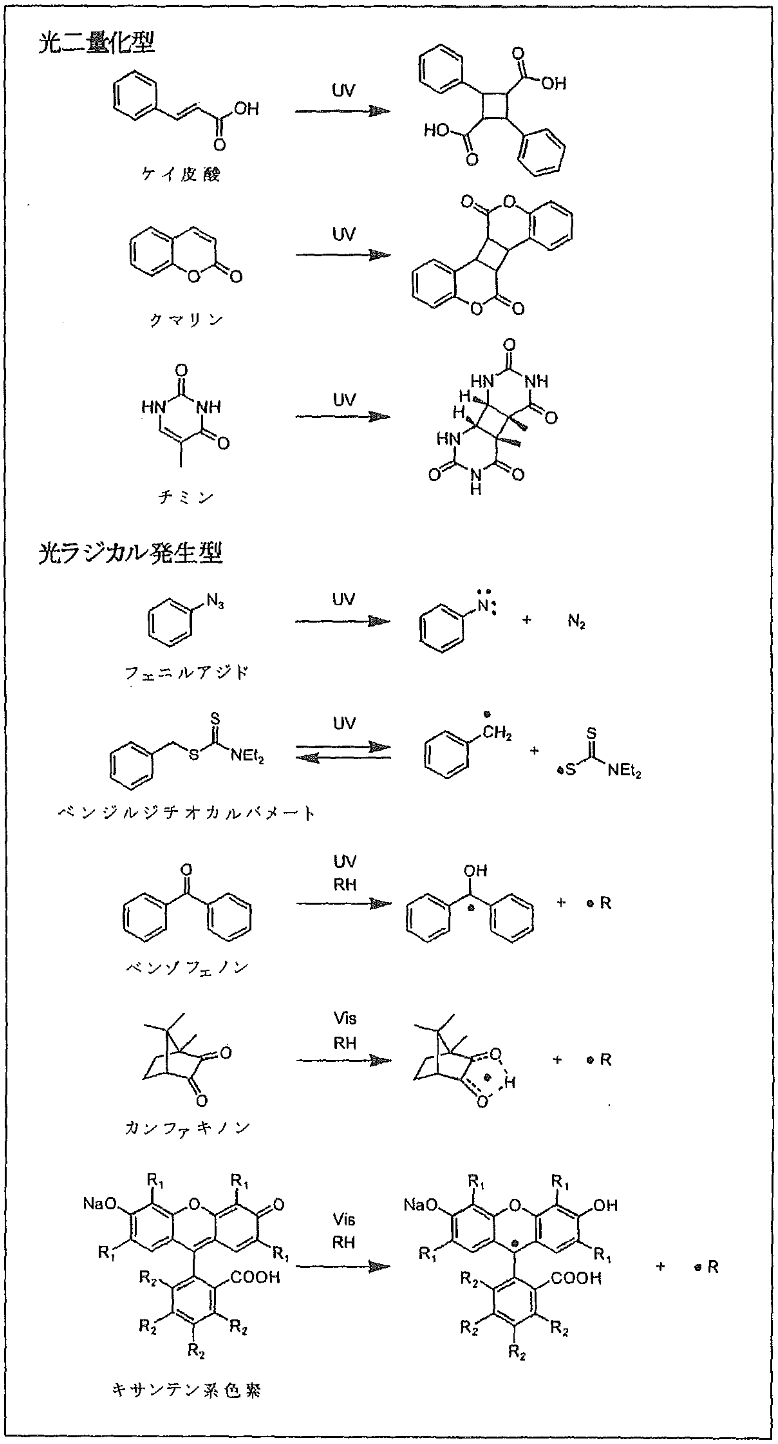

図1　界面設計に利用されている光反応性化合物

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

してポリウレタン基材の表面にあらかじめコーティングしておき，その上にさらに固定化させたい目的とする高分子，例えば，ポリ(*N*-ビニルピロリドン)をコーティングし，紫外光照射することで目的高分子を固定化させている6)。この方法は接着層が基材と目的高分子の両者に光架橋反応できるため，固定させる高分子種に特に制約はなく汎用性の高い界面設計法といえる。

一方，固定化を目的とする高分子の中にあらかじめフェニルアジドを導入しておくと，耐久性にすぐれ，かつ微細加工性を有する界面設計が可能となる。このためにアジドスチレンと各種ビニルモノマーとのラジカル共重合反応によって，親水性や疎水性など物性の異なる種々の光反応性高分子が分子設計されている7)。この光反応性高分子中のアジドスチレンの共重合比を高くすると一般に光反応効率は高くなるが，高分子の物性を保持させるため，アジドスチレン部の共重合比は約10mol%以下に調整されている。また，親水性高分子を合成する別の方法としてポリエチレングリコール(PEG)を主鎖に用いて末端にフェニルアジド基を導入することも行われている。これら光反応性の親水性高分子を基材表面にコーティングした後に紫外光を照射すると，照射領域のみが親水性化される。また，疎水性の光反応性高分子を用いると逆に照射領域のみが疎水性化される。このように，光反応性高分子を分子設計することで任意の物性を基材表面に容易に付与することが可能であり，またフォトマスクなどで照射領域を規制することによって親水－疎水パターン化回路などが自由に設計されている。この回路は細胞の2次元パターン組織体を作製する基材として利用されており，さらに，神経細胞のパターン化によって神経回路網の作製などへの応用も検討されている8)。また，フェニルアジドと同様に光照射によってラジカル発生能を有するベンゾフェノンやジチオカルバメートなどの光反応性化合物も同様な光固定化に利用されている。

5　ハイドロゲルの表面固定化による界面設計

ハイドロゲルは高い含水率を有するため，その表面ではタンパク質の吸着や細胞の接着がおこりにくい。従って，短期使用目的のデバイスの表面を生体適合化するのに適しているといえる。医療目的でハイドロゲルを光反応で作成する代表的な方法として，エオシンやアセトフェノンなどの光ラジカル重合開始剤が用いられ，主にPEGをベースとするジアクリレート体の3次元架橋が行われている。このシステムを用いてHubbellらは，創傷治癒促進剤への応用を検討している。そこでは，血管内腔の損傷部位で光ゲル形成させ，皮膜化することで，バルーンカテーテルやステントを用いた経皮的冠動脈形成術(PTCA)後の血栓形成と内膜肥厚の抑制への有効性を示している9)。

一方，光ラジカル発生基を側鎖に有する光反応性高分子を利用すると，ハイドロゲルの形成と同時にバイオマテリアル表面上に固定化させることが可能である。例えば，ポリ(*N*,*N*-ジメ

チルアクリルアミド）などの水溶性高分子と光ラジカル発生基との複合体を基材表面にサブmm以下の厚さでコーティングしておくと，光照射によって光反応性基に発生したラジカルの再結合が分子間で起こり，ハイドロゲルが形成されると同時に，基材との界面においては，連鎖移動によって基材表面に発生したラジカルと水溶性高分子側鎖のラジカルとの再結合が起こり，生成したハイドロゲルが基材表面に固定化される10)。ここで，コーティング層内にあらかじめ薬物を混合させておくと，生成したゲルの内部に薬物を包埋させることができる。

また，ハイドロゲルを生体内吸収性とするために，コラーゲンの熱変性体であるゼラチンを主鎖に用いてその側鎖にベンゾフェノン基を導入した光反応性ゼラチンが合成されている11)。この光反応性ゼラチンは小口径人工血管やステントグラフトの界面機能化材料として利用されている。両デバイスとも内腔の血液接触界面では抗血栓性が要求される。そのために，光反応性ゼラチンをコーティングする際にヘパリンやウロキナーゼなどを混合させておき，光形成されるゼラチンハイドロゲル内にそれらを包埋させている12)。一方，外側面には使用目的部位の要求に応じた機能を発揮する薬物が包埋されている。例えば，人工血管では，内腔面の内皮化を促進させることを目的として血管内皮増殖因子（VEGF）の包埋が行われている13)。また，ステントグラフトを用いた狭窄病変の血管形成術においては，平滑筋細胞の増殖抑制を目的として免疫抑制剤として知られているFK506や細胞外マトリックス分解酵素阻害剤（MMPI）などを，プラークの安定化・縮退化のためにはスタチンの包埋が検討されている14)。さらに，動脈瘤を閉鎖治療する場合には，瘤内での組織形成の促進を目的としてb-FGFなどの細胞成長因子が包埋されている。可視光に吸収帯域を有するキサンテン系色素を光ラジカル発生基として導入すると，可視光照射によってハイドロゲルを固定化させることも可能である。

高分子どうしのラジカル再結合反応によってハイドロゲルを作製する方法は，高分子上に発生したラジカルどうしが出会う必要が有り，また，不均化反応などによってラジカルが消失する可能性も有るため，必ずしも効率の良いゲル化方法とはいえない。そこで，光照射によって発生したラジカルを開始点としてラジカル重合によってゲルを固定化させる方法が考案された。そのための要素材料として，ゼラチンの側鎖にラジカル重合性基であるスチレン基を多数導入したスチレン化ゼラチン（ゼラチンマクロマー）が分子設計された15)。また，重合開始剤には歯科領域における光重合レジンの開始剤として実用化されているカンファキノンが選択された。これらの混合水溶液に歯科用のハロゲンランプからの青色光を照射すると，1分間ほどでゲルを生成した。可視光は紫外光に比べて物質透過性が極端に高いため，cmオーダーの立体光造形も可能であった。照射波長を400～500nmに制限して，さらに光量を低下させれば可視光に伴う熱の影響はほぼ無視できる。従って，紫外光硬化に比べて包埋物に与える障害性を大幅に低減させることができる。その結果，薬物以外にも，タンパク質やアデノウイルス，細胞

などをハイドロゲル内にほぼ非侵襲で包埋させることが可能となっている。薬物を包埋させたい場合，用いる光反応性高分子内の光反応性基の導入量や照射時間を変化させると，ハイドロゲル内の架橋量が調節できるので，ハイドロゲルからの薬物の放出挙動を制御することができる。

この方法を利用して，血管壁内への遺伝子導入デバイスへの応用が試みられている。すなわち，このスチレン化ゼラチンにLacZ遺伝子を組み込んだアデノウイルスを混合させて，ステントのストラット表面に光コーティングされた[16]。これを兎の頸動脈に留置すると，1ヵ月間にわたってLacZ遺伝子の発現が認められた。これはゲル内に包埋されたアデノウイルスがゲルの分解に伴って徐々に放出され，長期間にわたる持続的な遺伝子発現が起こったためと考えられる。また，このゼラチンマクロマーは細胞を包埋させることができるので，組織再生医療の分野において，*in situ* 硬化性の軟骨再生用のスキャホールド材，さらに組織接着剤への応用が検討されている。

6　光表面リビンググラフト重合による界面の3次元設計

高分子基材に高強度の紫外線を照射すると，γ線やグロー放電，コロナ放電処理した場合と同様に基材表面にパーオキサイドなどの高反応性基が生成する。従来この生成した高反応性種をラジカル重合開始種として表面グラフト重合が行われ，表面改質法として利用されてきた。しかし，この場合，複雑な形状を有するデバイスの内面を均質に，あるいは限定した特定部位のみを修飾することは困難である。また，高強度の紫外光照射などは基材内部の損傷や物性変化を伴う場合もある。さらに，一般にラジカルは多くのビニルモノマーの高分子化に有効な重合法であるが，フリーラジカルを連鎖てい伝体とする連鎖反応機構で進行するため，通常高分子表面への精密構造設計は極めて困難である。

ジチオカルバメートは，1980年代初めに大津らによって開発された光ラジカル重合開始剤の一種であり，これを用いるとスチレンやメタクリル酸メチルなどのビニルモノマーの光重合が見かけ上リビングラジカル重合機構で進行し，照射条件によってラジカル生長が制御できることが報告されている[17]。このジチオカルバメートは重合開始剤（initiator），連鎖移動剤（transfer agent），停止剤（terminator）として機能することから，イニファタ（iniferter）と名付けられている。イニファタの一種であるベンジルジチオカルバメートに紫外光を照射すると，ベンジル炭素と硫黄間の結合が可逆的に解離され，ベンジルラジカル（$Ph\text{-}CH_2\cdot$）とジチオカルバミルラジカル（$\cdot SCSNEt_2$）が生成する。ここで，ベンジルラジカルのみがビニルモノマーの重合開始剤として機能し，重合末端では常にジチオカルバミルラジカルによるキャッピングが起こる。従って，光照射時にのみモノマーを重合させることができ，照射条件（強度，時間）や溶液条件（モノマー濃度，モノマー/開始剤比）を選択すれば多くのモノマーに対してリビング

的にラジカル重合を進行させることができる。つまり，重合鎖長は照射時間によって，鎖の成分はモノマーの組成によって，また重合領域は照射領域によって規制することが可能であり，バイオマテリアル界面の3次元での精密設計に応用されている。

基材としてジチオカルバメート基を側鎖に有するポリスチレンのコーティング膜を用いて，モノマー溶液中で紫外光照射を行うと，基材表面に導入されたジチオカルバミル基から重合が起こる。水晶発振子（QCM）上で行うと，グラフト重合は光の照射時のみに限定して起こり，重合量はモノマー濃度と照射強度の増大に伴って増大し，さらに，重合速度はモノマーのQ値が約0.8で極大値をとることが報告されている[18]。従って，モノマー種に応じて重合挙動をある程度推定することができる。

照射領域はフォトマスクなどでサブミクロンレベルの精度で容易に規制することができるため，任意の微細領域のみで光重合を起こさせることができる。これを利用してWardらは，光リソグラフ技術を組み合わせることで，5～80ミクロン幅の構造物を作製している[19]。また，照射領域を段階的に横にずらせながらモノマー種を変化させて照射を繰り返すと，領域ごとに連続的に物性の異なる高分子鎖をグラフトすることが可能であり，異種高分子鎖のグラフトパターン表面が作製された[20]。デバイスの生体適合性は最表面層の性状と形状に依存することが知られており，目的に応じた生体適合性を獲得するためには適切な性状を有する高分子種を選択する必要がある。マイクロパターン化表面では微細領域ごとに化学組成を任意に変化させることが可能である。従って，タンパク質や細胞との相互作用の表面化学依存性を一度に同じ条件下で評価することができる。このパターン化表面を利用してAndersonらは，細胞応答性に関して表面化学依存性を調べており，マクロファージのアポトーシスは非イオン性，またはアニオン性の親水性表面において増加することなどが明らかにされ，医療デバイスの表面化学の最適化設計が検討されている[21]。また，照射量を調節することでグラフト重合のZ軸，つまり高さ方向の制御が可能である。例えば，一定時間毎に照射領域を段階的に，あるいは連続的に拡大させることで，それぞれ数十ナノメートルの段差を有する階段状の構造物や，高低差が数百ナノメートルのスロープ状の3次元構造物が作製されている。その他，グラフト鎖の密度や表面ブロック化（積層化）も実現されている[22]。

一方，イニファタのマルチ化とグラフト鎖長の制御能を組み合わせることによってグラフト鎖の分岐度と鎖長を自由に設計することが可能となり，多分岐型グラフト鎖が作製されている[23]。すなわち，基材表面に導入したジチオカルバミル基から光重合させる際にコモノマーとしてクロロメチルスチレンを共重合させておく。幹として形成されたグラフト鎖の側鎖に導入されたクロライド基は容易にジチオカルバミル基に置換できる。この後，先と同様に光共重合とジチオカルバミル化を繰り返すことで，枝さらに小枝となる分岐グラフト鎖が逐次的に形