Transferrin-appended β -(1 $\rightarrow$ 3)-D-glucan schizophyllan for antisense oligonucleotides delivery to enhance the cellular uptake*

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Schizophyllan (SPG) is a natural β -(1 $\rightarrow$ 3)-D-glucan existing as a triple helix in water and as a single chain in dimethylsulfoxide (DMSO), respectively. As we already reported, when a homo-phosphodiester polynucleotide [for example, poly(dA) or poly(C)] is added to the SPG/DMSO solution and subsequently DMSO is exchanged for water, SPG forms a complex with the polynucleotide. One of the potential applications of this novel complex is an antisense-oligonucleotide (AS ODN) carrier. The present paper describes a modification technique which enables us to introduce transferrin to the reducing end of SPG (Tf-SPG). We made a complex from Tf-SPG and AS ODN and carried out an in vitro antisense assay. We found that Tf-SPG dramatically enhances the antisense effect. Their superiority can be ascribed to enhancement of endocytosis due to the attached transferrin. One of the peculiar features of our system is that the complex (i.e., carrier + AS ODN) is charged negatively in total, because SPG itself is a neutral polysaccharide. This feature is different from the conventional systems such as cationic liposomes and polycations. The present work has thus clarified that SPG can act as a new potential candidate for AS ODN carriers. [DOI: 10.1380/ejsnt.2005.195]

Keywords: Biological molecules - nucleic acids; Biological molecules - proteins; Biological compounds; Compound formation; Biological aspects of nano-structures

I. INTRODUCTION

Antisense oligonucleotides (AS ODNs) are applied to silence single gene, exploiting their exquisite specificity to match the target mRNA [1, 2]. Principles and mechanisms of the antisense therapy are (1) to deliver AS ODNs to the target cells, (2) to induce the cellular uptake of AS ODNs, (3) to release AS ODNs to cytosol, (4) to bind to a particular mRNA to make a DNA/RNA duplex, and (5) to make RNase H cleave the duplex so as to inhibit the protein expression. However, there are two major issues to overcome; instability of AS ODNs in biological fluids and low uptake efficiency into the target cells [3]. The instability of AS ODNs is mainly ascribed to two factors; the hydrolysis mediated by deoxyribonuclease and non-specific binding to proteins. The hydrolysis can be considerably reduced by the use of oligonucleotide analogues such as phosphorothioates, phosphoramidates, and peptide nucleic acids. Especially, phosphorothioates [4] are the leading candidate among the first generation of antisense compounds and several of them are indeed in phase I/II clinical trial.

When phosphorothioates are used as an AS ODN, non-specific binding to proteins becomes a major and serious problem, because it sometimes induce another biological event other than antisense effects [2, 5]. It is reasonably

considered that the materials which can form a complex with the phosphorothioates can prevent the AS ODN from forming such undesirable interactions [3]. Cationic lipids can form a complex with AS ODNs and encapsulate them in the liposomes; however, there are some drawbacks in their use. For instance, cationic liposomes tend to accumulate in the reticuloendothelial system, leading to a short life time in the serum. Synthetic polycations, such as poly(L-lysine) and polyethyleneimine (PEI) have been studied as an AS ODN carrier. Although polycations have great advantage to improve the cellular uptake, serious drawbacks are pointed out, such as toxicity of the polycations and poor solubility of the resultant polyion complexes.

In order to enhance the uptake efficiency, many studies attached an acceptor to the AS ODN or AS ODN carrier which can lead ingestion through the receptor mediated (such as transferrin receptor-mediated) endocytosis.

The transferrins are a class of iron binding proteins. Cell-surface transferrin receptors deliver transferrin with its bound iron to early endosomes by receptor-mediated endocytosis [6]. To enhance the cellular ingestion, transferrin molecules are attached to the carrier, including poly(L-lysine), PEI, and cationic liposomes. In some case, these transferrin appended polycations have demonstrated high efficiency in tumor-targetting [7], AS ODN [8], and drug [9] delivery when compared with the polycation alone.

Utilizing natural polysaccharides is considered to be a good and new approach to design the AS ODN carrier, because natural polysaccharides can biodegrade into non-

*This paper is series of polysaccharide-polynucleotide complex (40).

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FIG. 1: Repeating units of schizophyllan: (a) and a schematic illustration of the complexation: (b). In (b), the capital letters of G and B represent the glucose and base, and the gray lines show the hydrogen bonds.

toxic components and can provide satisfactory solubility [10]. The recent finding that a polysaccharide of schizophyllan (SPG) can form a complex with polynucleotides suggested that SPG can be used as an AS ODN binding site as well as the carrier [11–16]. SPG [17–19] is an extracellular polysaccharide produced by the fungus *Schizophyllum commune* and the main chain consists of β -(1→3)-D-glucan and one β -(1→6)-D-glycosyl side chain links to the main chain at every three glucose residues (see Fig. 1(a) for the chemical structure). SPG adopts a triple helical conformation in water and a random coil in dimethylsulfoxide (DMSO). When water is added to the DMSO solution (renaturation), the triple helical structure can be partially retrieved through this process, although the entire chain structure is not the same as that of the original triple helix. Recently, Sakurai and Shinkai found that the single chain of schizophyllan (s-SPG) forms a macromolecular complex with some homo-phosphodiester polynucleotides [such as poly(C), poly(A), poly(U), poly(dA), and poly(dT)], when the polynucleotide is present in the renaturation process [see Fig. 1(b)] [15, 16]. Some of the novel features for this complex are (1) the complex is remarkably stable and considerably water-soluble in the physiological conditions, (2) the complexation occurs in a highly stoichiometrical manner and the stoichiometric number indicates that two SPG units and three base units are interacting with each other [(see Fig. 1(b)) [15, 16], (3) when a s-SPG/DNA complex meets the corresponding complementary sequence, for example s-SPG/poly(T) meets poly(dA), the complex is dissociated immediately and the hybridization takes place [20], (4) the complex inhibits (or reduces) non-specific interactions between the bound AS ODNs and serum proteins such as BSA [13, 21]. When SPG is used as an AS ODN carrier, the four features of (1)–(4) mentioned above seem greatly advantageous. In this paper, we have made attempts to introduce transferrin into the reducing end of SPG to enhance the cellular uptake and carry out an *in vitro* assay for this modified SPG as an antisense carrier.

II. SYNTHESIS, MATERIALS AND METHODS

A. Synthesis of Tf-SPG (Fig. 2)

1. Synthesis of *N*-(*tert*-butoxycarbonyl)-2-aminoethanthiol(**2**)

2-aminoethanthiol hydrochloride (3.41 g, 30 mmol) and Et₃N (4.2 ml, 30 mmol) were dissolved in 60 mL of dry-CH₂Cl₂ and the mixture was stirred under N₂ at room temperature. After 10 minutes stirring, di-*tert*-butyl dicarbonate (6.54 g, 30 mmol) was added to the solution, and the mixture was stirred for 16 h. The resulting solution was washed with water 2 times and the organic phase was dried over anhydrous magnesium sulfate. After removing the solvent, **2** was obtained as a oily liquid. The yield was 4.70 g (97%). ¹H NMR (600 MHz, CDCl₃, δ): 1.38 (t, 1H, -SH), 1.45 (s, 9H, -C(CH₃)₃), 2.65 (br, 2H, -CH₂-SH), 3.31 (br, 2H, -NH-CH₂-), 5.06 (br, 1H, NH). MALDI-TOF Mass (Dithranol): Calcd for $m/z = 177$, found 178 (M+H)⁺.

2. Synthesis of *N*-(*tert*-butoxycarbonyl)-*S*-(*N*-ethylcarbamoyl)-2-aminoethanthiol(**3**)

Ethyl isocyanate (4.8 ml, 54 mmol)/dry-CH₂Cl₂ (30 ml) solution was added to the mixture of compound **2** (4.60 g, 28 mmol) and Et₃N (1.2 ml). The mixture was stirred under N₂ for 2 days and the product was washed with saturated NaCl solution 5 times. The organic phase was dried over anhydrous magnesium sulfate. An analytical pure sample was obtained by recrystallization from benzene-hexane (1:1 (v/v)). The yield was 5.20 g (80%). ¹H NMR (250 MHz, CDCl₃, δ): 1.11 (t, 3H, -NH-CH₂-CH₃), 1.44 (s, 9H, -C(CH₃)₃), 3.02 (t, 2H, -NH-CH₂-CH₂-), 3.33 (m, 4H, -CH₂-CO-NH-CH₂-), 4.97 (br, 1H, -S-CO-NH-), 5.43 (br, 1H, -O-CO-NH-). MALDI-TOF Mass (Dithranol): Calcd for $m/z = 248$, found 271 (M+Na)⁺.

FIG. 2: Reaction scheme to introduce transferrin into the reducing end of s-SPG.

3. Synthesis of *S*-(*N*-ethylcarbamoyl)-2-aminoethanethiol trifluoroacetate (**4**)

A solution of **3** in CH₂Cl₂ (2.20 g, 9.5 mmol) was treated with 20 mL of 15% TFA. After 24 hours stirring, the mixture was evaporated to give a white solid. This crude product was purified by precipitation from a CH₂Cl₂/hexane mixture. The yield was 2.4 g (95%). ¹H NMR (250 MHz, DMSO-d₆, δ): 1.03 (t, 3H, -NH-CH₂-CH₃), 2.97 (s, 4H, -S-CH₂-CH₂-), 3.14 (m, 2H, -NH-CH₂-), 7.85 (br, 3H, -NH₃), 8.34 (br, 1H, -NH-). MALDI-TOF Mass (Dithranol): Calcd for m/z = 148, found 171 (M+Na)⁺.

4. Introduction of a thiol end into SPG (**5**)

SPG (300 mg, 2.0×10^{-3} mmol) was dissolved in 50 mL of DMSO-water (3:2 v/v) mixed solvent. To the SPG solution was added compound **4** (1.0 g, 3.8 mmol), followed by NaBH₃CN (300 mg, 4.7 mmol) and Et₃N, (3.0 mL). The resulting solution was stirred at 70°C under N₂ for 5 days. To remove the protecting group of the SH-group as well as inorganic by-product, the reaction mixture was subjected to dialysis under basic condition (MWCO = 14,000, pH = 8.0). After repeating the dialysis under neutral condition several times, freezing-and-pumping of the resulting solution gave **5** as a white powder. The yield was 270 mg (90%).

5. Synthesis of Tf-SPG (**7**)

Apo-transferrin (60 mg, 8.0×10^{-4} mmol) was dissolved in 30 mL of 0.1 M PBS buffer. To the transferrin solution was added 150 mL of 10 mM *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) ethanolic solution, and the resulting solution was stirred at room temperature for 50 minutes. To remove excess amount of SPDP, the reaction mixture was subjected to centrifugal filtration

FIG. 3: HPLC chromatographic chart after and before the purification.

(MWCO = 3000, 10°C), and the residue was washed with PBS buffer several times. The transferrin-SPG conjugate **7** was synthesized by following procedure: to the SPDP-modified schizophyllan **5** in water was added compound **6** (100 mg, 6.6×10^{-4} mmol) and the solution was incubated at 4°C for 1 day. The conjugate was purified by centrifugal filtration through a dialysis membrane filter (MWCO = 100,000, 10°C). Freezing-and-pumping of the resulting solution gave **7** (46 mg) as a white powder. The formation of the conjugate was confirmed by HPLC analysis. Unmodified SPG was taken out with a Sephadex G-100 column in 10 mM Tris buffer. Figure 3 compares the HPLC chromatographic chart before and after the purification.

B. Materials

Taito Co. Ltd., (Japan) kindly supplied the schizophyllan sample. The weight-average molecular weight

(M_w) and the number of repeating units were found to be 1.5×10^5 and 231, respectively. AS-c-myb, S-c-myb and MS-c-myb were synthesized at Hokkaido System Science (Hokkaido, Japan) and purified with high-pressure liquid chromatography. The fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco/BRL. Dulbecco's modified Eagle's medium (DMEM®) and RPMI1640 were obtained from Nissui Pharmaceutical Co. Ltd. Bovine serum albumin (BSA) were obtained from Sigma. Spermine was attached to the side chain of SPG according to the reported method and the modification level (defined by the molar percent of the modified side chain) was 4.6 mol% determined by elemental analysis. This sample was denoted by SP(4.6)-SPG. ODN samples were complexed with Tf-SPG (or SP(4.6)-SPG, SPG) according to the reported method. The molar ratio (M_{s-SPG}/M_{ODN}) was fixed at 1.5, where M_{s-SPG} and M_{ODN} are the repeating molar concentration of s-SPG and AS-c-myb, respectively.

C. Cell culture and the cell proliferation measurements

The leukemia cell lines HL-60 (human promyelocytic leukemia) and K562 (human proerythroblastic leukemia) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were maintained in DMEM® supplemented with 10% FBS, containing a 1 wt% penicillin/streptomycin mixture. The cell incubation was always carried out at 37°C in a fully humidified air containing 5 wt% of CO₂. After incubation for 3 days, the number of the cells was evaluated with Cell Counting Kit-8® (Dojindo, Japan), called WST-8 assay. Details of the experimental procedure was presented in the previous paper. Lipofectin/AS ODN complexed was made according to the recommended protocol. Competitive inhibition of transferrin up-take was carried out by adding free transferrin and deferoxamine to the culture media. Experimental details for RT-PCR was described by the previous paper.

III. RESULTS AND DISCUSSION

A. Complexation between phosphorothioate AS ODNs and schizopphyllan

The sequence of 5'-GTGCCGGGGTCTTCGGGC-3' is well known to bind to *c-myb* mRNA and to lead drastic depression of *c-myb* in many cell lines [22, 23]. As our previous work revealed, short and hetero ODNs cannot bind to s-SPG, so that we had to attach a poly(dA)₄₀ tail (which has 40 dA bases) at the 3' end of this sequence [12]. Thus, in this study, 5'-GTGCCGGGGTCTTCGGGC-(dA)₄₀-3' phosphorothioate was used as an AS ODN and denoted by AS-c-myb, hereinafter. Figure 4 presents the gel electrophoresis migration patterns for mixtures of AS-c-myb and transferrin-appended SPG (Tf-SPG), comparing with naked AS-c-myb and a mixture of AS-c-myb and s-SPG. AS-c-myb itself migrates, however, most of AS-c-myb in the Tf-SPG + AS-c-myb or s-SPG + AS-c-myb mixtures does not migrate at all. This is evidence that

FIG. 4: Confirmation of the complex formation between AS-c-myb and Tf-SPG sample by gel electrophoresis. -Lane 1; naked AS-c-myb, Lane 2; s-SPG + AS-c-myb, Lane 3; Tf-SPG + AS-c-myb, Lane 4; the extra amount of Tf-SPG + AS-c-myb. 2 w/t% NuSieve agarose gel (BMA) was used and the gel was stained with GelStarr (BMA).

Tf-SPG and AS-c-myb are complexed. Although the data are not presented, we confirmed the complexation by the use of UV spectroscopy.

B. Enhanced antisense effect by the transferrin modification

Figure 5 shows the K562 and HL60 cell growth after cultured for three days, comparing naked AS-c-myb and the complexed AS-c-myb with s-SPG, Tf-SPG, or spermine-attached SPG (SP(4.6)-SPG). Two AS-c-myb doses (25 and 50 $\mu\text{g}/\text{ml}$) were examined and the cell growth was defined by the number of cells normalized by that of the control (without any addition of AS-c-myb nor s-SPG). In all cases, the SPG : AS-c-myb molar ratio was fixed at 1.5:1.0. Figure 6 shows the corresponding experiment by the use of sense sequence (S-c-myb) and mismatched sequence (MS-c-myb) as negative controls instead of AS-c-myb.

When we exposed the cells to AS-c-myb itself, the cell growth decreased with increasing the AS-c-myb dose. This decrease should be mainly ascribed to the antisense effect. However, since when we exposed the cells with S-c-myb and MS-c-myb, there were some decreases observed in Fig. 6, some percent of the cell death in Fig. 5 may be caused by cytotoxicity of the phosphorothioate itself. Although SPG has no capability to be ingested by cells, the growth of the SPG complex was lower than that of the naked dose. This difference can be ascribed to the fact that the bound AS-c-myb is suffering from less adsorption by BSA in the culturing medium than that of naked AS-c-myb [13]. When we attached a functional group that can induce endocytic ingestion, such as sper-

FIG. 5: Cell growth when Tf-SPG/AS ODN was exposed to K562 and HL60 cells, including the Lipofectin and unmodified s-SPG systems. The cell numbers were determined with WST-8 assay, after we administrated AS-c-myb and incubated the cells for three days. The cell growth is defined by the number of cells normalized by that of the control. The s-SPG : AS-c-myb molar ratio was fixed at 1:1.5.

FIG. 6: Comparison of the cell growth for all samples in K562 cells, when we administrated the sense sequence (S-c-myb) and mismatched sequence (MS-c-myb). The other conditions were the exactly same as those in Fig. 5.

mine and transferrin, the cell growth was more reduced than the others. Furthermore, the Tf-SPG and SP-SPG complexes show almost the same growth as that of lipofectin dose. These features are consistent throughout the experiment for difference cell lines and different doses.

Polyamines such as putrescine, spermidine, and spermine are organic polycations present everywhere in cells and can interact with anionic biomolecules (e.g. DNA, RNA, proteins, and phospholipids) [10, 24]. Perhaps because of their ability to bind many biomolecules and their intrinsic toxicity, the transport of these compounds are tightly regulated. Although polyamine carriers have

not yet been molecularly identified, polyamines seem to first enter into the cytosol through a plasma membrane transporter, subsequently most polyamines are sequestered into intracellular vesicles which lead the ingested polyamines to late endosome. This ingestion pathway should be the major route for spermine itself, however, spermine-attached carrier may not be ingested by the same pathway. With increasing the molecular weight, it should be more difficult to pass the plasma membrane. In this case, the ingestion of larger subjects may be accomplished with regulatory endocytic pathway.

Transferrin [25], an iron-binding glycoprotein, is a well-

FIG. 7: Sequence specific mRNA silencing confirmed by RT-PCR, comparing between naked ODN and its complex. K562 was treated with 60 $\mu\text{g}/\text{ml}$ of AS ODN (targeting *c-myb* mRNA) or its Tf-SPG complex. PCR products were resolved by agarose gel electrophoresis and visualized by GelStarr. Amplified DNA fragments were quantified by a gel documentation system (Alpha DigiDOC gel documentation system, Alpha Innotech). The numbers under the gel image indicates the relative intensity of the mRNA band to the reference.

studied ligand for tumor targeting. Iron-loaded transferrin (ferrotransferrin) is recognized and binds to transferrin receptors on cell surfaces, then subsequently ingested by an endocytic pathway into acidic compartments. The drop in pH triggers the iron dissociation, and the iron-poor (apo-) transferrin is recycled to the cell surface and released. Expression of transferrin receptor is elevated in rapidly dividing cells due to a need for iron; therefore, it is often upregulated on surfaces of malignant cells and has been used as a tumor-targeting ligand for several drug delivery systems.

C. Confirmation of the antisense effect by reverse-transcriptase-mediated PCR

The enhanced antisense effect by AS-c-myb/Tf-SPG was confirmed with RT-PCR. The results are presented in Fig. 7. As a reference, we evaluated the amount of the β -actin mRNA, comparing naked AS-c-myb with the complexed AS-c-myb with Tf-SPG and s-SPG. As shown in the panel (a), there is no difference among the samples, indicating that administration of these ODN does not provide any effect on the amount of the β -actin mRNA. However, when we evaluated the amount of mRNA by the same manner, the transferrin complex decreased the amount of mRNA. These results evidence that AS ODN has silenced the target mRNA in a sequence-specific manner and the complex reduces the amount more drastically than the naked AS ODN, confirming that the antisense effect is enhanced in the complex system.

IV. COMPETITIVE INHIBITION OF TRANSFERRIN UP-TAKE

Figure 8 compares the cell growth when transferrin or deferoxamine was added to the culture media. The added free transferrin can act as an antagonist for the ingestion of Tf-SPG/AS ODN. Deferoxamine, a strong iron chelator [26], was added to the culture media to make the cell deficient in iron. Before exposing the cells to AS ODNs, the culture media were exchanged for fresh one. This treatment makes the cell active to ingest iron (i.e., transferrin) when Tf-SPG/AS ODN was added. As presented in Fig. 8, addition of transferrin or deferoxamine never interferes with the cell growth for the naked dose and the complexed dose with s-SPG and SP-SPG. The difference is within 5% for all cases. However, in the case of Tf-SPG/AS ODN, transferrin increased the growth back to 85% and 75% for 25 and 50 μg dose, respectively. On the other hand, deferoxamine decreased it by almost 10-20%. These results indicate that the enhanced antisense effect of Tf-SPG/AS ODN is ascribed to the fact that transferrin receptors on cell surfaces recognize the complex.

V. CONCLUSION

The present report describes a chemical modification technique of SPG to introduce transferrin to the reducing end of SPG. The resultant Tf-SPG can form a complex with AS ODN and we carried out an in vitro antisense assay. We found that Tf-SPG dramatically enhances the

FIG. 8: Competitive inhibition of transferrin up-take, comparing the naked dose and the complexes with s-SPG, Tf-SPG, SP-SPG in K562 cells. Medium + 50 μ M Deferrioxamine was added the culture media and the cells were exposed to it for 1 day. Before adding AS ODNs, the culture media was exchanged for fresh one.

antisense effect. This effect can be ascribed to enhancement of endocytosis due to the transferrin. The present work has thus clarified that SPG can act as a new potential candidate for AS ODN carriers, especially, presenting the synthetic method to attach proteins at the reducing end of SPG.

It should be emphasized that Tf-SPG/AS ODN complex is negatively charged in total because of the excess amount of AS ODNs. Therefore, the attractive Coulombic force should not work when the complexes approach to the negatively charged cell surface from far away. However, once the complexes are happened to be in the vicinity of the cell surface by the thermal motion or the flow of the culture medium, the attached transferrin can interact with the cell surface. It is interesting that the negatively charged complexes can show comparable efficiency in the cellular uptake with the positively charged Lipofectin. Negatively charged complexes should show a different performance from the conventional positively charged carries, such as a longer circulation in the blood. Therefore, we can propose that this is one of the peculiar features of our system, which is different from the conventional systems.

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Abbreviations

Special abbreviations and symbols used in this paper.

AS ODN; antisense oligonucleotide.

S ODN; sense oligonucleotide.

MS ODN; mismatched oligonucleotide.

AS-c-myb; 5'-GTGCCGGGGTCTTCGGGC-(dA)_{40-3'} phosphorothioate, AS ODN for *c-myb* mRNA.

S-c-myb; 5'-CACGGCCCCAGAAGCCCG-(dA)_{40-3'} phosphorothioate, S ODN for *c-myb* mRNA.

MS-c-myb; 5'-GTGTCGGAGTCTTTGGGC-(dA)_{40-3'} phosphorothioate, MS ODN for *c-myb* mRNA.

s-SPG; single chain of schizophyllan.

Tf-SPG; transferrin-attached SPG.

SP-SPG; spermine-attached SPG.

s-SPG/AS ODN or modified s-SPG/AS ODN; complexes made from s-SPG and AS ODN, or modified s-SPG and AS ODN. In this paper '/' means the complex.

s-SPG + AS ODN; mixture of s-SPG and AS ODN.

In this paper, s-SPG+AS ODN stands for a mixture of s-SPG + AS ODN; does not necessary mean the complex, it can be a just mixture of the two components without any interaction. After we confirmed the complex formation, we explicitly denote the complex as s-SPG/AS ODN.

- [1] E. Uhlmann and A. Peyman, Chem. Rev. **90**, 543 (1990).
- [2] C. A. Stein and Y. C. Cheng, Science **261**, 1004 (1993).
- [3] T. V. Chirila, P. E. Rakoczy, K. L. Garrett, X. Lou, I. J.

Constable, Biomaterials **23**, 321 (2002).

- [4] A. M. Krieg and C. A. Stein, Antisense Res Dev. **5**, 241 (1995).

- [5] C. A. Stein and A. M. Krieg, *Antisense Res Dev.* **4**, 67 (1994).
- [6] H. Sun, H. Li, P. J. Sadler, *Chem Rev.* **99**, 2817 (1999).
- [7] R. Kircheis, L. Wightman, A. Schreiber, B. Robitza, V. Rossler, M. Kursa, E. Wagner, *Gene Ther.* **8**, 28 (2001).
- [8] S. Vinogradov, E. Batrakova, S. Li, A. Kabanov, *Bioconjug Chem.* **10**, 851 (1999).
- [9] H. Iinuma, K. Maruyama, K. Okinaga, K. Sasaki, T. Sekine, O. Ishida, N. Ogiwara, K. Johkura, Y. Yonemura, *Int. J. Cancer* **99**, 130 (2002).
- [10] T. Azzam, H. Eliyahu, L. Shapira, M. Linial, Y. Barenholz, A. J. Domb, *J. Med. Chem.* **45**, 1817 (2002).
- [11] T. Hasegawa, T. Fujisawa, S. Haraguchi, M. Numata, R. Karinaga, T. Kimura, S. Okumura, K. Sakurai, S. Shinkai, *Bioorg. Med. Chem. Lett.* **15**, 327 (2005).
- [12] T. Matsumoto, M. Numata, T. Anada, M. Mizu, K. Koumoto, K. Sakurai, T. Nagasaki, S. Shinkai, *Biochim Biophys Acta* **1670**, 91 (2004).
- [13] M. Mizu, K. Koumoto, T. Anada, R. Karinaga, T. Kimura, T. Nagasaki, S. Shinkai, K. Sakurai, *Bulletin of the Chemical Society of Japan* **77**, 1101 (2004).
- [14] M. Mizu, K. Koumoto, T. Kimura, K. Sakurai, S. Shinkai, *Polym. J.* **35**, 714 (2003).
- [15] K. Sakurai, M. Mizu, S. Shinkai, *Biomacromolecules* **2**, 641 (2001).
- [16] K. Sakurai and S. Shinkai, *J. Am. Chem. Soc.* **122**, 4520 (2000).
- [17] T. Norisuye, T. Yanaki, H. Fujita, *J. Polym. Sci., Polym. Phys. Ed.* **18**, 547 (1980).
- [18] K. Tabata, W. Ito, T. Kojima, S. Kawabata, A. Misaki, *Carbohydr. Res.* **89**, 121 (1981).
- [19] K. Tabata, W. Itoh, A. Hirata, I. Sugawara, S. Mori, *Agric. Biol. Chem.* **54**, 1953 (1990).
- [20] K. Koumoto, M. Mizu, K. Sakurai, T. Kunitake, S. Shinkai, *Chemistry & Biodiversity* **1**, 520 (2004).
- [21] M. Mizu, K. Koumoto, T. Anada, K. Sakurai, S. Shinkai, *Biomaterials* **25**, 3117 (2004).
- [22] T. L. Burgess, E. F. Fisher, S. L. Ross, J. V. Bready, Y. X. Qian, L. A. Bayewitch, A. M. Cohen, C. J. Herrera, S. S. Hu, T. B. Kramer, *et al.*, *Proc. Natl. Acad. Sci. USA* **92**, 4051 (1995).
- [23] A. M. Gewirtz and B. Calabretta, *Science* **242**, 1303 (1988).
- [24] D. Soulet, B. Gagnon, S. Rivest, M. Audette, R. Poulin, *J. Biol. Chem.* **279**, 49355 (2004).
- [25] D. Trinder, O. Zak, P. Aisen, *Hepatology* **23**, 1512 (1996).
- [26] P. T. Doulias, S. Christoforidis, U. T. Brunk, D. Galaris, *Free Radic. Biol. Med.* **35**, 719 (2003).