1 脳卒中と血圧管理

—LIFE, ALLHAT, VALUE, SCOPE, PROGRESS, ACCESS—

はじめに

以下に最近5年間に発表された脳卒中の発症，再発を含む心血管イベント発症予防を目的とした大規模臨床試験の主なものを取りあげその概要を説明する．脳卒中の発症予防における降圧治療の重要性はすでに10年以上前から複数の臨床介入試験により確立されており，現在の議論の的は脳卒中予防における降圧薬の種類の間に差があるかどうかである．また，脳卒中再発予防における降圧治療のエビデンスは本稿で取り上げるPROGRESS研究で初めて確立されたものである．

1 脳卒中の発症予防

1. LIFE(Losartan Intervention for Endpoint Reduction in Hypertension)[1)]

55～80歳の高血圧患者で，心電図上左室肥大を有する9,193例を対象として，アンジオテンシンII受容体拮抗薬(ARB)ロサルタンとβ遮断薬アテノロールの2群に割り付け4年以上経過観察し，心血管死，心筋梗塞，脳卒中の発症を複合一次エンドポイントとした．降圧治療開始後，両群とも速やかな降圧がみられ，最終観察時点の血圧がロサルタン群で144.1/81.3 mmHg，アテノロール群で145.4/80.9 mmHgと差がみられなかったが，ロサルタン群では複合一次エンドポイントが13％低減し，なかでも致死性，非致死性脳卒中の発症が25％低下した．同程度の降圧効果が得られた場合，ARBロサルタンはβ遮断薬

に比し，心血管疾患，なかでも脳卒中発症予防により有効であることが示唆された．

2. ALLHAT(Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial)[2]

55歳以上で他の心血管危険因子を一つ以上有する高血圧症例33,357例(30％以上が黒色人種)を対象とし，利尿薬クロルタリドン(15,255例)，Ca拮抗薬アムロジピン(9,048例)，アンジオテンシン変換酵素(ACE)阻害薬リシノプリル(9,054例)に割り付け，一次エンドポイントを冠動脈疾患，二次エンドポイントを全死亡，脳卒中，血行再建術を含む冠動脈疾患，末梢動脈疾患，心不全を含む心血管イベントとし，平均4.9年追跡調査した．試験終了時の血圧は，クロルタリドン群133.9/75.4 mmHg，アムロジピン群134.7/74.6 mmHg，リシノプリル群135.9/75.4 mmHgと，クロルタリドン群で他の群に比しわずかではあるが有意に収縮期血圧が低下していた．一次エンドポイントである冠動脈疾患の発症リスクは3群間で差がなかったが，リシノプリル群はクロルタリドン群に比し全心血管イベント，脳卒中，心不全の発症リスクが有意に高かった．本研究結果より降圧薬としての利尿薬の有用性が改めて見直された印象がある．

EBM

3. VALUE(Valsartan Antihypertensive Long-term Use Evaluation)[3]

50歳以上の高血圧症患者で，糖尿病，喫煙，高脂血症，左室肥大の合併または心血管疾患の既往を有する高リスク症例15,245例を対象とした．ARBバルサルタン治療群もしくはアムロジピン治療群に割り付け，一次エンドポイントを心イベント，二次エンドポイントは心筋梗塞，心不全，脳卒中とし平均4.2年追跡調査した．治療開始後血圧

は両群とも低下したが，最初の1カ月ではアムロジピン群が4.0/2.1 mmHg有意に低く，以後も試験期間中2 mmHg程度アムロジピン群で低値であった．一次エンドポイントは両群で差がみられなかったが，二次エンドポイントではアムロジピン群で心筋梗塞の発症リスクが有意に低く，脳卒中の発症リスクも低い傾向が観察された．本試験は，もともと血圧値を同程度に保った場合のバルサルタンとアムロジピンの心血管疾患発症リスクの差をみるのが目的であったが，結果的にアムロジピン群で有意に血圧が低くなったため，脳卒中だけでなく心筋梗塞発症予防における降圧治療自身の重要性を示すものとなり，降圧を超えた両薬剤の臓器保護効果についての結論は得られなかった．

4．SCOPE(Study on Cognition and Prognosis in the Elderly)[4]

70～89歳の高齢者で収縮期血圧が160～179 mmHgまたは拡張期血圧が90～99 mmHgの4,964例を，ARBカンデサルタン群とプラセボ群に割り付けて一次エンドポイントを心血管イベント，二次エンドポイントを心血管死，致死性および非致死性心筋梗塞，脳卒中，MMSE(Minimental State Examination)の変化とし，3.7年追跡した．両群とも必要に応じて降圧薬の追加投与が認められ，その結果，治療開始後カンデサルタン群，プラセボ群ともに平均20/10 mmHg程度の降圧がみられた．カンデサルタン群ではプラセボ群に比し3.2/1.6 mmHgしか血圧値は低下していなかったにもかかわらず，非致死性脳卒中の発症率が28％も低減していた．一次エンドポイントでは両群間に差がなかった．レニン・アンジオテンシン系の抑制に降圧を超えた脳保護もしくは脳血管保護作用があることが示唆された．

2　脳卒中の再発予防

1. PROGRESS(Perindopril Protection Against Recurrent Stroke Study)[5]

過去5年以内に脳卒中または一過性脳虚血発作の既往のある6,105例を対象とし，ACE阻害薬ペリンドプリルと必要に応じて利尿薬インダパミドを加える治療群とプラセボ群に割り付け，一次エンドポイントを脳卒中発症，再発，二次エンドポイントを全心血管イベント，心血管死として4年間追跡調査した．観察期の血圧は両群とも147/86 mmHgであったが，試験開始後治療群ではプラセボ群に比し9.0/4.0 mmHg血圧が低下し，脳卒中の発症・再発率を28％，全心血管イベントを26％いずれも有意に低下させた．ペリンドプリルにインダパミドを併用した群では脳卒中発症率の低下は43％に及び有意であったが，インダパミドを併用しなかった群ではプラセボ群と脳卒中発症率に差がみられなかった．脳卒中の再発予防に降圧療法の有効性を初めて確立した点，国際的な大規模臨床試験にわが国からも多数の症例が登録された点で注目された研究である．

2. ACCESS(Acute Candesartan Cilexetil Evaluation in Stroke Survivors)[6]

高血圧を呈する急性期虚血性脳卒中症例339例を対象として，急性期からARBカンデサルタンを投与する群とプラセボ群に割り付け，7日目以後は両群とも高血圧を呈する例ではカンデサルタン投与を行うとし，3カ月後の機能予後，1年後の死亡率，心血管イベント発症率を両群間で比較した．両群間では試験開始後血圧値に有意な差はみられなかった．急性期からカンデサルタンで治療を開始した群では，1年後の死亡率が低下する傾向がみられ，心血管イベント(脳卒中と心イベントの総和)発症リスクを50％

EBM

近く有意に低下させた．脳梗塞急性期には降圧しないのが原則であるが，本研究の対象となった運動麻痺を有し内頸動脈に高度狭窄病変を有さない症例では，急性期から降圧薬ARBを投与することが有用である可能性が示された点で注目された研究結果である．

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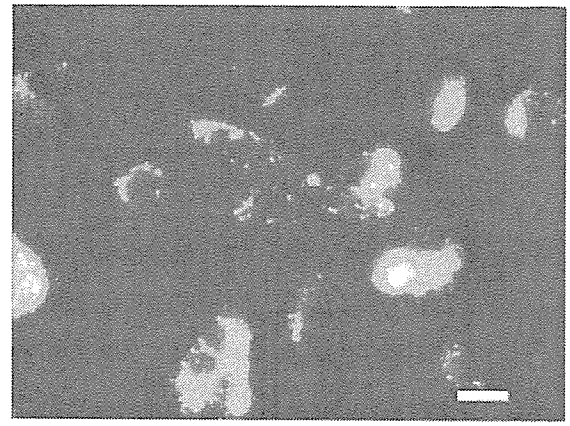
Summary: An amphiphilic poly(*N*-propargylamide) with galactose and lauryloyl groups was synthesized by copolymerization of the corresponding *N*-propargylamide monomers using a Rh catalyst. The obtained copolymer formed a one-handed helical conformation and molecular aggregates in water. The observations by fluorescence microscopy in a cell culture experiment in the presence of dye-labeled copolymer indicated that the copolymer was incorporated into the cells.



Localization of rhodamine B-labeled copolymer **8** in human aortic endothelial cells (fluorescence image).

Amphiphilic Poly(*N*-propargylamide) with Galactose and Lauryloyl Groups: Synthesis and Properties

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Introduction

Synthesis of polymers having sugar residues, so-called glycopolymers, has been widely investigated to seek biological applications because of their versatile functions.[1] It has been demonstrated that these glycopolymers can bind specifically to carbohydrate-recognition proteins, toxins, viruses, and cells, and, thus, these polymers can be utilized as cell culture substrates with specific cell recognition sites, as well as in targeting drug delivery systems.[2] The clustered saccharide ligands conjugated to the polymeric main chains are involved in these specific recognition processes. Most of the previously prepared glycopolymers have been based on a flexible polymer backbone, such as polystyrene and polyacrylamide.[3,4] This flexible nature of the glycopolymers causes the disordered orientation of the sugar residues in the glycopolymers. Regular orientation of the sugar residues is necessary for efficient interaction between the glycopolymers and receptor molecules. In this sense, the spatially regulated orientation of the sugar residues should be realized by attaching the sugar residues to a polymer backbone with a rigid conformation. The sugar residues attached to the rigid polymer backbone may give rise to the ordered orientation that can improve the molecular recognition of sugar residues by specific cell receptors. This is because the spatial regulation of the sugar residues is significant in molecular recognition as well as the chemical structure of the sugar molecules.[5]

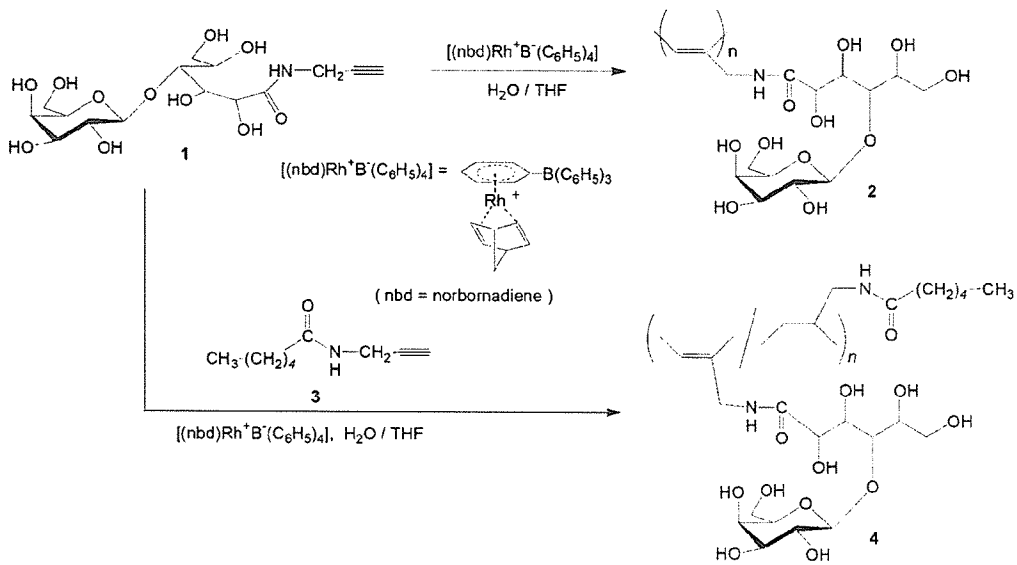
From the above viewpoints, rigid glycopolymers with π-conjugated polymer backbones would be a novel type of bio-inspired polymers, which could exhibit multiple valence states and interact specifically and firmly with targets such as cell surface receptors and biomacromolecules. In some previous works, rigid glycopolymers having various π-conjugated main chains, such as polythiophene,[6] poly(*p*-phenylene ethynylene),[7] polyisocyanide,[8] polyaniline,[9] poly(*p*-phenylene vinylene),[10] and poly(phenylacetylene),[11] have already been synthesized.[11] In the series of these studies, we reported the synthesis of poly(*N*-propargylamide) (**2**) with sugar residues. It contained a *cis*-polyacetylene main chain and was obtained by the rhodium-catalyzed polymerization of a *N*-propargylamide monomer (**1**) that had a galactose residue (Scheme 1).[12] Since polymerizations of the *N*-propargylamide monomers having various substituted groups using Rh catalyst have been widely reported to produce the corresponding poly(*N*-propargylamide) derivatives with *cis*-isomers,[13] we also investigated the copolymerization of **1** with *N*-propargylamide derivative **3** having a hexanoyl group to produce the amphiphilic glycopolymer **4**, as shown in Scheme 1. We tested the solubility of copolymer **4** in various solvents to confirm whether the copolymer exhibits an amphiphilic property. Although the homopolymer **2** is insoluble in common organic solvents, the copolymer **4** can be dissolved in some polar organic solvents, such as dimethyl sulfoxide (DMSO) and *N,N*-dimethylformamide (DMF), as well as in aqueous medium. However, the copolymer still exhibits a hydrophilic nature rather than an amphiphilic nature. We assumed that insufficient amphiphilicity of copolymer **4** could be attributed to poor hydrophobic property of the hydrophobic part.

In this study, we chose a more hydrophobic monomer; *N*-propargylamide monomer **5** having a longer alkyl chain, i.e., the lauryloyl group, as the hydrophobic part of the amphiphilic copolymer. The monomer **5** was copolymerized with **1** in the presence of Rh catalyst to give the corresponding amphiphilic copolymer **6** (Scheme 2). The resulting copolymer **6** can be expected to have the ability to conduct molecular aggregation in water, which is driven by intermolecular and intramolecular association of the hydrophobic lauryloyl groups.

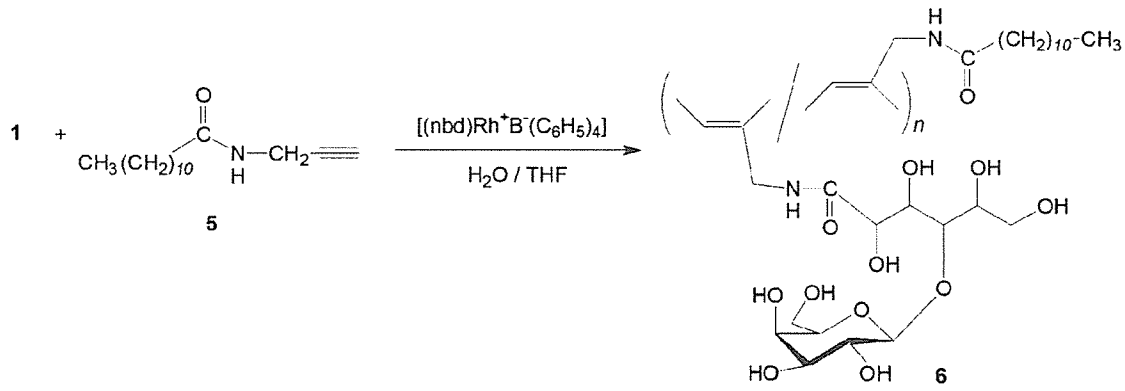
We believe that such molecular aggregates of the amphiphilic copolymer should play a significant role in the field of targeted drug delivery. The idea is supported by the following characteristics of the molecular aggregates:

1) Drug carriers with nanometer dimensions can be obtained by molecular aggregation of amphiphilic copolymers.
2) Nano-sized aggregates can remain in the bloodstream for an extended period because of the size-dependent uptake in the reticuloendothelial system.
3) Drug molecules with hydrophobic natures can be loaded into hydrophobic milieus formed by the association of hydrophobic long alkyl chains; otherwise the drug molecules could be directly attached to the constituent monomer molecules of the copolymer.
4) Sugar residues of the copolymer can function as recognition sites for target cells and tissues as well as providing the hydrophilic character of the amphiphilic copolymer.

Therefore, we studied the *in vitro* cell uptake of the molecular aggregates of the amphiphilic copolymer. For this purpose, the fluorescent marker, rhodamine B, was introduced into the amphiphilic copolymer. In this article, we report the synthesis of the amphiphilic



Scheme 1. Polymerization of **1** and copolymerization of **1** with **3**.



Scheme 2. Copolymerization of **1** with **5**.

poly(*N*-propargylamide) **6** by Rh-catalyzed copolymerization, evaluation of its molecular aggregation in water [gel permeation chromatography (GPC) characterization, scanning electron microscopy (SEM) observation, and dynamic light scattering (DLS) measurement] and secondary conformation [circular dichroism (CD) spectra], and cell uptake of the nanoaggregate of the rhodamine-labeled amphiphilic copolymer (fluorescence microscopy).

Experimental Part

Materials

Monomer **1** and catalyst (nbd)$Rh^+B^-(C_6H_5)_4$ were prepared according to the literature.[12,14] Tetrahydrofuran (THF) used as polymerization solvent was purified by distillation. Other reagents and solvents were used as received without further purification.

Synthesis of Monomer 5

Monomer **5** was synthesized by a method similar to that used for **3**.[15] Under argon, *N*-propargylamine hydrochloride (0.915 g, 10.0 mmol) was dissolved by slight warming in anhydrous acetonitrile (12.0 mL), and triethylamine (3.35 mL, 24.0 mmol) was added to the solution at room temperature. Then a solution of lauryloyl chloride (2.36 mL, 10.0 mmol) in anhydrous acetonitrile (12.0 mL) was added dropwise to the solution. After the mixture was stirred for 2 h, the reaction solution was concentrated by evaporation. The residue was dissolved in ethyl acetate and the solution was washed successively three times with 2 mol $\cdot L^{-1}$ hydrochloric acid and with saturated $NaHCO_3$ aqueous solution. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated. The residue was subjected to column chromatography on silica gel (hexane:ethyl acetate = 4:1, v/v) to isolate **5** (1.38 g, 5.82 mmol) in 58.2% yield.

^{1}H NMR ($CDCl_3$): $\delta = 0.88$ (t, $J = 7.2$ Hz, CH_3, 3H), 1.28 [m, $CH_3(CH_2)_8$, 16H], 1.62 (m, $CH_2CH_2C{=}O$, 2H), 2.19 (t, $J = 7.8$ Hz, $CH_2C{=}O$, 2H), 2.23 (t, $J = 2.4$ Hz, H−C≡C, 1H), 4.05–4.06 (m, CH_2N, 2H), 5.57 (s, NH, 1H).

Synthesis of Monomer 7

Under argon, triethylamine (0.836 mL, 6.00 mmol) was added to a solution of *N*-propargylamine hydrochloride (0.549 g, 6.00 mmol) and rhodamine B (1.92 g, 4.0 mmol) in anhydrous methanol (10.0 mL) at 0 °C. To the solution was added 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (1.15 g, 6.00 mmol) as a condensing agent at 0 °C and the mixture was stirred for 17 h at room temperature. The precipitated material was isolated by filtration and dried under the reduced pressure to give **7** (0.472 g, 0.915 mmol) in 23.0% yield.

^{1}H NMR ($CDCl_3$): $\delta = 1.16$ (m, CH_3, 12H), 1.77 (t, $J = 2.4$ Hz, H−C≡C, 1H), 3.33 (m, NCH_2CH_3, 8H), 3.95 (d, $J = 1.8$ Hz, $CH_2C{\equiv}$, 2H), 6.26–7.93 (m, aromatics, 10H).

Copolymerization of 1 with 5

A typical copolymerization procedure was as follows (entry 1, Table 1). Under argon, a solution of **5** (0.0356 g, 0.150 mmol) in THF (0.90 mL) and a solution of catalyst (0.00630 g, 0.0125 mmol) in THF (0.90 mL) were added to a solution of **1** (0.0397 g, 0.100 mmol) in water (0.20 mL) in this order at 30 °C. After the mixture was stirred at 30 °C for 140 min, the reaction mixture was concentrated by evaporation and dried under reduced pressure. The residue was dissolved in a small amount of DMSO and the solution was poured into a large amount of methanol to precipitate the polymeric product. The precipitate was isolated by filtration and was dried under reduced pressure to give **6** (0.0588 g) in 78.1% yield.

^{1}H NMR (DMSO-d_6): $\delta = 0.84$ (CH_3), 1.21 [$CH_3(CH_2)_8$], 1.45 ($CH_2CH_2C{=}O$), 2.15 ($CH_2C{=}O$), 3.0–3.9 [−CH(O-D-gal)−CH(OH)−CH_2OH, $=CCH_2-$, H2–H6 of D-gal], 4.04 [C(=O)CH(OH)CH(OH)−], 4.29 [C(=O)CH(OH)− and H1(β) of D-gal], 4.55, 4.79, 5.22 (OH), 6.11 (−CH=C−), 7.93 (NH).

Copolymerization of 1, 5, and 7

Under argon, a solution of **5** (0.0285 g, 0.120 mmol) and **7** (0.0929 g, 0.180 mmol) in THF (0.90 mL) and a solution of catalyst (0.0166 g, 0.0330 mmol) in THF (0.90 mL) were

Table 1. Results for copolymerization of **1** with **5** with Rh catalyst in THF–water (9:1) solvent.

Entry	Feed ratio[a]	Time	Yield[b]	Unit ratio[c]	$\overline{M}_n$[d]	$\overline{M}_w/\overline{M}_n$[d]	$[\alpha]_D$[e]
	1:**5**	min	%	**1**:**5**			degrees
1	1.0:1.5	140	78.1	1.0:0.92	9 100	1.30	
2	1.0:1.0	195	62.5	1.0:0.88	6 200	1.63	
3	1.0:0.67	240	57.0	1.0:0.35	5 200	1.60	+96.8
4	1.0:0.50	260	68.9	1.0:0.32	9 600	1.64	+142.3
5	1.0:0.33	200	69.9	1.0:0.26	7 100	1.50	+173.6
6	1.0:0.20	210	78.1	1.0:0.17	9 700	1.30	+185.6

[a] [Catalyst]/[**1** + **5**] = 0.05, reaction temperature: 30 °C.
[b] Fraction insoluble in methanol.
[c] Determined from ^{1}H NMR spectra.
[d] Determined by GPC with water as eluent using pullulan standards, sample concentration ≈ 0.1 mg · mL^{-1}.
[e] Measured by polarimetry in water, c = 1.0 g · dL^{-1} at 20 °C.

added to a solution of **1** (0.143 g, 0.360 mmol) in water (0.20 mL) in this order at 30 °C. After the mixture was stirred at 30 °C for 18 h, the reaction mixture was concentrated by evaporation and dried under reduced pressure. The residue was dissolved in a small amount of DMSO and the solution was poured into a large amount of methanol to precipitate the polymeric product. The precipitate was isolated by filtration and dried under reduced pressure to give **8** (0.164 g) in 62.0% yield.

^{1}H NMR (DMSO-d_6 + D_2O): δ = 0.93 ($CH_3CH_2CH_2$), 1.16 (CH_3CH_2N), 1.25 [$CH_3(CH_2)_8$], 1.50 ($CH_2CH_2C{=}O$), 3.0–4.5 (sugar protons and =C–CH_2), 6.16 (HC=), 7.0–8.0 (aromatics).

Cell Culture Experiment

Human aortic endothelial cells (HAECs) were purchased as cryopreserved samples of third passage (Lot: 3F1346) from Cambrex (Wakersville, MD, USA). The HAECs were subcultured once and stored in liquid nitrogen until cell culture experiment. The HAECs used in the experiment were fourth passage. Each well of a 12-well plate of polystyrene (Iwaki) was filled with 1 mL of a supplemented culture medium (EGM-2; Lot: 08103123, Cambrex) and equilibrated at 37 °C in a humidified incubator under 5% CO_2 for 30 min before cell seeding. After the frozen cells were thawed at 37 °C, 10 μL of the cell suspension was seeded in each well. The initial cell density was 2.2×10^3 cells · cm^{-2}. Cell viability assessed by the trypan blue exclusion test was 83% for the cell suspension. The cell seeded plates were placed in a humidified incubator at 37 °C under 5% CO_2. The HAECs were cultured for 48 h. Cell culture mediums were replaced with fresh medium 24 h after cell seeding. At 48 h after cell seeding, cell culture mediums were each replaced with an aqueous suspension of copolymer **8**. Then the HAECs were cultured in the polymer suspension for 1, 6, and 24 h in a humidified incubator at 37 °C under 5% CO_2 to study cellular uptake of nanoaggregates of copolymer **8**. For fluorescence microscopy observation, the cells were fixed by immersion in 10% formaldehyde neutral buffer solution (Nacalai Tesque) at room temperature (22 °C) for 15 min and were washed three times with phosphate-buffered saline (PBS; Gibco). Fluorescence images of the cells were taken with a fluorescence microscope (IX71; Olympus) equipped with a CCD camera (DP70; Olympus). Fluorescence intensity of the incorporated copolymer **8** was measured by integrating the fluorescence intensity observed at each pixel of the fluorescence images using image analysis software (Fluoview ver. 5.0; Olympus).

Measurements

NMR spectra were recorded on a JEOL ECA 600 spectrometer. Optical rotations were measured with a Jasco P-1030 digital polarimeter. GPC analyses were performed by using a TOSOH 8012 with refractive index detection under the following conditions: Shodex Asahipak GF-310HQ column with water as eluent at a flow rate of 0.5 mL · min^{-1}. The calibration curve was obtained using pullulan standards. CD and UV-vis spectra were measured in a quartz cell (thickness 1 cm) at room temperature using a Jasco J-820 spectropolarimeter and Shimadzu UV160A spectrophotometer, respectively. The SEM images were obtained using a Hitachi S-4100 electron microscope. The DLS measurement was performed on a Zetasizer 3000 (Malvern Instruments). Fluorescence spectra were obtained on a fluorescence spectrometer (Shimadzu) using a quartz cuvette (1-mm path length).

Results and Discussion

Copolymerization of 1 with 5

The polymerization of monosubstituted acetylene derivatives has been widely investigated using Rh complex catalysts, which enables stereoselective synthesis of the corresponding polyacetylenes of the *cis*-isomers.[16] As

already reported in our previous publication, **1** was polymerized using (nbd)$Rh^+B^-(C_6H_5)_4$ as the catalyst at around 25 °C to 50 °C in a mixed solvent of THF and water (9:1, v/v).[12] In this study, we performed the copolymerization of **1** with **5** under similar conditions. The copolymerization with various feed ratios of **1** to **5** was carried out using the Rh catalyst (5.0 mol-% for **1** + **5**) at 30 °C in a THF-water mixed solvent under argon. After polymerization, the resulting mixture was concentrated and then dissolved in DMSO. The solution was poured into a large amount of methanol to precipitate the polymeric product. The precipitate was isolated by filtration and dried under reduced pressure to give the copolymer **6** (Table 1). The copolymer was soluble in water and DMSO, and its $\overline{M}_n$ value was estimated by GPC analysis with water as eluent using pullulan standards. Figure 1a shows the ^{1}H NMR spectrum of the copolymer (entry 1, Table 1) measured in DMSO-d_6. The signals due to the sugar and alkyl protons are observed at around δ 3.0–4.3 (signals **e–h**) and δ 0.84, 1.21, 1.45, 2.15 (signals **a–d**), respectively. In addition to these signals, signal **i** ascribed to the main-chain proton of –CH=C– appears centered at δ 6.11. The chemical shift of this signal realistically corresponds to the *cis*-isomer. Furthermore, there is no signal due to the *trans*-isomer at lower magnetic field from the *cis*-signal. The NMR results support structure **6** as that of the copolymer, which is mainly composed of *cis*-isomer. The unit ratio of the copolymer is calculated by the integrated ratio of signal **a** and signal **i**.

Table 1 shows the copolymerization results obtained by the various feed ratios of **1** to **5**. The yields and the $\overline{M}_n$ values are 57.0–78.1% and 5 200–9 700, respectively. The unit ratios of **5** in the copolymers increase with increasing molar ratios of **5** in the feeds. In all cases, however, the ratios of **5** in the copolymers are lower than those in the feeds. This is probably because the copolymers with higher

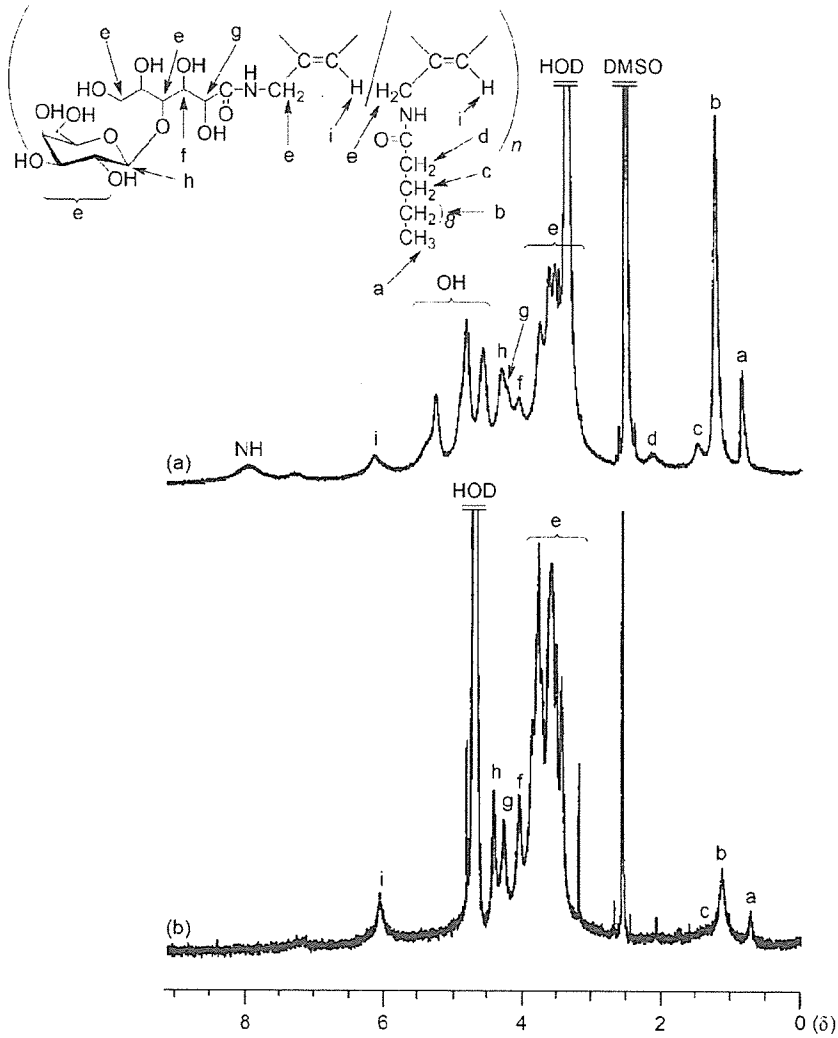


Figure 1. ^{1}H NMR spectra of copolymer **6** (entry 1, Table 1) in DMSO-d_6 (a) and D_2O (b).

contents of the unit **5** are lost as methanol-soluble fractions during the isolation procedure. The optical rotations of the copolymers with the higher contents of the sugar units were larger than those with the lower contents.

Formation of Molecular Aggregates in Water

When the copolymerization was followed by thin-layer chromatography (TLC) on silica gel (methanol: chloroform = 2:1 for **1**; hexane: ethyl acetate = 1: 1 for **5**), **1** appeared to be consumed at the early stage of the reaction; subsequently, the consumption of **3** at a later stage was confirmed. This indicated that **6** had the block copolymeric sequence between the unit **1** and the unit **5** rather than in random style. In fact, the intensities of the alkyl signals **a**–**d** in the ^{1}H NMR spectrum of **6** in D_2O (Figure 1b) are obviously lower than those of the same copolymer measured in DMSO-d_6 (Figure 1a). The NMR results suggest formation of micelle-like aggregates having the outer hydrophilic sugar residues and the inner hydrophobic lauryloyl groups in water, and this was attributed to the block copolymeric sequence. The formation of molecular aggregates of **6** in water was also confirmed by the GPC measurements on aqueous solutions of **6** (entry 5, Table 1, the unit ratio of **1** to **5** = 1.0:0.26) ranging in concentration from 0.05 to 9.0 mg · mL^{-1}. Figure 2 shows the relations of the $\overline{M}_n$ values to the sample concentrations in the GPC experiments. The $\overline{M}_n$ values increase from ca. 6300 to ca. 11000 for concentrations higher than 0.70 mg · mL^{-1}. These data suggest the formation of molecular aggregates for the higher concentrations of **6** in water. The molecular aggregates of **6** were directly observed by SEM. The SEM image of the spin-coated sample from the aqueous solution of **6** (entry 4, Table 1, the unit ratio of **1** to **5** = 1.0:0.32) on aluminium plate (Figure 3) shows the particle-type molecular aggregates with average diameters of 20–40 nm. The particle sizes were also confirmed by DLS measurement. The mean particle diameter of the sample shown as entry 4 in Table 1 was 85.2 ± 14.1 nm. The difference in the aggregate sizes obtained by SEM and DLS can be attributed to the difference in the sample condition: dry for SEM and wet for DLS.

Secondary Conformation of 6

We already reported in our previous report that the CD spectrum of the homopolymer **2** in water showed the positive Cotton effect at 330 nm corresponding to the main-chain UV-vis absorption.[12] This indicated the possibility for formation of a one-handed helical conformation in the main chain of **2**. In this study, the CD analysis was also performed to reveal the secondary conformation of copolymer **6**. Figure 4 shows the CD spectra of **6** (entry 5, Table 1, unit ratio of **1** to **5** = 1.0:0.26) in comparison with those of copolymer **4** (unit ratio of **1** to **3** = 1.0: 0.39) measured in DMSO and water at room temperature. The CD spectrum of **6** in DMSO (Figure 4a) shows the positive Cotton effect at 360 nm, corresponding to the main-chain UV-vis absorption. The positive Cotton effect also appeared at this region in the CD spectrum in water (Figure 4b), although its intensity was lower than that in DMSO. It has been reported that the helical structure of poly(*N*-propargylamide)s is stabilized by the intramolecular hydrogen bonds between the pendant amide groups.[17] In polar solvents such as DMSO and water, therefore, the hydrogen bonds are readily broken to effect destabilization of the helical structure. The helical conformation of **6** in polar solvents is probably stabilized by the bulky substituents in the side chains of sugar and lauryloyl groups. These bulky groups shield the hydrogen bonds from the solvents, which consequently stabilizes the helical structure. This reasoning is also supported by comparison of the CD spectra of copolymer **4** (gray lines in

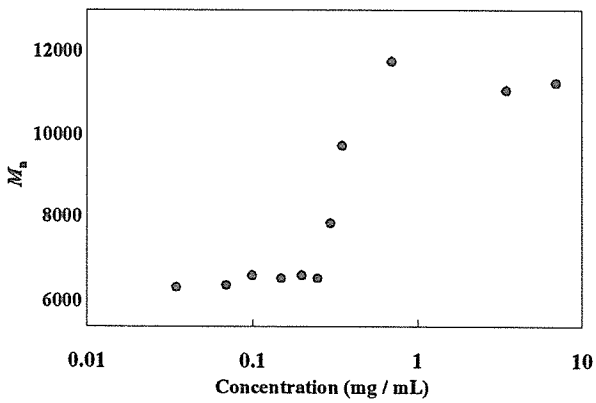


Figure 2. $\overline{M}_n$ values versus sample concentrations in GPC measurements with water as eluent (entry 5, Table 1).

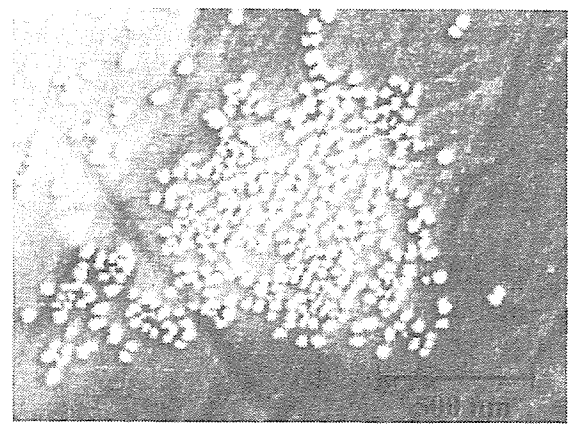


Figure 3. SEM image of **6**: the sample was prepared by spin coating of the dispersed solution of **6** (entry 4, Table 1) in water.

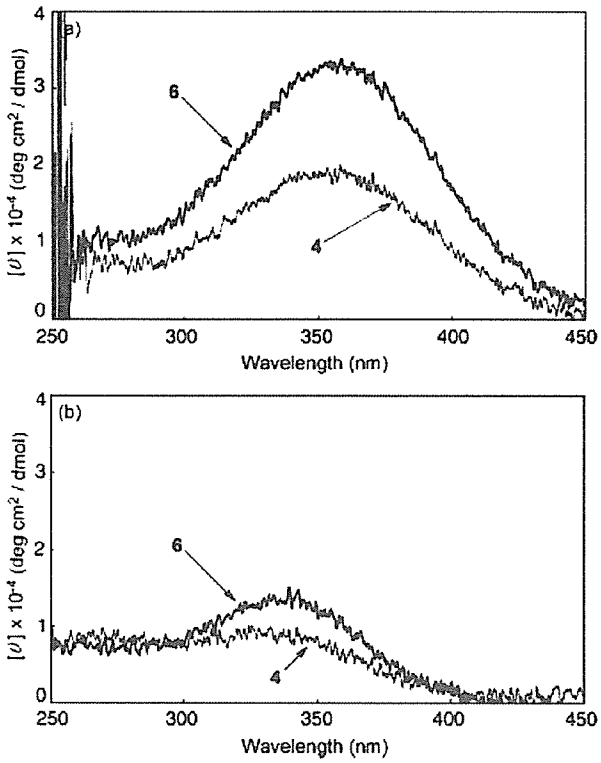
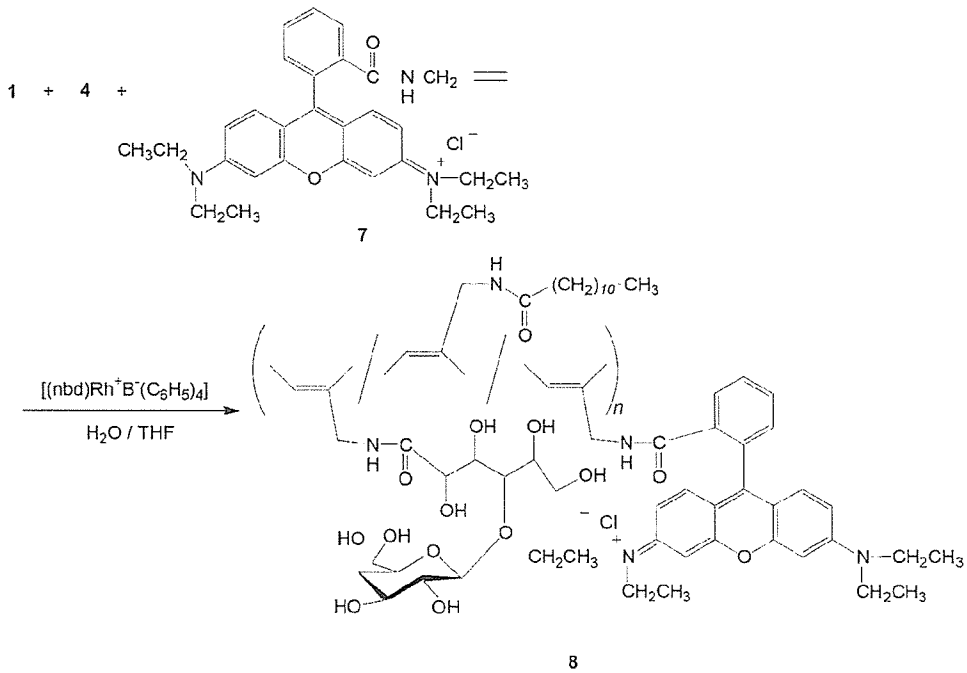


Figure 4. CD spectra of **6** (entry 5, Table 1, unit ratio; **1**:**5** = 1.0:0.26) and **4** (unit ratio; **1**:**3** = 1.0:0.39) in DMSO (a) and water (b) ($c = 0.2$ mmol · L^{-1}).

Figure 4a, b), which has the less bulky hexanoyl groups. The CD spectra of **4** show weaker Cotton effects than those of **6**, indicating the stabilization of the helical conformation as a result of the bulkiness of the pendant groups.

Cellular Uptake of Amphiphilic Copolymer 8

To evaluate cell uptake of the copolymer by fluorescence microscopy, the rhodamine B dye moiety was introduced into the amphiphilic copolymer. First, an *N*-propargylamide derivative **7** having a pendant rhodamine B moiety was prepared by condensation of *N*-propargylamine hydrochloride with rhodamine B in the presence of a condensing agent (1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride) in methanol. Then, the isolated **7** was copolymerized with **1** and **5** under conditions similar to those described above (Scheme 3). Although **7** did not have homopolymerizability by Rh catalyst, the unit from **7** was slightly incorporated into the resulting terpolymer by the copolymerization. The existence of the rhodamine B moiety in the obtained terpolymer **8** was confirmed by appearance of the signals due to methyl protons of N-CH_2CH_3 as well as the aromatic protons in the ^{1}H NMR spectrum of the product. However, the intensities of the signals were too weak to determine the exact content of the dye moiety in the copolymer by the integration ratio. For comparison, hydrophilic copolymer **9** was synthesized by copolymerization of monomer **1** with monomer **7** using Rh catalyst (Figure 5).



Scheme 3. Terpolymerization of **1**, **5**, and **7**

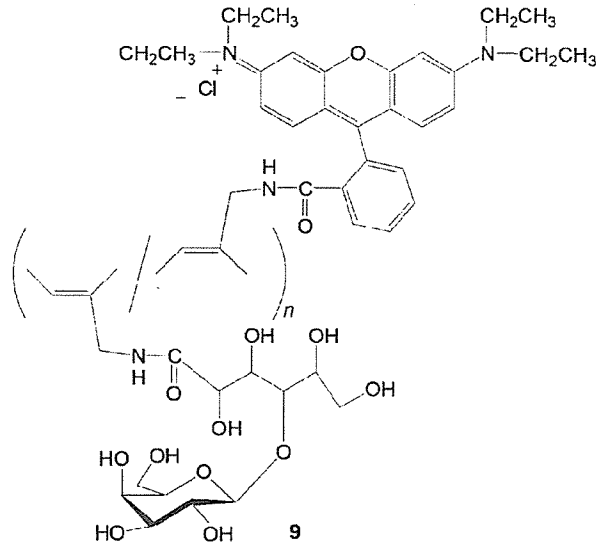


Figure 5. Structure of copolymer **9**.

The cell uptake of terpolymer **8** was studied by culturing human aortic endothelial cells (HAECs) in a culture medium containing the terpolymer **8**. Ten milligrams of **8** was suspended in 10 mL of culture medium EGM-2 and stirred for 3 h at room temperature. The suspension of **8** was sonicated at 25 W and 40 kHz for 5 min in an ultrasonic bath. The sonication was repeated twice. The obtained suspension was filtered through membrane filters with the pore sizes of 0.45 μm and 0.2 μm for sterilization. An aqueous solution of polymer **9** was prepared by following the above procedure. To evaluate the concentration of **8**, a calibration curve (data not shown) was obtained by using the aqueous solution of **9** (1 mg · mL^{-1}) as a standard polymer sample to relate concentration to fluorescence intensity. The concentration of **8** was estimated to be 0.09 mg · mL^{-1} by using the calibration curve. The DLS measurement revealed that the polymer aggregates (nanoparticles) of **8** have a mean diameter of 114.9 ± 32 nm in a culture medium containing 10% bovine serum. HAECs were exposed to the polymer aggregates of **8** while they were cultured in the culture medium containing the amphiphilic polymer **8**. After the prescribed period of culture, 1, 6, or 24 h, the HAECs were fixed in 10% formaldehyde neutral buffer solution for microscope observation. Figure 6 shows phase contrast (a), fluorescence (b), and merged (phase contrast + fluorescence) (c) images of HAECs after 24 h of incubation. The merged image demonstrates that red fluorescent light of rhodamine B was emitted from the sites where HAECs were located. This indicates that the polymer aggregates of **8** were incorporated into HAECs. The fluorescence images at each time of incubation are shown in Figure 7. The fluorescence images get brighter with the incubation time. To quantitatively evaluate cell uptake of nanoaggregates, fluorescence intensity per image (1 360 × 1 024 pixels) was determined by integrating the brightness at each pixel of the fluorescence image using image analysis software. The

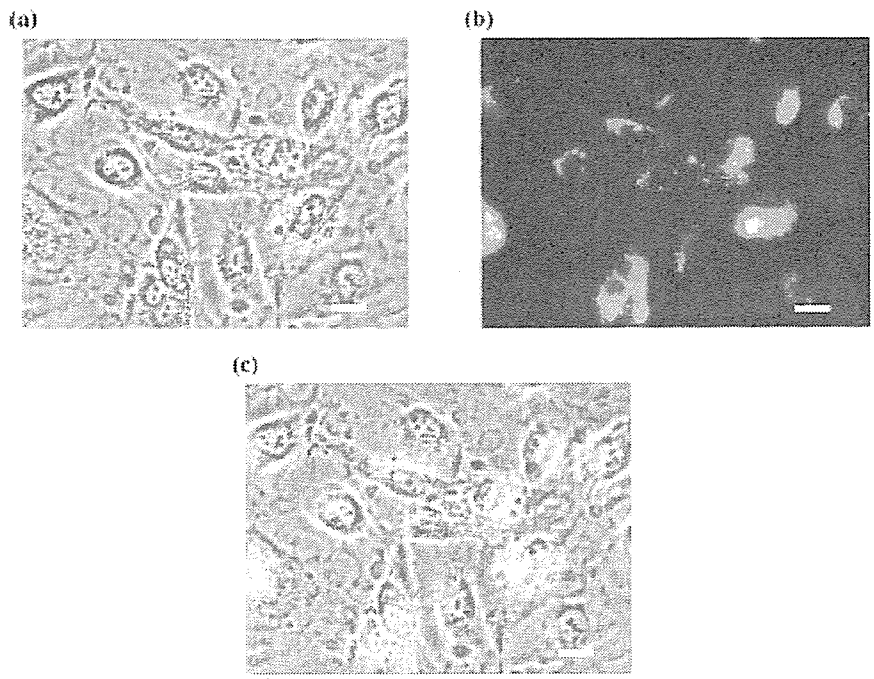


Figure 6a–c. Localization of rhodamine B-labeled copolymer **8** in human aortic endothelial cells. Phase contrast image (a), fluorescence image (b), and merged image of (a) and (b). Bars: 20 μm.

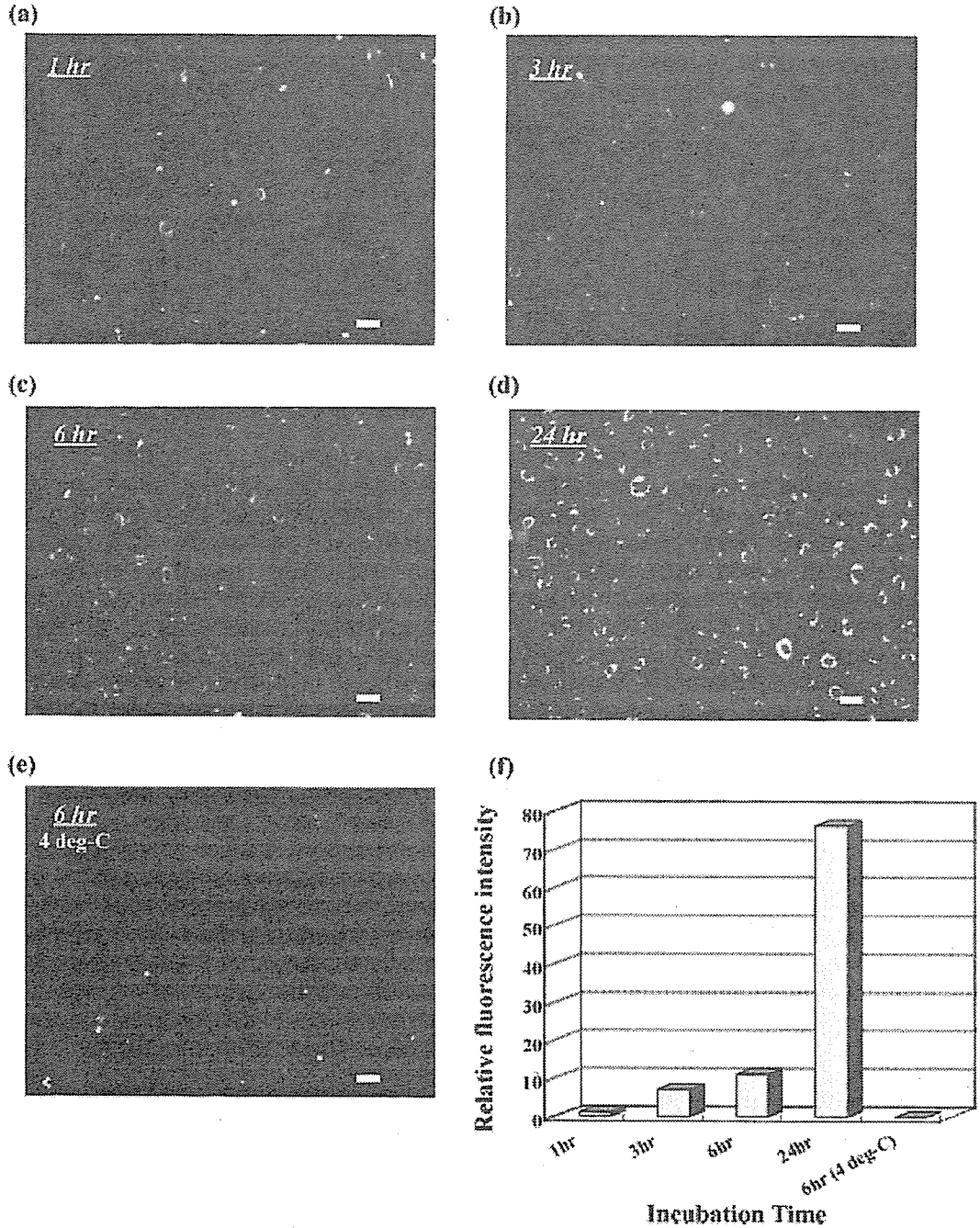


Figure 7a–f. Uptake of rhodamine B-labeled copolymer **8** by human aortic endothelial cells; incubation time, 1 h (a), 3 h (b), 6 h (c), 24 h (d) (incubation at 37 °C), and 6 h (incubation at 4 °C) (e). Bars: 20 μm. The uptake is quantitatively represented as relative fluorescence intensity in the bar graph (f).

fluorescence intensity was normalized in a ratio of the fluorescence intensity at each incubation time to that at 1 h of incubation. The ratio was termed as the relative fluorescence intensity. The time course of the relative fluorescence intensity is shown in Figure 7f and indicates that HAECs incorporated progressively more nanoaggregates of **8** during the incubation time. The relative fluorescence intensity was 7-fold at 3 h, 11-fold at 6 h, and 76-fold at 24 h of incubation. In contrast, the relative fluorescence intensity dropped considerably and was only 12% of the control level (1 h at 37 °C) when HAECs were exposed to the nanoaggregates of **8** at 4 °C for 6 h. The fact that the temperature triggered a dramatic decrease in the relative fluorescence intensity suggests that the nanoag-

gregates are incorporated into HAECs by endocytosis. Endocytosis is known as a cellular process that is coupled with temperature-dependent metabolic activities.[18] Furthermore, it is known that some receptors and membrane microdomains of endothelial cell are involved in endocytosis.[19] However, it is not clear which of the endocytic pathways is responsible for the uptake of the nanoaggregates. This issue is now under investigation.

Conclusion

In this study, we investigated synthesis of the amphiphilic poly(*N*-propargylamide) **6** containing both sugar residues (hydrophilic part) and long alkyl chains (hydrophobic part) in order to develop a novel nanoaggregate based on the self-organization of amphiphilic polymers with rigid backbone. The desired polymer was prepared by copolymerization of the two *N*-propargylamide monomers **1** and **5** having a galactose residue and a lauryloyl group, respectively, catalyzed by a Rh complex. The GPC, ^{1}H NMR, SEM, and DLS analyses of the resulting copolymers indicated formation of the nanoparticles in water. The formation of the one-handed helical conformation of the copolymer in both DMSO and water was confirmed by the CD spectra. An amphiphilic poly(*N*-propargylamide) containing fluorescent dyes was newly designed to evaluate cell uptake of nanoparticles of the amphiphilic copolymer by fluorescence microscopy. The *N*-propargylamide monomer **7**, having a rhodamine B dye moiety, was prepared and copolymerized with **1** and **5**. Human aortic endothelial cells (HAECs) were cultured in a medium containing the fluorescent-dye-labeled amphiphilic copolymer. Cell uptake of the copolymer was confirmed by red fluorescence emission from each of the HAECs. Progressive uptake was observed during the incubation period. When the cell culture experiment was conducted at 4 °C, the fluorescence intensity of the red emission was considerably lowered. This indicates that the cell uptake is inhibited at 4 °C and that this uptake process should occur in an endocytic pathway rather than by simple adsorption to the plasma membrane of HAECs. We are now synthesizing a fluorescent-dye-labeled hydrophilic copolymer of monomer **1** with monomer **7** to study preferential cell uptake of the nanoaggregates of the amphiphilic copolymer. In the future, we anticipate that nanoparticles will be able to be preferentially endocytosed into cells rather than monomeric chains of water-soluble copolymer. If this does occur, the nanoparticles of the amphiphilic copolymer will be a promising nanocarrier for drug delivery.

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